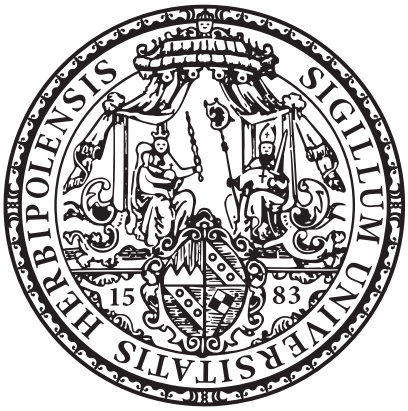
Color vision and retinal development of the compound eye in bees

Farbensehen und retinale Entwicklung des Komplexauges bei Bienen



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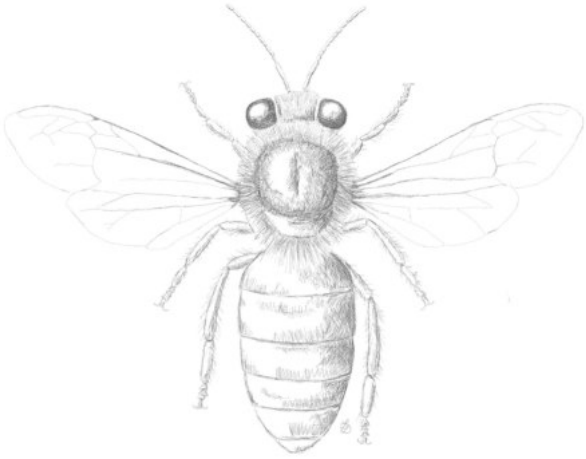

"Ihr Farbensehen, ihr Riechen und Schmecken und die Beziehungen ihrer Sinnesleistungen zur Blumenwelt, ihre "Sprache" und ihr Orientierungsvermögen — das war das rätselvolle Wunderland, das zu immer weiteren Vordringen lockte." [von Frisch, 1965]

Contents

List of Figures

List of Tables

SUMMARY

The superfamiliy of bees, Apiformes, comprises more than 20,000 species. Within the group, the eusocial species like honeybees and bumblebees are receiving increased attention due to their outstanding importance for pollination of many crop and wild plants, their exceptional eusocial lifestyle and complex behavioral repertoire, which makes them an interesting invertebrate model to study mechanisms of sensory perception, learning and memory. In bees and most animals, vision is one of the major senses since almost every living organism and many biological processes depend on light energy. Bees show various forms of vision, e.g. color vision, achromatic vision or polarized vision in order to orientate in space, recognize mating partners, detect suitable nest sites and search for rewarding food sources. To catch photons and convert light energy into electric signals, bees possess compound eyes which consists of thousands of single ommatidia comprising a fixed number of photoreceptors; they are characterized by a specific opsin protein with distinct spectral sensitivity. Different visual demands, e.g. the detection of a single virgin queen by a drone, or the identification and discrimination of flowers during foraging bouts by workers, gave rise to the exceptional sex-specific morphology and physiology of male and female compound eyes in honeybees. Since Karl von Frisch first demonstrated color vision in honeybees more than 100 years ago, much effort has been devoted to gain insight into the molecular, morphological and physiological characteristics of (sex-specific) bee compound eyes and the corresponding photoreceptors. However, to date, almost nothing is known about the underlying mechanisms during pupal development which pattern the retina and give rise to the distinct photoreceptor distribution. Hence, in Chapter 2 and 3 I aimed to better understand the retinal development and photoreceptor determination in the honeybee eye. In a first step, the intrinsic temporal expression pattern of opsins within the retina was evaluated by quantifying opsin mRNA expression levels during the pupal phase of honeybee workers and drones. First results revealed that honeybee workers and drones express three different opsin genes, *UVop*, *BLop* and *Lop1* during pupal development which give rise to an ultraviolet, blue, and green-light sensitive photoreceptor. Moreover, opsin expression patterns differed between both sexes and the onset of a particular opsin

