

Sexual dimorphism of the sensory systems in bees (Hymenoptera, Apoidea) and the evolution of sex-specific adaptations in the context of mating behavior

Sensorischer Geschlechtsdimorphismus bei Bienen (Hymenoptera, Apoidea) und die Evolution geschlechtsspezifischer Anpassungen im Kontext des Paarungsverhaltens

Dissertation zur Erlangung des  
naturwissenschaftlichen Doktorgrades  
der Julius-Maximilians-Universität Würzburg

vorgelegt von  
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aus Wien, Österreich  
Würzburg, 2013

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Tag des Promotionskolloquiums: .....

Doktorurkunde ausgehändigt am: .....

This thesis is based on the following manuscripts:

*Manuscript I:*

Streinzer, M., Brockmann, A., Nagaraja, N., Spaethe, J. (2013). Sex and caste-specific variation in compound eye morphology of five honeybee species. *PLoS One* 8(2):e57702. doi: 10.1371/journal.pone.0057702

*Manuscript II:*

Streinzer, M., Spaethe, J. (under review). A scientific note on peripheral compound eye morphology of small and normal-sized honeybee drones. *Journal of Apicultural Research*

*Manuscript III:*

Streinzer, M., Spaethe, J. (under review). The evolution of elaborate male visual systems in bumblebees (Hymenoptera, Apidae, *Bombus*). *Zoological Journal of the Linnean Society*

*Manuscript IV:*

Streinzer, M., Spaethe, J. (in preparation) Visual ecology of long-horned bee males (*Eucera berlandi*, Hymenoptera: Apidae): Adaptations of the visual system and mate detection.

*Manuscript V:*

Streinzer, M., Kelber, C., Pfabigan, S., Kleineidam, C.J., Spaethe, J. (in press). Sexual dimorphism in the olfactory system of a solitary and a eusocial bee species. *Journal of Comparative Neurology*. doi: 10.1002/cne.23312

## STATEMENT OF AUTHOR CONTRIBUTIONS

### *Manuscript I*

Streinzer, M., Brockmann, A., Nagaraja, N., Spaethe, J. (2013). Sex and caste-specific variation in compound eye morphology of five honeybee species. *PLoS One* 8(2):e57702. doi: 10.1371/journal.pone.0057702

<b>Contribution</b>	<b>Author initials; decreasing order of responsibility</b>				
Idea & study design	MS	AB	JS		
Material & data collection	MS	NN			
Data analysis & interpretation	MS				
Manuscript writing	MS	AB	JS		

### *Manuscript II*

Streinzer, M., Spaethe, J. (under review). A scientific note on peripheral compound eye morphology of small and normal-sized honeybee drones. *Journal of Apicultural Research*

<b>Contribution</b>	<b>Author initials; decreasing order of responsibility</b>				
Idea & study design	MS				
Material & data collection	MS				
Data analysis & interpretation	MS				
Manuscript writing	MS	JS			

### *Manuscript III*

Streinzer, M., Spaethe, J. (under review). The evolution of elaborate male visual systems in bumblebees (Hymenoptera, Apidae, Bombus). *Zoological Journal of the Linnean Society*

<b>Contribution</b>	<b>Author initials; decreasing order of responsibility</b>				
Idea & study design	MS	JS			
Material & data collection	MS				
Data analysis & interpretation	MS				
Manuscript writing	MS	JS			

### *Manuscript IV*

Streinzer, M., Spaethe, J. (in preparation). Visual ecology of long-horned bee males (*Eucera berlandi*, Hymenoptera: Apidae): Adaptations of the visual system and mate detection

<b>Contribution</b>	<b>Author initials; decreasing order of responsibility</b>				
Idea & study design	MS	JS			
Material & data collection	MS	JS			
Data analysis & interpretation	MS				
Manuscript writing	MS	JS			

### *Manuscript V*

Streinzer, M., Kelber, C., Pfabigan, S., Kleineidam C.J., Spaethe, J. (in press). Sexual dimorphism in the olfactory system of a solitary and a eusocial bee species. *Journal of Comparative Neurology*. doi: 10.1002/cne.23312

<b>Contribution</b>	<b>Author initials; decreasing order of responsibility</b>				
Idea & study design	MS	CJK	JS		
Material & data collection	MS	CK	SP		
Data analysis & interpretation	MS	CJK	JS		
Manuscript writing	MS	CJK	JS		

## TABLE OF CONTENTS

1. Summary	1
2. Zusammenfassung	3
3. Introduction	6
3.1. Bees (Hymenoptera: Apoidea: Apiformes)	6
3.2. Sexual dimorphism in bees	6
3.3. Bee mating	7
3.3.1. Mating systems	7
3.3.2. Signals and cues	8
3.4. Sensory systems investigated	9
3.4.1. Visual sense	9
3.4.1.1. Visual system	9
3.4.1.2. Adaptations of the compound eyes in bees	10
3.4.2. Olfactory sense	11
3.4.2.1. Olfactory system	11
3.4.2.2. Adaptations of the olfactory system in bees	13
3.5. Choice of study species	14
3.5.1. Honeybees (Hymenoptera: Apidae: Apinae: Apini: <i>Apis</i> LINNÉ)	14
3.5.2. Bumblebees (Hymenoptera: Apidae: Apinae: Bombini: <i>Bombus</i> LATREILLE)	15
3.5.3. The long-horned bee <i>Eucera berlandi</i> (DUSMET) 1926 (Hymenoptera: Apidae: Apinae: Eucerini)	16
4. Outline of the thesis	24
4.1. Aims and questions	24
4.2. Overview of manuscripts	25
5. Manuscript I: Sex and caste-specific variation in compound eye morphology of five honeybee species	30
6. Manuscript II: A scientific note on peripheral compound eye morphology of small and normal-sized honeybee drones	40
7. Manuscript III: The evolution of elaborate male visual systems in bumblebees (Hymenoptera, Apidae, <i>Bombus</i> )	45
8. Manuscript IV: Visual ecology of long-horned bee males ( <i>Eucera berlandi</i> , Hymenoptera: Apidae): Adaptations of the visual system and mate detection	75
9. Manuscript V: Sexual dimorphism in the olfactory system of a solitary and a eusocial bee species	92
10. Discussion	116
10.1. Visual system	116
10.1.1. Females and workers	116
10.1.2. Queens	117
10.1.3. Males	118
10.1.4. Evolution of elaborate visual systems in <i>Bombus</i>	119
10.1.5. Spatial vision in <i>Eucera</i> mating behavior and <i>Ophrys</i> pollination	119
10.2. Olfactory system	120
10.2.1. Sex-specific olfactory systems	120
10.2.2. Olfaction in bumblebee mating and foraging behavior	121
10.3. Degree of sexual dimorphism	122
11. Erklärungen	128
12. Danksagung	129

## 1. SUMMARY

Bees have had an intimate relationship with humans for millennia, due to their importance as pollinators of fruit, vegetable and other crops, as well as suppliers of honey, wax and other products. This relationship has led to an extensive understanding of their ecology and behavior that has attracted researchers from such diverse fields as ecology, economics, sensory ecology, neurobiology, sociobiology and many others. One of the most comprehensively understood species is the Western honeybee, *Apis mellifera*. Its behavioral repertoire is rich, often attributed to its social lifestyle, and its learning skills, memory and mating behavior have intrigued many researchers. The pronounced caste and sexual dimorphism has facilitated the study of the causes and consequences of sex differences on the evolutionary and developmental levels. Our understanding of sex-specific investment in bees, in general, has continued to increase, but has remained phenomenological and superficial in many respects. Signals and cues employed in bee foraging and mating behavior are reasonably well understood in only a handful of species. Functional adaptations are described in some species, but the knowledge, especially on male-specific adaptations, is fragmentary.

In this thesis I explored the variety of sensory adaptations for sex-specific behaviors in three model systems within the bees. Females share a similar ecology, engaging in nest construction, parental care and foraging, and therefore similar functional morphologies are to be expected. Males, however, engage mainly in mating behavior. A variety of male mating strategies has been described which differ in their spatiotemporal features and in the signals and cues involved, and thus selection pressures. As a consequence, males' sensory systems are more diverse than those of females.

In the first part of this thesis I studied adaptations of the visual system in honeybees. As a starting point I compared sex and caste-specific eye morphology among 5 species (*Apis andreniformis*, *A. cerana*, *A. dorsata*, *A. florea*, *A. mellifera*). While *A. mellifera* has been investigated in great detail, the Asian congeners have only recently received scientific interest. I found a strong correlation between body size and eye size in both female castes. Queens have a relatively reduced visual system which is in line with the reduced role of visual perception in their life history. Workers differed in eye size and functional morphology, which corresponds to known foraging differences among species. In males, the eyes are conspicuously enlarged in all species, but a disproportionate enlargement was found in two species. While it can be attributed to nocturnal mating activity in *A. dorsata*, the role of the eye enlargement in *A. florea* mating behavior is currently unknown. In addition, I demonstrate a correlation between male visual parameters and mating flight time, and propose that light intensities play an important role in the species-specific timing of mating flights.

In the second study I investigated eye morphology differences among two phenotypes of drones in the Western honeybee. Besides normal-sized drones, smaller drones are reared in the colony, and suffer from reduced reproductive success. My results suggest that the smaller phenotype does not differ in spatial resolution of its visual system, but suffers from reduced light and contrast sensitivity which may exacerbate the reduction in reproductive success caused by other factors.

In the third study I investigated the sex and caste-specific morphology of the visual system in bumblebees. I explored the association between male eye size and mating behavior and investigated the diversity of compound eye morphology among workers, queens and males in 11 species from 11 (of a total of 15) *Bombus* subgenera. I identified adaptations of workers that

correlate with distinct foraging differences among species (e.g. foraging environments). Bumblebee queens must, in contrast to honeybees, fulfill similar tasks as workers in the first part of their life, and correspondingly visual parameters are similar among both female castes. Enlarged male eyes are found in several subgenera and have evolved several times independently within the genus. Males of these species engage in visually guided mating behavior. I find similarities in the functional eye morphology among large-eyed males in four subgenera, suggesting convergent evolution as adaptation to similar visual tasks. In the remaining 7 species, males do not differ significantly from workers in their eye morphology. I further applied phylogenetic comparative methods to trace the character evolution of male eye enlargement. My results suggest enlarged male eyes to be the ancestral state, and that this phenotype has re-evolved several times within the genus.

In the fourth study I investigated the sexual dimorphism of the visual system in a solitary bee species. The long-horned bee *Eucera berlandi* lives a solitary life, i.e. females construct and provision their own nest. Males patrol nesting sites and compete for first access to virgin females. I chose this model system, due to its previous and ongoing use in behavioral and physiological studies related to orchid pollination. The signal and cues employed by the sexually deceptive orchid *Ophrys heldreichii* mimic real life mating signals and cues of *E. berlandi*, which facilitates the interpretation of observed morphological differences among the sexes. Males have enlarged eyes and better spatial resolution in their frontal eye region compared with females, which I determined using antidromic illumination technique. In a behavioral study, I further tested the effect of target size and speed on male mate catching success. Additional 3-D reconstructions of the chasing flights reveal that angular target size is an important parameter in male chasing behavior. I discuss similarities to other insects that face similar problems in visual target detection.

In the fifth study I examined to the olfactory system. Males of *E. berlandi* have extremely long antennae, among the longest in bees. The long antennae and the conspicuous sexual dimorphism of antenna size suggest that they constitute adaptations for mate detection. To investigate the anatomical grounds of this elongation and its possible functional consequences I studied antennal morphology in detail in the periphery and follow the sexual dimorphism into the brain where I reveal additional functional adaptations. In the course of this study I compared the adaptations with those of the Western honeybee, the only other bee species in which comparable comprehensive data are available. Functionally similar adaptations were found in males of both species (e.g. longer antennae, a multiplication of olfactory sensilla and receptor neurons, hypertrophied macroglomeruli in the antennal lobe, a numerical reduction of glomeruli in males and sexually dimorphic investment in higher order processing regions in the brain), which differed in their extent among the two species. The similarities and differences are discussed in the context of solitary vs. eusocial lifestyle and the corresponding consequences for selection acting on males.

To summarize, my thesis presents novel data on the functional aspects of sexual dimorphism in the visual system in three model groups and the first insights into the olfactory pathway of a solitary bee species. It increases our understanding of sensory adaptations to sex and caste-specific tasks and behaviors. My studies provide templates for future investigations in other bee taxa and I propose suitable model systems for future studies, which may reveal a surprising and remarkable magnitude of sex and caste-specific adaptations in sensory systems within this charismatic, important and interesting insect group.

## 2. ZUSAMMENFASSUNG

Bienen und Menschen verbindet eine lange andauernde und enge Beziehung, als Folge der Wichtigkeit der Bienen als Bestäuber von Früchten und Gemüse, und als Lieferanten von Honig, Wachs und anderen Produkten. Die enge Beziehung hat zu einem ausgeprägten Wissen über die Ökologie und das Verhalten der Bienen geführt und Wissenschaftler aus der Ökologie, Ökonomie, sensorischen Ökologie, Neurobiologie, Soziobiologie und vielen anderen Disziplinen angezogen. Die am besten untersuchte Bienenart ist die westliche Honigbiene, *Apis mellifera*. Ihr ausgeprägtes Verhaltensrepertoire, das oft mit ihrer sozialen Lebensweise in Verbindung gebracht wird, und ihre Lernfähigkeit und das auffällige Paarungsverhalten haben viele Wissenschaftler in ihren Bann gezogen. Der ausgeprägte Kasten- und Sexualdimorphismus hat außerdem das Studium der Geschlechterunterschiede aus evolutionärer und ontogenetischer Sicht vereinfacht und vorangetrieben. Unser Wissen über geschlechtsspezifische Investitionen ist stetig angewachsen, jedoch in vielerlei Hinsicht lückenhaft und oberflächlich geblieben. Die Signale und Achtungssignale die im Paarungsverhalten der Bienen eine Rolle spielen sind nur bei einer Handvoll Arten hinreichend bekannt. Funktionelle Anpassungen an diese sind in wenigen Arten beschrieben. Im Allgemeinen ist unser Wissen, vor allem über die Anpassungen der Männchen, äußerst lückenhaft.

In dieser Arbeit habe ich die Vielfalt sensorischer Anpassungen an geschlechtsspezifische Verhaltensweisen in drei Bienengruppen genauer untersucht. Weibchen und Arbeiterinnen haben im Allgemeinen eine ähnliche Lebensweise, sie suchen geeignete Nistplätze, konstruieren das Nest, foragieren und versorgen ihren Nachwuchs. Infolgedessen sind ähnliche morphologische Anpassungen der Sinnessysteme zu erwarten. Männchen beschäftigen sich fast ausschließlich mit der Partnersuche. Eine Vielzahl verschiedener Paarungsstrategien wurden bisher beschrieben die sich fundamental in ihrer zeitlichen und räumlichen Eigenschaften, den Signalen und Achtungssignalen und daher in den Selektionsdrücken auf die Männchen unterscheiden. Infolgedessen, zeigt die Sensorik der Männchen eine größere Vielfalt an morphologischen und funktionellen Anpassungen als die der Weibchen.

Im ersten Abschnitt dieser Arbeit habe ich mich mit Anpassungen des visuellen Systems von Honigbienen befasst. Als Ausgangspunkt habe ich die geschlechts- und kastenspezifische Augenmorphologie von 5 Honigbienenarten (*Apis andreniformis*, *A. cerana*, *A. dorsata*, *A. florea*, *A. mellifera*) untersucht. Während *A. mellifera* in vielerlei Hinsicht gut untersucht ist, erhielten die asiatischen Honigbienenarten erst in jüngerer Zeit erhöhte wissenschaftliche Aufmerksamkeit. In der Untersuchung habe ich eine deutliche Korrelation zwischen Körper- und Augengröße bei beiden weiblichen Kasten festgestellt. Königinnen haben relativ gesehen, kleinere Augen als Arbeiterinnen, was der verringerten Rolle visueller Wahrnehmung im Lebenszyklus dieser Kaste entspricht. Die Arbeiterinnen der 5 Arten unterschieden sich sowohl in ihrer Augengröße als auch in der funktionellen Morphologie. Die Unterschiede passen jeweils zu der artspezifischen Unterschieden im Foragierverhalten (z.B. zeitliche Einnischung). Drohnen aller Arten haben auffällig vergrößerte Augen, jedoch sind sie in zwei Arten überproportional vergrößert. Die vergrößerten Augen der Drohnen von *A. dorsata* können der Paarungszeit in der Dämmerung zugeschrieben werden. Die Rolle der vergrößerten Augen im Paarungsverhalten von *A. florea* ist hingegen unbekannt. Zusätzlich zeige ich, dass bestimmte Augenparameter (Facettengröße, Ocellengröße) mit dem artspezifischen Paarungszeitpunkt korrelieren, und



schlage vor, dass die absolute Lichtintensität eine wichtige Rolle bei der Feststellung des richtigen Paarungszeitpunktes spielen könnte.

In der zweiten Untersuchung habe ich die Augen von zwei Drohnenphänotypen der westlichen Honigbiene, *A. mellifera*, genauer untersucht. Neben normal großen Drohnen werden in der Kolonie auch kleinere Drohnen aufgezogen, die jedoch unter einem geringeren Fortpflanzungserfolg leiden. Meine Ergebnisse zeigen, dass sich die Phänotypen vermutlich nicht in der räumlichen Auflösungsfähigkeit, jedoch in der Lichtempfindlichkeit und Kontrastempfindlichkeit der Augen von den normalen Drohnen unterscheiden. Diese Unterschiede können, zusätzlich zu anderen Faktoren, zum geringeren Fortpflanzungserfolg beitragen.

In der dritten Untersuchung habe ich die geschlechts- und kastenspezifische Augenmorphologie bei Hummeln untersucht. Im Speziellen habe ich den Zusammenhang zwischen Augengröße der Männchen und der Paarungsstrategie, sowie artspezifische Unterschiede der Augenmorphologie innerhalb der Männchen und den beiden Weibchenkasten an 11 Arten aus 11 der 15 Hummel-Untergattungen untersucht. Ich beschreibe in dieser Studie Anpassungen der Arbeiterinnen, die vermutlich mit der Habitatwahl und den dort herrschenden Lichtverhältnissen im Zusammenhang stehen. Hummelköniginnen sind, im Gegensatz zu Königinnen der Honigbiene, in der ersten Zeit nach der Koloniegründung auf sich allein gestellt und müssen alle Aufgaben, die später von den Arbeiterinnen übernommen werden, selbst ausführen. Dementsprechend sind die Augen beider Weibchenkasten ähnlich in ihrer relativen Größe und funktionellen Morphologie. Vergrößerte Augen der Männchen können in Arten verschiedener Untergattungen gefunden werden und der Phänotyp ist im Laufe der Evolution mehrfach unabhängig entstanden. Männchen die solch vergrößerte Augen zeigen, warten normalerweise an bestimmten Orten und suchen die Umgebung visuell nach Jungköniginnen ab. Die Augenmorphologie der vier untersuchten großäugigen Arten ist sehr ähnlich, was auf konvergente Evolution aufgrund ähnlicher Signale und Achtungssignale und somit ähnliche Selektionsdrücke hinweist. Die Augenmorphologie der 7 restlichen Arten unterscheidet sich hingegen nicht deutlich von jener der Weibchen. Ich habe zusätzlich vergleichende Methoden unter Berücksichtigung der Phylogenie angewandt um die Evolution vergrößerter Männchenaugen zu verfolgen. Meine Ergebnisse zeigen, dass der gemeinsame Vorfahre aller existierenden Hummelarten vermutlich vergrößerte Männchenaugen hatte und dass die Augenvergrößerung mehrfach unabhängig innerhalb der Hummeln wieder entstanden ist.

In der vierten Untersuchung habe ich mich dem Sexualdimorphismus der Augen einer Solitärbienenart gewidmet. Die Langhornbiene *Eucera berlandi* verfolgt einen solitären Lebensstil, d.h. jedes Weibchen baut ihr eigenes Nest und ist für alle Aufgaben selbst verantwortlich. Männchen schlüpfen vor den Weibchen und suchen die Nestplätze nach paarungsbereiten Weibchen ab. Eine Vielzahl an Männchen konkurriert um den Zugang zu den Weibchen, und dieser Selektionsdruck fördert das Entstehen verbesserter Sinnesleistungen. Ich habe diese Bienenart ausgewählt, weil sie in einigen meiner früheren Untersuchungen zur visuellen Ökologie der Bestäubung einer Orchideenart als Untersuchungsobjekt fungiert hat. Die Sexualtäuschorchidee *Ophrys heldreichii* lockt mit einer perfekten Imitation der olfaktorischen, visuellen und taktilen Signale der Weibchen von *E. berlandi* die Männchen an, die bei ihren Paarungsversuchen für die Bestäubung sorgen. Diese Imitation der relevanten Signale und Achtungssignale erleichtert die Interpretation der morphologischen Unterschiede und männchenspezifischen Anpassungen von *E. berlandi*.

Männchen von *E. berlandi* haben größere Augen und sowohl größere Facetten als auch eine höhere räumliche Auflösung im frontalen Gesichtsfeld als Weibchen. In einem Verhaltensversuch habe ich die Auswirkungen der Größe von Weibchendummies auf die Detektion und Weibchenverfolgung getestet. In 3-D Rekonstruktionen der Weibchenverfolgung zeigte sich dass die Winkelgröße des Objektes, eine von der Distanz unabhängige Größe, eine wichtige Rolle spielt. Im Zusammenhang mit den gefundenen Daten diskutiere ich die Parallelen zu anderen Insektenarten, die mit ähnlichen Aufgaben während der Weibchen- bzw. Beuteerkennung und Verfolgung konfrontiert sind.

In der fünften Studie untersuche ich das olfaktorische System der Langhornbienen. *E. berlandi* Männchen haben extreme lange Antennen, die vermutlich die längsten innerhalb der Bienen darstellen. Der ausgeprägte Sexualdimorphismus der Antenne legt nahe, dass die langen Männchenantennen Anpassungen an die Partnerfindung darstellen. Um die anatomischen Grundlagen und funktionellen Konsequenzen der geschlechtsspezifischen Antennenmorphologie zu untersuchen habe ich die Antennen beider Geschlechter im Detail studiert. Zusätzlich bin ich dem Dimorphismus entlang der olfaktorischen Bahn bis ins Gehirn gefolgt, wo ich weitere funktionelle Anpassungen an die Partnerfindung beschreibe. Die gefundenen Anpassungen werden mit jenen der einzig ähnlich gut untersuchten Bienenart, der westlichen Honigbiene, verglichen. Bei Männchen beider Arten finden sich ähnliche funktionelle Anpassungen (z.B. längere Antennen, eine höhere Anzahl an olfaktorischen Sensillen und Rezeptorneuronen, stark vergrößerte Glomeruli im Antennallobus, eine zahlenmäßige Reduktion der Glomeruli und geschlechterspezifische Investition in höhere Integrationszentren im Gehirn), die sich jedoch in ihrer Ausprägung zwischen den Arten unterscheiden. Die Ähnlichkeiten und Unterschiede werden im Zusammenhang mit dem solitären bzw. sozialen Lebensstils und den daraus ableitbaren Konsequenzen für die Männchen diskutiert.

Zusammenfassend kann gesagt werden, dass meine Arbeit neuartige Ergebnisse zu den funktionellen Aspekten sexualdimorpher sensorischer Systeme in drei Bienengruppen liefert. Die Arbeit erweitert unseren Wissensstand sensorischer Anpassungen an geschlechts- und kastenspezifische Aufgaben und Verhaltensweisen. Meine Untersuchungen bieten eine Vorlagen für zukünftige ähnliche Untersuchungen in anderen Bienenarten. Ich schlage passende Arten und Artengruppen für zukünftige Untersuchungen vor, die neue, unerwartete und außergewöhnliche geschlechts- und kastenspezifische Anpassungen in dieser wichtigen, charismatischen und interessanten Insektengruppe zeigen können.

### 3. INTRODUCTION

Hymenoptera are one of the four speciose “super-orders” of insects (Coleoptera, Diptera, Hymenoptera and Lepidoptera) and approximately 125,000 species are described worldwide (Grimaldi and Engel, 2005). They inhabit all continents, except for Antarctica, and almost all terrestrial habitats, ranging from lowland tropical to high altitude and arctic environments, illustrating the enormous evolutionary success of this group. Hymenoptera have evolved an extremely diverse behavioral and ecological repertoire. Many species collect angiosperm products as larval and adult food, nesting material or even as basis for the cultivation of fungi while others are hunters, parasites and parasitoids (Grimaldi and Engel, 2005; Hölldobler and Wilson, 1990; Michener, 2007). Associated with this variety of behaviors, is a remarkable diversity in larval and adult morphologies (Chapman, 1998). Members of the section Aculeata (bees, wasps and ants) are very familiar to us, and are both loved as industrious sources of honey and wax, pollinators of flowers and crops, and scourges of pest insect populations, as well as hated as bringers of ecological and economic destruction, stinging nuisances, and perennial ruinners of picnics.

#### 3.1. Bees (Hymenoptera:Apoidea:Apiformes)

Bees are a subgroup of the Aculeata, which is easily recognized and highly important for their conspicuous flower visitation, and the concomitant pollination service. The monophyletic group comprises around 20,000 recognized extant species (Ascher and Pickering, 2013). Bees are, in contrast to their close relatives, the sphecoid wasps, vegetarian, i.e. they rely mainly on floral resources. Nectar, pollen and fatty oils are collected as a source of carbohydrates and protein for adult and larval nutrition. Resin and wax are collected as nest construction material and perfumes as pheromones (Eickwort and Ginsberg, 1980; Michener, 2007). The early diversification and fast radiation of the major bee families falls within the radiation of angiosperm plants in the Early Cretaceous (Cardinal and Danforth, 2013) and bees are generally considered the most important group of pollinating insects (Grimaldi and Engel, 2005). The phylogeny of the bees has been subject of many studies but there is still uncertainty about the relationship among certain groups (Cardinal et al., 2010; Plant, 2013), e.g. in the corbiculate Apinae (Euglossini, Apini, Bombini, Meliponini). In this group, molecular phylogenies on the one hand and morphology- and behavior based phylogenies on the other hand yield different results (Cameron and Mardulyn, 2001; Cardinal and Danforth, 2011; Noll, 2002; Serrão, 2001).

Another trait that makes bees highly fascinating is the remarkable social behavior of the usually better known species of honeybees, stingless bees and bumblebees (Michener, 1974). However, only a small percentage of all bee species is social or eusocial (Michener, 1974). The majority of species live a solitary life and females construct and provision their own nest. A considerable proportion of species, however, is parasitic and does not engage in construction and provisioning of their own nest (Cardinal et al., 2010; Michener, 2007). The activity of bees is mostly confined to sunny and warm days, although some species and entire bee groups are particularly adapted to temperate and arctic environments (e.g. *Bombus*, Heinrich, 1979) or show crepuscular or nocturnal foraging behavior (Wcislo and Tierney, 2009).

#### 3. 2. Sexual dimorphism in bees

In bees, both sexes usually differ in their lifestyle, behavior and morphology, sometimes to a degree that resulted in a classification of males and females in different genera (Eickwort and

Ginsberg, 1980; Stubblefield and Seger, 1994). Females usually perform a rich repertoire of behaviors, including nest construction, foraging, parental care, and in the case of social species, intra-specific communication (Eickwort and Ginsberg, 1980). Males are considered to have only a comparatively small behavioral repertoire and most of their lifetime is devoted to mate seeking and mating behavior (Alcock et al., 1978; Eickwort and Ginsberg, 1980).

The large difference in lifetime tasks is associated with often spectacular sexual dimorphism in Hymenoptera (Stubblefield and Seger, 1994). Sexual dimorphism, phenotypic differences among the sexes, evolves as a result of sex-specific selection pressures and can be found in a range of traits, e.g. body size, morphology, coloration, physiology, behavior and sensory systems. For instance, females of most bee species are adapted for foraging and brood rearing and have structures for pollen collection and transport, morphological adaptations for nest construction (Michener, 2007; Stephen et al., 1969). Their sensory system is generally tuned to the detection and discrimination of floral resources and nesting sites, spatial orientation and individual nest recognition (Dafni et al., 1997).

Males, in contrast, contribute little, if anything, to nest construction and parental care (Paxton, 2005). They lack the associated structures and their morphology and physiology is instead adapted to detect and securing females for mating (Stephen et al., 1969). Male-specific adaptations are usually found in the sensory and locomotive abilities for faster access to females, morphological traits that help to defend a territory, high quantity and quality of semen and morphological adaptations or behavioral strategies that ensure paternity (e.g. mating plugs and physical mate guarding; Boomsma et al., 2005; Michener, 2007).

The most commonly recognized selection pressure that leads to male specializations and thus also drives the development of sexual dimorphism is sexual selection (Andersson and Iwasa, 1996). Males compete for a limited number of (virgin) females. The adaptations of males are mostly determined by the mate choice system and the type of male-male interactions. Female choice, for instance, primes the evolution of (honest) signals such as conspicuous coloration, elaborate ornaments or conspicuous behavioral sequences. In contrast, male-male competition leads to the development of combat structures, and (often ritualized) fighting behavior. In the absence of female choice or male combat, males may gain access to females by arriving first and inseminating the female, before others can do (Thornhill and Alcock, 1983). Such scramble competition systems are often associated with adaptations of the male locomotion skills and improved sensory organs (Andersson and Iwasa, 1996). The two most important sensory systems in the context of mating are vision and olfaction, which are discussed in more detail below.

### 3.3. Bee mating

#### 3.3.1 Mating systems

Mating behavior can be quite conspicuous, and there is a large number of observations and phenomenological descriptions of mate seeking behaviors in bees (reviewed in Alcock et al., 1978; Ayasse et al., 2001; Eickwort and Ginsberg, 1980; Paxton, 2005).

Alcock *et al.* (1978) and Paxton (2005) reviewed the existing data and provided an evolutionary framework to interpret the different mating strategies. The most important factor that predicts male mate-searching behavior is the spatiotemporal distribution of females, followed by resource density and male density (Alcock et al., 1978; Paxton, 2005). When in search of females, males may either non-aggressively patrol nesting or foraging sites of the females, wait at resources or nest sites, or else both sexes meet in non-resource based rendezvous sites. Male density

influences whether it pays for a male to be territorial or not (Alcock et al., 1978; Paxton, 2005; Williams, 1991). Male mating strategies can be either species-specific with little variation, or show some degree of plasticity (Alcock et al., 1978). Alternate male strategies within species are often correlated with the presence of distinct morphological or body size differences (Paxton, 2005). Furthermore, the variation of female density and distribution over the mating season may result in a change of the location at which males seek for females or the used mating strategy (Leys, 2000).

Depending on the male mating strategy, various traits may be under selection. Body size is one trait that is sexually dimorphic in many animal species (Stillwell et al., 2010). In females, body size correlates with fecundity which favors larger individuals. In males, mating strategy determines whether large males have a reproductive advantage over smaller (cheaper) males. In territorial species, male body size is an important factor and males that are larger are more likely to hold a territory (Alcock, 1997; Alcock et al., 1978). In such a system males are usually similar or even larger in body size to females. However, territorial strategies are considered rare in bees and the majority of bee mating systems can be classified as non-territorial scramble competition systems (Paxton, 2005). Males patrol or perch in nesting and foraging sites or in non-resource based locations and body size is not necessarily a strong predictor of mating success. Accordingly, males are often smaller than females (Stillwell et al., 2010).

In scramble competition systems, males seek for females and the first male that detects and arrives at a female usually sires all of her offspring. This mating system thus promotes the evolution of improved sensory and locomotive abilities that help males to outrun competitors (Andersson and Iwasa, 1996; Thornhill and Alcock, 1983). Sensory adaptations should be widespread, judging by the often conspicuous size differences of antennae and eyes (see below).

### 3.3.2. Signals and cues

Several signals and cues are employed in bee mating behavior. Females of most bee species signal their location and mating status via sex pheromones that are produced in exocrine glands and usually secreted onto the body surface (Ayasse et al., 2001). Male pheromones are also described and used to scent mark territories, serve communication among males or attract virgin females. In many cases, the exact function of male produced pheromones is enigmatic (e.g. in *Bombus* Ayasse et al., 2001). In honeybees, the sex pheromone is also used in an additional behavioral context, e.g. as pheromone in the worker retinue behavior (Free, 1987; Jarriault and Mercer, 2012).

Females often alter the pheromone blend after mating to signal their mating status and avoid subsequent disturbance by males looking for mates (Schiestl and Ayasse, 2000). Collecting bee pheromones for chemical analyses is often difficult due to the spatiotemporal unpredictability of female emergence. Furthermore, females mate during or immediately after eclosion, and in some cases already are mated on emergence (Paxton, 2005). The study of bee sex pheromones has been much advanced by the discovery of sex pheromone mimicry by sexually deceptive orchids (Ayasse et al., 2001; Kullenberg and Bergström, 1976; Schiestl et al., 1999). The current knowledge on chemical communication in bee mating behavior has been reviewed by Ayasse *et al.* (2001).

Visual signals and cues are generally assumed to play a subordinate role in mating behavior, compared with olfactory signals (Eickwort and Ginsberg, 1980). Visual cues are used to determine location during the landing or grabbing response (Krieger et al., 2006) and may

further help to discriminate between conspecific males and the females, especially when male density around the female is very high and chemical discrimination is less reliable (Free, 1987). Visual cues may be important in the detection and discrimination of promising mating sites. Males of some oligolectic species, for instance, seek females at the food plants and color plays an important role in detection of these plants and forms part of a specific search image (Tengö et al., 1988, and own observations). Aerial flyways are used by some species, e.g. honeybees, and it has been suggested that the location is chosen by conspicuous visual properties (Pechhacker, 1994). However, the mechanism for the often remarkably stable mate congregation locations over many years is still a mystery. Many bees are sexually dichromatic, but the reasons for this color difference and whether it constitutes a signal used in mating behavior is currently unknown (Eickwort and Ginsberg, 1980; Leys and Hogendoorn, 2008; Michener, 2007). Additional signals and cues that are employed in the mating behavior may be tactile (Kullenberg and Bergström, 1976) and vibratory (Conrad et al., 2010). These signals and cues act only in the very short range, usually after contact between the mating partners. Moreover, they are little understood and little investigated, compared with chemical and visual signals and cues.

### 3.4. Sensory systems investigated

#### 3.4.1. Visual sense

##### 3.4.1.1. Visual system

Vision in bees is mediated by two systems. Three simple lens eyes on the vertex, the ocelli, produce a defocused image on the retina and their role is considered to be mainly in general light metering and in maintaining flight stability (Land and Nilsson, 2002). Moreover they may be important in the context of orientation and reception of celestial cues (Schwarz et al., 2011; Wellington, 1974). The more sophisticated visual tasks, e.g. form vision, motion vision and color vision are mediated by the two compound eyes (Land and Nilsson, 2002). Three major types of compound eyes can be distinguished; apposition eyes, neural superposition eyes and superposition eyes (Land and Nilsson, 2002). Since all bees possess apposition eyes I will focus on this eye type in the following. Bee compound eyes consist of several thousand ommatidia (Jander and Jander, 2002). Each ommatidium is equipped with a distal light focusing (dioptric) apparatus, formed by a corneal facet lens and a crystalline cone, and a proximal light receptive apparatus that is formed by 8-9 circularly arranged retinula cells (Land, 1989; Land and Nilsson, 2002). The fused rhabdomeres, the highly folded finger-shaped central membrane of the retinula cells, form a central light guiding structure, the rhabdom. The membrane holds the photoreceptive pigments, that are responsible for absorption of incoming photons and the starting point of the phototransduction cascade (Land and Nilsson, 2002). Each pigment consists of a chromophore that interacts with the photon and an enclosing protein, the opsin, that holds the chromophore in place and determines the spectral sensitivity of the pigment. The visual pigments belong to the large family of G-protein coupled receptors (Fain et al., 2010). Photoreceptor cells usually express only a single opsin protein (Pichaud et al., 1999, but see Kitamoto et al., 1998). Most bees express three different types of opsins in the compound eyes, which results in three spectral classes of photoreceptors, maximally sensitive in the UV (peak wavelength  $\lambda_{\max} \sim 350\text{nm}$ ), blue ( $\lambda_{\max} \sim 450\text{nm}$ ) and green ( $\lambda_{\max} \sim 550\text{nm}$ ) part of the light spectrum, which further enables them to perceive and discriminate chromatic information (Briscoe and Chittka, 2001; Peitsch et al., 1992).

Each ommatidium receives light from a small angular region of the surrounding space, which is determined by the angular acceptance function ( $\Delta\rho$ ) of the rhabdom (Land and Nilsson, 2002). Neighboring ommatidia point in slightly different directions. The angular separation between ommatidia, the interommatidial angle ( $\Delta\phi$ ), defines the sampling frequency of the ommatidial array (Land, 1997; Snyder et al., 1977). Optical isolation between ommatidia is achieved by pigment located in the retinula cells and in primary and secondary pigment cells which absorbs off-axis light (Land, 1989). There is generally only a little overlap between the sample region of neighboring ommatidia, since a larger overlap would compromise spatial resolution (Snyder et al., 1977).

Ommatidia in honeybees and bumblebee workers are equipped with different photoreceptor types and of their 8 long retinula cells, 6 are maximally sensitive in the green, 0-2 in the blue and the remaining 0-2 in the UV part of the spectrum (Spaethe and Briscoe, 2005; Wakakuwa et al., 2005). The spectral sensitivity of the short proximal 9<sup>th</sup> cell is unclear (Wakakuwa et al., 2005). This arrangement with different spectral classes of receptors allows the bee to perceive chromatic information within the sampled region. Upon its travel through the rhabdom any spatial detail is lost and one ommatidium thus samples only one 'pixel', with chromatic information but not spatial detail.

The two major features of the compound eye which are determined by the arrangement and dimensions of the ommatidia are spatial resolution and sensitivity. Spatial resolution depends on the angular separation of visual axes. Smaller interommatidial angles allow for resolution of finer detail (Snyder et al., 1977). The tiny facet lens apertures produce diffraction, which limits the minimum spatial resolution that can be achieved with an apposition eye (Land, 1989). Diffraction effects can be reduced by increasing the aperture, and therefore a decrease in interommatidial angles is in most cases accompanied by an increase in facet diameters. Based on this premise, a more acute eye needs a larger eye radius (Land, 1989). To achieve this enlarged radius, total eye size can be increased or spatial resolution can be improved only for a small region of the visual field while sacrificing resolution in the rest of the eye. Such 'acute zones' are indeed found in many insects and are often associated with regions that are used to detect flowers, prey or mating partners (Horridge, 1978; Land and Nilsson, 2002; Somanathan et al., 2009a). The sensitivity of the eye depends on the light collecting abilities of the photoreceptors. The sensitivity of an ommatidium is a function of the optic apparatus (facet diameter) and sensory apparatus (acceptance angle, rhabdom width and length; Land, 1989). Enlarged facets and acceptance angles are usual features found in crepuscular and nocturnal insects that have apposition eyes (see below).

In the apposition eye, resolution and sensitivity are not independent of each other. Since an increase in resolution demands larger facets, zones of small interommatidial angles are also more sensitive to light. Improved sensitivity, however, may exist without improved resolution. Such 'bright zones' have so far been described exclusively in flies (Straw et al., 2006; van Hateren et al., 1989). Sensitivity and resolution cannot be maximized at the same time and thus both parameters are traded-off against each other according to the ecological requirements of an animal (Greiner et al., 2007; Horridge, 1978; Snyder et al., 1977).

#### 3.4.1.2. Adaptations of the compound eyes in bees

Most investigations on the functional morphology and specific adaptations of the visual system in bees were performed in workers of eusocial species mainly in the context of foraging, learning

and memory (Dafni et al., 1997; Galizia et al., 2011). Honeybees and bumblebees are important model systems for vision and their eyes are relatively well investigated (Meyer-Rochow, 1981; Seidl, 1982). The interommatidial angles in the frontal eye region of bees are in the range of around 1-3° and were determined with various histological, physiological and behavioral methods (Macuda et al., 2001; Seidl, 1982; Somanathan et al., 2009a; Spaethe and Chittka, 2003; Theobald et al., 2006). Larger bees have been found to possess higher spatial resolution as a result of larger eyes, larger facets and a denser packing of ommatidial axes (Jander and Jander, 2002; Spaethe and Chittka, 2003). A few bee species (*Megalopta genalis*, *Xylocopa tranquebarica*, *X. leucothorax*, *X. tenuiscapa*, *Apis dorsata*) have been investigated in the context of dim-light foraging (Greiner et al., 2004; Somanathan et al., 2009a; Somanathan et al., 2009b). Apposition eyes are not well suited for dim-light foraging, but larger facet lenses and acceptance angles in addition to secondary neural summation strategies allow reasonable orientation and foraging in the dark (Greiner et al., 2004; 2005; Somanathan et al., 2009a; Theobald et al., 2006; Warrant, 2008).

In contrast to females, relatively little is known about the eye design and sex-specific adaptations of males. The only comprehensive data derive from the Western honeybee, *Apis mellifera*. Honeybee drones have remarkable visual organs. Their eyes are strongly enlarged and show a distinct dorso-ventral regionalization. The dorsal and ventral eye regions differ in facet diameters and interommatidial angles (Ribi et al., 1989; Seidl, 1982), spectral receptor composition (Autrum and von Zwehl, 1962; 1963; Peitsch et al., 1992), screening pigment (Menzel et al., 1991) and likely also in receptor cell physiology (Vallet and Coles, 1993a). Behavioral studies revealed a very high sensitivity of the upward-facing eye region for small objects viewed against the bright sky and confirmed the important function of this eye region in mate detection (Gries and Koeniger, 1996; Vallet and Coles, 1993b; van Praagh et al., 1980). Similar adaptations were described in eyes of Odonata, which face similar challenges in prey detection and capture (e.g. Labhart and Nilsson, 1995; Land, 1989).

Sex-specific investigations of the visual system are further available for one stingless bee (*Scaptotrigona postica debilis*; Ribi et al., 1989). Males of this species have slightly larger eyes, equipped with more and larger facets, and enlarged ocelli, but the functional and behavioral relevance has not been studied yet.

Besides these quantitative studies, male eyes of many species are described only qualitatively. Males of some taxa have strongly enlarged eyes, relative to females, which usually correlates with a visually guided mating strategy, e.g. in *Bombus* (Williams, 1991), *Xylocopa* (Hurd and Moure, 1962), *Exoneura* (Apinae: Allodapini; Michener, 2007), Andrenidae (*Melitturga* and *Oxaeinae*; Hurd and Linsley, 1976; Michener, 2007; Stephen et al., 1969), *Caupolicana* (Colletidae; Stephen et al., 1969) and *Xanthesma* (Colletidae: Euryglossinae; Michener, 2007). At least moderately enlarged eyes are found in many more taxa (e.g. Apinae: Eucerini; Michener, 2007, *Manuscript IV*).

### 3.4.2. Olfactory sense

#### 3.4.2.1. Olfactory system

The olfactory system evolved to detect volatile molecules and to convey chemosensory information from the environment to the brain, where it is integrated and used to elicit behavioral responses (Hansson and Stensmyr, 2011). In insects, odorants are detected by olfactory sensory neurons (OSNs). These are located in olfactory sensilla, cuticular structures of a variety of



shapes and sizes, that are usually found on the antennae and labial and maxillary palps (de Bruyne and Baker, 2008). The OSN dendrites are embedded in the sensillum lymph and odorants can enter the lymph through small pores in the sensillum wall (Steinbrecht, 1999). The biochemical pathway that leads to an excitation of the sensory neuron is still controversial (Pellegrino and Nakagawa, 2009; Wicher et al., 2008). Each OSN usually expresses only one olfactory receptor (OR) type which is coded by an olfactory receptor gene. ORs are a large and diverse family of receptor proteins (Robertson and Wanner). In insects they form a heteromeric complex with a co-receptor *Orco* (Galizia and Sachse, 2010; Vosshall and Hansson, 2011). Each OR responds to a limited range of molecules. The most specific ORs are usually pheromone receptors, while other receptor types are more generally tuned. Depending on the animal, tens or hundreds of different receptor types may be present (Hansson 2011, Robertson and Wanner, 2006). The axons of the OSNs project to the antennal lobe, where they make the first synaptic connections in spherical structures, called glomeruli (Hansson and Anton, 2000). Each individual receptor type targets only one glomerulus and all axons of the same receptor types terminate in the same glomerulus (Gao et al., 2000). By inference, the number of glomeruli serves as rough estimate about the number of receptor types expressed in the antennae, mouthparts and legs. Recent genome and transcriptome sequencing projects support this assumption and found a close correlation between the number of functional OR genes and the number of glomeruli (Grosse-Wilde et al., 2011; Robertson and Wanner, 2006; Vosshall, 2000).

In workers of eusocial hymenoptera, high numbers of glomeruli are found (~160 in the honeybee and up to 630 in ants; Arnold et al., 1985; Kelber et al., 2009; Kleineidam and Rössler, 2009), which is in contrast to the generally lower numbers of less than 100 in most other insects (Schachtner et al., 2005). The locust departs from the general insect olfactory system *bauplan* and is not discussed here (reviewed in Hansson and Stensmyr, 2011).

The high glomeruli numbers and large OR repertoire in Hymenoptera are often attributed to the demands for olfactory processing in eusocial communities (Robertson and Wanner, 2006). However, comparative data from solitary hymenoptera are only available for two parasitic wasps (Smid et al., 2003) and are entirely lacking for solitary bees (Kleineidam and Rössler, 2009).

Sex-specific organization of the antennal lobe is found in many insects. Sexual dimorphism in Hymenoptera are observed both in glomeruli number and size (Schachtner et al., 2005). Males have up to 60% fewer glomeruli than workers, which potentially limits the dimensionality of their odor coding (Arnold et al., 1985; Hoyer et al., 2005; Kuebler et al., 2010; Nishikawa et al., 2008; Stieb et al., 2011; Zube and Rössler, 2008). This strong sexual dimorphism in glomeruli number seems to be confined to Hymenoptera (Schachtner et al., 2005). A dimorphism in glomeruli size is much more widespread in insects. Extremely enlarged macroglomeruli or macroglomerular complexes are found in males of Hymenoptera (Arnold et al., 1988; Kuebler et al., 2010; Stieb et al., 2011), Lepidoptera (Rospars and Hildebrand, 2000) and Blattodea (Boeckh and Tolbert, 1993) and are usually considered adaptations for sex pheromone detection. The enlarged glomeruli usually illustrate an increased number of receptors for a particular substance (Lacher, 1964; Wanner et al., 2007). Such a multiplication seems to be important to increase the sensitivity of the antenna for a certain chemical by increasing the chance of a molecule making contact with a receptor. Increased sensitivity is highly important when the odors to be detected are present only in small amounts, or are sensed from a large distance. Males of many insects have enlarged antennae (Hansson and Stensmyr, 2011; Steinbrecht, 1999; Thornhill and Alcock, 1983) and a high number of receptor neurons (Esslen and Kaissling, 1976; Schachtner et al., 2005), which may increase the general sensitivity and improve the signal-to-noise ratio of the

antenna, and thus account for the high sensitivity for sex pheromones (Angioy et al., 2003; Brockmann et al., 1998; Verdugo-Dardon et al., 2011). In the female caste, enlarged glomeruli have so far only be found in the leaf-cutting ant *A. vollenweideri* where they process trail pheromones (Kleineidam et al., 2005; Kuebler et al., 2010). Enlarged female glomeruli were further found in *Cataglyphis*, but their function remains elusive (Stieb et al., 2011).

Odors elicit a response in a population of glomeruli and the odor is coded as a distinct spatiotemporal activation pattern of these glomeruli (Galizia and Menzel, 2000). Local interneurons enable communication between glomeruli and projection neurons relay information to higher brain centers, like the lateral horn and the mushroom bodies (Galizia and Sachse, 2010). In Hymenoptera, information is relayed to the higher centers via a dual pathway, which is assumed to improve olfactory perception via parallel processing of odor information (Rössler and Brill, in press).

The chemosensory and visual information from the first order neuropils is conveyed to the higher integration centers. The mushroom bodies (MB) in particular, have been shown to have an important function in multimodal integration (Strausfeld et al., 1998) and in learning and memory formation (Fahrbach and Robinson, 1995; Strausfeld et al., 1998). A high structural and neuronal plasticity of the MBs has been observed in many insect species and may have an important function in behavioral plasticity of the individual, e.g. associated with alloethism in honeybees (Fahrbach et al., 1997; Groh et al., 2012).

#### 3.4.2.2. Adaptations of the olfactory system in bees

The mechanisms underlying olfactory perception and coding are well investigated in the Western honeybee, which has become one of the most important model insects for the study of olfaction (Galizia et al., 2011; Galizia and Rössler, 2010; Rössler and Brill, in press). Our knowledge ranges from morphology of the antennae, sensilla and receptor cells, cell physiology, antennal lobe morphology to olfactory coding and processing in the central brain. *Apis* has a well developed olfactory system. The antennal sensilla and their innervations have been described in detail (Esslen and Kaissling, 1976). Olfactory sensilla (poreplates) are numerous and innervated by multiple OSNs (Kelber et al., 2006), which is a derived trait only found in Hymenoptera (Kleineidam and Rössler, 2009). *Apis* workers have approximately 160 glomeruli (Arnold et al., 1985) and queens about 150 (Arnold et al., 1988; Groh and Rössler, 2008). These relatively high numbers are usually attributed to their social lifestyle.

Adaptations of the olfactory system have been further described in workers and females of some bee species at various levels of the olfactory path. The majority of descriptions pertain to the periphery and the antennal surface; furthermore, sensilla types have been investigated in species of *Bombus* (Ågren and Hallberg, 1996; Fonta and Masson, 1982), Colletidae (Ågren, 1977), *Andrena* (Ågren, 1978), *Sphecodes* (Ågren and Svensson, 1982), Emphorini (Galvani et al., 2012), Epeolini (Galvani et al., 2008) and Meliponini (Johnson and Howard, 1987). The basic antennal organization and the observed sensilla types are similar across all species, and functional adaptations to a particular lifestyle are not obvious in the periphery of the olfactory system (e.g. Ågren and Hallberg, 1996; Johnson and Howard, 1987).

Sex-specific adaptations have been investigated in many of the above cited studies. Generally, the males possess an additional flagellum segment (Michener, 2007). Further, the sexes differ in the absolute and relative number of various sensilla types (Galvani et al., 2012; Johnson and Howard, 1987). In honeybees, drones have enlarged antennae and c. 7 times more poreplates than workers (Esslen and Kaissling, 1976). In other bee species, sex-differences are less

pronounced (Johnson and Howard, 1987). A comparison of six honeybee species suggests that *Apis mellifera* is an exception, even within the honeybees (Brockmann and Brückner, 2003). One common trait in male bees and most other Hymenoptera seems to be the complete absence of Sensilla basiconica (Ågren, 1977; Galvani et al., 2012), a sensillum type which has been suggested to process nestmate odors in a social insect (Ozaki et al., 2005, but see Brandstaetter and Kleineidam, 2011). However, this sensillum is not confined to females in social Apidae and other social Hymenoptera (Galvani et al., 2012).

It is more promising to detect functional differences between the sexes in the anatomy of the antennal lobes (AL). The number of glomeruli serves as an approximation for the number of olfactory receptors (OR) repertoire, while the presence/absence of macroglomeruli informs us whether certain substances are of particular importance. *A. mellifera* drones have fewer glomeruli than workers (Arnold et al., 1985), but four of the glomeruli are enlarged macroglomeruli that together account for c. 40% of the entire AL volume (Arnold et al., 1985). One macroglomerulus was shown to process 9-ODA (Sandoz, 2006) the major long-range attractant, for which the antenna is highly sensitive (Brockmann et al., 1998). Additionally, the antennal lobe morphology has been investigated in males of a second honeybee species, *A. florea*, the dwarf honeybee. It has only c. 90 glomeruli, and only two macroglomeruli that are smaller than those in *A. mellifera* (Brockmann and Brückner, 2001). The AL of the worker has not been studied, and thus conclusions about sexual dimorphism cannot be drawn. Bumblebee antennal lobe morphology resembles that of the honeybee (Fonta and Masson, 1985; Spaethe, 2001). Sex-specific macroglomeruli are present in *Bombus*, although the size differences are less pronounced (Fonta and Masson, 1985). However, detailed studies about the sex-specific differences in glomeruli number and size are lacking. Additional studies in other bee species have not been conducted.

Aside from these quantitative studies, qualitative differences in antennal morphology between the sexes and among bee species can be observed. Males of some bees have conspicuously enlarged antennae, e.g. most members of the tribe Eucerini (Michener, 2007) and males of *Ctenioschelus* (Hymenoptera: Apidae: Ericrocidini; Michener, 2007; Thiele, 2008), but the functional implications of these sexual dimorphisms are largely unknown. Apart from these extreme differences, males of many bee species have at least moderately enlarged antennae compared to females (e.g. some *Bombus* species, Williams, 1991).

### 3.5. Choice of study species

In this thesis, I chose three model taxa among the approximately 20,000 extant species of bees (Ascher and Pickering, 2013). The investigated taxa are described in the following.

#### 3.5.1. Honeybees (Hymenoptera: Apidae: Apinae: Apini: *Apis* LINNÉ)

Honeybees share the longest and most intimate relationship with humans of all species of bees. Their long-standing use as suppliers of honey, wax and propolis, as well as their pollination service, has led to the domestication of at least one species, *Apis mellifera* L. 1758. Honeybees are abundant, active throughout the warm season and can be easily trained (Frisch, 1914); these traits have made *A. mellifera* interesting and suitable for scientific investigations. Today, *A. mellifera* is one of the most comprehensively understood species in the animal kingdom. Our knowledge of the honeybee spans a wide range of disciplines from behavior and ecology,

molecular biology, genetics, neurobiology, developmental biology, sociobiology, sensory ecology to cognition (Galizia et al., 2011; Menzel, 2012; Srinivasan, 2010; Winston, 1991).

The mating biology of *A. mellifera* is also well investigated, including theoretical evolutionary backgrounds (Baer, 2005; Palmer and Oldroyd, 2000), behavioral and sensory adaptations for mating (Arnold et al., 1985; Esslen and Kaissling, 1976; Koeniger et al., 2011; Menzel et al., 1991; Vallet and Coles, 1993a; b; Wanner et al., 2007) and signals employed during the mating flights (Brockmann et al., 2006; Gary, 1962; Vallet and Coles, 1993b).

More recently, scientific interest has spread to other species of the genus. Michener (2007) lists 11 species of *Apis*, all of which except for *A. mellifera*, occur only in Asia. They differ largely in body size, ranging from small to very large. Although their behavior seems to be uniform, some differences exist in foraging preference, time of foraging activity, defense behavior, communication and colony construction (Hepburn and Radloff, 2011).

Males (drones) of all honeybee species have been described as “mating machines”, since they appear to consist only of eyes and antennae, flight muscles and an abdomen filled with enormous genitalia (Michener, 1944; Winston, 1991). They are incapable of surviving without the colony support and die after their first (and only) successful copulation. Sexual selection is remarkably strong in *Apis* males, mainly due to highly male biased operational sex ratios (Baer, 2005). *Apis* drones congregate in aerial spots and wait for the queens to visit these areas for mating. Fortunately, the mating behavior is comparatively well investigated. It is remarkably similar among *Apis* species, and even the main sex pheromone compound 9-ODA is used by most species (Free, 1987; Nagaraja and Brockmann, 2009). The major difference among species is the timing and location of the mating flights. Descriptions of the mating flight times of males are available for almost all species (Koeniger et al., 2011; Koeniger and Koeniger, 2000) and have even been used in taxonomy to separate species (Hadisoesoilo and Otis, 1996).

The sensory systems, as well as the signals and cues employed in mate detection are relatively well investigated for *A. mellifera*, but detailed and comparative studies are scarce for the Asian species (Brockmann and Brückner, 2001; 2003; Nagaraja and Brockmann, 2009). Conspicuously enlarged male eyes are found in all species, but the functional morphology of the eyes and its implications have yet to be evaluated. So far, comparative investigations have only been made for the worker caste (Somanathan et al., 2009b).

### 3.5.2. Bumblebees (Hymenoptera: Apidae: Apinae: Bombini: *Bombus* LATREILLE)

Bumblebees are conspicuous and ubiquitous insects that inhabit mainly the Northern Hemisphere. The genus *Bombus* is relatively large (~250 species; Williams, 1998) and their morphology is considered rather uniform (Michener, 2007). However, distinct morphological and behavioral traits are found among subgenera of *Bombus* (Williams et al., 2008). Several species are facultative or obligatory nest parasites and deviate slightly from the general *Bombus* morphology, e.g. reduction of pollen collection structures and more armor-like cuticle (Michener, 2007). Bumblebees are primitively eusocial, which means that the queen founds a new colony alone and, until the first workers emerge, is responsible for nest duties, foraging and parental care (Plowright and Laverty, 1984).

Bumblebee males are, in contrast to honeybee drones, mostly independent of the colony. They remain in the colony for a few days after eclosion but then usually leave without returning (except for species of the earliest diverging taxa; Haas, 1976). Natural selection pressures

associated with their own survival are thus likely to be higher than in *Apis* drones (for a review of sexual selection pressures in *Bombus* see Baer, 2003).

Male mating behavior is more diverse than in *Apis*. Several, well defined categories of mating strategies exist, which differ in the location of mating place, male behavior and the use of visual vs. olfactory information. Various studies have established that males that employ a visual mating strategy have enlarged eyes, while males that use a more olfactory guided strategy have smaller eyes (e.g. Franklin, 1954; Frison, 1927; Williams, 1991). Elaborate male visual systems have apparently evolved more than once within the genus (O'Neill et al., 1991), a condition similar to that in large carpenter bees (Apidae: Xylocopini; Hurd and Moure, 1962; Leys and Hogendoorn, 2008). Functional studies on the morphology and evolution of the male visual system have not been conducted so far. Recently published molecular phylogenies of the genus further facilitate phylogenetically controlled investigations, such as tracing character evolution of certain traits (Cameron et al., 2007; Hines, 2008).

### 3.5.3. The long-horned bee *Eucera berlandi* (DUSMET) 1926 (Hymenoptera: Apidae: Apinae: Eucerini)

Members of the large bee tribe Eucerini (~ 740 species; Michener, 2007) are commonly called long-horned bees. They are named after the exceptionally long male antennae, which is found in most species and makes them readily recognizable. The long male antennae are considered to be adaptations of the sensory system for mating behavior. Detailed investigations on the functional morphology and role of the long antennae in mate detection are lacking. Since males of *E. berlandi* and many other species of Eucerini also have enlarged eyes, it is highly likely that visual signals and cues are also important in their mating systems.

I chose *Eucera berlandi* to study the sexual dimorphism of the visual (*Manuscript IV*) and olfactory (*Manuscript V*) systems. My choice was influenced particularly by the fact that the males of the species acts as the pollinator of a sexually deceptive orchid (Paulus and Gack, 1990). Plants of *Ophrys heldreichii* (Orchidaceae) mimic the behaviorally active sex pheromone compounds of *E. berlandi* females to attract the males for pollination (Schaller, 2009). Males are lured to the flower through the scent and additionally rely on visual signals or cues in the close vicinity (Streinzer et al., 2009). To function as a mimic, the orchid must provide signals and cues that are relevant for the males in the context of mating. Studying orchid signals thus may permit easier identification of the signals and cues that are important for mate detection. *O. heldreichii* and *E. berlandi* have been used as model system to study visual and olfactory signals, and I was involved in several of these studies (Paulus, 2007; Rakosy et al., 2012; Spaethe et al., 2007; Spaethe et al., 2010; Streinzer et al., 2010; Streinzer et al., 2009).

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## 4. OUTLINE OF THE THESIS

### 4.1. Aims and questions

Bees (Hymenoptera: Apoidea: Apiformes) form a large monophylum of approximately 20,000 extant species. Bees have evolved a rich repertoire of morphological, neuronal and behavioral adaptations and traits. Over the last decades a large body of knowledge about their life histories, foraging preferences and mating biology have accumulated, while the sensory ecology has been investigated only in a handful of species. Peculiar morphological traits of the sensory apparatus are described in some species, and are mostly interpreted as adaptations to foraging, nocturnal lifestyle or mating behavior.

This thesis aims to explore the variety of sensory equipment in several bee species using a comparative and evolutionary approach, considering both sexes and, in the case of social species, both female castes.

The basic underlying questions addressed in this thesis are:

- Are there particular and pronounced sex-specific, or in the case of eusocial species, sex- and caste-specific, differences in the sensory equipment (*Manuscripts I-V*)?
- Can sex, caste and species specific morphological adaptations be correlated with distinct differences in ecology and mating biology (*Manuscripts I-V*)?

In this thesis, I focus mainly on the visual system (*Manuscripts I-IV*). In an additional study, I investigated the olfactory system in a solitary bee species (*Manuscript V*).

## 4.2. Overview of manuscripts

### Manuscript I

#### **Sex and caste-specific variation in compound eye morphology of five honeybee species**

Martin Streinzer, Axel Brockmann, Narayanappa Nagaraja, Johannes Spaethe  
*PLoS One* (2013) 8:2:e57702

#### ***Abstract***

Ranging from dwarfs to giants, the species of honeybees show remarkable differences in body size that have placed evolutionary constraints on the size of sensory organs and the brain. Colonies comprise three adult phenotypes, drones and two female castes, the reproductive queen and sterile workers. The phenotypes differ with respect to tasks and thus selection pressures which additionally constrain the shape of sensory systems. In a first step to explore the variability and interaction between species size-limitations and sex and caste-specific selection pressures in sensory and neural structures in honeybees, we compared eye size, ommatidia number and distribution of facet lens diameters in drones, queens and workers of five species (*Apis andreniformis*, *A. florea*, *A. dorsata*, *A. mellifera*, *A. cerana*). In these species, male and female eyes show a consistent sex-specific organization with respect to eye size and regional specialization of facet diameters. Drones possess distinctly enlarged eyes with large dorsal facets. Aside from these general patterns, we found signs of unique adaptations in eyes of *A. florea* and *A. dorsata* drones. In both species, drone eyes are disproportionately enlarged. In *A. dorsata* the increased eye size results from enlarged facets, a likely adaptation to crepuscular mating flights. In contrast, the relative enlargement of *A. florea* drone eyes results from an increase in ommatidia number, suggesting strong selection for high spatial resolution. Comparison of eye morphology and published mating flight times indicates a correlation between overall light sensitivity and species-specific mating flight times. The correlation suggests an important role of ambient light intensities in the regulation of species-specific mating flight times and the evolution of the visual system. Our study further deepens insights into visual adaptations within the genus *Apis* and opens up future perspectives for research to better understand the timing mechanisms and sensory physiology of mating related signals.

**Manuscript II****A scientific note on peripheral compound eye morphology of small and normal-sized honeybee drones**

Martin Streinzer, Johannes Spaethe  
*Journal of Apicultural Research* (under review)

***Abstract***

Honeybee drones face strong intra-sexual competition while searching for mates. Drones congregate in open areas, where they await a queen. Queens enter these areas and signal their presence and mating status using sex pheromones. The operational sex ratio in these congregation areas is extremely male-biased and only a handful of drones are successful. Males are usually reared within the colony in specialized drone cells. In some instances, drones develop in the smaller worker cells, which results in a smaller adult body size. Previous studies indicated a reduced mating success of small drones in terms of paternity share and access to queens. The latter may be due to differences in flight power and the potential to maneuver or a less sensitive olfactory system, which impair olfactory detection of the queen. In this study we investigated the peripheral eye morphology of small drones and compare it with normal sized drones. Smaller drones have smaller eyes equipped with smaller facet lenses, but not fewer ommatidia. Our results suggest that small males likely possess a similar spatial resolution as large males, but suffer from a c 13% reduced photon catch and thus light sensitivity. Drones are adapted to detect very small contrast differences while searching for the queen and this reduction is likely a serious disadvantage. We hypothesize that, among other factors, the reduced power of the visual system may account for the lower success rate of small honeybee drones, compared with normal sized drones.

**Manuscript III****The evolution of elaborate male visual systems in bumblebees (Hymenoptera, Apidae, *Bombus*)**

Martin Streinzer, Johannes Spaethe  
*Zoological Journal of the Linnean Society* (under review)

***Abstract***

Bumblebee mating behavior intrigued Darwin over 150 years ago and has elicited much interest since. Despite our increasing knowledge about male mating strategies, there is a general lack of knowledge about the signals and cues involved, the signal sender-receiver relationship and the functional adaptations of the involved sensory systems. While many authors have noticed a close relationship between the mating system and male-specific elaboration of the visual system, studies on the functional adaptations of the visual system and its evolutionary pattern have yet to be conducted. In this study we investigated the functional morphology of the compound eyes in 11 species of *Bombus* from various subgenera in detail. Of these, four species (*B. confusus*, *melaleucus*, *mendax*, *niveatus*) have enlarged male eyes, characterized by a higher number of ommatidia compared with worker eyes and a frontal zone with enlarged facets, which is likely associated with improved spatial resolution and contrast sensitivity. In these species, perching mating strategies are found. In contrast, males of species that patrol scent routes (*B. hortorum*, *lapidarius*, *pascuorum*, *pratorum*, *soroensis*, *terrestris*, *wurflenii*) show no distinct eye adaptations, and their eye morphology closely resembles that of the workers. A phylogenetic analysis of male eye structure reveals that enlarged males eyes are likely the ancestral state in *Bombus* and several independent transitions between enlarged and non-enlarged male eyes must have occurred. In addition, we found differences in the functional morphology of the worker visual system, which are consistent with known behavioral differences among the species, such as foraging mainly in forests vs. open environments.



**Manuscript IV****Visual ecology of long-horned bee males (*Eucera berlandi*, Hymenoptera: Apidae):  
Adaptations of the visual system and mate detection**

Martin Streinzer, Johannes Spaethe

***Abstract***

Males and females of the long-horned bee species *Eucera berlandi* show a remarkable sexual dimorphism in their sensory system. The elongated antennae of the males exhibit morphological traits that most likely improve olfactory detection of the females. During mating flights, males detect females both olfactorily and visually. Females emerging from the ground are immediately noticed and then chased by the males; only the fastest male achieves reproductive success. Therefore, a strong selection pressure acts on the males for good visual and olfactory abilities. In this study we focus on the visual system of *Eucera berlandi* and compared male and female eye morphology to identify adaptations for mating behavior in males. Additionally, we conducted a field behavioral experiment to study male chasing behavior in more detail. Males have enlarged compound eyes that result from enlarged frontal facets. The frontal eye region is characterized by small interommatidial angles and thus high spatial resolution. Further evidence for selection pressure on high resolution comes from the smaller eye parameter in the male frontal eye region compared to females. The behavioral experiment showed that the number of males attracted by a moving female dummy that was impregnated with a sex pheromone analogue correlated significantly with dummy size but not speed. Furthermore, males had a higher success rate when catching larger dummies as opposed to smaller ones. We discuss the morphological adaptations of the male visual system and possible mechanisms that allow males to detect and catch the female during the fast mating flights.

**Manuscript V****Sexual dimorphism in the olfactory system of a solitary and a eusocial bee species**

Martin Streinzer, Christina Kelber, Sarah Pfabigan, Christoph J. Kleineidam,  
Johannes Spaethe

*Journal of Comparative Neurology* (in press). Doi: 10.1002/cne.23312

***Abstract***

Sexually dimorphic sensory systems are common in Hymenoptera and are considered to result from sex-specific selection pressures. An extreme example of sensory dimorphism is found in the solitary bee tribe Eucerini. Males of long-horned bees bear antennae that exceed body length. This study investigated the pronounced sexual dimorphism of the peripheral olfactory system and its representation in higher brain centers of the species *Eucera berlandi*. *Eucera* males have elongated antennae, with 10 times more pore plates and three times more olfactory receptor neurons than females. The male antennal lobe (AL) comprises fewer glomeruli than the female AL (~100 vs. ~130), of which four are male-specific macroglomeruli. No sex differences were found in the relative volume of the mushroom bodies, a higher order neuropil essential for learning and memory in Hymenoptera. Compared with the Western honeybee, the degree of sexual dimorphism in *Eucera* is more pronounced at the periphery. In contrast, sex differences in glomerular numbers are higher in the eusocial honeybee and a sexual dimorphism of the relative investment in mushroom body tissue is observed only in *Apis*. The observed differences between the eusocial and the solitary bee species may reflect differences in male-specific behavioral traits and associated selection pressures, which are discussed in brief.

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*Manuscript I*

**Sex and caste-specific variation in compound eye morphology of five honeybee species**

Martin Streinzer, Axel Brockmann, Narayanappa Nagaraja, Johannes Spaethe  
*PLoS One* (2013) 8:2:e57702

# Sex and Caste-Specific Variation in Compound Eye Morphology of Five Honeybee Species

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## Abstract

Ranging from dwarfs to giants, the species of honeybees show remarkable differences in body size that have placed evolutionary constraints on the size of sensory organs and the brain. Colonies comprise three adult phenotypes, drones and two female castes, the reproductive queen and sterile workers. The phenotypes differ with respect to tasks and thus selection pressures which additionally constrain the shape of sensory systems. In a first step to explore the variability and interaction between species size-limitations and sex and caste-specific selection pressures in sensory and neural structures in honeybees, we compared eye size, ommatidia number and distribution of facet lens diameters in drones, queens and workers of five species (*Apis andreniformis*, *A. florea*, *A. dorsata*, *A. mellifera*, *A. cerana*). In these species, male and female eyes show a consistent sex-specific organization with respect to eye size and regional specialization of facet diameters. Drones possess distinctly enlarged eyes with large dorsal facets. Aside from these general patterns, we found signs of unique adaptations in eyes of *A. florea* and *A. dorsata* drones. In both species, drone eyes are disproportionately enlarged. In *A. dorsata* the increased eye size results from enlarged facets, a likely adaptation to crepuscular mating flights. In contrast, the relative enlargement of *A. florea* drone eyes results from an increase in ommatidia number, suggesting strong selection for high spatial resolution. Comparison of eye morphology and published mating flight times indicates a correlation between overall light sensitivity and species-specific mating flight times. The correlation suggests an important role of ambient light intensities in the regulation of species-specific mating flight times and the evolution of the visual system. Our study further deepens insights into visual adaptations within the genus *Apis* and opens up future perspectives for research to better understand the timing mechanisms and sensory physiology of mating related signals.

**Citation:** Streinzer M, Brockmann A, Nagaraja N, Spaethe J (2013) Sex and Caste-Specific Variation in Compound Eye Morphology of Five Honeybee Species. PLoS ONE 8(2): e57702. doi:10.1371/journal.pone.0057702

**Editor:** Eric James Warrant, Lund University, Sweden

**Received:** November 25, 2012; **Accepted:** January 24, 2013; **Published:** February 27, 2013

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**Funding:** M.S. is recipient of a DOC-fellowship of the Austrian Academy of Sciences at the Department of Behavioral Physiology and Sociobiology, University of Würzburg and the Department of Evolutionary Biology, University of Vienna. This publication was funded by the German Research Foundation (DFG) and the University of Würzburg in the funding programme Open Access Publishing. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Honeybee colonies comprise three adult phenotypes, males (drones), reproductive females (queens) and sterile females (workers). Drones, queens and workers differ in reproductive organs, as well as in the morphology of mouthparts, flight musculature, number of glands, sensory systems and structural organization of brains [1–5]. Morphological and physiological differences correlate with behavioral differences and result from different natural and sexual selection pressures.

The virgin queen leaves the colony for mating flights; after mating, she remains in the hive and lays eggs for most of her life [1,6]. The workers perform all tasks necessary to maintain the colony, e.g. brood care, foraging, and colony defense. Corresponding to this division of labor, queens show reductions in many morphological traits, e.g. mouthparts, pollen collecting structures, olfactory system and brain size [1,4]. In contrast, drones engage neither in social nor foraging tasks; they serve predominantly for reproduction, i.e. searching for and mating with queens. Drones show enlarged and elaborated olfactory [2,7,8] and visual systems

[9], as well as flight musculature that is adapted for fast pursuit flights [10].

Body size is considered the most characteristic morphological difference among honeybee species [11,12]. Presumably, body size affects all sex and caste-specific morphological traits and, most importantly, sensory organs, the brain and motor system [10,13,14]. Body size scaling in bumblebees, for instance, results in more sensitive olfactory systems [14] and more acute and sensitive visual systems [13,15] in larger bodied individuals, enabling foraging activities at lower light levels [13]. Large body size is further considered as an important pre-requisite for the behavioral transition to crepuscular activity [16].

Honeybees possess apposition compound eyes that consist of several thousand optically isolated ommatidia. Despite the general limitations given by the eye design, this eye type is well suited for orientation and object detection in bright daylight. Studies in sweat and carpenter bees show that, with some modification, the apposition compound eyes also enable reasonable visual orientation during night [17–20]. Eye design affects ecological success of a species, e.g. via improved flower detection capabilities in females

and improved mate detection in males. It also sets an important boundary to the specific timeframe in which the animal is able to operate [20]. Numerous studies in Hymenoptera (e.g. ants [21,22], bees [13,17–19,23,24] and wasps [25]) document the relation between the structure of the visual system and the specific light environment in which the animal is active. In a recent study, Somanathan et al. [24] document visual adaptations of honeybee workers in three Asian species and the Western honeybee and discuss the implications of the eye design in the context of photic niche utilization for foraging. Temporal niche partitioning in honeybees is further important in the context of mating. Due to their common behavioral pattern of long range sex-pheromone attraction by a similar odor bouquet and short range visual chasing, geographically co-occurring species are forced to temporally separate mating times [11].

As an initial step to explore the variability of sensory and neural structures and the interaction between size limitations and sex and caste-specific selection pressures (e.g. selection on fecundity in queens, efficient foraging in workers and mate detection in drones) in *Apis*, we studied the periphery of the visual system. We investigated all sexes and castes of five species (*Apis andreniformis*, *A. florea*, *A. dorsata*, *A. mellifera* and *A. cerana*), to contribute to our knowledge of caste and species-specific visual systems in the genus [17,26]. We compared number and arrangement of ommatidia and facet lens diameters in the compound eyes and ocellar size. In particular, we asked whether and how eye size, ommatidia number and facet size correlate with body size and differ among sexes and castes. We hypothesize that clear deviations from body size correlations indicate specific adaptations, either with respect to spatial resolution (ommatidia number) or light sensitivity (facet diameter), both of which are traded-off against each other in relation to the specific lifestyle of the animal.

## Materials and Methods

### Honeybee Specimens

We investigated queens, workers and drones of the two dwarf honeybee species (*A. andreniformis* SMITH, 1858 and *A. florea* FABRICIUS, 1787), the giant honeybee (*A. dorsata* FABRICIUS, 1793), the Western honeybee (*A. mellifera* LINNAEUS, 1758) and the Eastern honeybee (*A. cerana* FABRICIUS, 1793). Specimens were collected near Bangalore, India (Doddaballapur, 13°17'32"N, 77°32'35"E) between 2003 and 2012 (*A. florea*, *A. dorsata*, *A. cerana*), in Chiang Rai Province, Northern Thailand (Mae Sai, 20°25'60"N, 99°52'60"E; Mae Fang Luang, 19°52'25"N, 99°43'23"E) in 2011 (*A. andreniformis*), in Vienna, Austria (48°13'47"N, 16°21'32"E) and Würzburg, Germany (49°46'48"N, 9°58'25"E) between 2009 and 2011 (*A. mellifera carnica*) and obtained from the collection maintained at the bee research unit, Bremen, Germany (*A. florea*, obtained from Feyriz, Fars Province, Iran in 1991). Specimens were either pin-mounted or preserved in ethanol and pin-mounted prior to measurements. For each species a minimum of four males and workers and two queens, were examined (except for *A. dorsata*; see Table 1 for sample numbers in parentheses).

### Eye and Body Size Measurements

Size measurements of thorax, compound eyes and ocelli were performed on digital photographs using ImageJ (National Institute of Mental Health, Bethesda Maryland, USA). Photographs were taken with stereomicroscopes (Nikon SMZ-U equipped with DS-Fi1, Tokyo, Japan and Leica EZ4D, Leica Microsystems, Wetzlar, Germany) at different magnifications (4–16×). Size measurements were calibrated with respect to photographs of an object micrometer at the same magnification. As a measure of body size

we used intertegulae span, which was previously shown to be an appropriate estimate of body size in bees [15,27]. Eye length was measured as the longest linear measure across the eye from a frontal view. Eye surface area measurements were performed on eye surface replicas made of nail polish [26]. Replicas were photographed using light microscopes (Nikon Labophot equipped with DS-Fi1 and Zeiss Axiophot, Zeiss Germany equipped with Spot Insight Color, Diagnostic Instruments Inc., USA) at 100–400× magnifications in overlapping sections and subsequently stitched in Adobe Photoshop CS2 (Adobe Systems, San Jose, CA, USA). The eye surface was then measured by tracing the outlines in ImageJ. Measurements of the ocelli were performed as the longest linear measure across the median and the left lateral ocellus.

### Ommatidia Measurements

Ommatidia number was determined by manually marking all facet imprints of the eye replica in ImageJ. To measure the facet diameter, a row of 5 ommatidia in all three axes (x, y and z) was measured in ImageJ. We then calculated the mean diameter of a single ommatidium [13]. Measurements were performed on the largest facets. Additionally, eye maps were created to illustrate facet diameter distribution over the entire eye surface. ImageJ, Meshlab (Visual Computing Lab - ISRI - CNR, <http://meshlab.sourceforge.net/>) and CorelDraw X5 (Corel Corporation) was used to create the maps. In brief, ommatidia diameters were estimated from the distance between neighboring ommatidia centers. For visualization, ommatidia diameters across the eye surface were color coded.

### Mating Flight Activity

In addition to the size measurements, we analyzed published records of drone and queen flight activity of all investigated species. Reported flight times were corrected for solar azimuth differences according to the procedure employed by Otis et al. [28] when such a correction was not performed in the original study. For observations over several days we only report the total range of drone flight activity (e.g. [29]), and we aimed to avoid pseudo-replication from subsequent citations of the same original data set. Ambient light intensity is a function of solar elevation and not only depends on the time of the day but also on geographic latitude and time of the year. To transform daytime records of mating flight times to solar elevation information, we calculated the range of solar elevation for the flight period of all studies that reported location and date, using equations provided by the NOAA (U.S. Department of Commerce).

### Statistics

Body and eye parameters were compared between and within species with a Kruskal-Wallis H test. All P-values below the 5%-level were considered to be statistically significant. Statistical analyses were performed with Statistica 10 (StatSoft Inc., OK, USA).

## Results

### Body and Eye Size

The five investigated honeybee species differ with respect to body and eye size. The largest variation is found in workers and the smallest in drones (Table 1). Body size (intertegulae span) differs significantly, both between castes and sexes within species ( $H_{andreniformis}(2,11) = 8.6, p < 0.05$ ;  $H_{cerana}(2,17) = 12.3, p < 0.005$ ;  $H_{dorsata}(2,12) = 7.6, p < 0.05$ ;  $H_{florea}(2,13) = 8.6, p < 0.05$ ;  $H_{mellifera}(2,15) = 12.4, p < 0.005$ ) and within castes and sexes among

**Table 1.** Body and eye measurements of five honeybee species.

Species	Caste/Sex	Body size <sup>1</sup>	Eye length	Eye surface	Ommatidia	Facet diameter	Ocellus med.	Ocellus lat.
		mm	mm	mm <sup>2</sup>		μm	mm	mm
<i>Apis florea</i>	queen	3.1±0.1 (3)	2.1±0.1 (3)	1.9±0.0 (2)	4,036±54 (2)	24.9±0.3 (2)	0.27±0.02 (3)	0.26±0.02 (3)
	worker	2.0±0.0 (5)	1.8±0.0 (5)	1.5±0.0 (4)	4,394±29 (4)	22.1±0.3 (4)	0.20±0.00 (5)	0.20±0.00 (5)
	drone	3.1±0.1 (5)	3.2±0.1 (5)	8.1±0.3 (4)	9,434±334 (4)	38.0±0.5 (4)	0.32±0.01 (5)	0.28±0.01 (5)
<i>Apis andreniformis</i>	queen	2.9±0.0 (2)	2.0±0.0 (2)	1.6±0.0 (2)	3,965±93 (2)	24.1±0.1 (2)	0.24±0.00 (2)	0.23±0.01 (2)
	worker	1.8±0.0 (4)	1.6±0.0 (4)	1.3±0.0 (4)	3,851±110 (4)	21.6±0.3 (4)	0.19±0.01 (4)	0.18±0.01 (4)
	drone	3.2±0.1 (5)	2.8±0.0 (5)	5.5±0.2 (4)	7,351±225 (4)	34.4±0.2 (4)	0.29±0.01 (5)	0.26±0.01 (5)
<i>Apis dorsata</i>	queen	4.3 (1)	2.9 (1)	4.1 (1)	4,479 (1)	34.7 (1)	0.38 (1)	0.40 (1)
	worker	3.1±0.0 (8)	2.9±0.0 (8)	4.1±0.2 (5)	5,974±112 (4)	30.8±0.7 (5)	0.40±0.02 (8)	0.37±0.02 (8)
	drone	3.8±0.1 (3)	3.6±0.1 (3)	10.7±0.7 (3)	8,383±463 (3)	46.3±1.0 (3)	0.40±0.00 (3)	0.34±0.01 (3)
<i>Apis mellifera</i>	queen	3.5±0.1 (4)	2.4±0.1 (4)	2.2±0.0 (2)	4,460±55 (2)	26.1±0.2 (2)	0.30±0.01 (4)	0.30±0.01 (4)
	worker	2.9±0.0 (6)	2.4±0.1 (5)	2.5±0.1 (4)	5,375±143 (4)	25.2±0.3 (3)	0.30±0.01 (5)	0.28±0.01 (5)
	drone	4.3±0.1 (5)	3.6±0.1 (5)	9.4±0.4 (4)	9,993±483 (4)	40.1±0.7 (4)	0.36±0.02 (5)	0.34±0.02 (5)
<i>Apis cerana</i>	queen	3.2±0.1 (5)	2.1±0.1 (5)	1.8±0.1 (3)	3,582±106 (3)	25.9±0.3 (3)	0.27±0.01 (5)	0.26±0.01 (5)
	worker	2.6±0.1 (8)	2.1±0.0 (8)	2.3±0.0 (5)	4,921±88 (4)	25.4±0.1 (5)	0.25±0.01 (8)	0.23±0.01 (8)
	drone	3.2±0.1 (4)	2.8±0.1 (4)	5.9±0.3 (4)	7,994±167 (4)	35.8±1.1 (2)	0.30±0.01 (4)	0.26±0.00 (4)

Measured parameters are given as means±std.dev. Sample size is indicated in parentheses. <sup>1</sup>Body size is expressed as the distance between the wing bases (intertegulae span).

doi:10.1371/journal.pone.0057702.t001

species ( $H_{workers}(4,31) = 28.6, p < 0.005$ ;  $H_{queens}(4,15) = 12.2, p < 0.05$ ;  $H_{drones}(4,22) = 16.5, p < 0.005$ ). In all species, queens and males are larger than workers, whereas the polarity of size differences between males and queens varies among species. Males are larger than queens in *A. andreniformis* and *A. mellifera*, smaller than queens in *A. dorsata*, and similar in body size in *A. florea* and *A. cerana* (Fig. 1, Table 1). Among drones, *A. mellifera* drones are larger than *A. dorsata* drones, and the drones of *A. andreniformis*, *A. florea* and *A. cerana* are similar in size. Our results are consistent with previous weight measurements performed on several honeybee species [11].