occurred at different time points during retinal development. Immunostainings of the developing honeybee retina in Chapter 2 showed that at the beginning of pupation the retina consist only of a thin hypodermis. However, at this stage all retinal structures are already present. From about mid of pupation, opsin expression levels increase and goes hand in hand with the differentiation of the rhabdoms, suggesting a two-step process in photoreceptor development and differentiation in the honeybee compound eye. In a first step the photoreceptor cells meet its fate during late pupation; in a second step, the quantity of opsin expression in each photoreceptor strongly increase up to the 25-fold shortly after eclosion. To date, the underlying mechanisms leading to different photoreceptor types have been intensively studied in the fruit fly, *Drosophila melanogaster*, and to some extend in butterflies. Interestingly, the molecular mechanisms seemed to be conserved within insects and e.g. the two transcription factors, *spalt* and *spineless*, which have been shown to be essential for photoreceptor determination in flies and butterflies, have been also identified in the honeybee. In chapter 3, I investigated the expression patterns of both transcription factors during pupal development of honeybee workers and showed that *spalt* is mainly expressed during the first few pupal stages which might correlate with the onset of *BLop* expression. Further, *spineless* showed a prominent peak at mid of pupation which might initiates the expression of *Lop1*. However, whether *spalt* and *spineless* are also essential for photoreceptor determination in the honeybee has still to be investigated, e.g. by a knockdown/out of the respective transcription factor during retinal development which leads to a spectral phenotype, e.g. a dichromatic eye. Such spectral phenotypes can then be tested in behavioral experiments in order to test the function of specific photoreceptors for color perception and the entrainment of the circadian clock. In order to evaluate the color discrimination capabilities of bees and the quality of color perception, a reliable behavioral experiment under controlled conditions is a prerequisite. Hence, in chapter 4, I aimed to establish the visual PER paradigm as a suitable method for behaviorally testing color vision in bees. Since PER color vision has considered to be difficult in bees and was not successful in Western honeybees without ablating the bee's antennae or presenting color stimuli in combination with other cues for several decades, the experimental

setup was first established in bumblebees which have been shown to be robust and reliable, e.g. during electrophysiological recordings. Workers and drones of the buff-tailed bumblebee, *Bombus terrestris* were able to associate different monochromatic light stimuli with a sugar reward and succeeded in discriminating a rewarded color stimulus from an unrewarded color stimulus. They were also able to retrieve the learned stimulus after two hours, and workers successfully transferred the learned information to a new behavioral context. In the next step, the experimental setup was adapted to honeybees. In chapter 5, I tested the setup in two medium-sized honeybees, the Eastern honeybee, *Apis cerana* and the Western honeybee, *Apis mellifera*. Both honeybee species were able to associate and discriminate between two monochromatic light stimuli, blue and green light, with peak sensitivities of 435 nm and 528 nm. Eastern and Western honeybees also successfully retrieve the learned stimulus after two hours, similar to the bumblebees. Visual conditioning setups and training protocols in my study significantly differed from previous studies using PER conditioning. A crucial feature found to be important for a successful visual PER conditioning is the duration of the conditioned stimulus presentation. In chapter 6, I systematically tested different length of stimuli presentations, since visual PER conditioning in earlier studies tended to be only successful when the conditioned stimulus is presented for more than 10 seconds. In this thesis, intact honeybee workers could successfully discriminate two monochromatic lights when the stimulus was presented 10 s before reward was offered, but failed, when the duration of stimulus presentation was shorter than 4 s. In order to allow a more comparable conditioning, I developed a new setup which includes a shutter, driven by a PC based software program. The revised setup allows a more precise and automatized visual PER conditioning, facilitating performance levels comparable to olfactory conditioning and providing now an excellent method to evaluate visual perception and cognition of bees under constant and controlled conditions in future studies.

ZUSAMMENFASSUNG

Die Bienen umfassen weltweit mehr als 20000 Arten, aber besonders eusoziale Honigbienen und Hummeln gewinnen durch ihre essenzielle Rolle bei der Bestäubung vieler Wild- und Kulturpflanzen zunehmend an Bedeutung. Ihr einzigartiger eusozialer Lebensstil, aber auch ihr komplexes Verhaltensrepertoire macht sie zu einem interessanten Insektenmodel, um Mechanismen sensorischer Wahrnehmung, sowie Fähigkeiten des Lernens und Gedächtnisses näher zu untersuchen.

Da beinahe jeder lebende Organismus und viele biologische Prozessen durch Sonnenenergie beeinflusst werden, ist die Fähigkeit des Sehens im Tierreich weit verbreitet und zählt auch bei Bienen zu den wichtigsten sensorischen Sinnen. Um geeignete Nistplätze, Futterquellen oder auch Paarungsspartner zu finden, sowie zur Orientierung, nutzen Bienen verschiedenste Formen des Sehens, z. B. Farbensehen, achromatisches Sehen, oder auch das Polarisationssehen. Um Photonen einfangen und diese in ein elektrisches Signal für die weitere Verarbeitung umzuwandeln zu können, besitzen Bienen Komplexaugen, die sich aus mehreren tausend Einzelaugen, den sogeannten Ommatiden zusammensetzen. Jedes Ommatidium enthält eine festgelegte Anzahl an Photorezeptoren, welche durch ein spezifisches Opsin-Protein mit einer bestimmten spektralen Empfindlichkeit charakterisiert sind. Unterschiedliche visuelle Ansprüche wie zum Beispiel die Wahrnehmung einer einzelnen Königin während ihres Paarungsfluges durch einen Drohn oder die Identifizierung und Unterscheidung von Blüten während des Sammelflugs einer Arbeiterin, führten zu einer geschlechtsspezifischen Morphologie und Physiologie männlicher und weiblicher Komplexaugen.