Eye size (eye surface area) differs significantly between sexes and castes in all species except for *A. dorsata* ( $H_{andreniformis}(2,10) = 7.9, p < 0.05$ ;  $H_{cerana}(2,12) = 9.7, p < 0.01$ ;  $H_{dorsata}(2,9) = 5.4, p = 0.07$ ;  $H_{florea}(2,10) = 7.9, p < 0.05$ ;  $H_{mellifera}(2,10) = 7.9, p < 0.05$ ). Within species, eye size is similar between workers and queens (Fig. 1, Fig. 2, Table 1). However, due to larger body size, queen eyes appear relatively smaller. In contrast, drones have much larger and differently shaped eyes (Fig. 2). Differences between drone and female eyes range from 2.6-fold in *A. dorsata* to 5.4-fold in *A. florea* (Fig. 1, Table 1). While eye size of queens and workers positively scales with body size in all species, drone eye size does not simply so. In particular, *A. florea* and *A. dorsata* drone eyes are disproportionately enlarged relative to body size compared with drones of the other species (Fig. 1, Table 1).

### Ommatidia Numbers and Facet Size Distribution

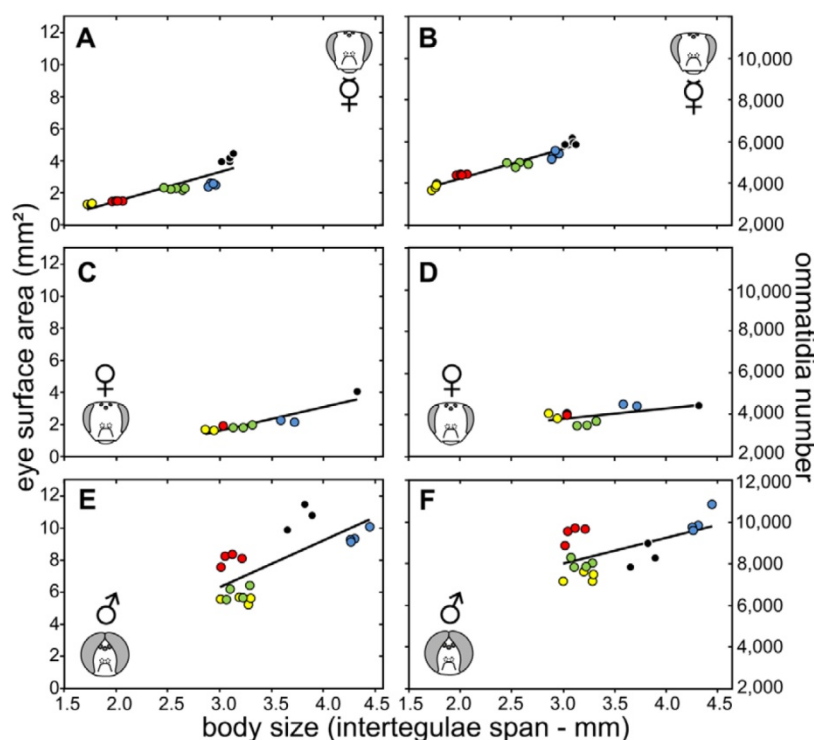
The number of ommatidia range from c. 3,500 in the queens of *A. cerana* to c. 11,000 in drones of the Western honeybee (Fig. 1, Table 1). In all species, except for *A. andreniformis*, queens possess the lowest, while drones possess the highest number of ommatidia (Table 1). The number of ommatidia differs significantly between castes and sexes in all species (Fig. 1, Table 1;  $H_{andreniformis}(2,10) = 6.9, p < 0.05$ ;  $H_{cerana}(2,9) = 7.0, p < 0.05$ ;  $H_{florea}(2,10) = 7.9, p < 0.05$ ;  $H_{mellifera}(2,10) = 7.9, p < 0.05$ ). For *A. dorsata*

( $H_{dorsata}(2,8) = 5.8, p = 0.054$ ) the p-value was marginal significant, due to the low sample size of queens (N = 1). Ommatidia numbers in worker and drone eyes differ among all species ( $H_{workers}(4,20) = 18.3, p < 0.005$ ;  $H_{drones}(4,17) = 14.3, p < 0.01$ ), whereas ommatidia number in queens do not differ among species ( $H_{queens}(4,10) = 8.1, p = 0.09$ ) despite strong variation in body size.

In apposition compound eyes facet diameters are usually not evenly distributed along the eye surface and the largest facets are commonly found in regions associated with high spatial acuity and light sensitivity [30]. In *Apis*, facet diameter frequency and distribution differs between sex and castes. Both female castes have a nearly Gaussian distribution of facet diameters and their largest facets are located in the fronto-ventral region of the eye (Fig. 2). In all species, except *A. mellifera*, queen eyes are composed of ommatidia with larger facet diameter compared with worker eyes. The largest difference in facet diameter between worker and queen eyes is found in *A. dorsata* (Fig. 2, Table 1). Drone eyes show a strong dorso-ventral regionalization, indicated by a steep transition in facet diameter (Fig. 2). The dorsal two thirds of the eye are equipped with large facets of which the largest are located in the dorso-lateral region. The ventral third is equipped with smaller facets that are similar in size to the largest facets in workers. The distinct dorso-ventral separation is also reflected in the diameter frequency distribution, which is flatter and shows more than one maximum in all species (Fig. 2).

### Ocelli

The three ocelli are located at the top of the head in queens and workers and frontal in drones (Fig. 2). Usually, the median ocellus is larger than the lateral ocelli (Table 1). Ocellus size differs significantly between castes and sexes in all species, both for the median ( $H_{andreniformis}(2,11) = 8.6, p < 0.05$ ;  $H_{cerana}(2,17) = 13.8, p < 0.005$ ;  $H_{dorsata}(2,12) = 2.6, p = 0.28$ ;  $H_{florea}(2,13) = 10.6, p < 0.01$ ;  $H_{mellifera}(2,14) = 9.6, p < 0.01$ ), and the lateral ocellus ( $H_{andreniformis}(2,11) = 8.6, p < 0.05$ ;  $H_{cerana}(2,17) = 12.4, p < 0.005$ ;  $H_{dorsata}(2,12) = 7.3, p < 0.05$ ;  $H_{florea}(2,13) = 10.6, p < 0.01$ ;  $H_{mellifera}(2,14) = 9.6, p < 0.01$ ).



**Figure 1. Morphological measurements of eye parameters in five honeybee species.** Eye surface area (left panel) and ommatidia number (right panel) measured in workers (A, B), queens (C, D) and drones (E, F) of the Western and four Asian honeybee species. Species are indicated by color (*A. andreniformis* – yellow, *A. florea* – red, *A. dorsata* – black, *A. mellifera* – blue, *A. cerana* – green). Each circle represents one measured individual (see Table 1 for sample sizes). Trend lines are based on all measured specimens (A:  $y = 1.82x - 2.15$ ,  $R^2 = 0.79$ ; B:  $y = 1,421.66x + 1,399.65$ ,  $R^2 = 0.94$ ; C:  $y = 1.46x - 2.75$ ,  $R^2 = 0.81$ ; D:  $y = 514.11x + 2,312.18$ ,  $R^2 = 0.38$ ; E:  $y = 2.88x - 2.29$ ,  $R^2 = 0.51$ ; F:  $y = 1,250.49x + 4,264.23$ ,  $R^2 = 0.34$ ). doi:10.1371/journal.pone.0057702.g001

$ea(2,13) = 9.5, p < 0.01$ ;  $H_{mellifera}(2,14) = 8.9, p < 0.05$ ), with the exception of the median ocellus in *A. dorsata*. The largest ocelli are found in *A. dorsata* and the smallest in workers of the dwarf honeybees *A. andreniformis* and *A. florea*. In general, ocelli are larger in queens than in workers of the small species (*A. andreniformis*, *A. florea*, *A. cerana*), similar in size in *A. mellifera*, and smaller in *A. dorsata*. In drones, ocellus diameters show the same trend as facet size; *A. dorsata* drones have the largest, followed by *A. mellifera*. Ocelli in *A. florea* are larger than in the similarly sized drones of *A. andreniformis* and *A. cerana* (Table 1).

## Discussion

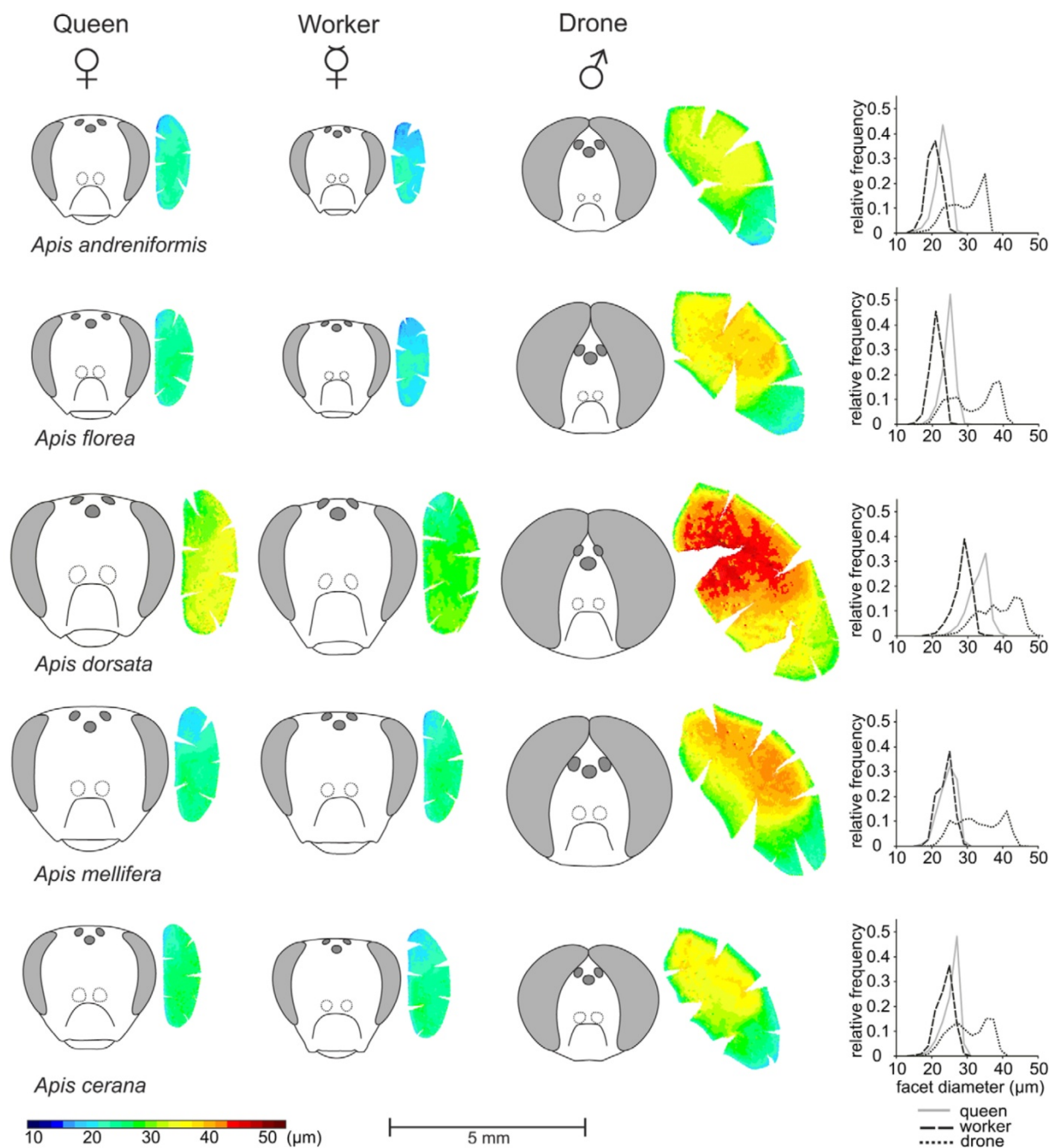
Our study documents sex and caste-specific variation in the compound eyes of five honeybee species. In queens and workers, eye size, ommatidia number, facet diameter and ocellus size positively correlate with body size among the species. Although queens are larger, queen and worker eyes are of similar size, but worker eyes on average comprise a higher number of ommatidia with smaller facets. Compared with the female castes, drones of all species show enlarged and highly modified compound eyes but drone eye size does not simply correlate with body size. Particularly, drones of the dwarf honeybee *A. florea* have disproportionately enlarged eyes in relation to body size and exhibit more ommatidia than drone eyes of the giant honeybee *A. dorsata* and almost as many ommatidia as drone eyes of the Western honeybee, *A. mellifera* (Figs. 1, 2; Table 1). Overall, the findings of our study indicate a greater variability in the design of

drone visual systems than previously assumed and this variability is probably the result of the interaction of species-specific body size limitations and sex-specific selection pressures.

## Female Eye Morphology and Female Behavior

In all species, eye size is similar between queens and workers, but queens usually possess less yet larger ommatidia (Fig. 1). Queens spend most of their lives in the colony where vision plays a minor role. The few occasions when they leave the hive (mating flights, swarming, absconding and migration) certainly require good spatial vision; however, the demands for visual acuity are likely less strong than for workers, which need to detect and identify flowers and orient themselves during foraging flights. Additionally, we found that the compound eyes of queens from the open nesting honeybee species (*A. andreniformis*, *A. florea* and *A. dorsata*) have relatively enlarged facets compared with workers, while such an enlargement is only marginal (*A. cerana*) or absent (*A. mellifera*) in the cavity nesting species. The two cavity nesting species are closely related [31], yet we do not know whether the smaller relative queen facet size (i) is related to the predominant life inside the nest, (ii) constitutes a phylogenetic constraint or (iii) is a byproduct of other selection pressures (e.g. [32]).

The largest caste difference in facet diameters is found in *A. dorsata*. Queen facets are enlarged, at the expense of ommatidia number, suggesting that queens trade-off spatial resolution for increased light sensitivity, a likely adaptation for crepuscular mating activity. In the two dwarf honeybee species, the decrease in worker body (and thus eye) size is accompanied by a reduction of



**Figure 2. Facet size distribution in compound eyes.** Eye maps illustrate eye size differences and facet size distribution between castes and sexes of the Western and four Asian honeybee species. Each circle represents one facet lens. Color indicates facet size (scale at the bottom). The largest facets in queens and workers are usually found in the fronto-ventral region of the eye. In drones, facet diameters are dorso-ventrally separated and the largest facets are found in the dorsal two-thirds of the eye. The eye maps are accompanied by line drawings of all individuals, which allow comparison of eye placement, eye size and ocellar size between species, sexes and castes (all to scale, scale bar 5 mm). Relative facet diameter frequencies are illustrated by histograms (right panel, bin width 2  $\mu\text{m}$ ) of one randomly selected queen (gray line), worker (dashed line) and drone (dotted line) from each species.  
doi:10.1371/journal.pone.0057702.g002

facet diameters, but not ommatidia number, suggesting that workers trade-off light sensitivity to retain spatial resolution, which is important in foraging tasks. The smaller facet diameters,

however, may limit their foraging abilities during the twilight hours, compared with the species that possess larger facet diameters [13]. Somanathan et al. [24] recently investigated the



worker eyes of four honeybee species. In concordance with our results, they found smaller eyes and facets in the dwarf species and enlarged facet and ocellar lenses in *A. dorsata*. Model calculation further suggest, that light sensitivity is highest in *A. dorsata* and lowest in *A. florea*, which correlates with the observed temporal foraging patterns ([24] and citations therein).

It must be noted that both spatial resolution and light sensitivity not only depend on the morphology of the peripheral optical system (ommatidia diameters and numbers), but also on the photoreceptor arrangement (interommatidial angles, rhabdom diameter) and potentially on neuronal computation strategies [18,19,23,33]. For instance, model calculations by Somanathan et al. [24] suggested that *A. dorsata* has the lowest spatial resolution, despite having the highest number of ommatidia and that their light sensitivity is additionally increased due to larger rhabdom diameters. So far, detailed measurements of the interommatidial and acceptance angles, light sensitivity, and behavioral assessment of the spatial resolution, object detection threshold and light intensity threshold are lacking for all Asian honeybee species.

Our measured eye parameters of *A. florea* differ from the previously published data [24]. We suggest that regional intra-specific variation in body (and thus eye) size may account for the c. 900 more ommatidia we find in *A. florea* workers. We investigated *A. florea* workers and drones from Iran, while Somanathan et al. [24] collected workers in India. No subspecies are officially recognized in *A. florea*, but morphometric studies revealed the existence of several morphotypes and workers from Iranian populations are larger than workers from Indian populations (own measurements and [34]). Similarly, our measurements on *A. mellifera* differ from earlier reports (e.g. [17,26]). The Western honeybee is widely distributed and comprises several distinct subspecies. For instance, three of the economically important subspecies, *A. m. mellifera*, *A. m. carnica* and *A. m. ligustica* differ with respect to body size (in this sequence, *A. m. mellifera* being the largest [35]). In addition, historical and regional differences in bee keeping management (e.g. the used foundation cell size) artificially constrain body size and may account for large intra-specific variation [36].

### Drone Eye Morphology and Drone Behavior

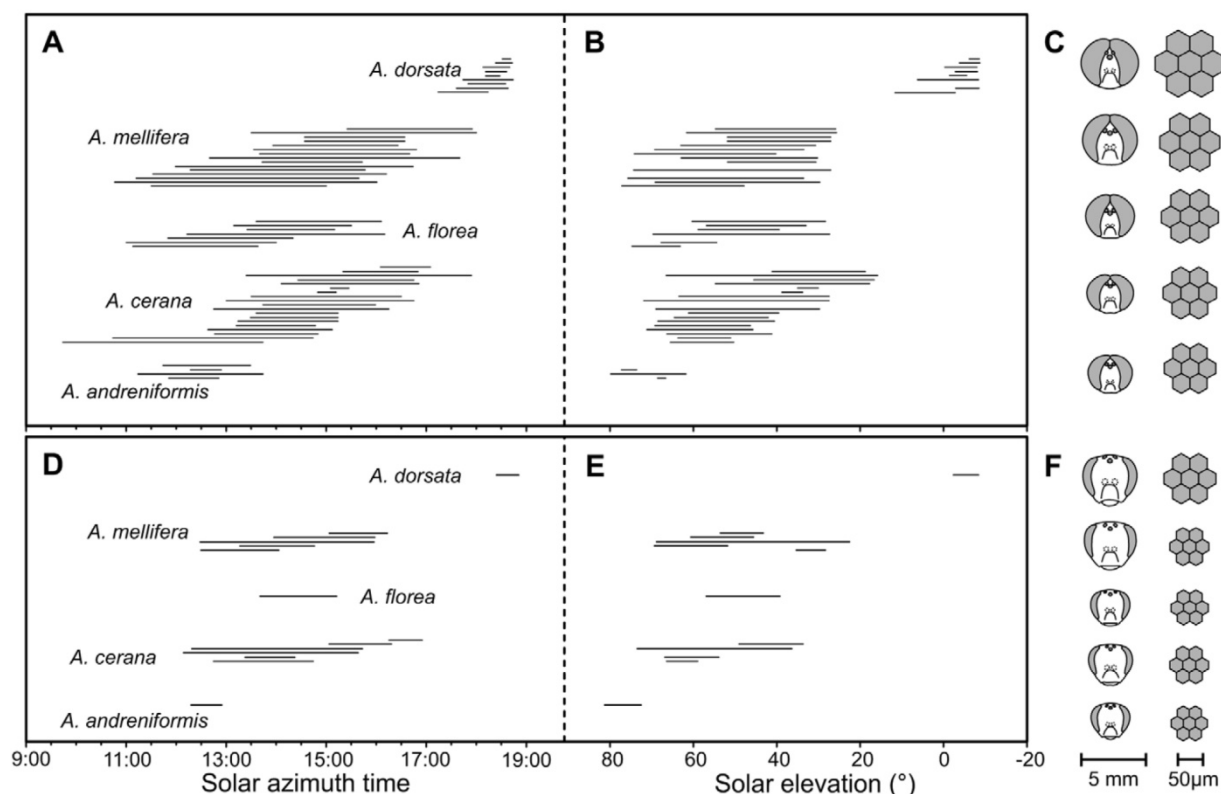
Several studies on eye morphology and drone chasing behavior in *A. mellifera* indicate that the drone compound eye is specialized to detect small moving objects against the bright blue sky [9,37–41]. The eyes show distinct regional specializations, e.g. extremely enlarged facets located in the dorsal region of the eye [26]. Our study demonstrates a similar organization in drone eyes of all investigated honeybee species (Fig. 2). However, the drone compound eyes of *A. florea* and *A. dorsata* apparently have evolved specific adaptations. *A. florea* drones have much larger compound eyes than drones of the other dwarf honeybee *A. andreniformis*. Furthermore, relative to body size, their eyes are larger than in drones of all other honeybee species. The enlargement of the compound eyes is accompanied by an increase of ommatidia number suggesting a substantial increase in spatial resolution. In contrast, the large compound eyes of *A. dorsata* drones consist of a lower number of ommatidia compared with *A. florea* drones, but these ommatidia exhibit much larger facet lenses, suggesting a significant increase in light and, probably more important, contrast sensitivity [20].

In most honeybee species drone mating flights start around noon or early afternoon at times of highest solar elevation and light intensities and last until late afternoon (Fig. 3). Mating flight times in *A. dorsata* diverge from this pattern; they occur at sunset and are much shorter in duration. Daily onset and end of mating flights are

strongly affected by changing ambient light and temperature conditions as well as the animal's motivation [42]. In *A. dorsata*, facet and ocelli diameters resemble those of some strictly nocturnal bee species [17,23], and may thus allow mating flights around sunset. *A. andreniformis* also seems to have a narrow mating flight period that is constrained to times of highest light intensities (Fig. 3). The findings of our study indicate a correlation between morphological characters of drone eyes and species-specific mating flight times, which reflects the importance of visual mate detection in honeybee mating behavior.

We find a similar correlation between facet and eye size and mating flight time in queens. Both sexes are active at the same time, ensuring successful meeting and mating. Queens, however, possess smaller facets than drones, suggesting that light levels alone may not account for the large facets in drone eyes. Drones face a tremendous challenge when detecting a fast moving queen from a distance. The visual acuity in the dorsal eye region is high [43] but probably even more importantly, the enlarged facet lenses give *Apis* drones an extremely high contrast sensitivity [39]. The large facet diameters of *A. dorsata* drones may thus be particularly important to maximize contrast sensitivity in low light environments that generally limit visual contrast [20]. The smaller facets and ocelli in *A. andreniformis* may constrain mating flights to high noon when light level and contrast ratios are sufficiently high (Fig. 2, Fig. 3). In the case of *A. dorsata*, body size might have been a pre-adaptation, which allowed a shift of mating flights to lower light levels [16]. Interestingly, the only report on drone mating flight times in *A. laboriosa*, the similar-sized Himalayan sister species of *A. dorsata*, indicated that mating flights start in early afternoon [44]. This shift is most likely a response to the harsh weather conditions at high elevations and a similar shift of mating flights is observed in the second Asian mountain honeybee *A. nuluensis* [45].

Based on our observations, we hypothesize that ambient light intensity is a major factor for the timing of honeybee mating flights. Although mating flight times exhibit a high degree of variability (Fig. 3, left panels), our calculations show that at least some of the variation is a result of differences in location [46] and time of year [42,47] and can be explained by differences in solar elevation (Fig. 3, right panels). Similar to hypotheses on worker foraging behavior [48], we suggest that eye morphology and ambient light intensity define a species-specific timeframe for mating behavior. In the case of geographically co-occurring honeybee species, mating flight times can be shortened and shifted within this basic timeframe according to sensitivity of the visual system [49,50]. Recent studies in Australian *Myrmecia* ants demonstrated that worker foraging activity is exclusively controlled by absolute light levels [21], and caste and species-specific activity schedules are determined by eye morphology [22]. However, at this time we cannot exclude the possibility that mating flight times in honeybees are also affected by other environmental parameters, such as ambient temperature and humidity, which correlate with light intensity. In addition, an impact of ambient light intensity levels on mating flight activity does not exclude that mating behavior is regulated by the circadian clock [51,52]. The clock likely regulates physiological processes involved in mating behavior in anticipation of the actual mating flight. Future experiments should focus on the hitherto unknown proximate physiological and neuronal mechanisms that generate narrow and temporally separated mating flight periods in *Apis*. The potential to quickly adapt the mating timeframe in response to sympatric honeybee species in order to avoid inter-specific interference provides an avenue for future research on the function and evolution of the mechanisms that regulate the timing of mating flights.



**Figure 3. Species-specific mating flight activity.** Drone (A, B, C) and queen (D, E, F) flight activity compiled from literature records [11,29,42,45,46,49,50,56,58–84]. (A, D) Temporal range and (B, E) corresponding solar elevation range of the flight time. When necessary, time was converted to solar azimuth time. For studies that did not report the date of observation, solar elevation could not be calculated (bars missing in B, E). (C, F) Graphical representation of the heads (left) and ommatidia diameters (right) for all species (scale bars below). Species are sorted in an ascending order of drone eye size, ommatidia and ocellar diameters. doi:10.1371/journal.pone.0057702.g003

### The Curious Case of *Apis florea* Drones

Characteristic of the honeybee mating behavior is that drones chase the queens [1]. Queens signal their presence by releasing their sex-pheromone, which triggers an upwind search by drones and also heightens their motivation to chase any small and dark object moving against the sky [39]. Although all *Apis* species are assumed to be highly sensitive to queen pheromone, differences in the number of olfactory sensilla suggest a unique exaggeration of the sex-pheromone specific olfactory system in *A. mellifera* [53,54]. In contrast, *A. florea* drones have the lowest number and density of olfactory sensilla [53] and much smaller sex-pheromone processing macroglomeruli compared with *A. mellifera* drones [54]. Neural tissue maintenance and information processing are energetically costly and thus may be particularly prone to counter selection [55]. This limitation certainly affects the trade-off between different sensory systems, e.g. an enlarged visual system comes at the cost of a poorer olfactory system and *vice versa*. The current data on the olfactory and visual sensory systems in drone honeybees suggest that *A. mellifera* drones have specifically improved the sensitivity of their olfactory system in their evolution, whereas *A. florea* drones invested particularly in their visual system. We can only speculate about the ultimate causes for the differences among honeybee species. Brockmann and Brückner [53] suggested that low mate density may have promoted the evolution of a particularly sensitive olfactory system in *A. mellifera*. The question

why drones of *A. florea* have evolved relatively enlarged eyes is of particular interest with respect to the fact that drones of the sister species *A. andreniformis* did not evolve similar traits. Almost nothing is known about the mating behavior and drone congregation areas of the latter two species [56]. Both species differ with respect to their preference for nesting and probably also mating areas [57]. However, current knowledge on mating related signals and cues and the specific tasks of the sensory system in honeybee mating behavior is limited and does not permit us to draw further conclusions about the evolution of their sensory systems.

### Conclusion and Future Perspective

Based on the assumption that body size differences interact with sex and caste-specific selection pressures, we compared four different characters of the visual system in drones, queens and workers of five honeybee species. This approach successfully identified common patterns of adaptation within castes and revealed distinct adaptations in the drone eyes of two species, *A. florea* and *A. dorsata*. In general, the variability among species seems to be caused by the interaction of different factors, such as body size limitations, different selection pressures (e.g. selection for mate detection, foraging efficiency and fecundity that are exclusive to drones, workers and queens, respectively), temporal activity pattern and different relative roles of the sensory systems (e.g. the importance of vision vs. olfaction during mate detection). In

the future, it will be interesting to test whether these morphological differences are accompanied by differences in the behavioral responses to visual and olfactory signals.

## Acknowledgments

We thank K. Möller for her invaluable help with data collection, J. Makinson for providing *A. andreniformis* samples and D. Brückner, F. Etl and D. Ahrens for providing additional specimens of the other *Apis* species.

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We further thank J. Plant for linguistic improvements. Thanks are also due to the two anonymous reviewers and their helpful comments on an earlier version of the manuscript.

## Author Contributions

Conceived and designed the experiments: MS AB JS. Performed the experiments: MS NN. Analyzed the data: MS. Wrote the paper: MS AB JS.

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***Manuscript II***

**A scientific note on peripheral compound eye morphology of small and normal-sized honeybee drones**

Martin Streinzer, Johannes Spaethe  
*Journal of Apicultural Research* (submitted)

## **A scientific note on peripheral compound eye morphology of small and normal-sized honeybee drones**

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Short title: Size dependent eye morphology in honeybee drones

Keywords: *Apis mellifera*, male-male competition, compound eye, ommatidia, visual physiology

Honeybee colonies comprise two sexes, males (drones) and two castes of females. The majority of females are sterile and perform colony maintenance and foraging tasks (the workers), while the sole reproductive female is involved in egg laying (the queen). Both female castes develop from diploid, fertilized eggs through differential nutrition, while drones develop from haploid unfertilized eggs. During the mating season, a restricted number of virgin queens and several hundreds of drones are reared for reproduction. While queens are usually reared in vertical queen cells at the bottom of the comb, drone-eggs are deposited in special drone-comb cells. These cells are, similar to worker-cells, hexagonal and horizontally arranged, but usually larger. In some instances, drone eggs are also deposited in the smaller worker-comb cells either by the queen or by workers that have developed ovaries. Drones that are reared in worker-comb cells (small drones, SD) are 7-15% smaller in linear body measures and weigh c. one third less than normal drones (ND) (Berg et al., 1997; Couvillon et al., 2010). Limitation of body size also affects morphological traits in the drone. For example, smaller drones possess smaller wings and wing muscles, which result in a reproductive disadvantage with respect to access to virgin females. Furthermore, they produce a lower number of spermatozoa. Collectively, these reductions lower the drones' reproductive success and paternity share (Berg et al., 1997; Couvillon et al., 2010).

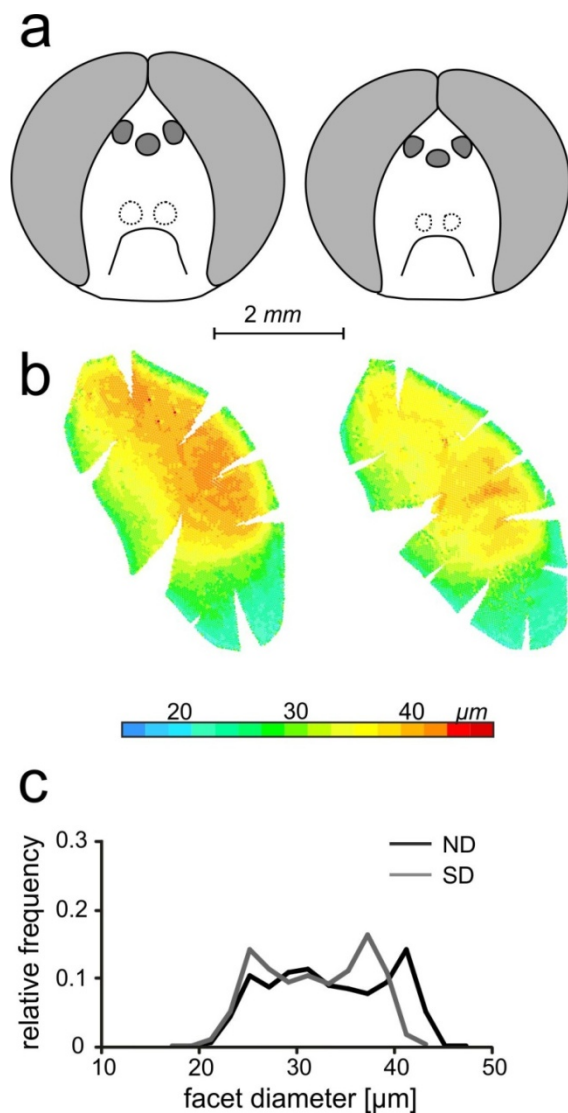
Honeybees mate on the wing. Drones congregate in aerial areas (drone congregation areas, DCA), where they await virgin queens. In the DCA, the operational sex-ratio is highly male-biased, resulting in strong male-male competition that selects for highly sensitive sensory

systems and strong locomotive abilities. Corresponding adaptations have been described in detail for *A. mellifera* L. concerning the olfactory system (Arnold et al., 1985), visual system (Menzel et al., 1991) and their flight musculature. Sensory organs and flight muscle usually scale with body size, which may in turn affect access to queens during the nuptial flight. Arnold et al. (1985), for example, found a reduced size of the olfactory neuropil in small drones.

In this study we investigated the impact of body size on compound eye morphology in *Apis mellifera carnica* drones (collected in Vienna, Austria, 48°13'47"N, 16°21'32"E in 2009). We measured body size (inter-tegulae span) using a stereomicroscope and eye morphology (eye surface area, facet number and size, facet size distribution maps) using microphotographs of nail-polish surface replicas of the compound eye. Drone eyes are greatly enlarged and possess enlarged facet lenses in the dorsal two-thirds, which differ with respect to their morphology and physiology from the ventral worker-like ommatidia (Streinzer et al., 2013). The large dorsal ommatidia allow for extremely high spatial resolution and contrast sensitivity (Menzel et al., 1991). We found no difference in the general organization of the compound eyes between ND and SD, regarding eye shape and dorso-ventral regionalization of facet diameters (Fig. 1). SD in our sample were c. 19% smaller (inter-tegulae span;  $its_{SD}: 3.5 \pm 0.1 \text{ mm}$ ;  $its_{ND}: 4.3 \pm 0.1 \text{ mm}$ ;  $means \pm st. dev.$ ;  $P < 0.05$ , Mann Whitney U-test;  $n=4$  each) and had significantly smaller eyes than ND (eye surface area;  $A_{SD}: 8.9 \pm 0.1 \text{ mm}^2$ ;  $A_{ND}: 9.4 \pm 0.4 \text{ mm}^2$ ;  $P < 0.05$ ). The decrease in eye size was not accompanied by a decrease in ommatidia number ( $Omm_{SD}: 10,098 \pm 157$ ;  $Omm_{ND}: 9,993 \pm 483$ ;  $P=0.31$ ). Instead, the eyes of SD are equipped with smaller facet lenses (largest facet diameter;  $D_{SD}: 37.5 \pm 0.1 \mu\text{m}$ ;  $D_{ND}: 40.1 \pm 0.7 \mu\text{m}$ ;  $P < 0.05$ ; Fig. 1). In apposition compound eyes, each ommatidium represents an optically isolated subunit that collects light from a certain solid angle in space. While the angular spacing between ommatidia determines spatial resolution, the facet lens aperture limits the amount of light collected by an individual ommatidium. In other words, more ommatidia usually result in higher spatial resolution and larger facets increase light catch of individual facets. Our findings implicate that spatial resolution is probably similar between SD and ND, assuming an isometric scaling of the head and a constant visual field. However, based on our facet size measurements we estimate that the dorsal ommatidia of SD suffer from a c. 13% reduced photon catch (Land, 1997). Drone eyes are adapted to detect small contrast differences (Vallet and Coles, 1993) and the smaller lenses found in SD may represent a serious disadvantage in the detection of the queen. No significant difference was found in ocellus diameter ( $Oc_{SD}: 0.36 \pm 0.01 \text{ mm}$ ;  $Oc_{ND}: 0.36 \pm 0.02$ ;  $P=0.89$ ).

Couvillon et al. (2010) found that small and large drones differ in the time of peak activity (but see Berg et al., 1997). In a recent study, Streinzer et al. (2013) suggested that eye morphology, and thus light sensitivity, may have an important role in the timing of the mating flight in *Apis*, and the differences in eye parameters between SD and ND (Fig. 1) may explain the observed difference in flight time. We suggest that eye morphology limits access of SD to queens during nuptial flights. The disadvantage posed by the reduction in visual power, however, is most likely only one factor, and is accompanied by a smaller olfactory system (Arnold et al., 1985), reduced flight power, size induced mounting limitations and potential post-copulatory mechanisms that

collectively lower mating success of SDs. Our findings bear further importance regarding the development of insect eyes. In social insects, worker body size usually correlates with ommatidia number (Kapustjanskij et al., 2007). In *Apis mellifera* drones, however, developmental plasticity seems to only affect qualitative (facet size) and not quantitative (ommatidia number) traits of the compound eye. A similar case exists in bumblebees, since ommatidia number correlates strongly with body size in workers, but not in males (Kapustjanskij et al., 2007). Our study opens up interesting perspectives for future research on the role of the sensory physiology in sexual selection and the developmental paths of the visual system in male Apoidea and, in particular, in the important model system *Apis mellifera*.



**Figure 1**

(a, b) Visual representation of the head (a) and eye (b) size of a normal drone (left panel) and a small drone (right panel) of *Apis mellifera*. Line drawings of the heads are accompanied by ommatidia size maps. On these, each circle represents an individual facet lens. The color-code helps to illustrate the gradual change of facet lens diameters across the eye surface. (c) Histograms of the distribution of facet lens diameters in one eye of a randomly chosen small (SD) and normal-sized drone (ND; bin width 2μm). Parts of this figure were previously published under the Creative Commons Attribution License (Streinzer et al., 2013).



### Acknowledgements

MS is recipient of a DOC fellowship of the Austrian Academy of Sciences at the Department of Behavioral Physiology and Sociobiology, University of Würzburg. The authors would like to thank K. Möller for help with data collection, F. Etl for providing bee samples and J. Plant for linguistic improvements.

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***Manuscript III***

**The evolution of elaborate male visual systems in bumblebees (Hymenoptera,  
Apidae, *Bombus*)**

Martin Streinzer, Johannes Spaethe

*Zoological Journal of the Linnean Society* (submitted)

**The evolution of elaborate male visual systems in bumblebees (Hymenoptera, Apidae, *Bombus*)**

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Running head: *Bombus* compound eye morphology

**Keywords:** ancestral state reconstruction- *Bombus*- mate detection- sexual selection- visual adaptation

## Abstract

Bumblebee mating behavior intrigued Darwin over 150 years ago and has elicited much interest since. Despite our increasing knowledge about male mating strategies, there is a general lack of knowledge about the signals and cues involved the signal sender-receiver relationship and the functional adaptations of the involved sensory systems. While many authors have noticed a close relationship between the mating system and male-specific elaboration of the visual system, studies on the functional adaptations of the visual system and its evolutionary pattern have yet to be conducted. In this study we investigated the functional morphology of the compound eyes in 11 species of *Bombus* from various subgenera in detail. Of these, four species (*B. confusus*, *melaleucus*, *mendax*, *niveatus*) have enlarged male eyes, characterized by a higher number of ommatidia compared with worker eyes and a frontal zone with enlarged facets, which is likely associated with improved spatial resolution and contrast sensitivity. In these species, perching mating strategies are found. In contrast, males of species that patrol scent routes (*B. hortorum*, *lapidarius*, *pascuorum*, *pratorum*, *soroensis*, *terrestris*, *wurflenii*) show no distinct eye adaptations, and their eye morphology closely resembles that of the workers. A phylogenetic analysis of male eye structure reveals that enlarged males eyes are likely the ancestral state in *Bombus* and several independent transitions between enlarged and non-enlarged male eyes must have occurred. In addition, we found differences in the functional morphology of the worker visual system, which are consistent with known behavioral differences among the species, such as foraging mainly in forests vs. open environments.

## Introduction

Sexual dimorphism, i.e. phenotypic differences of body size, morphology, physiology and behavior between the sexes of the same species, is widespread in the animal kingdom.

Hymenoptera show a remarkable diversity and expression of sex differences, which has been attributed to the particularly pronounced differences in the life histories of the sexes (Stubblefield and Seger, 1994). While females of many species build nests or seek for suitable places for egg deposition and forage for nectar, pollen or other nutrition to provision their offspring, males only invest in maximizing their access to virgin females. Additionally, competition among males for access to females, i.e. sexual selection, promotes the evolution of pronounced male-specific traits (Andersson and Iwasa, 1996).

Among Hymenoptera, bees historically have elicited enormous amounts of scientific attention, because of their conspicuous pollination behavior, intriguing social system, learning skills and as important suppliers of honey and wax (Frisch, 1914; Ruttner, 2003; Sprengel, 1793). This biased interest has resulted in a large body of knowledge about their physiology, morphology, sociobiology and behavior, and has also led to a comprehensive description of differences in mating systems and associated sex differences (Alcock et al., 1978; Paxton, 2005; Stubblefield and Seger, 1994). Bumblebees (Apidae: *Bombus* Latreille) are a particularly charismatic and easily recognizable group of bees (even for non-biologists) that has been collected, observed and studied by many researchers. Further, bumblebees have become an important model system for

such diverse fields as evolution, sociobiology, sensory ecology and sensory physiology (Baer, 2003; Plowright and Lavery, 1984; Real, 1991). Bumblebee colonies comprise two distinct female castes, reproductive queens and non-reproductive workers, as well as males. They differ in lifetime tasks, and thus show sex and caste-specific morphological, physiological and behavioral adaptations that are the result of counteracting and interacting natural and sexual selection pressures (Baer, 2003). Many of their behaviors are visually guided, e.g. nest site detection, spatial orientation, flower detection and recognition, predator avoidance and mate detection (Srinivasan, 2010). These caste and sex-specific tasks have resulted in various developments of sensory and neural traits among the sexes and castes (Fonta and Masson, 1985; 1987; Kapustjanskij et al., 2007; Shang et al., 2010). The visual system of bumblebees consists of three simple eyes (ocelli) and two large apposition compound eyes for spatial and color vision. Apposition compound eyes, however, are limited with respect in spatial resolving power and light sensitivity (Kelber et al., 2006; Land, 1997). To overcome at least some of the limitations of this eye type, regional specializations in eye design (e.g. acute zones; Land, 1997) and neural circuitry (Theobald et al., 2006) have evolved.

Workers of the c. 250 extant species of *Bombus* have been regarded as morphologically relatively homogenous (Michener, 2007), and there is no obvious difference in their visual system. However, interspecific differences in the visual system and its performance in workers have rarely been investigated (Kapustjanskij et al., 2007; Skorupski and Chittka, 2010). In contrast, the males' visual system differs among species and a distinct enlargement of the compound eyes has been described in some *Bombus* species (O'Neill et al., 1991; Williams, 1991). The enlarged visual system correlates with distinct mating strategies, as in other bee species (Hurd and Moure, 1962; Leys and Hogendoorn, 2008). Despite their abundance and long history of research, *Bombus* mating behavior has remained enigmatic. The first studies date back to Darwin, who studied male flight paths, although he did not realize that he was documenting mating behavior (Freeman, 1968). These flight paths were later identified as scent marked flight routes of males awaiting virgin queens (Krüger, 1951). In the meantime the mating behavior has been described for many species (reviewed in Goulson et al., 2011; Williams, 1991). The majority of species patrol scent marked flight routes (Bringer, 1973; Frank, 1941; Haas, 1949) or perch on elevated structures (Alcock and Alcock, 1983; Haas, 1976; O'Neill et al., 1991; Schremmer, 1972). Other strategies have been described, e.g. hovering in small territories (Williams, 1991), aggregating above nest entrances (Darvill et al., 2007) and hilltopping (Goulson et al., 2011). There is no clear phylogenetic pattern and some of the strategies apparently evolved repeatedly within the genus. Although our understanding of mating behaviors has increased since Darwin, and now includes knowledge about the origin and composition of species-specific marking pheromones (Ayasse et al., 2001; Kullenberg et al., 1970), female sex pheromones (Krieger et al., 2006) and male-male interactions (O'Neill et al., 1991; Williams, 1991) of many species, our knowledge about the underlying proximate mechanisms involved in mating behavior is still very patchy. Actual mating events are only rarely observed and there is very little direct evidence for the involved signals and cues, signaler-receiver relationship or

sensory specializations for mate finding behavior (Free, 1971; Krieger et al., 2006). Sex-specific and species-specific adaptations of the sensory systems have been described in some species (Ågren and Hallberg, 1996; Fonta and Masson, 1985; 1987; Williams, 1991), but systematic genus-wide studies are lacking.

The availability of a comprehensive molecular phylogeny (Cameron et al., 2007; Hines, 2008) provided a framework for a new comparative approach. Correlated changes in male eye morphology and mate finding behavior have been often suspected (O'Neill et al., 1991; Williams, 1991). However, systematic studies on the functional aspects and developmental differences of the enlarged male eyes and visual system differences among bumblebee species in general, have not been conducted. To trace the evolution of (male) visual adaptations in *Bombus*, we investigated male and female eye morphology of 11 taxa from 11 of the 15 subgenera, including 7 species with a patrolling and 4 species with a perching mating strategy in more detail. To detect differences in the functional morphology of the visual system, we measured parameters of the peripheral compound eye, such as eye size, ommatidia number and facet size. These parameters were then compared among the species and between the castes and sexes, and correlated to known behavioral differences of the respective subgenera (Williams et al., 2008).

## Material and Methods

### *Specimens studied*

We investigated queens, workers and males of 11 bumblebee species (*B. (Mendacibombus) mendax* Gerstaecker, *B. (Bombias) confusus* Schenck, *B. (Kallobombus) soroeensis* (Fabricius), *B. (Megabombus) hortorum* (Linnaeus), *B. (Thoracobombus) pascuorum* (Scopoli), *B. (Cullumanobombus) melaleucus* Handlirsch, *B. (Sibiricobombus) niveatus* Kriechbaumer, *B. (Melanobombus) lapidarius* (Linnaeus), *B. (Alpigenobombus) wurfleini* Radoszkowski, *B. (Bombus) terrestris* (Linnaeus) and *B. (Pyrobombus) pratorum* (Linnaeus). The species represent 11 of the 15 subgenera (excluding *Orientalibombus*, *Subterraneobombus*, *Alpinobombus* and the parasitic species of the subgenus *Psithyrus*), according to the recently simplified subgeneric classification of Williams *et al.* (2008).

### *Body size measurements*

Bumblebee workers show high intra-specific body size variation. To compare among the species, we aimed to reduce the inference of body size differences by correcting all eye parameters for individual body size. Several body size measures are commonly used in Hymenoptera (Cane, 1987; Ohl and Thiele, 2007). Especially intertegulae span (ITS) has been widely used in comparative studies on sensory systems (Kapustjanskij et al., 2007; Spaethe and Chittka, 2003). Recently, Hagen & Dupont (in press) reported differences in the ITS/weight ratio between male and female bumblebees. Moreover, the degree of dimorphism in thorax shape varies across species (Franklin, 1955) and seems to be associated with the mating system (Williams, 1991). Males of “perching” species have broader thoraces than “patrolling” species, probably to accommodate larger flight muscles, necessary for providing excess power for fast pursuit

maneuvers (Radloff et al., 2003). Therefore, intertegulae span appears to be critical as independent body size measure for our comparison. To reduce bias (which would overestimate sexual dimorphism of the visual system in patrolling species relative to perching species), we used the length of the 1<sup>st</sup> discoidal cell of the forewing. To ensure comparability between studies, we also measured ITS.

#### *Eye measurements*

Compound eye and ocelli size was measured from digital photographs using ImageJ (National Institute of Mental Health, Bethesda Maryland, USA). Photographs were taken by a stereomicroscope (Nikon SMZ-U equipped with DS-Fi1, Tokyo, Japan). For calibration, photographs of a stage micrometer were taken at the same magnification (Streinzer et al., 2013). Eye size was measured as the actual surface area of eye surface replicas made of nail polish (Ribi et al., 1989). Replicas were photographed using light microscopes (Nikon Labophot, Nikon, Japan, equipped with DS-Fi1 and Zeiss Axiophot, Zeiss, Germany, equipped with Spot Insight Color, Diagnostic Instruments Inc., USA) in overlapping sections and subsequently stitched in Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA). The eye surface area was then measured by tracing the outline of the replica in ImageJ. Measurements of the ocelli were performed as the longest linear measure across the median ocellus.

#### *Ommatidia measurements*

Ommatidia numbers were determined by manually marking all facets of the eye replica in ImageJ. To measure facet lens diameters we measured a row of 5 ommatidia in all three axes and calculated the mean diameter (Kapustjanskij et al., 2007). Measurements were performed on the largest facets, which are usually located in the fronto-ventral region of the eye.

To illustrate the facet size distribution we created eye maps of one worker and one male of all species. To create the maps, ommatidia center coordinates were used to estimate facet diameters from the distance between neighboring facets using customized workflows in ImageJ, Meshlab (Visual Computing Lab – ISRI – CNR, <http://meshlab.sourceforge.net/>) and CorelDraw X5 (Corel Corporation)(Streinzer et al., 2013).

#### *Statistics*

Body and eye parameters were compared between and within species using a non-parametric Kruskal-Wallis-H-test. Eye measures were divided by the 1<sup>st</sup> discoidal cell length to correct for body size differences between specimens. Values representing areas (eye surface area) were square-root transformed prior to body size correction. All P-values below the 5%-level were considered to be statistically significant. Statistical analyses were performed with Statistica 10 (StatSoft Inc., OK, USA).

### *Ancestral character state reconstruction*

The measured eye parameters and literature records were used to reconstruct the ancestral character state of female and male compound eyes in *Bombus*. Male eye size was categorized as “enlarged” (1) or “unenlarged” (0) relative to female eyes, based on our results (>50% larger eyes in similarly sized males, compared with workers; see below) and descriptions in identification keys, phylogenetic analyses, original descriptions and monographs on the genus *Bombus* (see Table S1 for full dataset and references). Ancestral characters were modeled using parsimony and maximum likelihood models (one-parameter Markov k-state model; Lewis, 2001) in Mesquite (Maddison and Maddison, 2011). Separate analyses were run for a tree that only included the 11 study species and a larger dataset using literature data on 198 taxa.

In addition, we modeled ancestral characters as continuous characters from our measured data on eye size, ommatidia number and facet size. Root node characters were calculated as phylogenetic independent contrasts (Felsenstein, 1985) using the APE package (Paradis et al., 2004) in R (version 2.15.2; Ihaka and Gentleman, 1996). Character evolution was reconstructed based on the molecular phylogeny published by Hines (2008). The phylogenetic tree differs only slightly from the equally comprehensive phylogeny published by Cameron *et al.* (2007), but includes divergence times and thus branch length information. Both phylogenies yield identical results in parsimony character evolution modeling (data not shown). The phylogeny was redrawn in Mesquite and pruned to include only the species used in the respective analysis.

## **Results**

### *Female eye morphology*

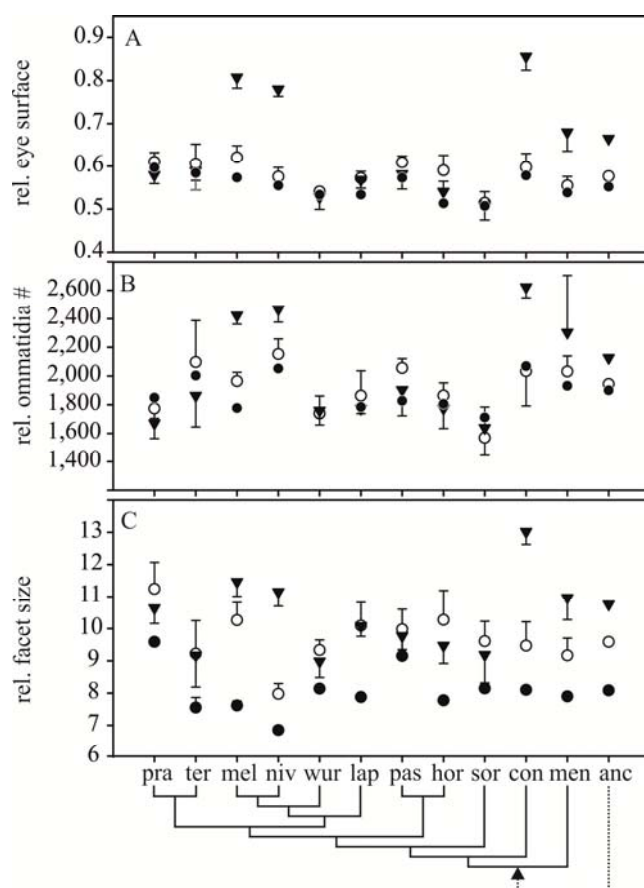
Workers differed significantly in absolute (abs:  $H_{10,66}=36.7$ ;  $p<0.001$ ) and relative eye size (rel:  $H_{10,65}=36.4$ ;  $p<0.001$ ) among species. While relatively large eyes were found e.g. in *B. melaleucus* and *B. pascuorum*, relatively small eyes were observed in *B. soroensis* and *B. wurflenii*. Significant differences between species were also noted for ommatidia number (abs:  $H_{10,52}=34.2$ ;  $p<0.001$ ; rel:  $H_{10,51}=32.7$ ;  $p<0.001$ ) and facet diameters (abs:  $H_{10,69}=41.2$ ;  $p<0.001$ ; rel:  $H_{10,68}=31.2$ ;  $p<0.001$ ). The highest relative number of ommatidia occurred in *B. niveatus*, *B. terrestris* and *B. pascuorum*, while *B. soroensis* workers had the least amount of ommatidia. *Bombus pratorum* workers possessed the largest facets, relative to body size, followed by *B. hortorum* and *B. melaleucus* (Fig. 1). Eye shape differed slightly between species and varied from nearly elliptic (e.g. *B. mendax*) to pear-like (e.g. *B. hortorum*; see Fig S1). The largest facets were usually located in the fronto-central and fronto-ventral regions of the eye (Fig. 2, Fig S1). Median ocellus size differed significantly among species (abs:  $H_{10,77}=40.2$ ;  $p<0.001$ , rel:  $H_{10,76}=30.3$ ;  $p<0.001$ ), with the largest ocelli found in *B. melaleucus* and *B. niveatus* (Table 1). In queens, similar values and differences among species were obtained (Table 1). Compared with workers, relative eye size was similar, but queens had smaller facets in relation to body size (Fig. 1). Due to the small sample size (usually  $n=1$  for each species, Table 1), no statistical comparisons were performed for queens.



**Table 1** Morphological measurements of 11 *Bombus* species

Species	sex/caste	its (mm)	1 <sup>st</sup> discoidal cell (mm)	median ocellus (mm)	eye surface (mm <sup>2</sup> )	ommatidia #	facet diameter ( $\mu$ m)
<i>B. pratorum</i>	queen	4.6 [1]	3.1 [1]	0.30 [1]	3.5 [1]	5,805 [1]	30.1 [1]
	worker	3.3 (0.2)[4]	2.4 (0.2)[4]	0.23 (0.01)[4]	2.2 (0.3)[4]	4,301 (373)[4]	27.1 (0.7)[4]
	male	3.2 (0.2)[4]	2.7 (0.1)[4]	0.25 (0.01)[4]	2.4 (0.3)[4]	4,492 (342)[4]	28.5 (0.7)[4]
<i>B. terrestris</i>	queen	5.4 (0.2)[8]	3.9 (0.1)[8]	0.38 (0.01)[8]	5.0 (0.4)[8]	7,691 [1]	29.3 (0.6)[8]
	worker	3.9 (0.6)[14]	2.7 (0.4)[14]	0.28 (0.04)[15]	2.8 (0.6)[15]	5,656 (475)[12]	25.1 (1.9)[15]
	male	4.0 (0.4)[13]	3.0 (0.3)[13]	0.31 (0.03)[13]	3.1 (0.4)[12]	5,624 (369)[8]	27.4 (1.3)[13]
<i>B. melaleucus</i>	queen	6.2 (0.1)[2]	4.8 (0.0)[2]	0.39 (0.0)[2]	7.5 (0.1)[2]	8,528 [1]	36.4 (0.7)[2]
	worker	4.3 (0.2)[3]	2.9 (0.1)[3]	0.30 (0.02)[3]	3.2 (0.3)[3]	5,659 (153)[3]	29.5 (0.9)[3]
	male	5.3 (0.2)[2]	3.4 (0.0)[2]	0.36 (0.00)[2]	7.7 (0.3)[2]	8,299 (127)[2]	39.3 (1.0)[2]
<i>B. niveatus</i>	queen	6.0 [1]	4.2 [1]	0.42 [1]	5.4 [1]	8,617 [1]	28.8 [1]
	worker	4.5 (0.4)[4]	3.4 (0.1)[4]	0.33 (0.03)[4]	3.7 (0.4)[4]	7,230 (437)[4]	26.8 (0.8)[4]
	male	4.5 (0.1)[4]	3.3 (0.1)[4]	0.34 (0.00)[4]	6.5 (0.3)[4]	8,051 (278)[4]	36.4 (0.8)[4]
<i>B. wurfleini</i>	queen	5.5 [1]	4.0[1]	0.34[1]	4.6[1]	6,960[1]	32.6[1]
	worker	3.8 (0.1)[4]	3.0 (0.1)[4]	0.27 (0.02)[4]	2.6 (0.2)[4]	5,213 (208)[4]	27.9 (0.6)[4]
	male	3.8 (0.3)[4]	3.2 (0.2)[4]	0.30 (0.01)[4]	2.8 (0.3)[4]	5,604 (275)[4]	28.6 (1.1)[4]
<i>B. lapidarius</i>	queen	4.9 [1]	3.8 [1]	0.38 [1]	4.1[1]	6,765[1]	29.9 [1]
	worker	3.5 (0.4)[4]	2.6 (0.2)[4]	0.3 (0.0)[4]	2.2 (0.4)[4]	4,800 (652)[4]	25.9 (1.5)[4]
	male	3.3 (0.2)[5]	2.9 (0.2)[5]	0.3 (0.0)[5]	2.7 (0.3)[5]	5,214 (336)[5]	29.3 (1.5)[5]
<i>B. hortorum</i>	queen	4.4 [1]	3.9 [1]	0.31 [1]	4.0 [1]	7,010[1]	30.2[1]
	worker	3.5 (0.3)[4]	2.8 (0.3)[4]	0.25 (0.02)[4]	2.7 (0.4)[4]	5,170 (443)[4]	28.4 (1.6)[4]
	male	3.1 (0.3)[4]	3.0 (0.3)[4]	0.25 (0.02)[4]	2.6 (0.5)[4]	5,232 (272)[4]	28.0 (1.9)[4]
<i>B. pascuorum</i>	queen	4.4[1]	3.5[1]	0.33[1]	4.1 (0.0)[1]	6,426 [1]	32.2 [1]
	worker	3.4 (0.4)[4]	2.8 (0.3)[4]	0.25 (0.02)[4]	3.0 (0.5)[4]	5,803 (519)[4]	28.0 (1.4)[4]
	male	3.4 (0.1)[4]	3.0 (0.2)[4]	0.27 (0.02)[4]	3.0 (0.1)[4]	5,666 (268)[4]	29.1 (0.7)[4]
<i>B. soroeensis</i>	queen	4.4 [1]	3.5 [1]	0.31[1]	3.2 [1]	6,042[1]	28.8 [1]
	worker	3.4 (0.2)[4]	2.7 (0.2)[4]	0.24 (0.00)[4]	1.9 (0.1)[4]	4,250 (155)[4]	26.0 (0.5)[4]
	male	3.4 (0.1)[4]	3.1 (0.3)[4]	0.26 (0.00)[4]	2.4 (0.2)[4]	4,968 (131)[4]	27.8 (0.8)[4]
<i>B. confusus</i>	queen	5.1[1]	3.7 [1]	0.35[1]	4.5 [1]	7,569 [1]	29.7 [1]
	worker	3.9 (0.2)[8]	2.8 (0.2)[8]	0.25 (0.01)[8]	2.9 (0.3)[8]	5,870 (400)[4]	26.7 (0.9)[8]
	male	4.9 (0.1)[6]	3.0 (0.1)[6]	0.33 (0.01)[6]	6.6 (0.6)[6]	7,821 (145)[4]	39.2 (0.9)[6]
<i>B. mendax</i>	queen	4.5 [1]	3.6 [1]	0.35 [1]	3.7 [1]	6,868 [1]	28.1 [1]
	worker	3.7 (0.2)[11]	2.7 (0.2)[11]	0.25 (0.01)[11]	2.2 (0.2)[11]	5,375 (357)[4]	24.5 (0.8)[11]
	male	4.1 (0.4)[5]	3.1 (0.3)[5]	0.28 (0.01)[5]	4.4 (0.4)[5]	7,032 (260)[4]	34.0 (1.6)[5]

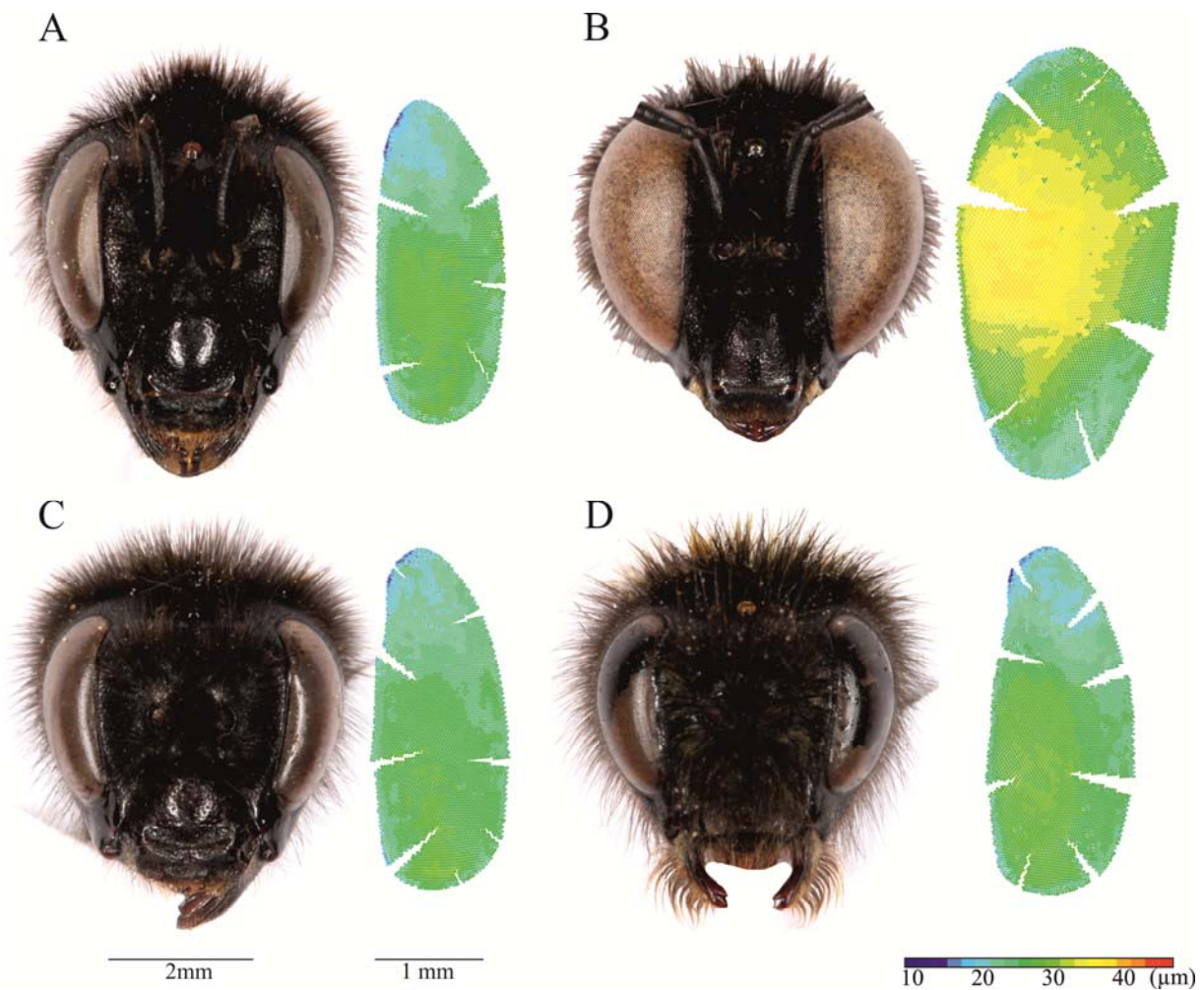
Values are reported as means, S.D. is given in parenthesis, followed by sample size in brackets. its – intertegulae span.



**Figure 1** *Relative eye parameters in Bombus*  
 Relative eye size (a), ommatidia number (b) and facet diameter (c), of females (queens - open circles, workers - filled circles) and males (filled triangles) in *Bombus*. All parameters were corrected for individual body size (1<sup>st</sup> discoidal cell length of the forewing). Error bars denote standard deviation and are only presented for one direction to improve the readability of the figure (black for workers and males, white for queens). The species are sorted according to their phylogenetic relationship (see cladogram at the bottom). 'pra' – *Bombus pratorum*, 'ter' – *B. terrestris*, 'mel' – *B. melaleucus*, 'niv' – *B. niveatus*, 'wur' – *B. wurflenii*, 'lap' – *B. lapidarius*, 'pas' – *B. pascuorum*, 'hor' – *B. hortorum*, 'sor' – *B. soroensis*, 'con' – *B. confusus*, 'men' – *B. mendax*. 'anc' refers to the reconstructed parameters for the common ancestor of the study species.

### Male eye morphology

Similar to workers, males of all species differed with respect to eye size (abs:  $H_{10,53}=41.3$ ;  $p<0.001$ ; rel:  $H_{10,53}=43.6$ ;  $p<0.001$ ), ommatidia number (abs:  $H_{10,46}=38.0$ ;  $p<0.001$ ; rel:  $H_{10,46}=32.0$ ;  $p<0.001$ ), facet diameters (abs:  $H_{10,57}=41.7$ ;  $p<0.001$ ; rel:  $H_{10,57}=42.8$ ;  $p<0.001$ ) and ocellus size (abs:  $H_{10,62}=47.5$ ;  $p<0.001$ ; rel:  $H_{10,62}=47.7$ ;  $p<0.001$ ). Males of four species (*B. mendax*, *B. confusus*, *B. niveatus*, *B. melaleucus*) had distinctively enlarged compound eyes compared with workers, which were between c. 50% (*B. mendax*) and c. 100% (*B. confusus*) larger in relative surface area (Fig. 1, Fig S1). The enlargement of the male eyes is a result of having more and larger facets compared with workers; however, the contribution of both factors differed among species (Fig. 1). While *B. mendax* and *B. confusus* had both larger



**Figure 2** Eye morphology and facet size distribution

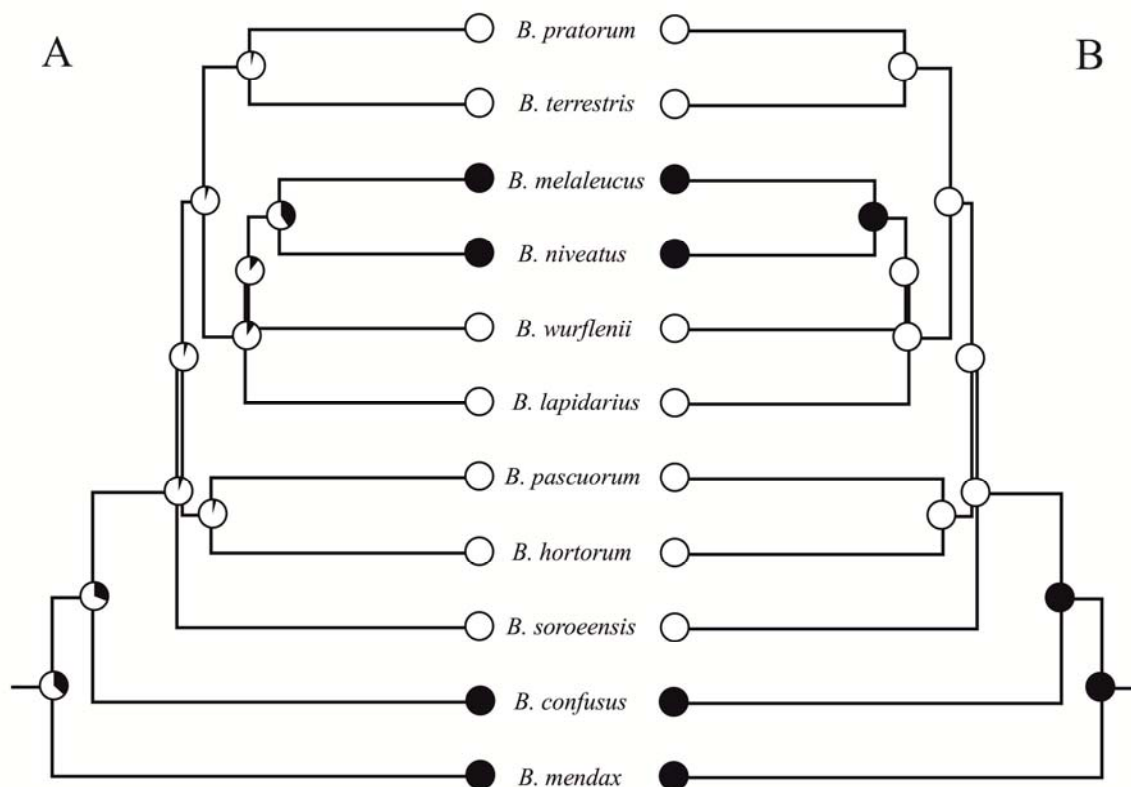
Representatives of the two major mating strategies within the genus *Bombus*. *Bombus confusus* (A, B) adopts a visually guided perching strategy, while *B. terrestris* (C, D) patrols scent routes. Males of *B. confusus* (B) have strongly enlarged eyes, relative to workers (A), while males (D) and workers (C) of *B. terrestris* do not differ in their appearance. Eye maps illustrate the distribution of facet lens diameters across the eye surface. While in *B. terrestris* (both sexes) and *B. confusus* workers the largest facets are found in the fronto-ventral region of the eye, males of *B. confusus* have an extended acute zone located in the frontal region of the eye, which is characterized by increased facet diameters.

Heads and eye maps, respectively, are to scale (left scale bar in C – heads; right scale bar in C – eye maps). Note that the individuals used for the photographs of the head and for production of the eye maps must not necessarily match.

and more facets, the enlargement of the compound eye is more greatly attributed to enlarged facets in *B. niveatus* and increased ommatidia numbers in *B. melaleucus*. However, this observation may be an artifact, owing to the fact that *B. niveatus* workers also have a higher number of ommatidia compared with workers of other species, and *B. melaleucus* workers have relatively large facets. In absolute terms, males of all four species have distinctively larger facets ( $>33\mu\text{m}$ ) and more ommatidia ( $> 7,000$ ) than males of the remaining species ( $<30\mu\text{m}$  and  $< 6,000$ , respectively). The largest facets in males were found in the same eye regions as in workers, except for the four large-eyed species, where they are located in a fronto-central acute zone (Fig. 2, Fig. S1).

*Ancestral character state reconstruction*

The ancestral character state reconstruction yielded different results with respect to the root node character. While parsimony reconstruction suggested that male enlarged eyes are the ancestral state in both datasets (11 and 198 taxa, respectively, Fig. 3, Fig. S2), the results of the maximum likelihood analysis are less clear. Maximum likelihood modeling resulted in a 36.6% likelihood (small dataset, Fig. 3) or a 70.6 % likelihood (large dataset, Fig. S3) of enlarged male eyes, respectively. In both cases the probability of the ancestral character is not statistically significant (1:7.4 ratio criterion; Pagel, 1997).



**Figure 3** *Ancestral character state reconstruction*

Reconstruction of the sexual eye dimorphism based on categorical eye size information. Ancestral character states were reconstructed using maximum likelihood (a) and parsimony (b) in the 11 study species, based on a molecular phylogeny by Hines (2008). Branch lengths are proportional to divergence time. Note that branch lengths are ignored in the parsimony analysis. Filled circles indicate species with enlarged male eyes, and open circles species with unenlarged eyes. (a) Pie charts at the nodes illustrate the proportional probabilities for either state at the nodes in the maximum likelihood analysis. (b) Circles indicate the character for each node, leading to the most parsimonious reconstruction.

Based on the analysis of the majority of *Bombus* species, the number of transitions between enlarged and not enlarged male eyes is 8 (parsimony reconstruction; gains and losses equally weighted) or 8-9 (maximum likelihood analysis; Figs. S2, S3). While assuming the independent origin of enlarged male eyes in the early diverging taxa (*Mendacibombus* + *Bombias*) and members of the clade “*Alpigenobombus* + *Melanobombus* + *Sibiricobombus* + *Cullumanobombus*” appears safe (Fig 3, Figs. S2, S3), assumptions on the exact number of transitions within the latter clade are vague.

In addition to categorical character modeling, we estimated the ancestral characters of relative eye size, ommatidia number and facet size of all three castes from continuous data using phylogenetic independent contrasts (Felsenstein, 1985). The obtained values suggest that male of the common ancestor had slightly enlarged compound eyes (m:w-ratio 1.15, which corresponds to c. 30% larger eye surface than in workers of the same body size), as a result of both enlarged facet diameters and increased ommatidia numbers compared with workers (Fig. 1).

## Discussion

In our study we investigated eye size and compound eye morphology between the castes and sexes in 11 subgenera of bumblebees. In females, eye size is similar among species, but differences in the functional morphology were found that match species specific lifestyles. Males differ more clearly across species and, most importantly, between mating systems. Males that adopt a visually guided perching strategy are characterized by enlarged compound eyes with many and large facets, while patrolling species do not show a distinct sexual dimorphism in eye size, facet size or ommatidia number. Phylogenetic reconstructions suggest that the common ancestor of all extant bumblebee species had similar eye morphology with the male eyes being slightly enlarged, as in the earliest diverging subgenus *Mendacibombus*. Furthermore, enlarged male eyes and visually guided mating strategies must have re-evolved several times independently within *Bombus*.

### *Female visual ecology and eye morphology*

Bumblebee colonies consist of two female castes, a reproductive queen and several hundreds of sterile workers. Both forage for nectar and pollen, orient between food sources and the nest location and face predation risk; their sensory and neural systems have correspondingly adapted to these challenges (Ings and Chittka, 2008; Morawetz and Spaethe, 2012; Spaethe et al., 2001). Queens only forage during the initial colony phase which may explain the slightly smaller relative eye size in all investigated species (Table 1, Fig. 1). However, the eye size differences between the female castes are small compared with honeybees (Streinzer et al., 2013). In workers, body size differs strongly within a colony. The sensory organs scale with body size, and larger workers have more sensitive and acute olfactory and visual systems (Kapustjanskij et al., 2007; Spaethe et al., 2007; Spaethe and Chittka, 2003). After correcting for intra-specific body size differences, we found variation in compound eye parameters among species. *B. pratorum* and *B. melaleucus* have relatively large facets (Fig. 1). Both species forage in forests and at forest borders (Hoffmann et al., 2004; Reinig, 1970, and own observations by Streinzer M.), and the larger facets may constitute an adaptation to low light environments. In contrast, the smallest relative facet size is observed in *B. niveatus*, which inhabits high altitude steppic environments, characterized by higher ambient light levels (Reinig, 1930). Such environments with their characteristic light intensity and spatial frequencies determine the optimum ratio of ommatidia number and facet diameters, to maximize information capacity of the eye (Snyder et al., 1977). Our results emphasize that the visual system of bumblebees is adapted to the local environment

in which the species in question typically nests and forages. Experimental work on workers of *B. hortorum*, *B. terrestris* and workers and males of *B. lapidarius* has revealed differences in light intensity thresholds at which they were able to fly (Kapustjanskij et al., 2007). These observations are consistent with the observed differences in facet diameters (Fig. 1, Table 1). Foraging environments not only differ in their ambient light levels, but also in the spectral composition of the light, and thus further species-specific adaptations of the physiology and neural circuitry of the compound eye can be expected.

Bumblebees became an important model system for learning and memory, visual and olfactory perception, natural and sexual selection and pollination ecology (Baer, 2003; Plowright and Lavery, 1984). Several species are now commercially available for pollination purposes, and used in scientific investigations. Sensory ecology and perception have been studied in *B. terrestris* (Morawetz and Spaethe, 2012; Spaethe et al., 2001), *B. occidentalis* (Riveros and Gronenberg, 2009), *B. ignitus* (Pfeiffer and Kinoshita, 2012) and *B. impatiens* (Macuda et al., 2001; Riveros and Gronenberg, 2012). The former species belong to the subgenus *Bombus s. str.* while *B. impatiens* is a member of *Pyrobombus*. We found relatively large differences in facet size, ommatidia number and relative eye size between members of both subgenera which suggest that whenever visual resolution or light sensitivity plays a role in study design and analysis, visual parameters should be evaluated for the species that is studied.

#### *Male mating behavior and eye morphology*

Male mating strategies in *Bombus* are usually species-specific, despite some degree of plasticity (Williams et al., 2008). In the majority of species, males follow scent marked routes. The scent marks likely function to help males keep track of their route, but it has also been suggested that they attract and ‘arrest’ queens until the next transit by the male (Free, 1971). Detecting the queen in a spatially restricted area is not a heavily demanding task for the visual system and likely does not differ substantially from flower detection in workers. When male individuals with improved vision have no reproductive advantages that outweigh the costs of maintaining an enlarged visual system, then improved vision is not likely to persist in a population. Generally, relative eye size does not differ between sexes in those species that show patrolling behavior (*B. hortorum*, *B. lapidarius*, *B. pascuorum*, *B. pratorum*, *B. soroensis*, *B. terrestris* and *B. wurfleini*).

The second major mating strategy of bumblebees is mainly visually guided (“racing” and “territorial”, *sensu* Williams, 1991)). Males usually perch on stationary, elevated or otherwise distinctive spots and wait for passing queens. Detection of a small, moving, spatially and temporally unpredictable object is much more demanding for a visual system and thus selection that favors elaboration of the visual system can be expected (Vallet and Coles, 1993). Improved visual abilities of the “racing/territorial” males are indicated by their enlarged eyes. In our study such enlarged eyes are found in males of *B. mendax*, *B. confusus*, *B. niveatus* and *B. melaleucus*. Males of the first two named species are known to perch on the ground or on elevated structures and dart toward flying queens, as well as other moving objects (Aichhorn, 1976; Haas, 1976;

Schremmer, 1972, and own observations by Streinzer M.). The mating behavior strategy of *B. niveatus* has not been investigated but is likely to be similar to that of the closely related *B. asiaticus* and *B. sulfureus* (“racing/cruising”; Rasmont et al., 2005; Williams, 1991). Likewise, the mating behavior of *B. melaleucus* is probably similar to that of the closely related species *B. robustus*, *B. griseocollis* and *B. rufocinctus* (“racing/territorial”; O'Neill et al., 1991; van der Pijl and Dodson, 1966).

Functionally, enlarged eyes permit a general improvement of both spatial resolution and contrast sensitivity. The large frontal facets further suggest the development of an acute zone with very high spatial resolution and contrast sensitivity (Land, 1997). Additionally, neuronal pooling strategies and differences in photoreceptor physiology may improve the target detection abilities of males in perching species (Menzel et al., 1991; Straw et al., 2006). Enlarged male eyes evolved several times independently within *Bombus* (see below) and differences in the developmental basis for the enlargement relative to workers are observed (predominantly increased ommatidia numbers vs. facet diameters). Despite these relative differences, all four species have remarkably similar eye morphologies with a high number of ommatidia (>7,000) and large frontal facets (>33 $\mu$ m). These similarities indicate convergent evolution to very similar visual tasks, despite small variation in the mating behavior (Williams, 1991).

Aside from these two broad mating behavior categories, several other strategies have been described for a few species. For example, Williams (1991) noted that *B. (Alpigenobombus) kashmirensis* differs from its close relative *B. wurflenii* in both mating strategy and the dimorphism of the visual system. *Bombus kashmirensis* males have slightly enlarged eyes and a cruise mating strategy, i.e. hover in small area, while *B. wurflenii* males have unenlarged eyes and patrol scent routes. Consistent with this observation, we found no striking sex differences in *B. wurflenii* with respect to eye size, facet diameters and ommatidia numbers (Table 1, Fig.1, Fig. S1). Behavioral and morphological differences among closely related species also evolved repeatedly within the subgenus *Melanobombus* (Williams, 1991). In our study species, *B. lapidarius* males have a similar eye size as workers, but relatively enlarged facets. The scent routes of the males are unusual in being located in the crown regions of trees and shrubs which are approached with a rapid circling flight (Bringer, 1973, and own observations by Streinzer, M.). The male behavior thus superficially resembles the cruising behavior described for *B. kashmirensis* (Williams, 1991) and may represent an intermediate step between the two strategies.

#### *Visual vs. olfactory adaptations*

Most likely, males of all bumblebee species utilize visual and olfactory signals and cues for mate detection. Species that patrol use olfactory signals to keep track of the route and discriminate between queens and workers through sex pheromones, but also react to visual signals that are presented close to the scent marks (Free, 1971; Krieger et al., 2006). Males that perch mark their perch with glandular secretions (Hovorka et al., 1998; Kindl et al., 1999; O'Neill et al., 1991), but visual cues are sufficient to trigger chasing behavior (Schremmer, 1972). However, since

objects other than queens are usually followed for a short distance only, sex pheromones presumably also play a role in their mating behavior (Kindl et al., 1999).

Depending on the relative importance of visual vs. olfactory signals and cues, we expect trade-offs between the sensory systems of the males to have occurred. Maintenance of sensory organs and neural tissue is metabolically extremely costly (Niven and Laughlin, 2008) and the development of certain structures is constrained by nearby developing tissue (Emlen, 2001). While perching males have enlarged compound eyes, a reciprocal dimorphism of the olfactory system may be expected in patrolling species. However, Williams (1991) and Ågren *et al.* (1996) did not find any significant correlation between mating strategy and antennal size or functional morphology. Functional adaptations of male olfactory system are often found at higher levels of the olfactory pathway. Investigations of the antennal lobe glomeruli allow estimates about the complexity of the olfactory coding and relative sensitivity for odorants (Hansson and Stensmyr, 2011). Male honeybees (Apini) and long-horned bees (Eucerini) have a few hypertrophied olfactory glomeruli, which are beneficial in long-distance olfactory detection (Arnold et al., 1985; Streinzer et al., in press), while species that detect females in spatially restricted rendezvous areas lack such apparent adaptations (e.g. *Andrena nigroaenea*, Andrenidae, unpublished data Streinzer, M.). We suggest that investigations of the antennal lobe may reveal interesting associations between mating behavior and olfactory adaptations in *Bombus*.

#### *Ancestral character state and the evolution of the male visual system*

Based on our measurements and literature data, we reconstructed the ancestral state of the visual system in bumblebees. Although the basal node character is undecided in the maximum likelihood analyses (~40% and ~70% likelihood of enlarged male eyes in the small and large dataset, respectively; Fig. 3, Fig. S3), both parsimony analysis and modeling of the eye parameters using phylogenetic independent contrasts favor an ancestor that had enlarged male eyes and thus was potentially adapted to a perching or cruising mating strategy (Fig. 1, Fig. 3, Fig. S2). Multiple transitions between enlarged and non-enlarged male eyes occurred, but there is some uncertainty about their exact number. The reasons for this uncertainty are three-fold. First, there is some ambiguity in the topography of the phylogeny at some of the nodes where transitions are likely to have occurred (Cameron et al., 2007; Hines, 2008). Second, not all extant species are represented in the phylogeny; e.g. Williams (1991) notes that *B. (Ml.) semenovianus* (Skorikov) has enlarged male eyes and groups this species with *B. lapidarius*, which would result in one additional independent occurrence of enlarged male eyes in *Melanobombus*. Third, eye dimorphism is a continuous trait, although our morphological data suggests that there are only enlarged (>50% larger eye size in males compared to females) and normal eyes with no intermediate forms (Fig. 1). Several bumblebee species show only slightly enlarged male eyes (Richards, 1968; Williams, 1991) and their mating strategy may also be intermediate between the major strategies (see above). Slightly enlarged male eyes evolved in several species of *Alpigenobombus* and *Melanobombus*, but are also found in the early diverging species of the



subgenera *Cullumanobombus* and *Sibiricobombus*, suggesting an independent evolution of enlarged male eyes in the latter two subgenera.

Irrespective of the uncertainty in the reconstruction, three major conclusions can be drawn. First, enlarged male eyes are not present in any member of the two large clades “*Orientalibombus* + *Subterraneobombus* + *Megabombus* + *Thoracobombus* + *Psithyrus*” and “*Pyrobombus* + *Alpinobombus* + *Bombus*”. Males of these species patrol scent marked routes close above the ground or aggregate at the nest entrance to await emerging queens (Williams et al., 2008). Second, enlarged eyes are likely the ancestral state being found in the early diverging species of the subgenera *Mendacibombus* and *Bombias*. The visual system of the common ancestor of all extant *Bombus* species likely resembled the eyes of the subgenus *Mendacibombus* (Fig. 1). Third, all other species with enlarged or slightly enlarged male eyes are members of a clade uniting “*Alpigenobombus* + *Melanobombus* + *Sibiricobombus* + *Cullumanobombus*”. In this group several independent transitions occurred between normal and enlarged male eyes and between patrolling and racing/territorial/cruising mating strategies (Figs. S1, S2, Williams, 1991). The only slightly enlarged male eyes and cruising strategies in some species further suggest that these traits may constitute an important intermediate step between strictly patrolling (no eye dimorphism) and perching (large eye dimorphism) strategies.

Why this clade has been more susceptible to these transitions than the large clades of strictly patrolling species cannot be presently explained and is not correlated with any known distinct behavioral, metabolic or otherwise phylogenetically constrained trait.

Many studies have so far found an association between male size and mating tactics in bees. Alcock (1994) showed that in *Hylaeus alcyoneus* (Colletidae), body size is a strong predictor for the ability to defend a territory which ultimately results in intra-specific plasticity in the mating strategy. Large males perch on flowers while smaller males patrol regular routes. In *Bombus*, males of perching species are relatively large compared with most patrolling species (Table 1; del Castillo and Fairbairn, 2012). However, large males are also found in other subgenera (e.g. *Thoracobombus*), while males of *Alpigenobombus* + *Melanobombus* are only intermediate in body size (del Castillo and Fairbairn, 2012; Medler, 1962). The large body size in perching species may thus be a result of the demands for fast pursuit flights, rather than a pre-requisite for the evolution of a perching strategy. Rather than absolute male body size, the magnitude of size differences among males in a population may constitute an important prerequisite for the evolution of alternative male tactics (Cade, 1980). The only available data on size variation among males include members of only few subgenera and fail to show a clear pattern (Medler, 1962).

In carpenter bees (Xylocopini), comparative correlated changes of male eye dimorphism and mating strategy are found. Leys & Hogendoorn (2008) traced the correlated evolution of the morphological and behavioral traits and concluded that changes from non-defense (patrolling) and resource-defense strategies to non-resource defense strategies (territorial, racing) are more

likely to be preceded by eye enlargement than *vice versa*. Furthermore, some combinations (e.g. non-defense and enlarged eyes) were presumably instable and thus either resulted in reversal of the mating strategy or eye reduction. In bumblebees, we suggest that the large clade “*Alpigenobombus* + *Melanobombus* + *Sibiricobombus* + *Cullumanobombus*” may represent an interesting comparative study system to investigate how changes in the functional adaptations of the eye (facet size and number) and mating systems correlate with each other.

### **Conclusion and future perspective**

In a survey covering the female castes and males in most subgenera of the genus *Bombus*, we report functional adaptations of the visual system. We identified adaptations that increase the power of the visual system with respect to spatial resolution and sensitivity in males of four species that adopt a visual mate detection strategy, while members of patrolling species show no distinct eye size sexual dimorphism. Differences in worker eye morphology generally correlated with foraging preferences. These differences in functional adaptation of the worker eye should be taken into account when behavioral and physiological studies are compared among *Bombus* species. We advocate future research, in particular, to increase our knowledge about mating behavior and the associated signals but also to widen our knowledge of sex-specific sensory adaptations to generate a more comprehensive picture about the evolutionary history of mating behavior within bumblebees.

### **Acknowledgements**

We thank K. Möller for her invaluable help with data collection, T. Küpper, J. Neumayer, H.F. Paulus, F. Etl and S. Pfabigan for providing bumblebee specimens and J. Plant for linguistic help. Further thanks are due to D. Zimmermann (Naturhistorisches Museum Wien) and F. Gusenleitner (Oberösterreichisches Landesmuseum, Linz) for access to museum collections. MS is recipient of a DOC-fellowship of the Austrian Academy of Sciences at the Department of Behavioral Physiology and Sociobiology, University of Würzburg and the Department of Evolutionary Biology, University of Vienna.

### **Author contributions**

MS and JS designed the study and collected samples, MS collected and analyzed the data and drafted the manuscript. JS commented on the draft. Both authors read and approved the final version of the manuscript.

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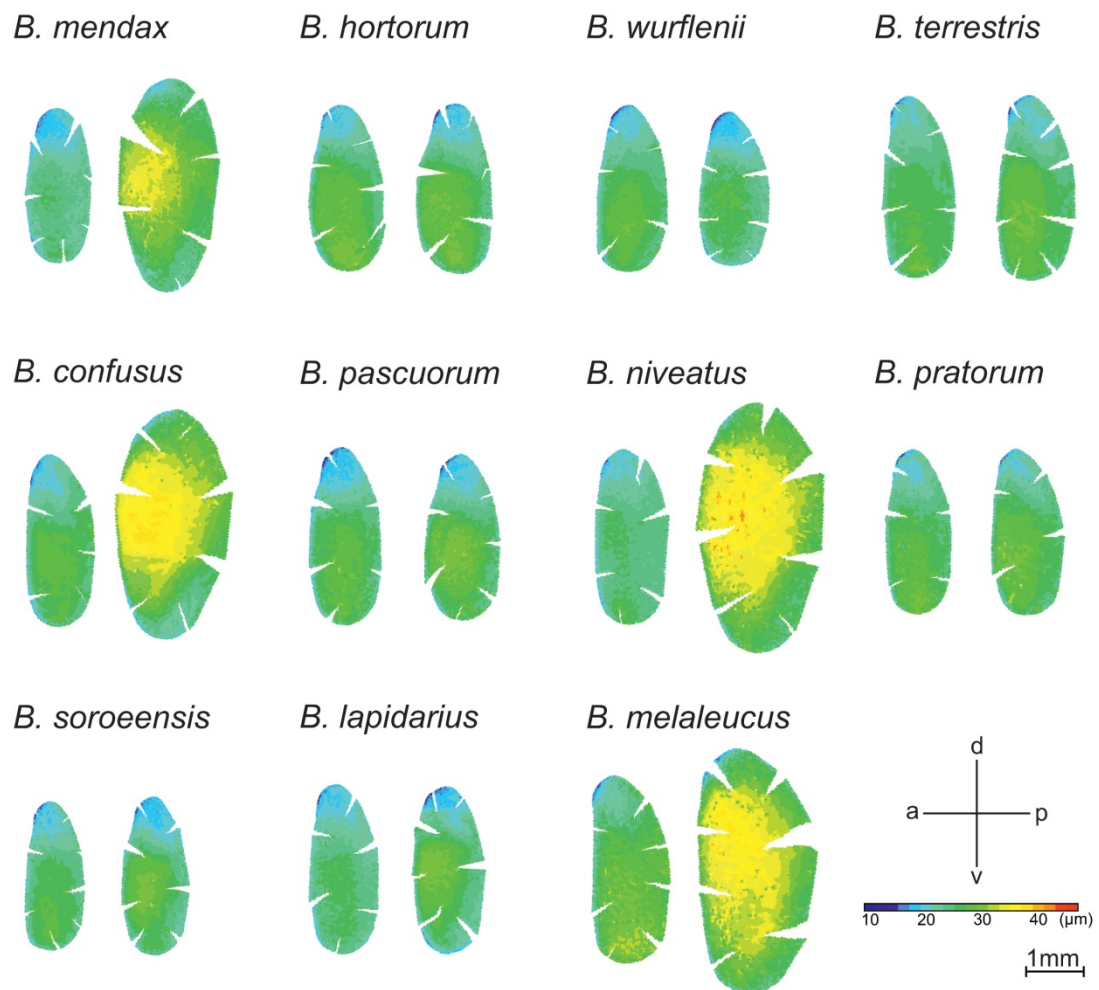
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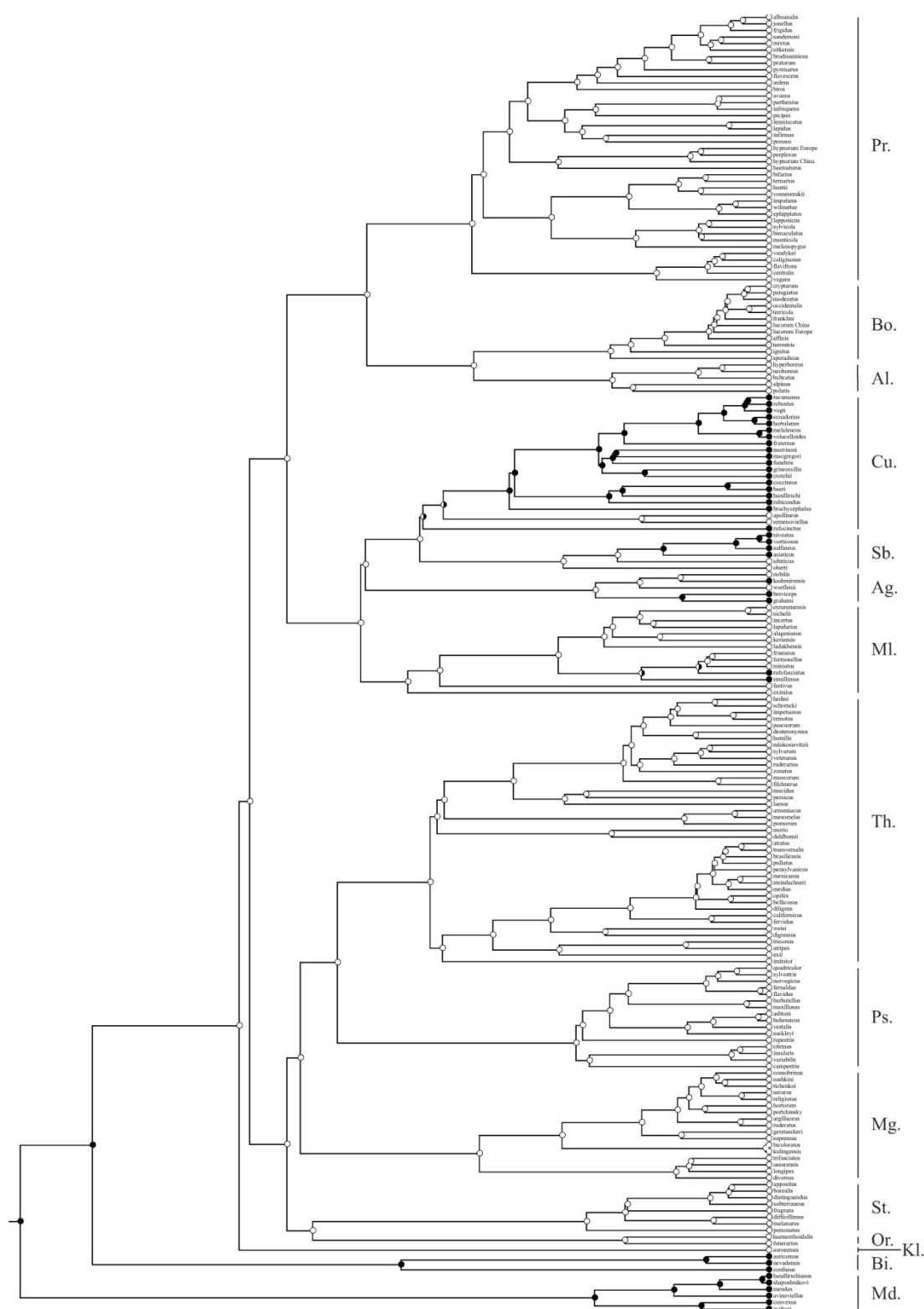
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## Supporting information



**Figure S1** Eye maps of all species

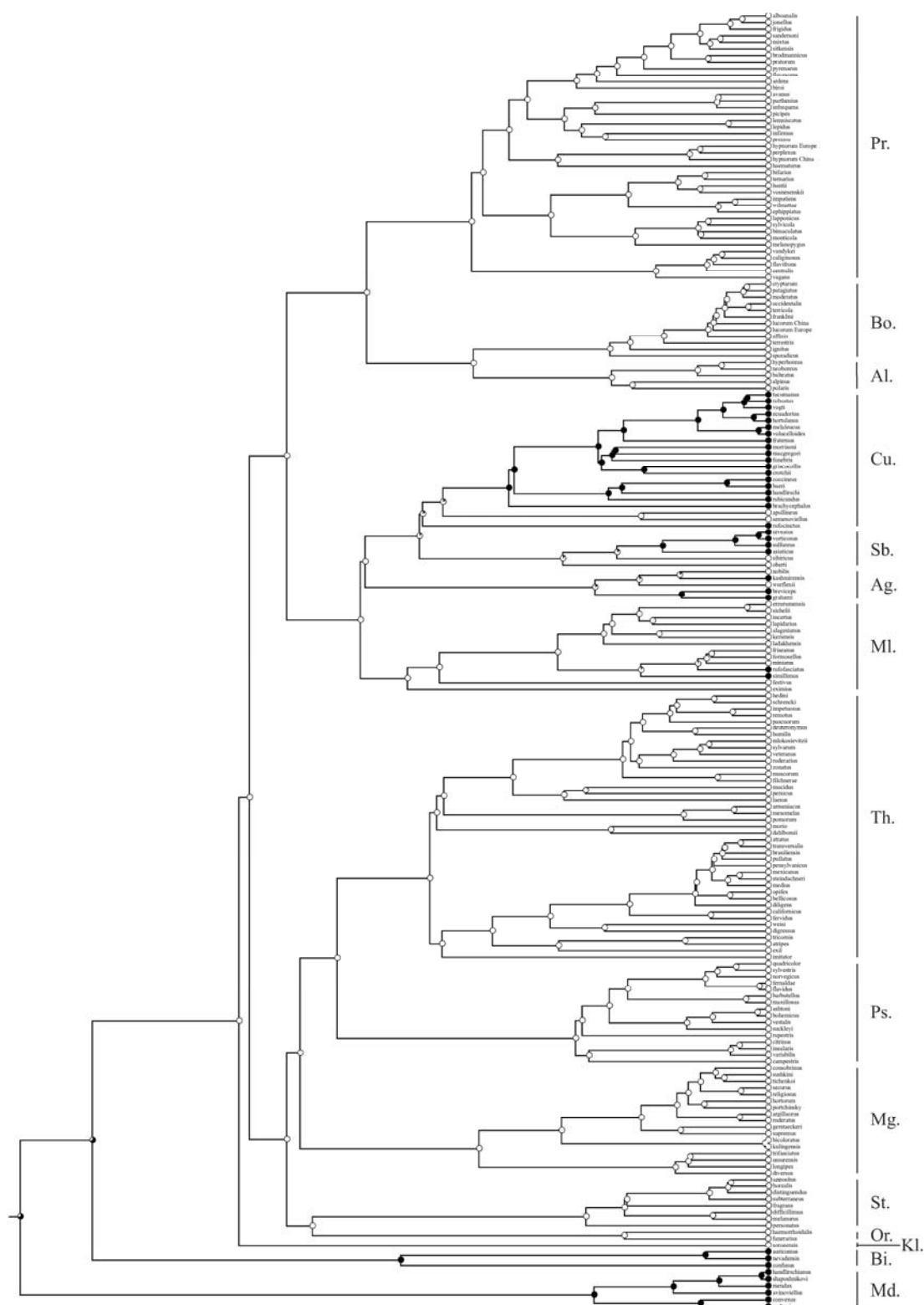
Eye maps of one worker (left) and a male (right) illustrating the distribution of facet lens diameters across the eye surface in all study species. All maps are to scale. The orientation bar applies to all maps, f-frontal, l-lateral, d-dorsal, v-ventral.



**Figure S2** Parsimony ancestral character state reconstruction from a larger dataset

Ancestral character state reconstruction of sexual eye dimorphism in 198 *Bombus* taxa using parsimony modeling in Mesquite. Male eye size information was scored as un-enlarged (white circles) and enlarged (black circles) relative to females, based on literature data and own observations (see Table S1). Branch lengths of the phylogeny correspond to the divergence times of the species (Hines, 2008). Note that the parsimony analysis ignores branch length differences. Subgeneric classification presented at the right according to (Williams *et al.* 2008). Md. - *Mendacibombus*, Bi. - *Bombias*, Kl. - *Kallobombus*, Or - *Orientalibombus*, St. - *Subterraneobombus*, Mg - *Megabombus*, Ps. - *Psithyrus*, Th. - *Thoracobombus*, MI. - *Melanobombus*, Ag. - *Alpigenobombus*, Sb. - *Sibiricobombus*, Cu. - *Cullumanobombus*, Al. - *Alpinobombus*, Bo. - *Bombus s.str.*, Pr. - *Pyrobombus*.





**Figure S3** Maximum likelihood ancestral character state reconstruction from a larger dataset  
 Ancestral state reconstruction of sexual eye size dimorphism in 198 *Bombus* taxa using maximum likelihood in Mesquite. Male eye size information was scored as un-enlarged (white circles) and enlarged (black circles) relative to females, based on literature data and own observations (see Table S1). Branch lengths of the phylogeny correspond to the divergence times of the species (Hines, 2008). Pie charts at the nodes illustrate proportional likelihood for either state. A likelihood ratio of 7.4:1 is considered a statistically significant support for a character state at a given node (Pagel, 1996). Subgeneric classification presented at the right according to (Williams *et al.* 2008). Md. - *Mendacibombus*, Bi. - *Bombias*, Kl. - *Kallobombus*, Or - *Orientalibombus*, St. - *Subterraneobombus*, Mg - *Megabombus*, Ps. - *Psithyrus*, Th. - *Thoracobombus*, Ml. - *Melanobombus*, Ag. - *Alpigenobombus*, Sb. - *Sibiricobombus*, Cu. - *Cullumanobombus*, Al. - *Alpinobombus*, Bo. - *Bombus s.str.*, Pr. - *Pyrobombus*.

**Table S1** *Bumblebee species and their eye size dimorphism*

Literature data on eye size dimorphism for *Bombus* species represented in the phylogeny of Hines [1]. Species names, synonyms, authority and subgenera according to the world bumblebee species list maintained by PH Williams (<http://www.nhm.ac.uk/research-curation/research/projects/bombus/> accessed 14.03.2013). Synonyms are indicated when either the current species name differs from the name published by Hines [1] or two species have since been synonymised.

Male eye size was scored as '0' (male eyes relatively un-enlarged, compared with females) or '1' (male eyes relatively enlarged), without further sub-classification of the extent of eye size dimorphism. Eye size dimorphism information was taken from the stated literature. When no literature record was available, eye size was judged by the first author (indicated as 'MS'), based on material from various collections (coll. M Streinzer; Naturhistorisches Museum Wien; Oberösterreichisches Landesmuseum, Linz). Whenever possible the literature information was double checked with museum material by MS (not specifically mentioned).

species	subgenus	eyes	reference
<i>Bombus avinoviellus</i> (Skorikov), 1914	<i>Mendacibombus</i>	1	[2-4]
<i>Bombus convexus</i> Wang, 1979	<i>Mendacibombus</i>	1	[2; 4; 5]
<i>Bombus handlirschianus</i> Vogt, 1909	<i>Mendacibombus</i>	1	MS
<i>Bombus mendax</i> Gerstaecker, 1869	<i>Mendacibombus</i>	1	[6-10]
<i>Bombus waltoni</i> Cockerell, 1910	<i>Mendacibombus</i>	1	[5]
<i>Bombus auricomus</i> (Robertson), 1903	<i>Bombias</i>	1	[6; 7; 11-15]
<i>Bombus confusus</i> Schenck, 1859	<i>Bombias</i>	1	[2; 4; 6-8; 10; 16; 17]
<i>Bombus nevadensis</i> Cresson, 1874	<i>Bombias</i>	1	[2; 4; 7; 10; 12; 13; 15; 18-20]
<i>Bombus soroeensis</i> (Fabricius), 1777	<i>Kallobombus</i>	0	[2; 4; 8; 10; 16; 17]
<i>Bombus funerarius</i> Smith, 1852	<i>Orientalibombus</i>	0	[5; 21]
<i>Bombus haemorrhoidalis</i> Smith, 1852	<i>Orientalibombus</i>	0	[2; 4; 21]
<i>Bombus appositus</i> Cresson, 1878	<i>Subterraneobombus</i>	0	[13; 15]
<i>Bombus borealis</i> Kirby, 1837	<i>Subterraneobombus</i>	0	[11; 13; 15]
<i>Bombus difficillimus</i> Skorikov, 1912	<i>Subterraneobombus</i>	0	[5]
<i>Bombus distinguendus</i> Morawitz, 1869	<i>Subterraneobombus</i>	0	[8; 16; 17]
<i>Bombus fragrans</i> (Pallas), 1771	<i>Subterraneobombus</i>	0	[16]
<i>Bombus melanurus</i> Lepeletier, 1835	<i>Subterraneobombus</i>	0	[2-4]
<i>Bombus personatus</i> Smith, 1879	<i>Subterraneobombus</i>	0	[5]
<i>Bombus subterraneus</i> (Linnaeus), 1758	<i>Subterraneobombus</i>	0	[16; 17]
<i>Bombus argillaceus</i> (Scopoli), 1763	<i>Megabombus</i>	0	[16]
<i>Bombus bicoloratus</i> Smith, 1879	<i>Megabombus</i>	0	[2; 4; 5]
<i>Bombus diversus</i> Smith, 1869	<i>Megabombus</i>	0	MS
<i>Bombus gerstaeckeri</i> Morawitz, 1881	<i>Megabombus</i>	0	[8; 16]
<i>Bombus hortorum</i> (Linnaeus), 1761	<i>Megabombus</i>	0	[8; 16; 17]
<i>Bombus longipes</i> Friese, 1905	<i>Megabombus</i>	0	[5]
<i>Bombus portchinsky</i> Radoszkowski, 1883	<i>Megabombus</i>	0	MS
<i>Bombus religiosus</i> (Frison), 1935	<i>Megabombus</i>	0	[5]
<i>Bombus ruderatus</i> (Fabricius), 1775	<i>Megabombus</i>	0	[16; 17; 22]
<i>Bombus securus</i> (Frison), 1935	<i>Megabombus</i>	0	[5]
<i>Bombus supremus</i> Morawitz, 1887	<i>Megabombus</i>	0	[2; 4; 5]
<i>Bombus tichenkoi</i> (Skorikov), 1923	<i>Megabombus</i>	0	[5]
<i>Bombus trifasciatus</i> Smith, 1852	<i>Megabombus</i>	0	[2-5]
<i>Bombus ussurensis</i> Radoszkowski, 1877	<i>Megabombus</i>	0	MS
<i>Bombus armeniacus</i> Radoszkowski, 1877	<i>Thoracobombus</i>	0	[2; 4]
<i>Bombus atratus</i> Franklin, 1913	<i>Thoracobombus</i>	0	[22; 23]
<i>Bombus atripes</i> Smith, 1852	<i>Thoracobombus</i>	0	[5]
<i>Bombus bellicosus</i> Smith, 1862	<i>Thoracobombus</i>	0	[22; 23]
<i>Bombus brasiliensis</i> Lepeletier, 1835	<i>Thoracobombus</i>	0	[22; 23]
<i>Bombus brevivillus</i> Franklin, 1913	<i>Thoracobombus</i>	0	[23]

<i>Bombus consobrinus</i> Dahlbom, 1832	<i>Thoracobombus</i>	0	MS
<i>Bombus dahlbomii</i> Guérin-Méneville, 1835	<i>Thoracobombus</i>	0	[22; 23]
<i>Bombus deuteronymus</i> Schulz, 1906	<i>Thoracobombus</i>	0	MS
<i>Bombus digressus</i> (Milliron), 1962	<i>Thoracobombus</i>	0	[24]
<i>Bombus diligens</i> Smith, 1861	<i>Thoracobombus</i>	0	[23]
<i>Bombus exil</i> (Skorikov), 1931	<i>Thoracobombus</i>	0	[2; 4]
<i>Bombus fervidus</i> (Fabricius), 1798	<i>Thoracobombus</i>	0	[2; 4; 11; 13; 15; 18; 23; 25]
<i>Bombus filchnerae</i> Vogt, 1908	<i>Thoracobombus</i>	0	[2; 4; 11]
<i>Bombus hedinii</i> Bischoff, 1905	<i>Thoracobombus</i>	0	[5]
<i>Bombus humilis</i> Illiger, 1806	<i>Thoracobombus</i>	0	[5; 16; 17]
<i>Bombus imitator</i> Pittioni, 1949	<i>Thoracobombus</i>	0	[5]
<i>Bombus impetuosus</i> Smith, 1871	<i>Thoracobombus</i>	0	[5]
<i>Bombus inexpectatus</i> (Tkalcu), 1939	<i>Thoracobombus</i>	0	[26]
<i>Bombus laesus</i> Morawitz, 1875	<i>Thoracobombus</i>	0	[2; 4; 5; 16]
<i>Bombus medius</i> Cresson, 1863	<i>Thoracobombus</i>	0	[23]
<i>Bombus mesomelas</i> Gerstaecker, 1781	<i>Thoracobombus</i>	0	[16]
<i>Bombus mexicanus</i> Cresson, 1878	<i>Thoracobombus</i>	0	[23]
<i>Bombus mlokosievitzii</i> Radoszkowski, 1877	<i>Thoracobombus</i>	0	[26]
<i>Bombus morio</i> (Swederus), 1787	<i>Thoracobombus</i>	0	[22; 23]
<i>Bombus mucidus</i> Gerstaecker, 1869	<i>Thoracobombus</i>	0	[2; 4; 16]
<i>Bombus muscorum</i> (Linnaeus), 1758	<i>Thoracobombus</i>	0	[16; 17]
<i>Bombus opifex</i> Smith, 1879	<i>Thoracobombus</i>	0	[22; 23]
<i>Bombus pascuorum</i> (Scopoli), 1763	<i>Thoracobombus</i>	0	[16; 17]
<i>Bombus pennsylvanicus</i> (DeGeer), 1773	<i>Thoracobombus</i>	0	[11; 13-15; 18; 20; 23]
<i>Bombus persicus</i> Radoszkowski, 1881	<i>Thoracobombus</i>	0	[2; 4]
<i>Bombus pomorum</i> (Panzer), 1805	<i>Thoracobombus</i>	0	[8; 16; 17]
<i>Bombus pullatus</i> Franklin, 1913	<i>Thoracobombus</i>	0	[23]
<i>Bombus remotus</i> (Tkalcu), 1968	<i>Thoracobombus</i>	0	[5]
<i>Bombus ruderarius</i> (Müller), 1776	<i>Thoracobombus</i>	0	[16; 17]
<i>Bombus schrencki</i> Morawitz, 1881	<i>Thoracobombus</i>	0	MS
<i>Bombus steindachneri</i> Handlirsch, 1888	<i>Thoracobombus</i>	0	[23]
<i>Bombus sylvarum</i> (Linnaeus), 1761	<i>Thoracobombus</i>	0	[2; 4; 16; 17; 26]
<i>Bombus transversalis</i> (Olivier), 1789	<i>Thoracobombus</i>	0	[23]
<i>Bombus tricornis</i> Radoszkowski, 1888	<i>Thoracobombus</i>	0	[2; 4]
<i>Bombus veteranus</i> (Fabricius), 1793	<i>Thoracobombus</i>	0	[16; 17]
<i>Bombus weisi</i> Friese, 1861	<i>Thoracobombus</i>	0	[23]
<i>Bombus zonatus</i> Smith, 1854	<i>Thoracobombus</i>	0	MS
<i>Bombus barbutellus</i> (Kirby), 1802	<i>Psithyrus</i>	0	MS
<i>Bombus bohemicus</i> Seidl, 1837	<i>Psithyrus</i>	0	[11; 15]
<i>Bombus campestris</i> (Panzer), 1801	<i>Psithyrus</i>	0	MS
<i>Bombus citrinus</i> (Smith), 1854	<i>Psithyrus</i>	0	[11; 15]
<i>Bombus flavidus</i> Eversmann, 1852	<i>Psithyrus</i>	0	[11; 15]
<i>Bombus insularis</i> (Smith), 1861	<i>Psithyrus</i>	0	[2; 4; 11; 15]
<i>Bombus intrudens</i> (Smith), 1861	<i>Psithyrus</i>	0	[15]
<i>Bombus norvegicus</i> (Sparre-Schneider), 1918	<i>Psithyrus</i>	0	MS
<i>Bombus rupestris</i> (Fabricius), 1793	<i>Psithyrus</i>	0	MS
<i>Bombus suckleyi</i> Greene, 1860	<i>Psithyrus</i>	0	[15]
<i>Bombus sylvestris</i> (Lepelletier), 1832	<i>Psithyrus</i>	0	[2; 4]
<i>Bombus vestalis</i> (Geoffroy), 1785	<i>Psithyrus</i>	0	MS
<i>Bombus ardens</i> Smith, 1879	<i>Pyrobombus</i>	0	MS
<i>Bombus avanus</i> (Skorikov), 1938	<i>Pyrobombus</i>	0	[5]

<i>Bombus bifarius</i> Cresson, 1878	<i>Pyrobombus</i>	0	[13; 15; 18]
<i>Bombus bimaculatus</i> Cresson, 1863	<i>Pyrobombus</i>	0	[11; 13-15]
<i>Bombus biroi</i> Vogt, 1911	<i>Pyrobombus</i>	0	[3]
<i>Bombus brodmannicus</i> Vogt, 1909	<i>Pyrobombus</i>	0	[16]
<i>Bombus caliginosus</i> (Frison), 1927	<i>Pyrobombus</i>	0	[18]
<i>Bombus centralis</i> Cresson, 1864	<i>Pyrobombus</i>	0	[13; 15; 18]
<i>Bombus ephippiatus</i> Say, 1837	<i>Pyrobombus</i>	0	[23]
<i>Bombus flavescens</i> Smith, 1852	<i>Pyrobombus</i>	0	[5]
<i>Bombus flavifrons</i> Cresson, 1863	<i>Pyrobombus</i>	0	[11; 13; 15; 18]
<i>Bombus frigidus</i> Smith, 1854	<i>Pyrobombus</i>	0	[11; 13; 15]
<i>Bombus haematurus</i> Kriechbaumer, 1870	<i>Pyrobombus</i>	0	MS
<i>Bombus huntii</i> Greene, 1860	<i>Pyrobombus</i>	0	[13; 15; 18; 23]
<i>Bombus hypnorum</i> (Linnaeus), 1758	<i>Pyrobombus</i>	0	[2-5; 16; 17; 19; 27]
<i>Bombus impatiens</i> Cresson, 1863	<i>Pyrobombus</i>	0	[11; 13; 15]
<i>Bombus infirmus</i> (Tkalcu), 1968	<i>Pyrobombus</i>	0	[5]
<i>Bombus infrequens</i> (Tkalcu), 1989	<i>Pyrobombus</i>	0	[5]
<i>Bombus jonellus</i> (Kirby), 1802	<i>Pyrobombus</i>	0	[13; 16; 17]
<i>Bombus lapponicus</i> (Fabricius), 1793	<i>Pyrobombus</i>	0	[2; 4; 19]
<i>Bombus lemniscatus</i> Skorikov, 1912	<i>Pyrobombus</i>	0	[5]
<i>Bombus lepidus</i> Skorikov, 1912	<i>Pyrobombus</i>	0	[3; 5]
<i>Bombus melanopygus</i> Nylander, 1848	<i>Pyrobombus</i>	0	[11; 13; 15; 18]
<i>Bombus mixtus</i> Cresson, 1878	<i>Pyrobombus</i>	0	[11; 13; 15; 18]
<i>Bombus monticola</i> Smith, 1844	<i>Pyrobombus</i>	0	[16]
<i>Bombus parthenius</i> Richards, 1934	<i>Pyrobombus</i>	0	MS
<i>Bombus perplexus</i> Cresson, 1863	<i>Pyrobombus</i>	0	[11; 13; 15]
<i>Bombus picipes</i> Richards, 1934	<i>Pyrobombus</i>	0	[5]
<i>Bombus pratorum</i> (Linnaeus), 1761	<i>Pyrobombus</i>	0	[8; 16; 17]
<i>Bombus pressus</i> (Frison), 1935	<i>Pyrobombus</i>	0	[2; 4]
<i>Bombus pyrenaeus</i> Pérez, 1880	<i>Pyrobombus</i>	0	[16]
<i>Bombus sandersoni</i> Franklin, 1913	<i>Pyrobombus</i>	0	[11; 13]
<i>Bombus sitkensis</i> Nylander, 1848	<i>Pyrobombus</i>	0	[13; 15; 18]
<i>Bombus sylvicola</i> Kirby, 1837	<i>Pyrobombus</i>	0	[11; 13; 15; 18]
<i>Bombus ternarius</i> Say, 1837	<i>Pyrobombus</i>	0	[11; 13; 15]
<i>Bombus vagans</i> Smith, 1854	<i>Pyrobombus</i>	0	[11; 13-15]
<i>Bombus vandykei</i> (Frison), 1927	<i>Pyrobombus</i>	0	[18]
<i>Bombus vosnesenskii</i> Radoszkowski, 1862	<i>Pyrobombus</i>	0	[13; 15; 18; 23]
<i>Bombus alpinus</i> (Linnaeus), 1758	<i>Alpinobombus</i>	0	[13; 16]
<i>Bombus balteatus</i> Dahlbom, 1832	<i>Alpinobombus</i>	0	[11; 13; 15; 18; 19]
<i>Bombus hyperboreus</i> Schönherr, 1809	<i>Alpinobombus</i>	0	[2; 13; 19]
<i>Bombus neoboreus</i> Sladen, 1919	<i>Alpinobombus</i>	0	[13; 15]
<i>Bombus polaris</i> Curtis, 1835	<i>Alpinobombus</i>	0	[11; 13; 15]
<i>Bombus affinis</i> Cresson, 1863	<i>Bombus</i>	0	[11; 13; 15]
<i>Bombus cryptarum</i> (Fabricius), 1775	<i>Bombus</i>	0	[13; 15-17]
<i>Bombus franklini</i> (Frison), 1921	<i>Bombus</i>	0	[13; 18]
<i>Bombus ignitus</i> Smith, 1869	<i>Bombus</i>	0	[5]
<i>Bombus lucorum</i> (Linnaeus), 1761	<i>Bombus</i>	0	[3; 5; 17]
<i>Bombus occidentalis</i> Greene, 1858	<i>Bombus</i>	0	[13; 15; 18]
<i>Bombus patagiatus</i> Nylander, 1848	<i>Bombus</i>	0	[5]
<i>Bombus sporadicus</i> Nylander, 1848	<i>Bombus</i>	0	[2; 4]
<i>Bombus terrestris</i> (Linnaeus), 1758	<i>Bombus</i>	0	[2; 4; 8; 16; 17; 19; 28]
<i>Bombus terricola</i> Kirby, 1837	<i>Bombus</i>	0	[11; 13; 15]

<i>Bombus breviceps</i> Smith, 1852	<i>Alpigenobombus</i>	1	[5]
<i>Bombus grahami</i> (Frison), 1933	<i>Alpigenobombus</i>	1	[5]
<i>Bombus kashmirensis</i> Friese, 1909	<i>Alpigenobombus</i>	1	[3; 5; 7]
<i>Bombus nobilis</i> Friese, 1905	<i>Alpigenobombus</i>	0	[2; 4; 5]
<i>Bombus wurflenii</i> Radoszkowski, 1860	<i>Alpigenobombus</i>	0	[3; 8; 16; 17]
<i>Bombus eximius</i> Smith, 1852	<i>Melanobombus</i>	0	[2; 5]
<i>Bombus festivus</i> Smith, 1861	<i>Melanobombus</i>	0	[2; 4; 5]
<i>Bombus friseanus</i> Skorikov, 1933	<i>Melanobombus</i>	0	[3; 5]
<i>Bombus incertus</i> Morawitz, 1881	<i>Melanobombus</i>	0	MS
<i>Bombus keriensis</i> Morawitz, 1887	<i>Melanobombus</i>	0	[3; 5]
<i>Bombus ladakhensis</i> Richards, 1928	<i>Melanobombus</i>	0	[2-5]
<i>Bombus lapidarius</i> (Linnaeus), 1758	<i>Melanobombus</i>	0	[8; 16; 19]
<i>Bombus miniatus</i> Bingham, 1897	<i>Melanobombus</i>	0	[3; 27]
<i>Bombus pyrosoma</i> Morawitz, 1890	<i>Melanobombus</i>	0	[3]
<i>Bombus rufipes</i> Lepeletier, 1835	<i>Melanobombus</i>	0	MS
<i>Bombus rufofasciatus</i> Smith, 1852	<i>Melanobombus</i>	1	[3; 5; 7; 10]
<i>Bombus sichelii</i> Radoszkowski, 1860	<i>Melanobombus</i>	0	[5; 16]
<i>Bombus simillimus</i> Smith, 1852	<i>Melanobombus</i>	1	[2-4]
<i>Bombus asiaticus</i> Morawitz, 1875	<i>Sibiricobombus</i>	1	[3; 7; 8]
<i>Bombus morawitzi</i> Radoszkowski, 1876	<i>Sibiricobombus</i>	1	[3]
<i>Bombus niveatus</i> Kriechbaumer, 1870	<i>Sibiricobombus</i>	1	[2; 6; 8; 29]
<i>Bombus oberti</i> Morawitz, 1883	<i>Sibiricobombus</i>	0	[3]
<i>Bombus sibiricus</i> (Fabricius), 1781	<i>Sibiricobombus</i>	0	[3; 8; 10]
<i>Bombus sulfureus</i> Friese, 1905	<i>Sibiricobombus</i>	1	[29]
<i>Bombus baeri</i> Vachal, 1904	<i>Cullumanobombus</i>	1	[22; 23; 30]
<i>Bombus brachycephalus</i> Handlirsch, 1888	<i>Cullumanobombus</i>	1	[2; 4; 20; 23; 30]
<i>Bombus coccineus</i> Friese, 1903	<i>Cullumanobombus</i>	1	[2; 4; 23; 30]
<i>Bombus crotchii</i> Cresson, 1878	<i>Cullumanobombus</i>	1	[2; 4; 13; 15; 18; 19; 23; 25;
<i>Bombus cullumanus</i> (Kirby), 1802	<i>Cullumanobombus</i>	0	[17]
<i>Bombus ecuadorius</i> Meunier, 1890	<i>Cullumanobombus</i>	1	[23; 30]
<i>Bombus fraternus</i> (Smith), 1854	<i>Cullumanobombus</i>	1	[2; 4; 11; 13-15; 19; 23; 30]
<i>Bombus funebris</i> Smith, 1854	<i>Cullumanobombus</i>	1	[2; 23; 30]
<i>Bombus griseocollis</i> (DeGeer), 1773	<i>Cullumanobombus</i>	1	[2; 4; 7; 11; 13; 15; 18; 19; 30]
<i>Bombus handlirschi</i> Friese, 1903	<i>Cullumanobombus</i>	1	[2; 4; 30]
<i>Bombus haueri</i> Handlirsch, 1888	<i>Cullumanobombus</i>	1	[11; 23; 31]
<i>Bombus hortulanus</i> Smith, 1904	<i>Cullumanobombus</i>	1	[23; 30]
<i>Bombus macgregori</i> Labougle & Ayala, 1985	<i>Cullumanobombus</i>	1	[2; 4]
<i>Bombus melaleucus</i> Handlirsch, 1888	<i>Cullumanobombus</i>	1	[23; 30]
<i>Bombus morrisoni</i> Cresson, 1878	<i>Cullumanobombus</i>	1	[2; 4; 13; 15; 18; 19; 23; 30]
<i>Bombus robustus</i> Smith, 1854	<i>Cullumanobombus</i>	1	[6; 23; 30]
<i>Bombus rubicundus</i> Smith, 1854	<i>Cullumanobombus</i>	1	[2; 4; 23; 30]
<i>Bombus rufocinctus</i> Cresson, 1863	<i>Cullumanobombus</i>	1	[2; 4; 11; 13; 18; 30; 32] [15]
<i>Bombus semenoviellus</i> Skorikov, 1910	<i>Cullumanobombus</i>	0	MS
<i>Bombus tucumanus</i> Vachal, 1904	<i>Cullumanobombus</i>	1	[22; 23; 30]
<i>Bombus vogti</i> Friese, 1903	<i>Cullumanobombus</i>	1	[2; 4; 30]

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*Manuscript IV*

**Visual ecology of long-horned bee males (*Eucera berlandi*, Hymenoptera:  
Apidae): Adaptations of the visual system and mate detection**

Martin Streinzer, Johannes Spaethe  
(in preparation)



## **Visual ecology of long-horned bee males (*Eucera berlandi*, Hymenoptera: Apidae): Adaptations of the visual system and mate detection**

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### ***Abstract***

Males and females of the long-horned bee species *Eucera berlandi* show a remarkable sexual dimorphism in their sensory system. The elongated antennae of the males exhibit morphological traits that most likely improve olfactory detection of the females. During mating flights, males detect females both olfactorily and visually. Females emerging from the ground are immediately noticed and then chased by the males; only the fastest male achieves reproductive success. Therefore, a strong selection pressure acts on the males for good visual and olfactory abilities. In this study we focus on the visual system of *Eucera berlandi* and compared male and female eye morphology to identify adaptations for mating behavior in males. Additionally, we conducted a field behavioral experiment to study male chasing behavior in more detail. Males have enlarged compound eyes that result from enlarged frontal facets. The frontal eye region is characterized by small interommatidial angles and thus high spatial resolution. Further evidence for selection pressure on high resolution comes from the smaller eye parameter in the male frontal eye region compared to females. The behavioral experiment showed that the number of males attracted by a moving female dummy that was impregnated with a sex pheromone analogue correlated significantly with dummy size but not speed. Furthermore, males had a higher success rate when catching larger dummies as opposed to smaller ones. We discuss the morphological adaptations of the male visual system and possible mechanisms that allow males to detect and catch the female during the fast mating flights.

### ***Introduction***

The females of solitary bees perform many tasks, such as nest construction, foraging and parental care, while males only invest in finding virgin females and mating. Competition for access to virgin females results in strong sexual selection pressures in males that have led to the development of conspicuous adaptations in many species (Andersson and Iwasa, 1996; Stubblefield and Seger, 1994), for example, improved sensory systems (enlarged eyes and antennae) and adaptations of the flight apparatus.

Sensory adaptations of males have been comprehensively investigated in the Western honeybee. Compared with females, the drones show enormously enlarged compound eyes that result in accurate spatial and temporal vision (Menzel et al., 1991; Ribi et al., 1989; Seidl, 1982). Further, the male antennae are equipped with a high number of olfactory sensilla and receptors for long-distance pheromone detection (Esslen and Kaissling, 1976; Streinzer et al., in press). Adaptations

of the receptor physiology and neural circuitry further improve visual and olfactory mate detection (Arnold et al., 1985; Vallet and Coles, 1993a), and the role of these sensory adaptations in queen detection has been thoroughly investigated (Gries and Koeniger, 1996; Vallet and Coles, 1993b; van Praagh et al., 1980). Although sensory adaptations for mate detection have been superficially described in other bee taxa (Ågren and Hallberg, 1996; Hurd and Moure, 1962; Tengö et al., 1990; Williams, 1991), comprehensive morphological, physiological and behavioral studies on sensory adaptations of mating behavior are lacking so far. Aside from the honeybee, the olfactory system of a second bee species has been recently described in more detail (Streinzer et al., in press). Males of the solitary long-horned bee *Eucera berlandi* have extremely long antennae that are densely covered with olfactory sensilla and have a high number of receptor neurons. The morphology of the antennal lobe further suggests that the bees are particularly adapted to fast and accurate olfactory long-distance detection of the female sex pheromone (Streinzer et al., in press). *Eucera* males patrol nesting sites where they wait for virgin females to emerge. They first detect them olfactorily (Schaller, 2009), but their visual system plays an important role in visual detection and discrimination of the female during the subsequent chase (Shimron and Hefetz, 1985; Streinzer et al., 2009). Aside from the clearly visible sexual dimorphism of the olfactory system, males have enlarged eyes compared with females (Streinzer, 2008). The enlargement of the visual system is not as distinct as in the species of *Apis* (Streinzer et al., 2013), but is comparable to other bees that visually search for their mates (Hurd and Moure, 1962 and *Manuscript III*). The enlarged compound eyes of the males accommodate large facet lenses, but do not differ in the number of ommatidia (Streinzer, 2008). However, the functional implications of the enlarged eyes and facets have not been adequately investigated. The species of *Eucera*, like all other bees, possess apposition compound eyes consisting of several thousand optically isolated units. Each ommatidium is equipped with its own dioptric apparatus and nine photoreceptor cells (Land and Nilsson, 2002). Apposition compound eyes are generally limited with respect to light sensitivity and spatial resolution. To overcome some of these limitations, spatially restricted regions of the eye (acute zones) may increase spatial resolution but at the expense of resolution in the remainder of the eye (Land, 1989). These acute zones are usually associated with flower and mate detection, as well as with the habitat in which the animal lives (Land and Nilsson, 2002; Snyder et al., 1977). In honeybee drones an extended acute zone is found in the dorsal eye region, characterized by small interommatidial angles and large facet lenses (Seidl, 1982; Streinzer et al., 2013). The enlarged facets in male *Eucera berlandi* also suggest the presence of a region of improved spatial resolution. To better understand the functional adaptation of the (male) visual system in mating behavior, in a first step we expanded our existing knowledge on the sexual dimorphism of *Eucera* eyes (Streinzer, 2008). In particular, we mapped the distribution of facet diameters in the eye of both sexes and measured interommatidial angles in the frontal eye region. In a second step, we studied target detection abilities of males chasing a moving female dummy of different sizes and at different speeds under natural conditions. In a final step, we undertook a more close

inspection of the chase flights and modeled the excitation pattern of the frontal ommatidia during the chase of a dummy target.

## ***Material & Methods***

### Study species

*Eucera (Synhalonia) berlandi* (DUSMET) is a large eucerine bee, abundant in the Mediterranean region. Animals used for anatomical measurement were collected in Neapolis, Crete, Greece (35°15'N, 25°38'E) and Elassona, Thessaly, Greece (39°48'N, 22°09'E) in 2010 and 2011. All behavioral experiments were carried out near Neapolis, Crete, Greece in 2010.

### Eye anatomy

The sexual dimorphism of the peripheral visual system in *E. berlandi* was described previously (Streinzer, 2008). Males have enlarged compound eyes compared with females. The enlargement is due to larger facet diameters in males and not to a higher number of ommatidia. To study the sex specific distribution of facet sizes across the eye surface, surface replicas were made using nail polish (Ribi et al., 1989). The replicas were photographed using a light microscope (Nikon SMZ-U equipped with DSFi1, Tokyo, Japan) in overlying sections, which were stitched together using Adobe Photoshop CS 2 (Adobe Systems, San Jose, CA, USA). Facet lens centers were then marked manually in ImageJ (National Institute of Mental Health, Bethesda Maryland, USA). Customized workflows in Meshlab (Visual Computing Lab - ISRI - CNR, <http://meshlab.sourceforge.net/>) and CorelDraw X5 (Corel Corporation) were used to produce color-coded maps of facet size distribution (Streinzer et al., 2013).

### Interommatidial angles

Interommatidial angles were measured under antidromic illumination following the methods described by (Seidl, 1982; Spaethe and Chittka, 2003). In brief, the diverging rays of light, emerging from the facet lenses of the back-illuminated eye, were photographed at several known distances from the eye surface. The distance between the centers of the rays was then measured from the photographs to calculate the angle between rays of neighboring ommatidia (Spaethe and Chittka, 2003). Prior to the measurements, *E. berlandi* males and females were cooled to immobilize them. The head was then separated from the thorax with a sharp razor blade. To increase the amount of light that enters the rhabdoms, the occipital foramen was slightly enlarged using a microscalpel. The head was then mounted onto a diode emitting red light (LED;  $\lambda_{\text{max}}=630$  nm) using adhesive putty (UHU patafix; UHU GmbH & Co KG, Bühl, Germany). Petroleum jelly was applied to prevent desiccation of the tissue. Subsequently, the LED and head were mounted in the center of a goniometer apparatus (Leitz, Germany) and adjusted so that the backplane of the head was aligned with the goniometer stage at latitude=0°, longitude=0°, i.e. that the head was upright and facing upward. In this position the bright pseudopupils of both eyes appeared symmetrical with respect to the dorso-ventral axis of the head. The bright pseudopupil, a result of the light travelling through the rhabdoms and exiting through the dioptric

apparatus, was viewed at approximately 250x magnification using a digital microscope camera (DigiMicro 2.0 Scale; DNT, Dietzenbach, Germany). The pseudopupil was photographed at several (5-6) distances  $H$  from the eye surface. For each image we measured the distance  $W$  between the light rays in all three axes (x,y,z) of the hexagonal lattice and additionally in the vertical plane. For each axis we took 4-6 measurements in a different part of a small region (5x5x5 facet rows) of the pseudopupil. The mean of these measurements was then used for the calculation of the interommatidial angle. For each individual we then calculated the slope  $M$  of a regression line through the point set  $(H, W/2)$ , which represents  $1/2\Delta\phi$ . The interommatidial angle  $\Delta\phi$  was then determined by eqn 1:

$$\Delta\phi = 2 \arctan (M) \quad [\text{Eqn. 1}]$$

Interommatidial angles are reported as horizontal ( $2\Delta\phi_h$ ) and vertical ( $2\Delta\phi_v$ ) interommatidial angles, as well as mean interommatidial angle ( $\Delta\phi$ ), calculated from angular separation of adjacent ommatidia rows in the x,y and z-axis, and the vertical ommatidia rows (Land and Nilsson, 2002). Interommatidial angles were only measured in the frontal region, where the pseudopupil appeared the largest. Based on the measured parameters we calculated the eye parameter  $D\Delta\phi$ . The eye parameter is a measure to which extent the eye reaches its diffraction limit, and allows for conclusions to be made about the trade-off between resolution and sensitivity in the eye. The theoretical limit (at  $\lambda=500\text{nm}$ ) is  $0.25\mu\text{mrad}$ , but diurnal insects usually have slightly higher values, around  $0.50\mu\text{mrad}$  (Land, 1989).

#### Visual attraction and success of grabbing a moving female dummy

To study male attraction to a visual stimulus (female dummy), we used a rotating carousel (Brockmann et al., 2006). The carousel consisted of a wiper motor mounted on a tripod at a height of c. 1.3m. The electric motor was powered by a 12V lead battery. A control unit allowed adjustment of the rotation speed. The rotating axis of the motor held two horizontal arms perpendicular to each other. One arm ( $\varnothing=10\text{mm}$ ,  $l=0.3\text{m}$ ) carried a camcorder (Sony DCR-SR50, Tokyo, Japan) mounted on a ball head. The second arm held a thin aluminum pipe ( $\varnothing=5\text{mm}$ ,  $l=0.7\text{m}$ ). At the end we inserted a thin metal wire ( $\varnothing=2\text{mm}$ ,  $l=0.5\text{m}$ ). The wire was bent upwards and covered with white PTFE band (mean reflectance  $>95\%$ ) to reduce visual contrast to the background. Female dummies were attached at the top of the thin wire at a height of c. 1.8m above the ground. Dummies consisted of three different sized wooden spheres ( $\varnothing_{\text{small}}=10\text{mm}$ ,  $\varnothing_{\text{medium}}=14\text{mm}$ ,  $\varnothing_{\text{large}}=18\text{mm}$ ) painted with matte black paint (Plaka 070; Pelikan, Hannover, Germany; mean reflectance  $<5\%$ ). Additionally, we filmed approaches to a real female dummy, which was made odorless to remove potential sex pheromones by washing it in solvent for several days and regularly replacing the solvent. Due to the non-spherical appearance of the female, we approximated its size by using the mean of its length and height ( $\varnothing_{\text{female}}=12\text{mm}$ ). To elicit a chase of the dummies, we presented males with a putative sex pheromone mixture made from washed labella *Ophrys heldreichii* in solvent (40 flower labella in 4ml of pentane; 24 hours

at RT). This orchid mimics the original sex pheromone of *E. berlandi* females to attract males for pollination (Schaller, 2009). 10  $\mu\text{l}$  of this mixture (equivalent to 0.1 flower labellum) was applied to a piece of filter paper attached near the dummy before the start of each trial to ensure that identical amounts of chemical attractant were present in all trials.

Since we had no prior information on the appropriate dummy speed we chose a speed that was previously used in honeybee experiments ( $3\text{m}\cdot\text{s}^{-1}$ ; Brockmann et al., 2006), as well as a faster ( $4\text{m}\cdot\text{s}^{-1}$ ) and a slower speed ( $2\text{m}\cdot\text{s}^{-1}$ ) in preliminary experiments. The test revealed that males were not able to follow the dummy at  $4\text{m}\cdot\text{s}^{-1}$  and we therefore discontinued using this preset.

We filmed approaches and contacts of male *E. berlandi* to the dummies in 6 trials (2 dummy speeds x 3 dummy sizes) for 3 minutes at  $25\text{ frames}\cdot\text{s}^{-1}$ . The area viewed by the camera measured  $0.31\text{m}^2$  in the plane of the dummy. For each trial we counted the number of males that made contact with the female dummy. Since varying numbers of males were attracted in each trial we corrected contacts for male activity. Therefore, we counted all males that appeared on single images taken from the video sequence (two images/s, 350 images/trial). We then standardized the number of contacts using eqn 2:

$$C_s = C / T * 100 \quad [\text{Eqn. 2}]$$

where  $C_s$  is the number of standardized contacts,  $C$  is the total amount of contacts in a 3 minute trial and the male activity  $T$  is the sum of males that were counted on the images of the trial.

### Approach behavior

To study the approach behavior of *E. berlandi* males toward moving dummies, we filmed approaches with two identical cameras (Sony DCR-SR50) simultaneously to allow for a three dimensional reconstruction of the flight path. The cameras were aligned approximately perpendicular at a distance of c. 5m. A precise alignment was not necessary, since the position of the cameras was calibrated using the Camera Calibration Toolbox (Bouquet, 2010) in Matlab (version 2009a, MathWorks Inc., Massachusetts, USA). The cameras were temporally synchronized using light flashes at the beginning of each recording. For 3D reconstruction, the dummy and the approaching bee head were tracked manually in SkillSpector 1.3.0 (Video4coach, Svendborg, Denmark). The extracted coordinates were then used to calculate the position in the 3D space in Matlab.

Two different dummies were used in this experiment, a large black sphere ( $\text{Ø}=29.5\text{mm}$ ) and a medium sized black sphere ( $\text{Ø}=14\text{mm}$ ). The objects were fixed to a long piece of string and swung manually on a circular path ( $\text{Ø}=1.5\text{m}$ ;  $\text{speed}=3.5\text{m}\cdot\text{s}^{-1}$ ). The reconstructed approach sequences started when a male entered the filmed area and lasted for 2s. Distances between the bee and dummy were converted to the visual angle  $\alpha$  that the dummy subtended at the bees' eye using eqn. 3:

$$\alpha = 2 * \text{arc tan} (r/s) \quad [\text{Eqn. 3}]$$

where  $r$  is the radius of the dummy and  $s$  is the distance between dummy and bee head.

### Number of ommatidia involved in object tracking

To estimate the number of ommatidia involved in target tracking, we first calculated the median distance of the bee during its approach to the dummy. We then calculated the excitation of the ommatidial array by the angular size, which was determined by the size of the object and the distance between object and bee. To calculate ommatidia excitation, the angular sensitivity function of the ommatidia was integrated over the (angular) area of the target. For details on the calculation see (Giurfa et al., 1996; Spaethe and Chittka, 2003). Since no data on the acceptance angles of *Eucera* are available we used data from *Apis mellifera* ( $2.6^\circ$ ; Laughlin and Horridge, 1971). The excitation of all ommatidia involved in target detection was calculated relative to the ommatidium that directly faces the target.

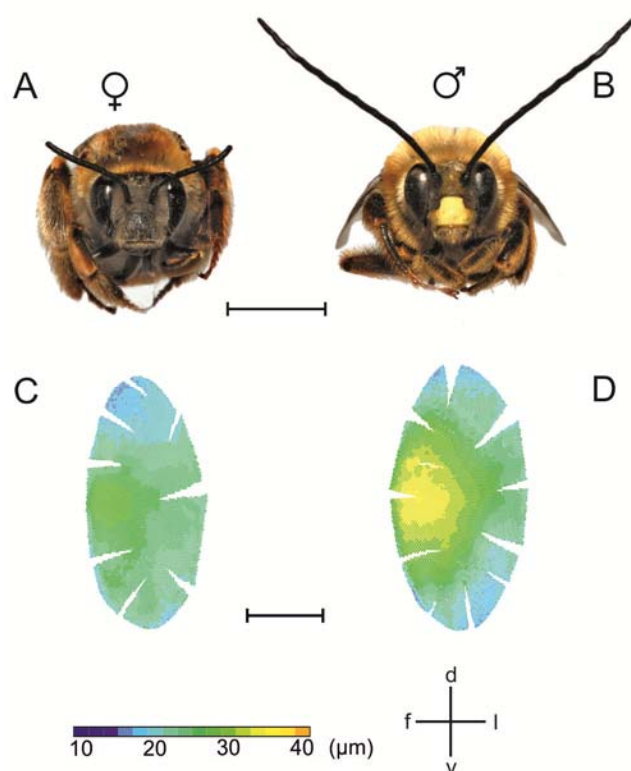
### Statistics

Statistical calculations were performed with SigmaPlot 11.0 (Systat Software Inc.). All  $P$ -values above 0.05 were considered statistically not significant. To identify the factors that affect male attraction and success rate in making contacts with the dummy, we used a multiple linear regression analysis with  $T$  and  $C_s$  as the dependent variable and dummy size and speed as independent variables.

## **Results**

### Eye anatomy

Peripheral parameters of the compound eye in *Eucera berlandi* have been previously investigated in part by Streinzer (2008) and are summarized in Tab. 1. In this study, eye maps were constructed to study the spatial variation of facet sizes in the compound eye (Fig. 1). The maps show a gradual change of facet sizes over the eye surface with the largest facets located in the frontal eye region in both sexes. Small facets (c.  $15\mu\text{m}$ ) are found in the dorsal region in both sexes, and in the ventral eye region in males. The largest facets are located in both sexes in the frontal eye region and measure  $30\text{-}32\mu\text{m}$  and  $36\text{-}38\mu\text{m}$  in females and males, respectively (Tab. 1, Fig. 1). The largest facets are thus located in the region that faces upward during patrol flights of the males, which suggests that males detect the female against the sky, rather than against the ground (Fig. 2).



**Figure 1** Eye maps of *Eucera berlandi*

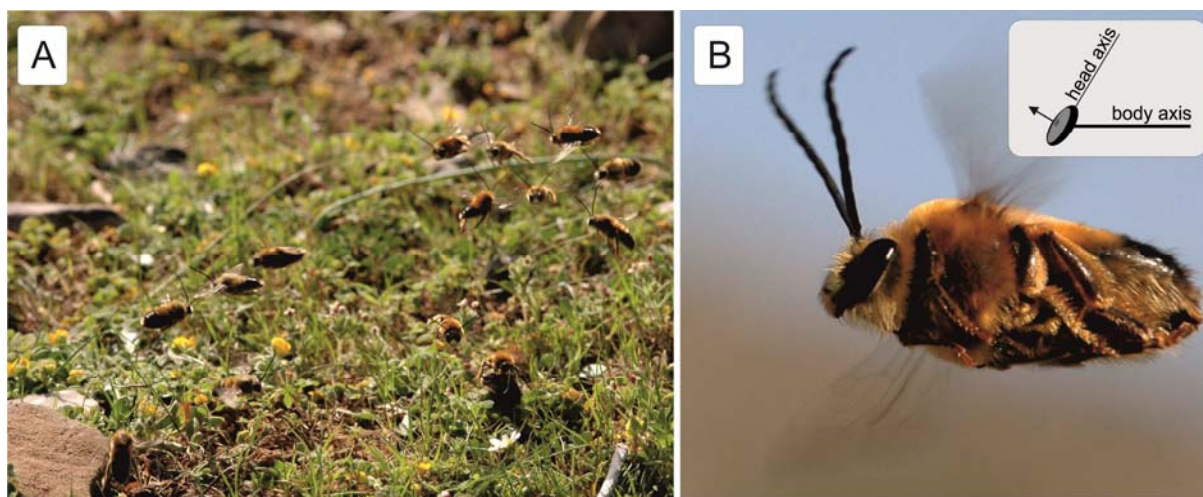
Photographs of a female (A) and a male (B) specimen of *E. berlandi* illustrate the apparent sexual dimorphism of the olfactory and visual system. Eye maps (C – female, D – male) show the size of facet diameters across the eye surface. Each circle represents an individual facet lens and the color indicates facet diameter (bin width 2 $\mu$ m). Scale bars 5mm (A, B) and 1 mm (C, D). Orientation and color code of the eye maps are indicated in the legend. f – frontal, l – lateral, d – dorsal, v – ventral. The entomological pins in have been digitally removed from the pictures (A, B).

Antidromic illumination resulted in a very bright pseudopupil that could be easily observed through the microscope. Interommatidial angles change over the eye surface, which was apparent by a pronounced size change (in terms of the number of bright facets) of the pseudopupil. The interommatidial angles were measured in the frontal region of the eye, where the pseudopupil appeared largest. The values for  $2\Delta\phi_v$ ,  $2\Delta\phi_h$  and  $\Delta\phi$  are 0.83, 1.36 and 0.93 in females, respectively, and 0.65, 0.83 and 0.61 in males (means,  $N=3$  each, Tab. 1). Therefore, males have a much higher spatial resolution compared with females due the smaller interommatidial angles. The eye parameter  $D\Delta\phi$  was also smaller for males (0.39  $\mu$ mrad) than females (0.49 $\mu$ mrad), further suggesting that the eye design is more biased towards high resolution in males (Tab. 1).

**Table 1** Eye anatomy of *Eucera berlandi*

	unit	female	male
its	mm	4.57 $\pm$ 0.17 <sup>a</sup>	4.24 $\pm$ 0.17 <sup>a</sup>
$A_{\text{eye}}$	mm <sup>2</sup>	4.84 $\pm$ 0.15 <sup>a</sup>	5.40 $\pm$ 0.37 <sup>a</sup>
# <sub>ommatidia</sub>		8,426 $\pm$ 237 <sup>a</sup>	8,354 $\pm$ 362 <sup>a</sup>
$D_{\text{facet}}$	$\mu$ m	32.2 $\pm$ 0.6 <sup>a</sup>	38.0 $\pm$ 1.2 <sup>a</sup>
$2\Delta\phi_v$	°	0.83 $\pm$ 0.02 [3]	0.65 $\pm$ 0.05 [3]
$2\Delta\phi_h$	°	1.36 $\pm$ 0.21 [3]	0.83 $\pm$ 0.12 [3]
$\Delta\phi$	°	0.93 $\pm$ 0.04 [3]	0.61 $\pm$ 0.09 [3]
$D\Delta\phi$	$\mu$ mrad	0.49 $\pm$ 0.02 [3]	0.39 $\pm$ 0.06 [3]

Values represent means  $\pm$  SD, sample size is given in brackets. <sup>a</sup>Measurements from (Streinzer, 2008); its – intertegulae span,  $A_{\text{eye}}$  – Eye surface area; #<sub>ommatidia</sub> – ommatidia number;  $D_{\text{facet}}$  – facet diameter;  $\Delta\phi_h$ – horizontal and  $\Delta\phi_v$ –vertical interommatidial angle,  $D\Delta\phi$  – eye parameter.



**Figure 2** Male competition and body position during the flight  
 (A) *Eucera berlandi* males patrolling nesting sites and waiting for females to emerge. The highly male biased operational sex ratio results in strong competition among males. (B) Male during a chasing flight. The head is vertically tilted with respect to the body axis. The frontal eye region, and thus the acute zone, is directed upward during flight.

### Male attraction

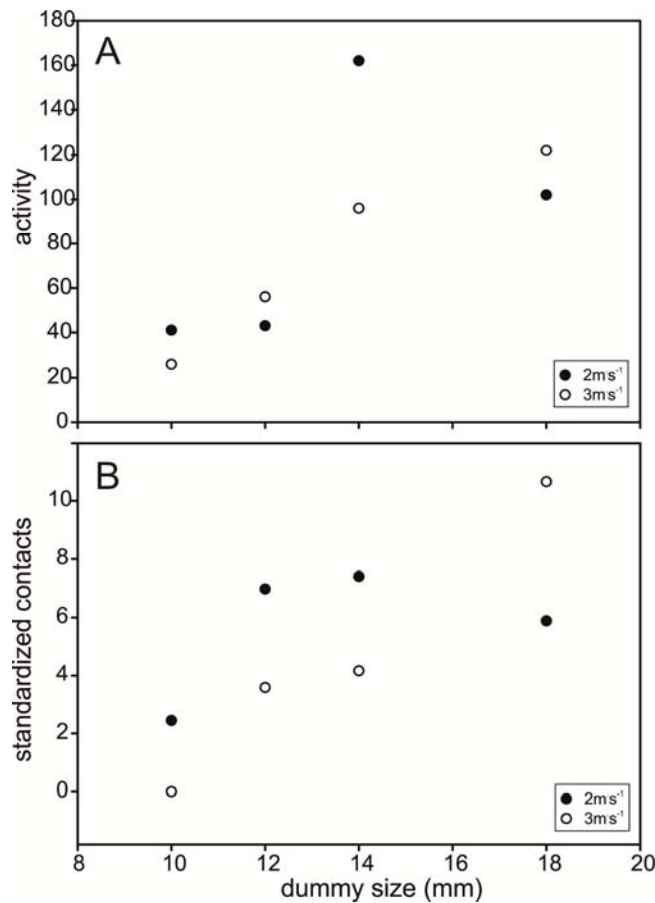
The moving female dummy, in conjunction with the putative sex pheromone mixture, was able to attract high numbers of males to the carousel and elicit a chase of the dummy that resulted in touching and grabbing the dummy (Fig. 3). In total, 41 successful approaches (i.e. approaches that resulted in contact with the dummy) were recorded. In general, larger dummies attracted more males and allowed a higher proportion of males to make contact with the dummies. The contribution of dummy size to the contacts made by males was statistically significant ( $P < 0.05$ ), while the contribution to male attraction was marginally not ( $P = 0.06$ , Tab. 2). There was no significant difference between the two dummy speeds  $2\text{m}\cdot\text{s}^{-1}$  and  $3\text{m}\cdot\text{s}^{-1}$  (Tab. 2).

**Table 2** Effects of dummy size and dummy speed on male activity and standardized contacts  $C_s$

dependent variable	explanatory factor	$\beta$	$P$
activity	constant	-34.41	0.72
	size	10.77	0.06
	speed	-12.00	0.67
$C_s$	constant	-2.94	0.64
	<b>size</b>	<b>0.80</b>	<b>&lt;0.05</b>
	speed	-1.08	0.56

Significant factors in bold. Activity:  $N=6$ ;  $R^2 = 0.54$ , Contacts:  $N=6$ ;  $R^2 = 0.61$ .



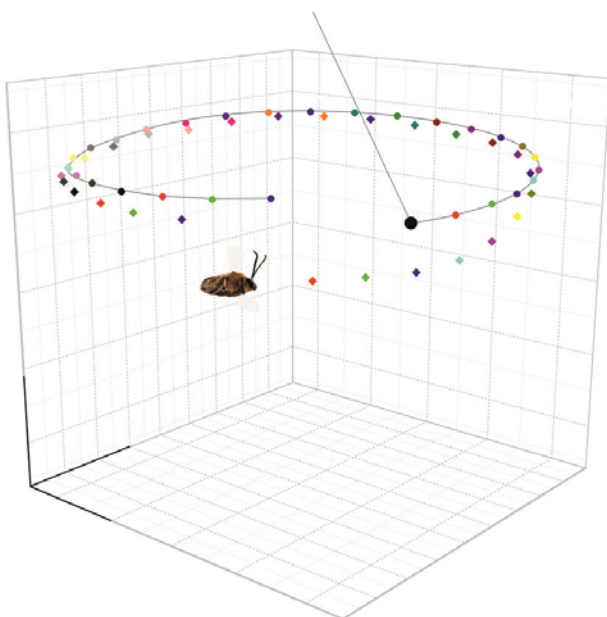


**Figure 3** Number of attracted males (A) and the proportion of successful contacts (B) as a function of dummy size

Raw male numbers (A) and standardized male contacts (B) increase with dummy size, while the effect of dummy speed is not significant in both cases (circles – 2ms<sup>-1</sup>; diamonds – 3ms<sup>-1</sup>; Tab. 2). Contacts are measured as the sum of all bees appearing on images taken every half second from the 3 minute video sequence. Standardized contacts are calculated from the number of males that made contact with the dummy in a trial divided by (activity\*100).

### Chasing behavior and ommatidia excitation

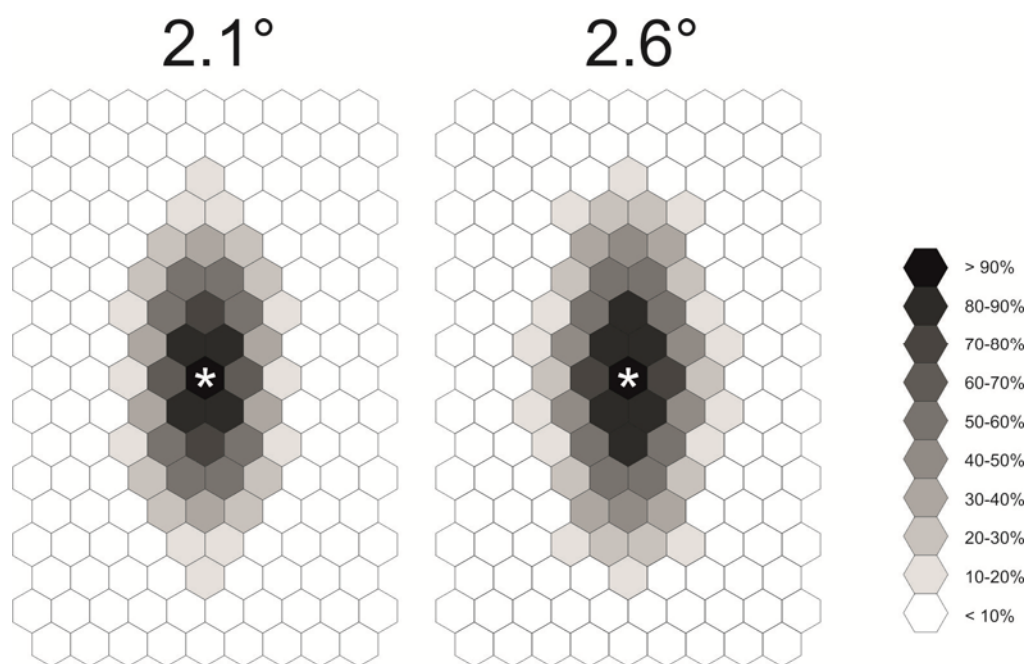
*E. berlandi* males were attracted to the moving dummy and kept a certain distance from the dummy during the chase (Fig. 4). In total, we analyzed six approaches, three to the medium sized dummy and three to the large dummy.



**Figure 4** Reconstruction of a chase

Schematic drawing of a 3-D reconstructed flight path of a male *E. berlandi* chasing a small black female dummy ( $\varnothing=14\text{mm}$ ). The dummy was attached to a piece of string and manually moved on a circular path. Depicted is a sequence of 1s, showing the position of the dummy (upper path, circles connected by a black line) and the bee head (lower path, diamonds) for each frame (40ms). Identical colors indicate the position of the dummy and the male at the same point in time. A scale is given in the lower left corner (50cm in each direction). Note that the insets that depict the dummy and the bee are for illustrative purposes only and are not drawn to scale.

The median distance during the approach was c. twice as large for the three approaches to the large sphere (661mm, 648mm, 764mm) compared with the approaches to the small sphere (353mm, 349mm, 389mm). However, when the median angle was converted to the visual angle that the object subtends at the bee's eye, it was similar for the large (2.6°, 2.6°, 2.2°) and small (2.3°, 2.3°, 2.1°) spheres, suggesting that the angular size of the target plays a more important role than absolute distance during the chase. Based on our interommatidial angle measurements and the median distances during the chase, we estimate that 17 ommatidia are excited to at least 50% relative to the central one (Fig. 5).



**Figure 5** Ommatidial excitation pattern

Excitation of the ommatidial array caused by the dummy as seen from the median distance during the chase. The smallest (2.1°) and largest (2.6°) median angular sizes of the dummy during the chases were used to calculate the excitation pattern (see results). Excitation was calculated to be relative to the central ommatidium (marked with an asterisk).

## Discussion

We investigated the visual system and visual target detection abilities of a solitary bee species. *Eucera berlandi* is a large Eucerini bee which shows a distinct sexual dimorphism in its sensory system (Streinzer, 2008; Streinzer et al., in press). Males have larger eyes than females, equipped with larger, but not more, facets. The interommatidial angles in the frontal eye region are smaller in males than in females. Male of *E. berlandi* are attracted by the scent of the virgin female, and once in close vicinity, chase a moving object for some time. During the chase they appear to hold the object at a fixed angular size. Our calculations revealed that several ommatidia are involved in this phase of target detection. Larger dummies are more attractive to male bees and receive a higher proportion of successful approaches. Our findings are discussed in more detail in the following.

### Adaptations of the compound eye

Apposition compound eyes, found in most diurnal insects including all bees, allow only limited spatial resolution and are relatively insensitive to light compared with other eye types, such as superposition compound eyes or lens eyes (Land, 1997). Spatial resolution is mostly determined by the angle between neighboring ommatidia, while light sensitivity depends on the angular acceptance function of the ommatidium and the lens diameter. To improve resolution, ommatidial axes have to move closer together. Since the eye is highly diffraction limited, a simultaneous increase in facet diameter is necessary to actually improve spatial resolution (Land and Nilsson, 2002; Snyder et al., 1977). Due to this relationship, an improvement of spatial resolution demands an increase of the local eye radius.

Improved spatial resolution can be achieved by increasing overall eye size or by increasing the eye radius and facet lenses (and decreasing the interommatidial angles) only in a small region, which comes at an expense of the resolution in the rest of the eye. Both strategies interact; usually larger bees have larger eyes with better resolution, but acute zones are also found in nearly all species (Jander and Jander, 2002; Kapustjanskij et al., 2007; Somanathan et al., 2009a; Somanathan et al., 2009b; Spaethe and Chittka, 2003). *Eucera berlandi* is a relatively large bee, and consequently has large eyes equipped with more than 8,000 facets. The interommatidial angles of females are smaller than in honeybees (Seidl, 1980) and bumblebees (Spaethe and Chittka, 2003), and similar compared with females of the large carpenter bees *Xylocopa leucothorax*, *X. tenuiscapa* and *X. tranquebarica* (Somanathan et al., 2009a).

Male eye design departs from the females' and distinct adaptations for mate detection are found. They have enlarged frontal facets with a facet size reaching that of *Apis* drones (Ribi et al., 1989; Streinzer et al., 2013; van Praagh et al., 1980) and interommatidial angles that are considerably smaller than in females, but also slightly smaller than in *Apis mellifera* drones (Seidl, 1980). Vallet and Coles (1993b) noted that honeybee drones have interommatidial angles that appear relatively large compared with even smaller individuals of Diptera and the authors concluded that this may be due to a larger size of the acute zone in *Apis*. In *Eucera*, the region where large facets are found in males is smaller and restricted to the frontal facets. Future mapping of the interommatidial angles in the entire visual field of *Eucera* should reveal the exact extent of the zone of high spatial acuity. We cannot entirely exclude that the differences among species are to some extent due to differences in the methods used to map the ommatidial angles (see discussion in Land, 1997). However, independent of small differences among species, the comparison between both sexes of *Eucera* reveals a highly acute and most likely contrast sensitive region in the frontal visual field of male *Eucera*, which is associated with mate detection. The eye parameter is a measure of the trade-off between resolution and sensitivity, and further emphasizes the importance of high resolution in males compared to females (Tab. 1). While females have a value ( $0.49\mu\text{mrad}$ ) typical of diurnal flying insects, males possess a lower value ( $0.39\mu\text{mrad}$ ). Such low values are not typical for fast flying insects, such as *Eucera* males. Fast flight introduces motion blur, which can be reduced by increasing the light catch of the facets, i.e. sensitivity rather than resolution (Land, 1989). However, as stated above, the acute

zone is restricted to the frontal eye region, which receives little motion blur during forward flight compared with the periphery of the visual field. Based on our observations of the facet diameters and pseudopupil size, we estimate that the eye parameter in the peripheral visual field in *Eucera* males is larger, i.e. it is enhanced for fast flight (Warrant et al., 2004).

Comparative data on the male-specific adaptations of the visual system in other bee species are lacking, although enlarged male eyes have evolved in many bee species, e.g. *Apis* (Streinzer et al., 2013), several times within *Bombus* (see *Manuscript III*) and *Xylocopa* (Leys and Hogendoorn, 2008), as well as in many other bee taxa (Michener, 2007). We suggest that collecting such data may greatly improve our understanding of male adaptations for visual mate detection in bees.

### Mate detection

Male *Eucera berlandi* patrol the nesting site close to the ground and wait for the females to emerge (Fig. 2; Streinzer et al., 2009). Upon emergence, females signal their presence and mating status with a sex pheromone (Schaller, 2009) which lures males to the vicinity. Males approach and start to chase a female usually in the sky (own observations). We attracted males to a moving target with olfactory (a sex pheromone mimic) and visual (a highly contrasting female dummy) signals, and compared attraction and success rates at three different dummy sizes and two dummy speeds. We found a significant contribution of dummy size but not speed (Fig. 3, Tab. 2). Larger dummies attracted a higher number of males, most likely because they were better able to detect the larger dummies visually. It is important to note that we did not directly assess the absolute distance from which males were attracted to the dummy. It is conceivable that males are first lured to the vicinity by the sex pheromone and use visual cues only at shorter distances, which is also suggested by previous behavioral studies on *Eucera berlandi* (Streinzer et al., 2009). Similar to honeybees (Vallet and Coles, 1993b), males may be able to react to the very small brightness modulation of single ommatidium, but this was not tested with the designed setup.

We applied an identical amount of sex pheromone in each trial, and thus differences in the number of attracted males can be interpreted as a result of better visibility of the target. Males fly very fast and during these flights are easily distracted by other males or flying objects, which are then chased instead of the original target (own observation). We hypothesize that a large visual target (which corresponds to a short distance to the female in real life situations) minimizes the probability of being lured away by another moving object. This hypothesis may be further supported by the fact that the males' rate of making contact is higher for larger objects (Tab. 2). The differences in success rate between dummy sizes may also be a result of a specific mechanism that males use to visually fixate and manually grab the female. To be successful, males must effectively estimate the distance to the dummy/female and decide when to start the motor pattern to grab the female. Such a mechanism is likely to be hard-wired in the brain, but the exact mechanism is unknown. Behavioral and neuronal target detection mechanisms are better studied in other insect orders, e.g. Diptera (Egelhaaf, 2002; Hornstein et al., 2000) or

Odonata (Olberg, 2012; Olberg et al., 2005). Boeddeker *et al.* (2003) showed in a similar setup that blowflies use a smooth pursuit system that relies on the angular size of the target. As a result, males had the highest success rate when they are confronted with a dummy of the correct size (i.e. similar to the original female). These results differ from our findings. In *Eucera*, male success was correlated with dummy size, even if it was larger than the original female, suggesting a different mechanism than in blowflies.

Our second behavioral experiment revealed that males follow the dummy for a short time, apparently at a distinct distance. This distance depends on the visual angle of the target rather than the absolute distance to the object. This result suggests that distance is coded by retinal size rather than mechanisms such as motion parallax or stereopsis which are observed in Odonata (Olberg et al., 2005). Such a mechanism may be disadvantageous when the object to be detected varies in size (such as prey size of dragonflies), but not when the target size is constant (such as female size in *Eucera*). The distance at which males follow the target is relatively large, compared with shorter distances found in male honeybees (van Praagh et al., 1980), which is probably the result of differences in the flight pattern. Drones exhibit slow forward flight and hovering movements behind the queen, at least in an artificial experimental setup (van Praagh et al., 1980), while male *Eucera* follow the target in a fast flight without hovering elements. The fact that both species keep the target at a fixed distance before attempting to finally catch it highlights the apparent importance of this phase during the approach. In the chase phase, males may discriminate the female/queen from con-specific males, a distinction that demands a different choice of the appropriate subsequent motor patterns. Furthermore, males may wait for the best moment to grab the object. We estimated that the number of ommatidia involved (i.e. they are excited to at least 50% compared with the excitation of the central one) during this phase is 17. This amount of ommatidia is greater than that involved in flower detection by worker honeybees (Giurfa et al., 1996) and bumblebees (Dyer et al., 2008; Spaethe and Chittka, 2003). This fact underpins the importance of resolving details, rather than mere detection of the object. Future experiments should reveal the mechanism for target detection and discrimination in males and show whether it is distinct from the flower detection mechanism in female bees (Dyer et al., 2008; Giurfa et al., 1996; Spaethe and Chittka, 2003).

### Conclusions

In this study we identified adaptations of the male visual system in a solitary bee species, which are most likely associated with visual mate detection. Strong sexual selection among males results in enlarged eyes that provide superior spatial acuity, and probably also a greater contrast sensitivity compared to females. Males utilize visual signals and cues during their approach flight toward a putative female and most likely employ visual cues to activate their motor pattern when finally grabbing the female. Our limited observations may be taken as a starting point for future and more sophisticated and controlled analyses of the approach pattern toward a dummy target. In particular, an analysis of the adjustment of body axis and the control of acceleration

and turning responses toward the target may reveal parallels and differences in the mechanisms that are at work in bees, flies and dragonflies.

### Acknowledgements

We want to thank T. Ellis for help during field work and J. Plant for linguistic improvements.

### Author contributions

MS and JS designed the study and collected the data, MS analyzed and interpreted the data, MS wrote the manuscript and JS commented on the manuscript draft.

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***Manuscript V***

**Sexual Dimorphism in the Olfactory System of a Solitary and a Eusocial Bee  
Species**

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*Journal of Comparative Neurology* (in press). doi: 10.1002/cne.23312

**Sexual Dimorphism in the Olfactory System of a Solitary and a Eusocial Bee Species**

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Received December 22, 2011; Revised August 31, 2012; Accepted January 18, 2013

DOI: 10.1002/cne.23312

INDEXING TERMS: *Eucera berlandi*; *Apis mellifera*; mate detection; olfaction; antennal lobe

Grant sponsor: Forschungsstipendium; Grant number: F-444 (to M.S.); Grant sponsor: Austrian Academy of Sciences at the Department of Evolutionary Biology, University of Vienna (to M.S.).

**ABSTRACT**

Sexually dimorphic sensory systems are common in Hymenoptera and are considered to result from sex-specific selection pressures. An extreme example of sensory dimorphism is found in the solitary bee tribe Eucerini. Males of long-horned bees bear antennae that exceed body length. This study investigated the pronounced sexual dimorphism of the peripheral olfactory system and its representation in higher brain centers of the species *Eucera berlandi*. *Eucera* males have elongated antennae, with 10 times more pore plates and three times more olfactory receptor neurons than females. The male antennal lobe (AL) comprises fewer glomeruli than the female AL (~100 vs. ~130), of which four are male-specific macroglomeruli. No sex differences were found in the relative volume of the mushroom bodies, a higher order neuropil essential for learning and memory in Hymenoptera. Compared with the Western honeybee, the degree of sexual dimorphism in *Eucera* is more pronounced at the periphery. In contrast, sex differences in glomerular numbers are higher in the eusocial honeybee and a sexual dimorphism of the relative investment in mushroom body tissue is observed only in *Apis*. The observed differences between the eusocial and the solitary bee species may reflect differences in male-specific behavioral traits and associated selection pressures, which are discussed in brief.

## INTRODUCTION

Insects sense odors with olfactory sensilla located on the antenna. Individual sensilla house a few to several dozen olfactory receptor neurons (ORNs), which usually express only a single olfactory receptor gene, coupled with the coreceptor *Orco*; together they account for odor specificity (de Bruyne and Baker, 2008; Vosshall and Hansson, 2011). ORNs project into the antennal lobes (ALs), where they synapse with local interneurons and projection neurons, thereby forming functional units called glomeruli (Boeckh and Tolbert, 1993). The number of glomeruli is assumed to reflect the number of olfactory receptor types expressed in the antenna (de Bruyne and Baker, 2008). Olfactory information is subsequently conveyed to higher processing centers, such as the lateral protocerebrum and the mushroom bodies (MBs), where sensory integration and memory formation occur (Farris, 2005; Strausfeld et al., 1998). Specializations along the olfactory pathway are common and generally reflect specific odor-guided behaviors that are performed throughout life (Hansson and Stensmyr, 2011). Selection pressure on various traits of the olfactory system (and other sensory systems) vary between taxa, sexes, and life styles (e.g. social vs. solitary life) and cause variation in the organization and complexity of the olfactory system (Hansson and Stensmyr, 2011; Kleineidam and Rössler, 2009; Strausfeld and Reisenman, 2009) and higher brain centers (El Jundi et al., 2009; Gronenberg, 2008). The largest body of knowledge on sex-specific odor and pheromone detection and processing originates from Lepidoptera (Hansson and Stensmyr, 2011). Adaptations of the olfactory system have further been described from a wide range of insect taxa, such as Diptera, Coleoptera, Hymenoptera, and Blattodea (Hansson and Stensmyr, 2011; Kelber et al., 2010; Schachtner et al., 2005). Differences between sexes usually result from species-specific selection pressures and task differences during life. In bees, females usually seek to find or build nests, forage for food, and provision the brood, whereas males focus predominantly on the search for females with which to mate (Alcock et al., 1978). As a consequence, adaptations of the olfactory system differ between the sexes, which are often apparent in a distinct external sexual dimorphism. An outstanding example of such a dimorphism is observed in the solitary bee tribe Eucerini (Hymenoptera, Apidae). Male long-horned bees bear antennae that exceed body length, whereas those of the female are relatively short (Michener, 2007). The physiological and functional consequences of the long antennae, as well as the consequences of the pronounced morphological dimorphism on the olfactory pathway, are hitherto unexplored. Solitary bees are vastly underrepresented in comparative studies on the olfactory system of insects and only one of the ~20,000 species worldwide, the Western honeybee, has been comprehensively investigated. *Apis mellifera* also shows an extreme sexual dimorphism of the olfactory system. Drones possess enlarged antennae that house a higher number of olfactory sensilla than workers (Esslen and Kaissling, 1976; Nishino et al., 2009). The additional bias in multiplication of sex-pheromone-sensitive ORNs in honeybee drones causes an enlargement of the corresponding glomeruli in the AL (Arnold et al., 1985; Brockmann et al., 1998; Wanner et al., 2007). As a consequence, the antenna is selectively tuned to the detection of the long-distance sex pheromone component (2E)-9-oxodecenoic acid (9-ODA; Brockmann et

al., 1998; Sandoz, 2006; Wanner et al., 2007). Furthermore, honeybee sexes differ greatly in the number of glomeruli in the antennal lobe, a feature that seems to be unique in Hymenoptera (Schachtner et al., 2005). In *Apis*, the overall number of AL glomeruli in drones is about 40% lower than that in workers (Arnold et al., 1985). This article describes the conspicuous olfactory dimorphism of the solitary long-horned bee species *Eucera berlandi* and compares it with the olfactory system of the eusocial *Apis mellifera*. Males of both species presumably face strong sexual selection pressures, and their scramble competition mating system is known to promote the evolution of enhanced sensory and locomotion capabilities (Andersson and Iwasa, 1996). The elaboration of sexually selected traits, however, is assumed to counter and interact with natural selection and is thus limited. In honeybees, drones are provided with food and shelter by their colony, whereas *Eucera* males live fully autonomous lives. We speculate that additional selection pressures in *Eucera* may limit the allocation of sensory resources for mate detection. Therefore, we expect that the investment in olfactory systems and higher order processing is less dimorphic in the solitary bee species than in *Apis*. To test our prediction, we measured and compared the numbers of olfactory sensilla and ORNs, the presence/absence of macroglomeruli, the numbers of glomeruli in the AL, and the volumes of the mushroom bodies.

## MATERIALS AND METHODS

### Study species

*Eucera (Synhalonia) berlandi* (Dusmet, 1926)

The long-horned bee *E. berlandi* (for simplification referred to as *Eucera* throughout this article) is a solitary eucerine bee common to the Mediterranean region. Long-horned bees show an extreme sexual dimorphism, and the males' antennae rank among the longest in bees (Michener, 2007). Males emerge from underground nests prior to females and patrol the nesting sites, awaiting the appearance of the females. Once females have emerged, males rely on olfactory and visual signals to detect females with which to mate (Danesch and Danesch, 1976; Shimron and Hefetz, 1985; Streinzer et al., 2009). Most female solitary bees are assumed to mate only once, so the first successful male receives full reproduction success from that female (Paxton, 2005; Shimron and Hefetz, 1985). The males of *E. berlandi* serve as specific pollinator of a Mediterranean sexually deceptive orchid and have been extensively used for behavioral studies in this context (e.g. Streinzer et al., 2009). Furthermore, the chemical mimicry of the sex pheromones by the plant allows for future characterization of the behaviorally active semiochemicals. All male and female specimens used in this study were collected on Crete (Greece) and Istria (Croatia).

*Apis mellifera* Linné, 1758

The Western honeybee (for simplification referred to as *Apis* throughout this article) is a highly eusocial bee. Colonies persist for several years. To reproduce, drones and virgin queens are produced only during a short period in the summer season. Males temporarily leave the colony, congregate at prominent landmarks, and wait for queens to enter these congregations during their

mating flight (Winston, 1991). When a queen enters such a congregation, the drones detect her by both olfactory and visual means. Queens usually mate multiple times, whereas drones mate only once and die shortly after (Winston, 1991). All honeybee specimens used in this study were obtained from the bee facility at the University of Würzburg. In the honeybee colony, the majority of tasks (e.g. nest site selection and nest construction, foraging, orientation and flower detection while foraging, thermoregulation, and brood care) are performed by workers (Winston, 1991). Although the queen is the reproductive female, the sterile workers represent the functional equivalent to the female in solitary bees, and we thus compare our data on the solitary bee species with that on the honeybee workers. Previous studies in eusocial and solitary bees indicate that the volume of the brain and brain structures, in particular the mushroom bodies, increases with age and experience (Withers et al., 1993, 2008). We did not control for individual age and experience in the specimens used. However, all individuals were collected at an age when they were already foraging for food and nectar (females) or performing mating activities (males). We are thus confident that the major volume change, which is associated with the transition from indoor tasks to outdoor foraging and mating behavior (Fahrbach et al., 1997; Withers et al., 1993, 2008), has already taken place.

#### Antennal morphology

Ethanol-stored antennae of *Eucera* males and females were mounted on a microscope slide and photographed at x15 magnification using a stereo microscope (Nikon SMZ-U, Tokyo, Japan) equipped with a digital camera (Nikon DS-Fi1). Antennal length and width were measured from photographs in ImageJ (National Institute of Mental Health, Bethesda, MD). To compare the total number of olfactory sensilla between sexes, we counted sensilla placodea (pore plates; PPs) on the antenna. PPs constitute the most abundant olfactory sensilla, and their role in sensing odors and sex pheromone components has been demonstrated in the honeybee (Kaissling and Renner, 1968; Vareschi, 1971). One antenna was cut from male and female individuals of *Eucera* and covered with nail polish. After drying, the nail polish coat was removed from the antenna, and the negative imprint was used for further investigations. The imprint was cut with a microblade along the length axis, flattened, and mounted onto a microscope slide and subsequently photographed with a microscope (Nikon LaboPhot II equipped with a DS-Fi1 camera) at x40 and x100 magnifications. The overlapping sections were stitched together in Adobe Photoshop CS2 (Adobe Systems, San Jose, CA). PPs could be easily recognized on the imprint surface (see Fig. 1) and were counted manually in ImageJ. Additionally, we measured PP size (length and width) of 30 randomly selected PPs in one individual of each sex. Some, but not all, of the other sensilla types could also be unambiguously classified with the technique used (see Fig. 1; see also Riveros and Gronenberg, 2010). The antennal surface was measured from the antennal imprint by tracing the outlines. Surface measurements and PP counts were performed on the flagellum segments 2–10 in females and 2–11 in males, because only these segments are equipped with PPs. Antennal measurements of *Apis* were taken from the literature (Esslen and Kaissling, 1976).

### Number of ORNs

Antennae were cut from immobilized *Eucera* bees at the base of the most basal flagellum segment. To facilitate perfusion of the fixative (Karnovsky solution: 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer), only a short piece of two or three basal segments was fixed for 1 hour at RT. Antennae were rinsed three times in 0.1 M sodium cacodylate buffer and postfixed with 1% osmium tetroxide for 2 hours. After thoroughly rinsing in cacodylate buffer, segments were dehydrated in an ascending alcohol series (50%, 70%, 80%, 96%, 3 x 100%, 10 minutes each) and embedded in Agar Low Viscosity Resin (Agar Scientific, Essex, United Kingdom). Semithin (1–2  $\mu\text{m}$ ) and ultrathin (70 nm) sections were cut with glass or diamond knives (type ultra, size 2.5, 45° knife angle; Diatome, Biel, Switzerland) on an EM UC6 (Leica Microsystems, Wetzlar, Germany). The sections were stained for light microscopy using 1% azure II and 1% methylene blue in an aqueous 1% borax solution (diluted 1:9 in aqua bidest) for 15 seconds at 60°C. Sections were photographed on a light microscope (Nikon LaboPhot II equipped with a DS-Fi1 camera). Ultrathin sections were contrasted (25 minutes uranyl acetate, 5 minutes lead citrate) and subsequently viewed and photographed with a Zeiss EM 902 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) equipped with a slow-scan CCD camera (TRS, Tröndle). Images were corrected for brightness and contrast in Adobe Photoshop CS4. To quantify the total number of ORNs, we first calculated the area of both antennal nerves from the light microscopic sections of the antennae by tracing the outlines in ImageJ (Fig. 1H). EM photographs at magnifications between x4,400 and x12,000 were used to count all individual ORNs on five randomly chosen areas of  $80.2 \pm 46.2$  (SD)  $\mu\text{m}^2$ . ORNs were distinguished from other sensory neurons by their small diameter (Nishino et al., 2009). Total ORN number was then calculated by multiplying the mean ORN density of the five areas with the total antennal nerve cross-section area. The only published ORN numbers for *Apis* are extrapolations derived from multiplication of sensilla numbers by mean receptor neuron number/sensillum ratios (R/S ratio). Furthermore, estimates for drone antennae were obtained using worker R/S ratios (Esslen and Kaissling, 1976). For reliable comparisons, we therefore measured total ORN number also for workers and drones of *Apis* using the same procedure as for *Eucera*.

### Brain reconstruction

*Eucera* and *Apis* specimens were cooled in a freezer for several minutes. The heads were removed with a sharp razor blade and mounted in wax dishes for preparation. The head capsule was opened, and the brain was dissected in ice-cold phosphate-buffered saline (PBS; pH 7.4). Brains were subsequently transferred to 4% paraformaldehyde in PBS and prefixed for 2 hours. The brains were then transferred to a second fixative (FixMix: 2% paraformaldehyde, 2% glutaraldehyde in PBS; Kuebler et al., 2010) and postfixed for several days at 4°C. For confocal microscopy, the brains were first dehydrated in an ascending series of ethanol (50%, 70%, 90%, 96%, 3 x 100%, 10 minutes each) and subsequently transferred to methylsalicylate (M-2047; Sigma-Aldrich, Steinheim, Germany). Whole brains were mounted in customized aluminum

slides and scanned with a laser scanning confocal microscope (Leica TCS SP2 AOBS; Leica Microsystems AG, Wetzlar, Germany) equipped with an argon/krypton laser ( $\lambda_{\text{ex}} = 561 \text{ nm}$ ). Appropriate objective lenses were used to scan brains (HC PL APO CS 10.0 x 0.40 UV; 10.0 x 0.40 IMM/CORR) at intervals of 4–5  $\mu\text{m}$  and ALs (10.0 x 0.40 IMM/CORR, HC PL APO lbd.BL 20.0 x 0.70 IMM/ CORR UV) at 1  $\mu\text{m}$ . z-Axis refractive index mismatches were corrected manually before 3-D reconstruction. Reconstruction and volumetric analyses were carried out with Amira 3.1 (Mercury Computer Systems, Berlin, Germany). We reconstructed the neuropil volume (without cell bodies) of the third optic lobe neuropil, ALs, central complex, and MBs of 16 brains (*E. berlandi*: females and males,  $n = 4$  each; *A. mellifera*: workers and drones,  $n = 4$  each). Because of the large size of the brains, only a part of the optical lobes could be scanned in most of the preparations. For our analysis, we therefore included only the third optical lobe neuropil, the lobula, which could be completely reconstructed in all preparations. As a measure of total brain size, we also reconstructed the remainder of the supraesophageal ganglion (SEG; excluding cell bodies) and calculated the sum of all reconstructed neuropils. To distinguish the SEG from the subesophageal ganglion, all neuropil located dorsal of the esophageal foramen was arbitrarily assigned to the SEG. ALs of *Eucera* (females  $n = 3$ , males  $n = 4$ ) were scanned at a higher magnification and reconstructed. All individual glomeruli were segmented and subsequently reconstructed in 3D. Volumetric data were calculated in Amira 3.1 and exported to Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA) and SigmaPlot 11.0 (Systat Software, Chicago, IL) for further analysis. AL data for *Apis* were obtained from the literature (Arnold et al., 1985; Brockmann and Brückner, 2001). Previously published volume data on brain neuropils in *Apis* may not be comparable because of methodological differences (see comments in Fahrbach et al., 1995). We therefore decided to repeat brain neuropil measurements in *Apis* using the same method as for *Eucera*.

#### Definition of a macroglomerulus

Numerous studies reported the presence of conspicuously large glomeruli, so-called macroglomeruli (MG) or macroglomerular complexes (MGCs), and several measures are used to define an MG (Kelber et al., 2009; Kuebler et al., 2010). Here, we followed the definition by Kuebler et al. (2010), using descriptive statistics of the distribution of glomeruli volumes in each individual and treating all glomeruli as MGs that exceed the upper percentile by more than three times the interpercentile range (using the 10<sup>th</sup> and 90<sup>th</sup> percentile, i.e., the range that includes 80% of the measured values).

#### Statistical analysis

To compare absolute brain volume between sexes, a nonparametric Mann-Whitney U-test was applied. For comparison of the relative neuropil volumes, percentage values were first arcsine-transformed. All P values below the 5% level were considered to be statistically significant. All statistical calculations were performed in SigmaPlot 11.0. For data visualization we used SigmaPlot 11.0 and Microsoft Excel 2007.

## RESULTS

### *Eucera berlandi*

#### Antennal morphology

The antenna of *Eucera* consists of a scape, pedicel, and flagellum, which in males has 11 and in females 10 segments, a common trait in bees (Michener, 2007). The male flagellum is more than three times as long as the female flagellum ( $FL_{\text{male}}$ :  $12.1 \pm 0.1$  [SD] mm, and  $FL_{\text{female}}$ :  $3.7 \pm 0.2$  [SD] mm;  $n = 3$  each) and is about one third wider in males ( $0.45 \pm 0.00$  [SD] mm) than in females ( $0.34 \pm 0.02$  [SD] mm;  $n = 3$  each; Table 1). The larger size in males results from one additional segment and overall elongated individual flagellum segments. Total surface area, measured from surface imprints, is  $15.4 \pm 0.5$  (SD)  $\text{mm}^2$  for the males, nearly four times the area of the female antenna ( $3.9 \pm 0.1$  [SD]  $\text{mm}^2$ ;  $n = 3$  each; Table 1). The male antenna appears dorsoventrally flattened, whereas the female antenna is round in cross section.

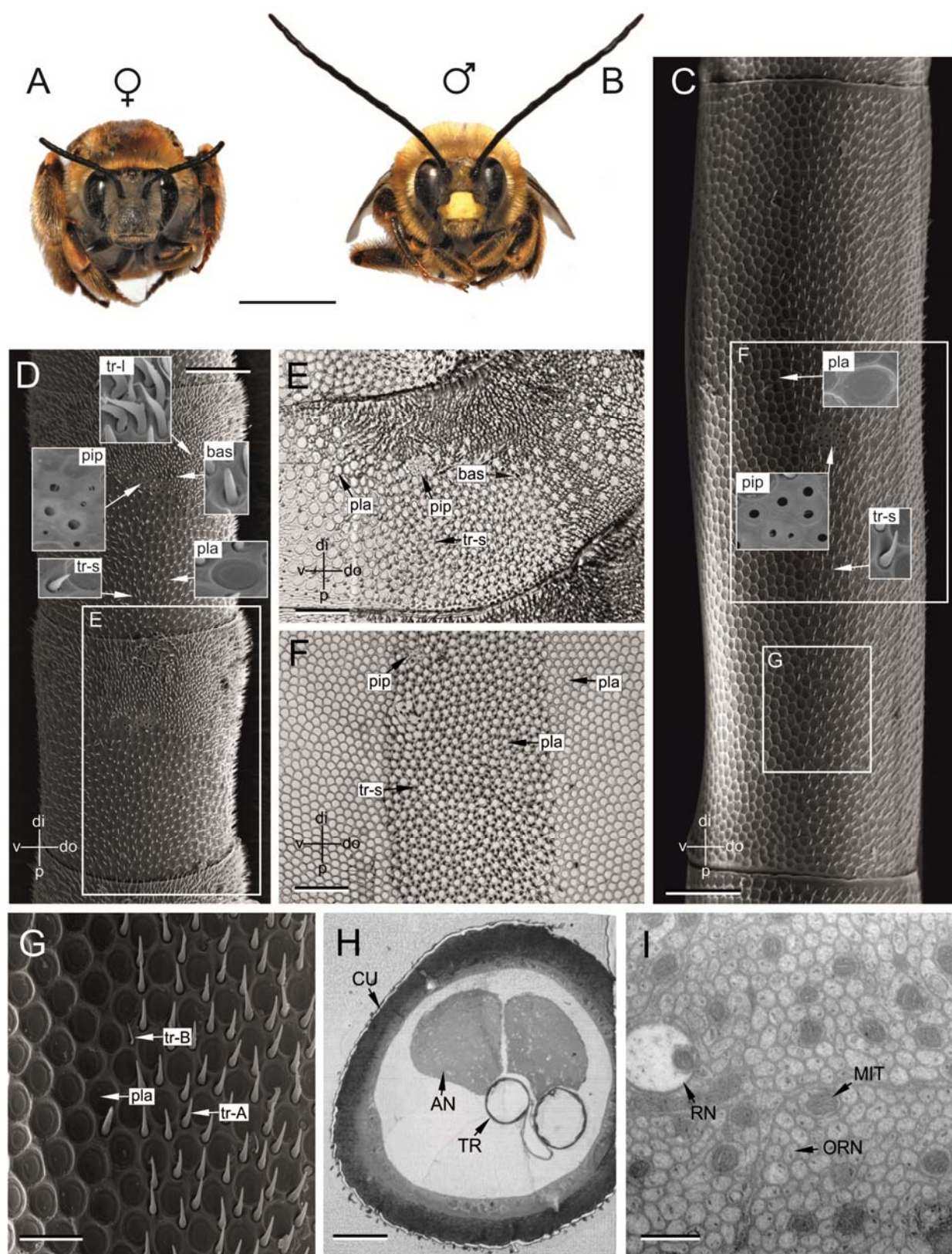
#### Sensilla types and distribution on the antenna

Several types of sensilla are present on the antenna in both sexes (Fig. 1). We classified them based on their gross morphology into placoid, basiconic, trichoid, and peg-in-pit sensilla. The focus of our study was to assess the number of the main olfactory sensilla, the placoid sensilla (PPs); however, the presence and topography of the other types are described for both sexes in brief.

#### *Sensilla placodea*

The total number of PPs on each antenna is  $35,578 \pm 1,099$  for males and  $3,371 \pm 112$  for females (means  $\pm$  SD;  $n = 3$  each; Table 1). PPs are present on all flagellar segments, except for the most proximal segment, and their numbers vary between flagellar segments in both sexes. PP topography differs between sexes. In the female, patches of PPs are present at the anterior-distal part of each segment. Only few PPs are scattered throughout the rest of the segment (Fig. 1D,E). On the male antenna, PPs cover the whole antennal surface except for a small band on the posterior surface, which contains no sensilla at all. The posterior flagellum surface consists of very thick cuticle, which probably serves to increase stability during flight. Although the dorsal and ventral surfaces of the male antenna are exclusively equipped with PPs, the anterior surface shows a conspicuous band with a mixture of PPs and trichoid sensilla (see below; Fig. 1C, F, G). In males, the total PP density is between two and three times higher than in females ( $2,289 \pm 58$  and  $856 \pm 38$  PP/ $\text{mm}^2$  antennal surface, respectively; mean  $\pm$  SD;  $n = 3$  each). Individual PPs measure  $15.7 \pm 2.0 \times 13.3 \pm 2.0$   $\mu\text{m}$  ( $n = 30$ ) in females ( $n = 1$ ) and  $15.4 \pm 1.2 \times 14.0 \pm 1.0$   $\mu\text{m}$  ( $n = 30$ ) in males ( $n = 1$ ).





**Figure 1**

A,B: Dried specimens of *Eucera berlandi* illustrating the pronounced sexual dimorphism of the peripheral olfactory system. The male (B) antenna possesses about three times the surface area compared with the female (A). Scanning electron microscopic images (C,D,G) and light microscopic images (E,F) of replicas of the female (C,E) and male (C,F,G) antennal surface. The approximate regions of the details in E–G are indicated by boxed areas in C,D. A variety of sensilla types is present on the antenna in both sexes. Pore plates (pla) were found on all flagellar segments except for the most proximal one. Their number is about 10 times higher in males compared with females. In males, poreplates are distributed almost uniformly over the antennal surface, whereas, in females, their distribution is patchy (C,D). Peg-in-pit sensilla (pip) are present as a single cluster with few individual sensilla outside the cluster. They could not be further

subclassified with the applied technique. Short trichoid sensilla were found in both sexes (tr-s; C–G). Higher magnifications (G) allows us to subclassify them into sensilla trichodea A (tr-A; sensu Esslen and Kaisling, 1976) and sensilla trichodea B (tr-B). They are located mainly in the PP-rich region in females and form a conspicuous band along the anterior rim of the antenna in males. Longer trichoid sensilla (tr-l) are present in females but are lacking in males. Basiconic sensilla (bas) were found only on the female antenna (D,E) and are clearly lacking in males. H: Semithin section at the base of flagellum segment 1 of a male *E. berlandi*. The two antennal nerves (AN) are almost identical in cross section area. Two large tracheae (TR) are present in the antennal lumen. CU, cuticle. I: Transmission electron microscopic images of the antennal nerve. The antennal nerve tissue comprises mainly ORNs; other receptor neurons (RN) such as mechano-, thermo-, or hygrosensitive neurons are present at much lower numbers. Mitochondria (MIT) are also abundant in the antennal nerve. ORNs can be distinguished from other sensory neurons by their small diameter (Nishino et al., 2009). di, Distal; do, dorsal; p, proximal; v, ventral. All images were adjusted for brightness and contrast in Adobe Photoshop CS 4. The entomological pins were digitally removed from the images in A,B. Scale bars = 5 mm in A,B; 100  $\mu$ m in C,D,F,G (20 $\mu$ m for sensilla insets); 25  $\mu$ m in E; 50  $\mu$ m in H; 500 nm in I.

**Table 1** Morphological Features of Antennae and Antennal Lobe of *Eucera berlandi* and *Apis mellifera* <sup>1</sup>

	<i>Eucera berlandi</i>		<i>Apis mellifera</i>	
	Female	Male	Worker	Drone
Flagellum surface	3.94 (0.07) n = 3	15.38 (0.45) n = 3	1.58 <sup>2</sup>	3.55 <sup>2</sup>
No. pore plates	3,283/3,334/3,497	34,604/36,769/35,362	2,530/2,672 <sup>2</sup>	18,153/19,035 <sup>2</sup>
Pore plate dimensions	15.7 (2.0) x 13.3 (2.0)	15.4 (1.2) x 14.0 (1.0)	16 x 9 <sup>2</sup>	14 x 9 <sup>2</sup>
Antennal nerve area	4,472	11,713/12,591	4,393/5,268	9,290
No. ORNs	98,601	268,265/298,767	73,309/99,332	288,966
No. glomeruli	132/133/133	95/95/98/103	165/166/166/	102/102/104/
No. MGs	0	4	0 <sup>3</sup>	4 <sup>3</sup>
Proportion MGs/	—	0.36–0.44	—	0.44 <sup>3</sup>
Total glomerular	6.2 (1.1)	14.4 (2.7)		

<sup>1</sup>When means are presented, standard deviation is given in parentheses.

<sup>2</sup>From Esslen and Kaisling (1976).

<sup>3</sup>From Arnold et al. (1985).

### *Sensilla trichodea*

Trichoid sensilla occur on the antennal surface of both sexes. Two types of trichoid sensilla were distinguished. One type, short trichoid sensilla, is present on flagellar segments 3–10 in females and 2–11 in males. In females, this type is mainly located in the PP-rich region between the PPs and on the ventral surface of the antenna. In males, short trichoid sensilla are present only on the anterior rim of the antenna (except for a few single sensilla that are found outside this region), where they form a conspicuous band (Fig. 1D, G). The second type, long trichoid sensilla, is present only on the female antenna. These sensilla are located primarily on flagellar segments 3–10 mainly distal and dorsal to the PP-rich region. In males, long trichoid sensilla are absent in the flagellum segments 2–11. With the applied technique, they appear similar to long, stiff setae, which are located on the basal segments in males (flagellum segment 1) and females (flagellar segments 1–2), and thus the presence of long trichoid sensilla in these segments cannot be entirely excluded.

### *Peg-in-pit sensilla*

Peg-in-pit sensilla are present on the antennal surface in both sexes. In both sexes, they are clustered as a single patch on each segment, and few single peg-in-pit sensilla are present outside this patch (Fig. 1C, D). In females, the patch is located distal to the PP-rich area, at the anterior

antennal surface (Fig. 1C, F). In males, the patch is centered at the lower rim within the conspicuous band of trichoid sensilla (Fig. 1D, G). The three types of peg-in-pit sensilla (*S. ampullaceum*, *S. coeloconicum*, and *S. coelocapitulum*) are morphologically indistinguishable on the imprint and thus could not be further classified.

### *Sensilla basiconica*

Basiconic sensilla occur only in females on flagellar segments 3–10. They are located mainly in the distal region of the anterior surface of each segment, scattered among trichoid sensilla (Fig. 1C, F). Their number was not assessed. In male *E. berlandi*, no basiconic sensilla are found on the antennal surface, a trait that seems to be common to male bees.

### Antennal nerve size and ORN numbers

The receptor cell axons form two antennal nerves, which are almost identical with respect to their cross-sectional area. The cross-sectional area of the antennal nerve in male *E. berlandi* is  $12,152 \pm 621 \mu\text{m}^2$  (mean  $\pm$  SD;  $n = 2$ ), almost three times the area of the female ( $4,472 \mu\text{m}^2$ ;  $n = 1$ ; Tables 1, 2). The calculated number of ORNs per antenna is about three times higher in males (298,767 and 268,265 ORNs/antenna) than in females (98,601 ORNs/antenna). We additionally measured ORN diameters in all preparations. We found variation (range of means 121–173 nm,  $n = 50$  per individual); however, it is not clear whether these differences reflect variation between sexes and/or individuals. Additional measurements on a larger sample are therefore needed to elucidate the source of this variation.

**Table 2** Sex-Specific Ratios of Selected Traits of the Olfactory Pathway of *Eucera berlandi* and *Apis mellifera*<sup>1</sup>

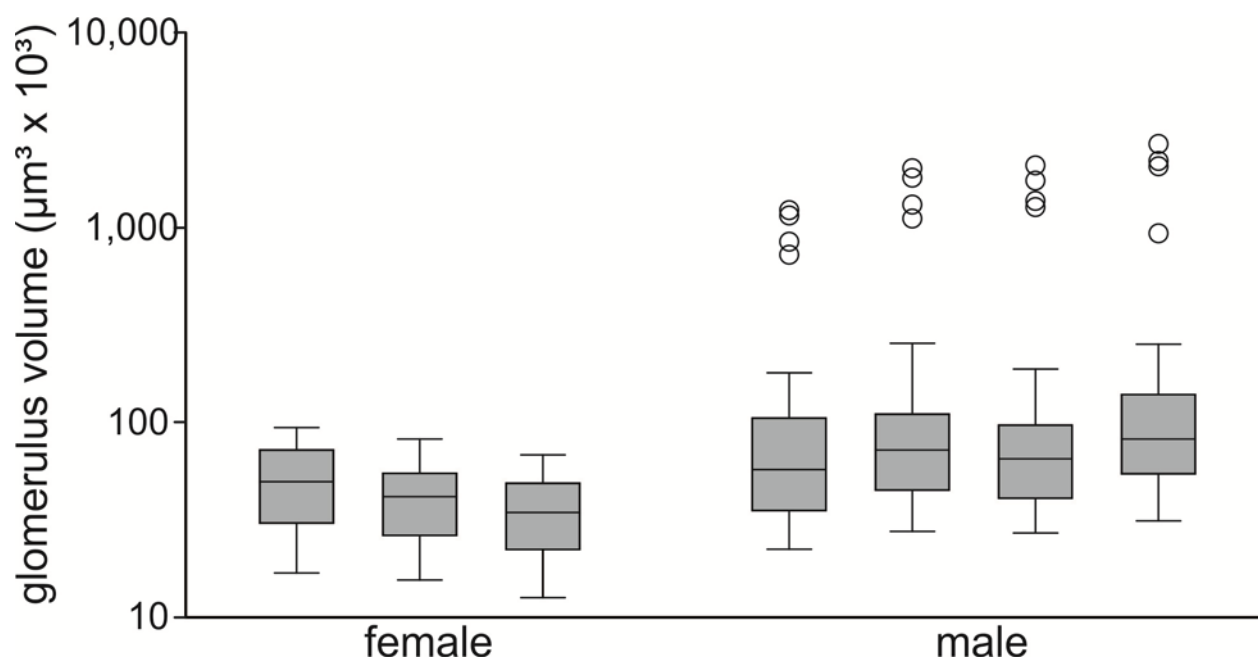
	<i>Eucera berlandi</i>	<i>Apis mellifera</i>
No. of PP sensilla (m:f)	10.6	7.2 <sup>2</sup>
Antennal nerve area (m:f)	2.7	1.9
ORN number (m:f)	2.9	3.3
Rel. AL volume (m:f)	1.6	0.9
Rel. MB volume (m:f)	0.9	0.7

<sup>1</sup>Means were used to calculate ratios (Table 1). No statistical comparisons were performed. Relative volume of neuropils is calculated relative to total brain size. m, Male; f, female; PP, pore plates; ORN, olfactory receptor neuron; MB, mushroom body; AL, antennal lobe; Lo, lobulae. <sup>2</sup>From Esslen and Kaisling (1976).

### AL morphology

The antennal nerves terminate in the AL, where ORNs are sorted and converge onto glomeruli. The number of glomeruli is ~26% (35 glomeruli) lower in males (95, 96, 98, 103) compared with females (132, 133, 133; Table 1). For the male AL, we found four strikingly large glomeruli that, according to our definition, are to be considered as macroglomeruli (MG; Figs. 2, 3A, B). Two of the MGs are located at the anterior side of the AL near the entrance of the antennal nerve. The other two MGs are located at the posterior part of the AL (Fig. 3B). We termed them MG 1–4, with MG 1 lying anterior and proximal, MG 2 anterior and distal, MG 3 posterior and proximal,

and MG 4 posterior and distal from the antennal nerve entrance (Fig. 3A, B). The volume of the four MGs accounts for 36–44% of total glomerular volume (Table 1). The largest glomeruli are MG 2 and MG 4; they are between 20 and 32 times larger than the median glomerulus volume. In two preparations, the frontal MG 2 was found to be the largest MG; in two other preparations, the anterior MG 4 was the largest. MG 1 and MG 3 are smaller, with MG 1 being the larger of the two in three of four preparations. MG 1 and MG 3 are between 11 and 25 times the volume of the median glomerulus. In comparison, only regularly sized glomeruli are present in the female AL, with the largest glomerulus having five to six times the volume of the median ( $n = 3$ ; Figs. 2, 3D,E). Glomeruli volumes are not normally distributed in all preparations, and range from 6.2 to  $269.4 \times 10^3 \mu\text{m}^3$  in females (medians 34.5, 41.4,  $49.3 \mu\text{m}^3$ ;  $n = 3$ ) and from 7.5 to  $2,677.1 \times 10^3 \mu\text{m}^3$  (medians 56.9, 64.6, 71.9,  $81.7 \mu\text{m}^3$ ;  $n = 4$ ) in males. The total glomerular volume in males is more than twice that of females.



**Figure 2** Glomeruli volume distribution in female (left) and male (right) *Eucera berlandi*.

Males exhibit extremely enlarged glomeruli (macroglomeruli, MGs; open circles), which are defined as outliers exceeding three times the interpercentile range ( $10^{\text{th}}$  and  $90^{\text{th}}$  percentile) from the upper percentile. In females, no MGs are found, whereas all males possess four MGs. The total number of glomeruli is higher in females (132–133, see Results and Table 1) than in males (95–103). Note that the y-axis is scaled logarithmically, so the difference between median and MGs is greater than it appears. Lines represent the median, boxes the upper and lower quartiles, and the whiskers the  $10^{\text{th}}$  and  $90^{\text{th}}$  percentiles.

### Selected brain neuropils

3-D reconstructions of male and female brains allowed volumetric comparison of selected neuropils. As a measure of total brain size, we calculated the sum volume of all reconstructed neuropils. In *Eucera*, brain volume differs significantly between sexes and is larger in females ( $\text{Vol}_{\text{female}}: 401.5 \pm 29.3$  [SD]  $\mu\text{m}^3$ ,  $\text{Vol}_{\text{male}}: 368.6 \pm 42.7$  [SD]  $\mu\text{m}^3$ ;  $n = 4$  each;  $P < 0.05$ , Mann-Whitney U-test). Among the individual neuropils, only the ALs and the remainder of the supraesophageal ganglion (SEG) differ significantly in their relative volume. While the ALs are

larger in males (13% of total brain volume compared with 8% in females), the SEG is larger in females (Fig. 4). The MBs account for 30% of the total brain volume in females and 26% in males.

### *Apis mellifera*

#### Antennal nerve size and ORN numbers

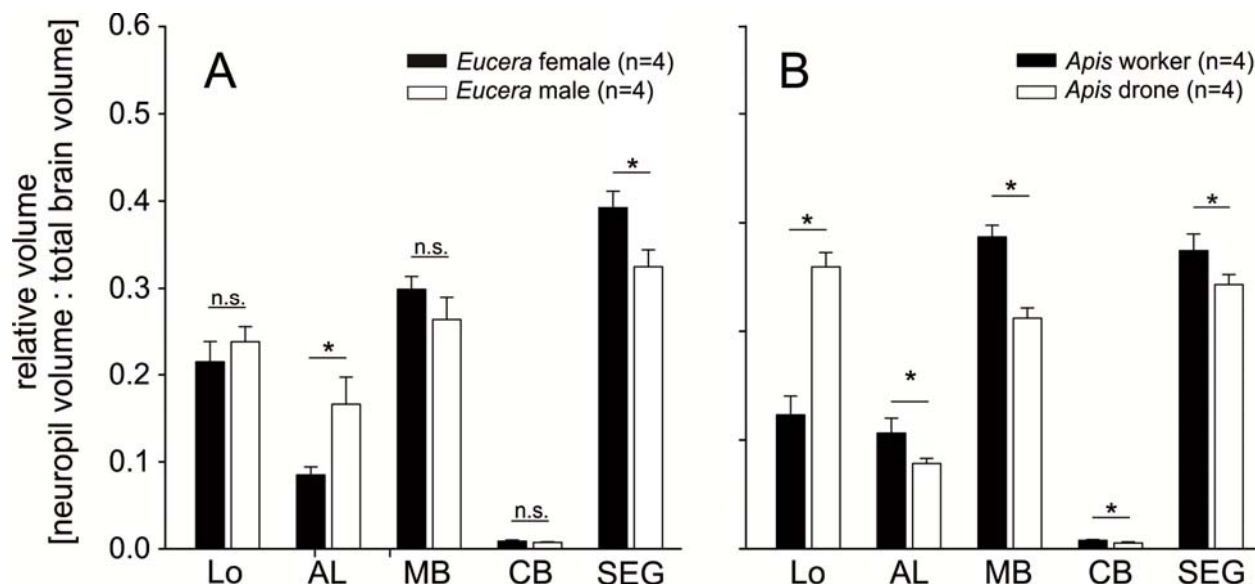
The antennal nerve cross-sectional area of *A. mellifera* drones is  $9,290 \mu\text{m}^2$  ( $n = 1$ ), about twice that of the workers ( $4,831 \pm 619$  [SD]  $\mu\text{m}^2$ ;  $n = 2$ ; Tables 1, 2). The number of ORNs per antenna in drones (288,966 ORNs/ antenna,  $n = 1$ ) is about three times higher compared with workers (99,332 and 73,309 ORNs/antenna;  $n = 2$ ; Table 2). As in *Eucera*, ORN diameters vary between samples (range of means 115–150 nm;  $n = 50$  per individual), without a clear caste-specific separation (see above).

#### Selected neuropils

In *Apis*, absolute brain volume is significantly larger in drones compared with workers ( $\text{Vol}_{\text{worker}}$ :  $209.7 \pm 21.4$  [SD]  $\mu\text{m}^3$ ,  $\text{Vol}_{\text{drone}}$ :  $220.1 \pm 15.4$  [SD]  $\mu\text{m}^3$ ;  $n = 4$  each;  $P < 0.05$ , Mann-Whitney U-test). All neuropils differ in their relative size between sexes. The lobulae occupy about twice the relative volume in drones, but all other investigated neuropils were relatively smaller than in workers. The ALs cover ~13% of the brain volume in workers and 10% in drones, and the MBs account for 36% of the brain volume in workers, compared with only 27% in drones.



transparent in all views. The nerve is larger in diameter in males compared with females, a result of the higher number of ORNs. E,F: 3-D reconstruction of selected neuropils of the brain in male and female *E. berlandi*. Lo, lobula; CB, central body; MB, mushroom bodies; SEG, remainder of the supraesophageal ganglion. The medulla is depicted for visualization only but not included in the analysis. G,H: 3-D reconstruction of selected neuropils of drone and worker honeybees. a, Anterior; d, dorsal; l, lateral; m, median; p, posterior; v, ventral. Scale bars = 200  $\mu\text{m}$  in A (applies to A,C); 200  $\mu\text{m}$  in B (applies to B,D); 500  $\mu\text{m}$  in H (applies to E–H).



**Figure 4** Comparison of relative neuropil volumes in *Eucera* (A) and *Apis* (B). All neuropil volumes were calculated relative to total brain volume (sum of all neuropils). Lo, lobulae; AL, antennal lobes; MB, mushroom bodies; CB, central body; SEG, remainder of the supraesophageal ganglion. Bars represent means  $\pm$  SD ( $n = 4$  each). Relative volume differences were compared between sexes using a nonparametric Mann-Whitney U-test after arcsine transformation of percentage values; n.s.,  $P > 0.05$ ; \* $P < 0.05$ .

## DISCUSSION

We investigated the peripheral olfactory system and associated neuronal brain structures of the highly sexually dimorphic solitary bee species *Eucera berlandi* and compared it with the well-described olfactory system of *Apis mellifera*. Extreme sex differences in the size and sensillar equipment of the antennae (e.g. *Eucera* males have 10 times more PPs), total ORN number (males have three times more ORNs), and the presence of four macroglomeruli in the *Eucera* male AL suggest that high sensitivity for a few selected substances are of great importance for mate detection in *E. berlandi*. Although the extent of dimorphism in the periphery resembles or even exceeds that described for *Apis*, the relative investment in higher order processing neuropils was found to be not significantly different between sexes in *Eucera*, which contrasts with the condition observed in *Apis*. We initially speculated that *Eucera* males are restricted in the allocation of neural resources for mate detection, whereas honeybee drones can afford a higher degree of specialization because of reduced natural selection associated with the safety provided by their colony. The supporting and opposing evidence regarding these predictions is discussed in more detail in the following.

### Origin of highly modified male sensory systems

The origin of highly modified male sensory systems and sexual dimorphism in insects is considered to be driven by sexual selection (Allen et al., 2011; Andersson and Iwasa, 1996). Males compete for access to receptive females, and adaptations are favored that increase the chance of successful mating. Sexual selection is assumed to both counter and interact with natural selection (Allen et al., 2011; Andersson and Iwasa, 1996). Accordingly, male insects often invest more in improved, sex-biased sensory systems (Hansson and Stensmyr, 2011), but the elaboration of a biased sensory system is assumed to be limited (Andersson and Iwasa, 1996; Knell et al., 2004). *Apis* and *Eucera* males both certainly face strong male–male competition (Alcock et al., 1978; Danesch and Danesch, 1976; Shimron and Hefetz, 1985; Winston, 1991; and our observations); however, measuring the absolute strength of sexual selection is impossible, and our knowledge on the behavioral repertoire of males in solitary bees, and even in the well-investigated honeybee, is very limited (Ohtani, 1974).

### Sex-specific adaptations of the antenna

Our investigations of antennal parameters in *Eucera* show a multiplication of PP sensilla and ORNs in males, illustrating the importance of olfactory sensing in mate detection (Tables 1, 2; Esslen and Kaissling, 1976). Such multiplications increase the likelihood of detecting odor molecules, lowering the detection threshold of the antenna. *Eucera* males seem to invest more in olfactory sensing than *Apis* drones, which is suggested by the longer antennae and higher sensilla numbers. Nonetheless, males of both species have very similar numbers of ORNs (Table 1). In honeybees, individual PPs are innervated by 15–30 individual ORNs (Esslen and Kaissling, 1976; Kelber et al., 2006). Based on total ORN numbers divided by the number of PPs in *Eucera*, PPs are innervated by ~29 ORNs in females and ~8 in males. These numbers probably overestimate the actual numbers of ORNs per individual PP, because the contributions of other sensilla types to the total ORN number were ignored. However, our results illustrate that, in *Eucera* males, PPs are innervated by fewer (less than half as many) ORNs compared with the PPs of *Apis* drones (Esslen and Kaissling, 1976). To accommodate the ~300,000 ORNs found in males of both species, the antennae in *Eucera* must be equipped with twice as many PPs and are almost three times as long as in *Apis* drones. The long antennae likely produce considerable costs associated with development (Emlen, 2001), energy consumption (Chittka and Niven, 2009), and locomotion/aerodynamic drag (Swallow et al., 2000). On the other hand, long antennae increase the detection range for simultaneous odor tracking and may thus improve the detection of the female. A similar tradeoff has been described for Lepidoptera. Symonds et al. (2012) found antennal length to be negatively correlated with male abundance and molecular weight of the sex pheromone, suggesting a role of both detection range and chemical structure of the pheromone in the evolution of elongated antennae. At the present stage, the specific adaptive value of the long antennae is still unclear; they could be adaptations to the chemical structure of the sex pheromone, adaptations for elaborated orientation tasks, or merely a byproduct of a phylogenetic constraint associated with the increase of ORN numbers and thus antennal sensitivity in *Eucera*



males. In *Eucera* females, PP and ORN numbers are comparable to the numbers found in *Apis* workers (Esslen and Kaissling, 1976; this study). Although the exact contribution of each sensillum type to total ORN number is unknown for *Eucera*, we suppose that PPs possess multiple ORNs in both sexes. Multiple ORNs are a common trait in Hymenoptera, but the role of this organizational principle is unknown (Kelber et al., 2006; Kleineidam and Rössler, 2009). Aside from PPs we found high numbers of trichoid sensilla in both sexes. The short trichoid sensilla appear similar to Sensilla trichodea A and B of *A. mellifera* workers and drones (Fig. 1D, F, G; Esslen and Kaissling, 1976). Their function is unclear, but at least S. trichodea A (one class of short trichoid sensilla) is assumed to sense odors (Esslen and Kaissling, 1976). Peg-in-pit sensilla have been shown to be thermo-, hygro-, and CO<sub>2</sub>-sensitive (Kleineidam et al., 2000; Lacher, 1964; Ruchty et al., 2009) and were found in both sexes in *Apis* (Nishino et al., 2009) and *Eucera* (this study). Sensilla basiconica were found only in females and were entirely absent in males. This sensilla type is also female specific in other Hymenoptera (Ågren, 1977, 1978; Ågren and Hallberg, 1996; Esslen and Kaissling, 1976; Mysore et al., 2010; Nakanishi et al., 2009; Nishino et al., 2009). A role in gustatory and olfactory chemoreception of colony-specific cuticular hydrocarbon profiles has been proposed for S. basiconica, but conclusive studies on their function are still lacking (Ozaki et al., 2005; but see Brandstaetter and Kleineidam, 2011). S. basiconica innervate glomeruli clusters in the female AL that are lacking in male ants (Kelber et al., 2010; Nakanishi et al., 2010) and bees (Nishino et al., 2009) and thus may contribute to the marked sexual dimorphism of glomeruli numbers observed in Hymenoptera (Schachtner et al., 2005).

### AL morphology

Sex-specific adaptations of the ALs have been described for many insect species (Hansson and Stensmyr, 2011). Sex differences regard mostly the size of individual glomeruli, whereas numerical differences in glomeruli number are rare, with the exception of Hymenoptera (Schachtner et al., 2005; Strausfeld and Reisenman, 2009). The number of associated olfactory-guided behaviors, e.g. detection and discrimination of suitable larval nutrition, seeking nest sites, nest recognition, evaluation of brood viability, and in the case of social Hymenoptera intraspecific communication, may relate to the typically high number of glomeruli in Hymenoptera (Kleineidam and Rössler, 2009; Schachtner et al., 2005). The elaborate brood care performed by females, but not males, has been proposed to be a key factor for the evolution of the pronounced sexual dimorphisms (Stubblefield and Seger, 1994). Hymenopteran males usually have fewer glomeruli than females, indicating that lower numbers of functional receptor types are expressed in the antenna. The lower number theoretically reduces the dimensionality of odor coding and thus complexity of the male odor space. We found about 34 (26%) fewer glomeruli in *Eucera* males, so glomeruli numbers are more similar between sexes compared with *Apis* (~64–69 or ~38–40% fewer glomeruli in males than in workers and ~52–62 or ~33–38% less than in queens [Arnold et al., 1985, 1988; Groh and Rössler, 2008]). Compared with *Apis*, similar or even greater sex-specific differences in glomeruli number have been described for

other eusocial insects (Kuebler et al., 2010; Mysore et al., 2009; Nishikawa et al., 2008; Stieb et al., 2011; Zube and Rössler, 2008) and reach up to 60% in the ant *Harpegnathos saltator* (Hoyer et al., 2005). In most eusocial species, males are embedded in a protective social network and leave the colony only for short, synchronized mating flights (Ayasse et al., 2001). The solitary life style of *Eucera* males likely imposes higher demands on orientation, food detection and sheltering, which distinguishes them from eusocial species and may have resulted in the evolution of relatively more complex (or less reduced) olfactory systems. Preliminary data from another solitary bee species further support our interpretation. *Andrena nigroaenea* males have ~23% fewer glomeruli than females (M.S., unpublished data). Alternatively, it can be argued that the larger sex differences and high numbers of glomeruli in honeybee workers and other eusocial Hymenoptera result from an increased demand for olfactory communication within the colony and are thus specific adaptations of the worker caste in eusocial Hymenoptera. Within bees, however, *Apis* worker do not have particularly many glomeruli, compared with other social and solitary species (M.S. and C.K., unpublished data).

### MGs

The high sensitivity of honeybee drone antenna for the sex pheromone compound 9-ODA is considered to be the result of multiplication of PPs and a sex bias in the equipment with different receptor types. In *Apis*, the presence of four hypertrophied glomeruli coincides with the highly male-biased expression of four receptor genes, and for one of them the odor specificity has been demonstrated (Arnold et al., 1985; Sandoz, 2006; Wanner et al., 2007). In *Eucera* males, the presence of four MGs, occupying about 40% of the total glomerular volume, suggests a male bias in the equipment with corresponding receptor types, analogous to *Apis* drones. The involvement of the MGs in long distance sex pheromone detection is likely, and some evidence indicates the presence of long-distance attractants in *Eucera* (Priesner, 1973; and personal observations by M.S. and J.S.). Whether the putative pheromone receptors in *Eucera* are sex-specific receptor types, as in some moth species, or result from male-biased expression of a shared receptor type, as in *Apis*, remains to be investigated (Galizia and Rössler, 2010). Additional chemical signals play an important role in close-range communication in *A. mellifera* mating behavior (Brockmann et al., 2006; Renner and Vierling, 1977), and previous studies suggest the involvement of “ordinary” glomeruli rather than MGs in processing these close-range substances (Brockmann et al., 1998; Sandoz, 2006). Evidence is still lacking that all MGs are involved in sex pheromone processing, and, as yet, the function of three of the four MGs in *Apis* remains unknown (Sandoz, 2006). In *Eucera*, the chemistry of sex communication has not yet been investigated. The exploitation of males by sexually deceptive orchids for the purpose of pollination (Streinzer et al., 2009) will facilitate future characterization of the behaviorally relevant pheromone components and thus evaluation of the role of MGs and ordinary glomeruli in mate detection.

### Higher processing centers in the brain

In the insect brain, sensory input is processed in distinct modality-specific primary and integrative higher order neuropils (Strausfeld et al., 1998). Although recent studies challenge the use of absolute brain size as an estimator for behavioral complexity and computational power (Chittka and Niven, 2009, and references therein), many studies have shown correlations between brain size and performance (Deaner et al., 2007; Gronenberg and Couvillon, 2010; Lefebvre and Sol, 2008). Among related species and between sexes, comparisons of the relative size of brain regions are commonly used to quantify differential investment in brain tissue (Ehmer and Gronenberg, 2004; El Jundi et al., 2009; Ott and Rogers, 2010). In *Eucera* males, relatively more neural tissue is devoted to the AL than in females, underpinning the importance of this modality in mate detection (Table 2, Fig. 4). In *Apis*, the ALs are relatively smaller in males. In contrast, the lobulae are relatively larger, suggesting differences in the relative importance of vision vs. olfaction between the two species (Gronenberg and Hölldobler, 1999). The MBs are considered as centers of multimodal integration and memory. They have been shown to be relatively larger in generalist feeders (Farris and Roberts, 2005; Ott and Rogers, 2010) and socially dominant caste members (Molina and O'Donnell, 2007). Larger MBs are assumed to reflect behavioral repertoire rather than social organization per se (Farris, 2005; Gronenberg, 2009; Smith et al., 2010). Sexual dimorphism in MB size is observed in many Hymenoptera (Ehmer and Gronenberg, 2004; Kuebler et al., 2010; Mysore et al., 2009) but not in other insect taxa (Dreyer et al., 2010; El Jundi et al., 2009). The observed sexual dimorphism in hymenopteran MBs probably illustrates the striking differences in ecology and life history. The reduced relative MB volume correlates with the probably smaller and less plastic behavioral repertoire in males (Ehmer and Gronenberg, 2004; Ohtani, 1974). Consistent with our original interpretation, investment in MB tissue is more similar between sexes in *Eucera* compared with *Apis*. Males of the latter species have smaller MBs, which possibly reflects the relaxed natural selection pressure in conjunction with a more stereotyped mating behavior in honeybee drones, compared with *Eucera* males (Ohtani, 1974; Shimron and Hefetz, 1985; Winston, 1991). Further support comes from another solitary bee species, *A. nigroaenea*, which also shows no difference in relative MB investment (M.S., unpublished data). Alternatively, the larger MBs of honeybee workers, relative to *Apis* drones and both *Eucera* sexes, may illustrate the demands for higher behavioral repertoires in workers of eusocial species compared with solitary species of bees (Smith et al., 2010).

### **CONCLUSIONS AND FUTURE PERSPECTIVE**

In this study, we document the extraordinary sexual dimorphism of the olfactory system in a solitary bee species. We found a high degree of dimorphism at the periphery and in first-order neuropils. Surprisingly, the sexual dimorphism is not as pronounced in the central brain of *Eucera*. In comparison, the sexual dimorphism in *Apis* at the periphery is comparable to that of *Eucera*, and it is present along the entire olfactory pathway. In *Apis* drones, a stronger reduction in glomerular numbers and less investment in MB tissue was found compared with *Eucera*

drones. We initially expected, based on differences in the life style of males, that sexual dimorphism and allocation of brain resources to mating-related behaviors should be less elaborate in *Eucera* males than in *Apis* drones. Our findings support our prediction; however, alternative explanations limit our conclusions drawn from the observed differences between only two species of Hymenoptera. Further studies, using an approach comparable to that in our investigation, are necessary to understand better the origin and extent of male-specific adaptations in bees. We suggest comparative sampling across the various families of bees, including species with various mating strategies and life styles. In particular, taxa that differ with respect to male ecology but not social structure are of great interest, because this allows one to separate male- and female-specific adaptations better. Bumblebees (*Bombus*) constitute good candidates. They show a eusocial life style, but males lack a colony support and have to forage for food and shelter by themselves (as with *Eucera* males).

### ACKNOWLEDGMENTS

We thank M. Eberhard and J. Bauder for help with histological procedures, J. Plant for linguistic improvements, and the anonymous reviewers for their helpful comments. We further thank the Core Facility for Cell Imaging and Ultrastructural Research, University of Vienna, for providing facilities and support.

### CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

### ROLE OF AUTHORS

All authors read and approved the final manuscript. Study concept and design: MS, CJK, JS. Acquisition of data: MS, CK, SP. Analysis and interpretation of data: MS, CJK, JS. Statistical analysis: MS; Drafting of the manuscript: MS, CJK, JS.

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## 10. DISCUSSION

Sex and caste specific sensory systems are widespread among bees. This conclusion is based on a comparison of the gross morphology between the sexes and between the castes (Michener, 2007; Stephen et al., 1969). However, the functional consequences of the often conspicuous sexual dimorphisms are poorly understood. It is surprising that only a single bee species has been comprehensively investigated, despite the long-lasting relationship between bees and humans and their use as models in diverse fields of research.

In this thesis, I investigated sensory adaptations in three model taxa and add to the existing knowledge on sex and caste-specific morphologies of the visual system in honeybees, bumblebees and solitary bees. Further, I provide the first comprehensive investigation about sex-specific morphological features of the olfactory system in a solitary bee. The latter is especially valuable, since many of the elaborate olfactory capabilities of bee workers are discussed and interpreted in context of eusociality, although we lack any knowledge about such capacities in solitary species (see discussion in Kleineidam and Rössler, 2009). My study thus provides a template for future research on sex-specific sensory systems, and how mating behavior, mating signals and system of sociality shape the sensory and neuronal systems. This template and procedures can be readily applied to a wider array of species to detect underlying patterns. In the following, I discuss the findings of my studies and suggest particular studies for future research questions.

### 10.1. Visual system

#### 10.1.1. Females and workers

The functional morphology of the visual system in bees has been previously investigated only in honeybees and bumblebees (e.g. Seidl, 1982; Spaethe and Chittka, 2003), both of which are commonly used taxa for lab-controlled and field studies in diverse fields of research. Other bee species have served as model systems to study adaptations for low-light environments (e.g. Greiner et al., 2004; Somanathan et al., 2009a). Opposed to parasitic bee species, females of solitary and social bee species fulfill relatively similar tasks, e.g. nest construction and maintenance, parental care and foraging (Michener, 1974; 2007). The visually guided behaviors are thus rather similar among females and workers in the various species. In correspondence with this, I found similar eye structures in the five investigated species of *Apis*, 11 of *Bombus* and in the solitary bee *E. berlandi* (*Manuscripts I, III, IV*). Their eyes are similar in shape and organization, and their absolute size correlates to the body size of the bees (*Manuscripts I, III, IV*). Larger individuals are generally equipped with more ommatidia and larger facets, and therefore likely have higher spatial resolution and sensitivity (Jander and Jander, 2002; Somanathan et al., 2009a; Spaethe and Chittka, 2003). In one species, *E. berlandi*, I directly measured the interommatidial angles, eye parameter and facet sizes, and the results fit the scheme of a generally tuned apposition eye of a large bee species that flies during the daytime (see discussion in *Manuscript IV*; Horridge, 1978; Seidl, 1982).

To investigate trade-offs between resolution and sensitivity, I measured facet size and number in all investigated species. These measures serve as an initial approximation to detect trade-offs between the two visual parameters. Direct measurements of the spatial resolution and sensitivity are now necessary to back up these predictions (see discussion in *Manuscript I, III* and *VI*). My investigations of honeybee worker eyes obtained similar general conclusions as in the more in-depth study by Somanathan (2009b). These joint conclusions strengthen the validity of the assumptions which can be drawn by examining the external morphology.

Workers of some studied species were found to possess relatively enlarged facets, at the expense of total ommatidia number, which suggests that they trade-off resolution for increased sensitivity (*Manuscript I, III*). This observation correlates with flight activity in low light environments typical for these species. For example, workers of *Apis dorsata* are known to be facultatively nocturnal (Dyer, 1985), and they have larger facets and increased acceptance angles which improve the capture of light (*Manuscript I*; Somanathan et al., 2009b). In bumblebees, large facets were found, e.g. in *B. pratorum* and *B. melaleucus*; both species forage mainly in forests and thus in low light environments. In contrast, females of *B. niveatus* are active in open environments, and they possess smaller facets and a high number of ommatidia (see discussion in *Manuscript III*). They may thus benefit from better spatial resolution, which may improve their flower detection abilities and orientation skills. Future studies must focus on assessing the impact of these differences on individual foraging success and efficiency. One important aspect of my results should be kept in mind in future investigations. Since various bumblebee species, from several subgenera, are currently used in studies associated with visual ecology, I strongly advise that one should consider the differences in their visual systems when designing future studies and when interpreting the data (see discussion in *Manuscript III*).

### 10.1.2. Queens

In eusocial species, workers can be regarded as functional equivalent to the female in solitary species, since they perform analogous tasks, such as nest site selection, nest construction, parental care, foraging, and defense behavior (see *Manuscript I*). In *Apis* and *Bombus*, I additionally investigated the eye morphology of the queens. Queens of honeybees and bumblebees differ in one important aspect. In the highly eusocial honeybee, the queen depends on the help of workers for colony foundation and survival. The only outdoor tasks that she accomplishes are mating and swarming flights, and she lacks adaptations for foraging, e.g. the corbicula (Winston, 1991). In the primitively eusocial bumblebees, the queens are functionally and morphologically more similar to workers. They independently found a new colony from the start and are responsible for all colony tasks during the first colony phase (Michener, 1974; Plowright and Lavery, 1984). This major difference between honeybee and bumblebee queens is reflected in their visual system. In *Apis*, the queen eyes are relatively smaller compared to workers. They have less ommatidia, which illustrates a reduced need for high spatial resolution and vision in general (*Manuscript I*). Eyes are energetically costly (Niven and Laughlin, 2008) and a reduced or absent selection pressure for accurate vision usually results in a decrease in eye

size over several generations (Tan et al., 2005). However, honeybee queens have a visual system that is sufficient for spatial vision during mating flights and swarming events (Hepburn, 2011; Koeniger et al., 2011). To minimize the costs of the eye during the periods that are spent in the dark, the size and thus energetic requirements of the visual neuropils and other structures may be reduced (Julian and Gronenberg, 2002, Gronenberg and Liebig, 1999, but see Fahrbach et al., 2003). In bumblebees, queens and workers have a similar eye size relative to their body size, which illustrates the need for acute vision during the first phase of the colony cycle when the queen is responsible for foraging and nest initiation. During the latter colony phases, the costs of maintaining the large visual system may also be minimized, but this hypothesis remains to be tested.

In summary, my results support the view that the female visual morphology is matched to the ecology and photic foraging environment of the animals, similar to what is found in other bee species (Somanathan et al., 2009a; Somanathan et al., 2009b) and bull ants (*Myrmecia*; Greiner et al., 2007; Narendra et al., 2011). Future studies may focus on the physiological adaptations of the eyes and thus further increase the understanding of sensory adaptations in insects.

### 10.1.3. Males

In males, the morphology of the visual system is more diverse than in females. The variety of mating systems, signals and cues deployed during mate search, and thus diverse selection pressures on the sensory systems are responsible for this larger diversity (Alcock et al., 1978). The selection pressures that favor sensory elaborations are discussed in detail in the *Manuscripts I, II, III*. The functional morphology of the male eyes differs among the studied taxa, and more importantly, correlates with the mating strategy. Honeybee drones possess extremely enlarged eyes and show a distinct dorso-ventral regionalization of the compound eye. The eye is functionally separated in an upper region for queen detection and a lower region for the detection and landing in the hive (Menzel et al., 1991; van Praagh et al., 1980). The high number of ommatidia, the large acute zone and the large field of view seem to be particularly important in detection of the queen and discrimination among the high number of other drones (Free, 1987; Vallet and Coles, 1993). The high number of ommatidia and high spatial resolution may further improve stability during the hovering movements in the drone comet (Theobald et al., 2010; van Praagh et al., 1980). Similarly enlarged eyes are found in other bee species from which hovering mating behavior is described (Hurd and Linsley; Stephen et al., 1969; and see *Introduction*).

Males of all investigated honeybee species showed a similar general organization of their eyes, but differences in the trade-off between facet size and number. These are likely related to differences in mating time and location (see discussion in *Manuscript I*). The strong selection pressures acting in *Apis* (Baer, 2005) obviously lead to the development of an ideal male phenotype, and small deviations, e.g. in body size (Berg et al., 1997; Couvillon et al., 2010) or wing symmetry (Jaffé and Moritz, 2010), have great influence of their mating success. Small drones, a male phenotype that is up to 20% smaller because it is reared in worker cells (see *Manuscript II*), have a lower reproductive success than normal sized drones and the reasons are

likely multifactorial. In *Manuscript II*, I reported a reduced facet size but equal ommatidia numbers in the small phenotype. I suggested that a reduced sensitivity of the eyes substantially reduces the abilities to visually detect a queen, a prediction that has to be tested in future studies. In *Bombus*, two general male eye types were found; enlarged eyes that presumably have high spatial resolution are found in visually searching species, and normal, worker-like, eyes in patrolling species. The enlarged male eyes do not show a distinct dorso-ventral regionalization, like in honeybee drones, and the largest facets are found in the region that faces forward. The eye morphology is similar to *Eucera berlandi*, suggesting that similar signals and cues play a role in target detection (*Manuscript IV*). The functional measurements in *Manuscript IV* confirmed the high spatial resolution that was previously predicted from the large facets in the frontal eye region of male *E. berlandi* (Streinzer, 2008). The measurements further show that the eye design is more biased towards high spatial resolution in males.

In *Bombus* and *Eucera* (and many other bee species) the discussion of adaptations is complex due to the fact that mostly phenomenological observations, but little direct investigations of the signals, cues and the signaler-receiver relationship are available. Therefore, future studies have to focus on studying these aspects of the mating behavior in more detail. In the following, two approaches are discussed that may deepen our understanding of the adaptations of the visual system in males of *Bombus* and *Eucera*.

#### 10.1.4. Evolution of elaborate visual systems in *Bombus*

In *Manuscript III*, I investigated the functional morphology of *Bombus* eyes and traced the evolution of sexually dimorphic visual systems. My data show that, in particular, in one derived clade of bumblebees, *Alpigenobombus* + *Melanobombus* + *Sibiricobombus* + *Cullumanobombus*, a high number of transitions between large eyed and normal eyed males is found. Furthermore, some species seem to take an intermediate position, both in the enlargement of the male visual system and their mating strategy (discussed in *Manuscript III*).

This clade might be interesting for a comparative investigation that aims to trace the correlated evolution of mate searching behavior and the visual and olfactory system, male pheromone compounds and other traits, in order to identify the factors that facilitate or precede changes in the mating system (see Leys and Hogendoorn (2008) for an example in carpenter bees, *Xylocopa*). To achieve that, we need detailed investigations on the morphology of these species, but also have to increase our knowledge about the mating behavior and the signals and cues that are important. Males of some perching bumblebee species have been reported to engage in their typical mating behavior also in captivity, and their conspicuous behavioral reaction to moving stimuli opens up the perspective of controlled lab-studies to identify the important visual cues (Aichhorn, 1976; Hobbs, 1965).

#### 10.1.5. Spatial vision in *Eucera* mating behavior and *Ophrys* pollination

Orchids of the Mediterranean genus *Ophrys* mimic sex pheromones of female Hymenoptera in order to attract males. During copulation attempts, they get attached with pollen packages and

pollinate flowers in subsequent visits (Paulus and Gack, 1990). The flowers attract males by means of a perfect mimicry of the olfactory, visual and tactile signals and cues that are usually employed in the species-specific mating behavior and that are necessary and sufficient to trigger male mating activity (Mant et al., 2005; Schiestl, 2005; Streinzer et al., 2009). Males of *E. berlandi* are the sole pollinator of *Ophrys heldreichii*, and this species pair has been widely used in field studies on visual and olfactory signaling (Paulus and Gack, 1990; Rakosy et al., 2012; Schaller, 2009; Spaethe et al., 2007; Spaethe et al., 2010; Streinzer et al., 2010; Streinzer et al., 2009). Previous studies show that males rely on strong achromatic visual cues for reliable target detection, and, given the choice, prefer large targets over smaller ones (Streinzer et al., 2009). These results agree with the results presented in *Manuscript IV*. In this study, larger targets attracted more males and lead to a higher catching success. The mechanism of target detection, chase and catching behavior is not yet known and additional studies, e.g. detailed 3-D reconstructions of the approaching flights, could help to identify this mechanism (see discussion in *Manuscript IV*, and e.g. Boeddeker et al., 2003).

In *Manuscript IV*, I further report the spatial resolution of the compound eye in *E. berlandi* males. Observation of the male behavior, after they have been tricked by the orchid, suggest that the bees try to memorize the complex labellum pattern, a trait that is only found in *Ophrys* species pollinated by male long-horned bee (Stejskal, 2011). The high spatial resolution of the male eyes may be important in memorizing the pattern to avoid future approaches to deceptive flowers (Stejskal, 2011; Streinzer et al., 2010). Furthermore, these predicted learning abilities could be related to the behavioral flexibility that is suggested by the female-like investment in higher order processing neuropils (see *Manuscript V*).

## 10.2. Olfactory system

### 10.2.1. Sex-specific olfactory systems

In *Manuscript V*, I described the organization of the antenna as well as adaptations along the olfactory path in a solitary bee species. This investigation can be considered important, since it is the first description in a solitary bee species. *Eucera berlandi* males show remarkable sex-specific traits in their olfactory system, which may be considered adaptations for long-distance pheromone detection. Such adaptations are suggested from a pronounced dimorphism of the external antenna morphology that is observed in many Hymenoptera and other insects (Hansson and Stensmyr, 2011; Thornhill and Alcock, 1983). In bees, the functional adaptations for sex pheromone detection have been described in detail only in the honeybee drone. Deciphering the functional adaptations is complicated and demands many small steps, e.g. the identification of the sex pheromone, the corresponding sensilla, glomeruli and receptors (Arnold et al., 1985; Brockmann et al., 1998; Esslen and Kaissling, 1976; Free, 1987; Lacher, 1964; Sandoz, 2006; Wanner et al., 2007). In the investigated *Eucera* species, we are only at the beginning of this cascade. However, we benefit from the deceptive mimicry of the sex pheromone by the orchid species *Ophrys heldreichii*. *Ophrys* species have already been used to facilitate and speed up the identification of sex pheromones in solitary bee species (Gögler et al., 2011; Mant et al., 2005;

Schiestl et al., 2000). Male antennae have been used in electrophysiological setups to identify the substances that can be perceived by the males (Ayasse et al., 2000; Krieger et al., 2006; Mant et al., 2005). Ongoing experiments in the species pair *O. heldreichii* – *E. berlandi* have so far revealed a similarity in chemical composition of the original and the mimic of the sex pheromone, and the behavioral responses are found to be similar (Schaller, 2009). The next step could now be the isolation and identification of the behaviorally active substances that are perceived by the antenna and the identification of those substances and receptors that target the macroglomeruli in *E. berlandi* males.

Sex pheromones are diverse among bees and have been identified in several species, in many cases by means of the corresponding deceptive *Ophrys* species (Ayasse et al., 2003; Gögler et al., 2011; Mant et al., 2005; Schiestl et al., 2000; Stoekl et al., 2005). In *Andrena* and *Colletes* they consist of hydrocarbons comprising a defined blend ratio (Mant et al., 2005; Schiestl et al., 1999; Stoekl et al., 2005). In contrast to these unpolar substances, polar sex pheromones are found e.g. in *Apis* (Free, 1987) and probably also in bumblebees (Gögler et al., 2011). In *Eucera*, preliminary experiments also suggest that the behaviorally active substances is contained in the polar fraction (own unpublished data by Ayasse M, Spaethe J, Streinzer M). The remarkable adaptation for long distance pheromone detection in *Apis mellifera* (Arnold et al., 1985; Brockmann et al., 1998; Esslen and Kaissling, 1976; Wanner et al., 2007) and *Eucera* (*Manuscript V*) and the description of male-specific glomeruli in *Bombus* (Fonta and Masson, 1985) contrast with a less elaborate olfactory system and the absence of similarly enlarged macroglomeruli in the antennal lobe of males in *A. nigroaenea* (own unpublished results). Furthermore, long-distance attraction, which is observed for *Apis* and *Eucera* (Loper et al., 1993; Priesner, 1973 and own observations), seems to be absent in *Colletes* and *Andrena* (Ayasse et al., 2001; Peakall and Schiestl, 2004). We currently do not know whether and how the chemical structure of the pheromone (e.g. polar vs. unpolar), the operating range (long distance vs. short distance) and the adaptations of the sensory system (macroglomeruli vs. ordinary glomeruli) are interrelated (Ayasse et al., 2001). Furthermore, phylogeny may be an important factor to consider in future comparisons among species. Future anatomical studies in other bee species, maybe initially in those where the identity of the sex pheromone has already been described (e.g. *Colletes cunicularius*; Mant et al., 2005) will be necessary to understand these interrelations.

### 10.2.2. Olfaction in bumblebee mating and foraging behavior

Bumblebee species differ in their mating behavior and can be categorized in two broad categories; perching and patrolling. In the perching males, improved vision seems to be important and their mating behavior is often described as ‘visually guided’ (see *Manuscript III*). Patrolling males, however, follow scented routes and their strategy is often regarded as ‘olfactory guided’. Males of either strategy are known to scent mark certain spots (Kindl et al., 1999; Kullenberg et al., 1973; O'Neill et al., 1991), but nothing is known about the role of olfactory perception in males and whether it differs among mating strategies (Ayasse et al., 2001). A comparative study on the antennal lobe morphology may reveal functional differences in the

olfactory system among the two major strategies, e.g. a higher repertoire for odor coding (more glomeruli) or adaptations for long distance perception (macroglomeruli). Noteworthy in this context is a small group of three *Thoracobombus* species (formerly *Rhodobombus*, Williams et al., 2008). Males of these species have reduced head labial glands and lack major putative pheromone compounds, and their scent deposition apparatus is lacking (Terzö et al., 2007a; Terzö et al., 2007b). Future investigations could clarify whether this reduction is correlated with differences in the AL anatomy of males or queens of this species.

Besides male-specific adaptations, worker-specific adaptations to foraging and nest tasks may be present in the olfactory system. Moreover, there may be differences among species. Workers are similar in their morphology (Michener, 2007) and life time tasks (Plowright and Lavery, 1984). In *Manuscript III*, I report only relatively minor differences in their visual system, which are likely related to foraging environments rather than e.g. flower choice. Although bumblebees are relatively generalist flower visitors, there are differences in the repertoire of visited plants. These have so far been mainly attributed to differences in their proboscis length (Brian, 1957). The visited range of nectar and food plants may, however, also influence selection pressures on the olfactory system. In future studies, known generalist foragers (e.g. *Bombus terrestris*) may be compared with relatively specialized species. For example, *Bombus (Kallobombus) soroeensis* is the only species that regularly and extensively collects pollen from Campanulaceae ( J Neumayer, personal comment); or *B. (Megabombus) consobrinus* and *B. (Megabombus) gerstaeckeri*, two species which have a very narrow food plant spectrum and are associated with *Aconitum* (Loken, 1973; Ponchau et al., 2006).

### 10.3. Degree of sexual dimorphism

In general, the types of adaptations for mate detection differ among species and can be categorized into several categories. In the scramble competition system, elaborate sensory abilities are favored, which is illustrated by the described adaptations in honeybees (*Manuscript I, II*), bumblebees (*Manuscript III*) and long-horned bees (*Manuscript IV, V*). Depending on the sexual selection pressure, which cannot be easily quantified, the elaboration of sensory equipment may differ (Thornhill and Alcock, 1983). However, there is likely an upper limit of the elaboration of sensory equipment. That is, when the costs for developing, maintaining and carrying the sensory organs exceed the benefits (discussed in *Manuscript V*). It is tempting to regard the extant male phenotype of any species as the balanced state between the sexually selected traits and the counteracting natural selection. In *Manuscript V* I present a testable hypothesis about the role of a social backup system in the evolution of exaggerated sensory systems and male behavioral plasticity. I propose that, in particular, honeybee drones may benefit from a social security net, which reduces the natural selection pressure (since they do not need to forage and seek shelter). As a consequence, it would allow males to invest even more in sexually selected traits (e.g. sensory system elaboration). Indeed, there is a smaller investment in mushroom body neuropil in drones, while, in contrast, the sexes do not significantly differ in their relative investment in MB tissue in solitary species, e.g. *E. berlandi* (*Manuscript V*) and

*Andrena nigroaenea* (Andrenidae; own unpublished data). As discussed in *Manuscript V*, the performed comparison does not permit final conclusions. Instead, some of the observations may be linked to the eusocial lifestyle of honeybee workers, to which drones are compared. Two strategies may help to solve this dilemma in future studies (see also *Manuscript V*). First, a more widespread survey across bee species can be performed, using the methods outlined in *Manuscript V*. This strategy may reveal patterns of visual and olfactory dimorphisms among solitary species, communal species and social species. It may also include some of those species that show extreme male-specific eyes or antennae, e.g. *Melitturga* or *Ctenioschelus* (see *Introduction*).

Second, suitable study species may be chosen. In *Manuscript V*, I propose *Bombus* as a comparative system. Bumblebees are eusocial and the demands for visual and olfactory perception and processing are likely similar among honeybee and bumblebee workers. Males, however, live a solitary life and thus selection pressures should more strongly resemble those acting on males of solitary bee species. Some species of the earliest diverging subgenera (*Mendacibombus* and *Bombias*) differ in their nest organization and male behavior. Males of these species apparently return to the nest, which contrasts with the normal observation of male behavior in bumblebees (Haas, 1976; Hobbs, 1965). A study incorporating species of either male foraging strategy can help to pinpoint the role of a social security system for a reduced investment in higher cognitive abilities in male bees, which I proposed in *Manuscript V*. A broader survey of the brain structures within bumblebees may further add to the knowledge about the (relative) investment in sensory neuropil in general and trade-offs between the sensory systems (olfaction vs. vision), also in relation to differences in the mating system of the species of interest (Gronenberg and Hölldobler, 1999).

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## 12. DANKSAGUNG

Nach einigen Jahren - genau will ich sie gar nicht zählen - ist diese Arbeit nun fertig geworden. An diesem Erfolg waren viele Menschen direkt und indirekt beteiligt, denen ich meinen herzlichen Dank aussprechen möchte.

Am längsten und direktesten involviert in meine Forschungsarbeit war Johannes. Danke für die nunmehr 7 Jahre andauernde Zusammenarbeit. Du hast immer neue Ideen für Projekte gehabt (zusätzlich zu unseren bereits vorhandenen), fast alle haben wir in den unzähligen Kaffeepausen weiter entwickelt, die meisten haben erfolgversprechend geklungen, viele sind wir angegangen, einige haben funktioniert, und ein paar sind wirklich fertig und gut geworden. An den anderen arbeiten wir noch. So hab ich mir Wissenschaft eigentlich immer vorgestellt. Du hast mich immer wieder animiert doch dies oder jenes auszuprobieren und schließlich hab ich neben meiner Arbeit in der Wissenschaft, Wissenschaft auch als Hobby machen müssen, weil mit sonst ja noch langweilig geworden wäre. Zum Glück gab's bei dir immer Stärkung in Form von Kaffee und Schokoriegeln (wobei eigentlich esse ich ja keine Schokolade...). Danke für die vielen unvergesslichen Feldaufenthalte, Exkursionen und Konferenzen.

Danke an Hannes Paulus und Wolfgang Rössler, die mir während meiner Doktorarbeitszeit Ressourcen, Arbeitsplätze und Arbeitsmittel zur Verfügung gestellt haben und immer an meiner Arbeit interessiert und für Diskussionen zu haben waren.

Bei Thomas Schmitt, meinem Zweitgutachter, möchte ich mich für die Bereitschaft bedanken die Arbeit neben Umzugs- und Zeitstress zu lesen und zu beurteilen.

Tina, du hast mich herzlich in Würzburg aufgenommen, mir beigebracht wie man Böbbel malt und Pilzkörper wrappt. Vielen Dank für die gemeinsame Zeit im Labor, im Feld und in der Stadt.

Christoph, du hast mich in die Olfaktorik eingeführt, danke für deine Erklärungen und Diskussionen über ALs, ORNs, MGs, MBs, Sensillen usw. es hat mir viel gebracht.

Während meiner Freilandaufenthalte hatte ich Hilfe von Tom, Alex, Demetra und Kerstin. Gerald, Marc, Sarah, Tina, Moni, Julia, Conny, Norbert und Linde ihr habt mir bei den Labortechniken unterstützend zugesehen, Tipps gegeben, Reagenzien vorbereitet und bei der Statistik geholfen.

Karin, ein riesiges Dankeschön für die Punkte!

Für viele der verwendeten Bienen bedanke ich mich bei Hannes, Flo, Sarah, John, Hans, N. Nagaraja, T. Küpper, J. Makinson und D. Brückner; außerdem bei D. Zimmermann (NHM Wien) und F. Gusenleitner (OÖ Landesmuseum, Linz) für den Zugang zu ihren wissenschaftlichen Sammlungen. Hans, Josef, Flo, John, Johannes, Hannes, Tina mit euch konnte man immer über Bienen sprechen, nach ihnen suchen und Tipps bekommen wo man sie finden könnte.

Danke an Christoph, Almut und Adrian, dass ihr mich in euren Labors arbeiten gelassen und mir neue Techniken gezeigt habt.

Kollektiv bedanke ich mich bei allen Mitglieder der Arbeitsgruppen Spaethe, Kleineidam, Paulus und Rössler für arbeitsbezogene Diskussionen und private Aktivitäten. Oli, Ilija und

Sonja, ihr hattet immer eine Lösung, wenn ich schnell Computer, Bildschirme oder was auch immer brauchte.

John und Tom, vielen Dank für das Glattbügeln meiner englischen Texte, Manuskripte, Poster und Abstracts, die ich euch meist spät geschickt und schnell wieder gebraucht habe. Ihr habt sie trotzdem ausgebessert, auch wenn ihr eigentlich gar keine Zeit hattet.

Wissenschaft kostet natürlich auch Geld, für Material, Reisen und das restliche Leben. Für finanzielle Unterstützung danke ich meine Eltern, die mir immer einen Zuschuss für Reisen gegeben haben und dafür gesorgt haben, dass ich weiter machen konnte, auch wenn ich gerade keine Finanzierung hatte. Meine Forschungsfinanzierung kam von der Uni Wien (Forschungsstipendium F-444), der Akademie der Wissenschaften (Doc-Stipendium), dem Department für Evolutionsbiologie, sowie dem Lehrstuhl für Zoologie II. Während meiner Besuche in Würzburg durfte ich immer wieder bei Christoph und Anja, Johannes und Caro, sowie Jan übernachten, danke!

Zu guter Letzt hatten viele Menschen in meinem Privatleben Anteil am Gelingen dieser Arbeit. Meine Eltern haben mich immer unterstützt und mir ermöglicht und mich ermutigt diesen Weg einzuschlagen. Ohne euch wäre ich jetzt nicht da wo ich bin. Meinen Brüdern und Freunden danke ich für die offenen Ohren, wenn ich versucht habe zu erklären was ich eigentlich mache, sowie für Verständnis, wenn ich vor lauter Arbeit mal wieder keine Zeit hatte.

Sarah, wir haben uns auf diesem Weg getroffen und gefunden. Du hast mich in vielerlei Hinsicht erwachsen und vollständig gemacht und immer Verständnis für meinen Weg gehabt. Ich danke dir für so viel, im Moment aber vor allem für dein Verständnis und deine Ermutigung während meiner psychischen und physischen Abwesenheit während der Endphase dieser Arbeit.