Seit Karl von Frisch vor mehr als 100 Jahren zeigen konnte, dass Honigbienen Farben wahrnehmen können, wurden viele Anstrengungen unternommen, ein besseres Verständnis für die molekularen und physiologischen Eigenschaften des (geschlechtsspezifischen) Bienenkomplexauges zu entwickeln. Dennoch ist bis heute wenig über die zugrundeliegenden Mechanismen bekannt, die während der Puppenentwicklung der Biene zur Bildung der Retina und der spezifischen Verteilung der Photorezeptoren innerhalb der Retina führen. Daher wurde in Kapitel 2 dieser Thesis das Ziel verfolgt, die retinale Entwicklung sowie die Determinierung der Photorezeptoren im Honigbi-

enenauge weiter aufzuschlüsseln. In einem ersten Schritt wurde das zeitliche Opsinexpressionsmuster während der Puppenentwicklung von Drohnen und Arbeiterinnen der Honigbiene durch Quantifizierung der Opsin-mRNA Expression, untersucht. Erste Ergebnisse zeigten, dass Drohnen und Arbeiterinnen während ihrer Puppenentwicklung drei verschiedene Opsin-Gene, *UVop*, *BLop* und *Lop1* exprimieren, welche letztendlich drei verschiedene Photorezeptortypen hervorbringen, einen ultraviolett-, blau- und grün-sensitiven Photorezeptor. Die Opsin-Expressionsmuster unterschieden sich nicht nur zwischen de Geschlechtern, sondern auch im Expressionsbeginn der jeweiligen Opsine während der Retinaentwicklung. Immunfärbungen der sich entwickelnden Retina zeigten außerdem, dass die Retina von Honigbienen zu Beginn ihrer Entwicklung zunächst nur aus einer sehr dünnen Hypodermis besteht, jedoch bereits alle retinale Strukturen enthält. Die Photorezeptordeterminierung bei Honigbienen lässt auf einen zweistufigen Prozess schließen, da ab etwa der Mitte der Verpuppung die Opsinexpression signifikant zunimmt und Hand in Hand mit der Differenzierung der Rhabdome verläuft. Im ersten Schritt, während der späten Puppenphase, erfolgt die Festlegung des Photorezeptortyps in den jeweiligen Photorezeptorzellen. Im zweiten Schritt, kurz nach dem Schlupf der Biene, nimmt dann die Quantität der Opsinexpression stark zu, nämlich bis um das 25-fache. Bisher wurden die zugrundliegenden Mechanismen, die die verschiedenen Photorezeptortypen determinieren, zum Teil in Schmetterlingen, aber besonders intensiv in der Taufliege, *Drosophila melanogaster*, untersucht. Interessanterweise scheinen die molekularen Mechanismen innerhalb der Insekten konserviert zu sein und beispielsweise die zwei Transkriptionsfaktoren, *spalt* und *spineless*, welche während der Photorezeptordeterminierung in Fliegen und Schmetterlingen eine essenzielle Rolle spielen, auch in der Honigbiene identifiziert. In Kapitel 3 habe ich die Expressionsmuster dieser beiden Transkriptionsfaktoren während der Puppenentwicklung von Honigbienenarbeiterinnen untersucht und konnte zeigen, dass *spalt* hauptsächlich in den ersten Puppenstadien exprimiert wird was vermutlich mit dem Beginn der *BLop* -Expression korreliert. *Spineless* zeigte hingegen in der Mitte der Puppenentwicklung einen markantes Maximum in seiner mRNA Expression, was mit der Expression von *Lop1* zusammenhängen könnte. Ob *spalt* und *spineless* jedoch auch in der Honigbiene eine Rolle in

der Photorezeptordeterminierung spielen, bleibt noch zu untersuchen. Zum Beispiel durch einen Knockdown/out des jeweiligen Transkriptionsfaktors während der Retinaentwicklung, der zu einem spektralen Phänotyp, beispielsweise einem dichromatischen Auge, führt. Solche spektralen Phänotypen könnten dann in Verhaltensexperimenten getestet werden, um Aufschluss über die Funktion einzelner Photorezeptoren für das Farbensehen und die Synchronisierung der inneren Uhr gewinnen zu können. Um jedoch die Farbunterscheidungsfähigkeiten von Bienen und die Qualität in der Farbwahrnehmung evaluieren zu können ist ein zuverlässiger Verhaltensversuch vonnöten. Daher war es in Kapitel 4 mein Ziel, das visuelle PER Paradigma als passende Verhaltensmethode für das Testen von Farbensehen in Bienen zu etablieren. Seit mehreren Jahrzehnten gilt die visuelle Konditionierung der PER bei Bienen als schwierig und war bei der europäischen Honigbiene bisher ohne ein Abschneiden der Antennen oder ohne Präsentation weiterer Cues, wie Duft oder Bewegenung, nicht erfolgreich. Daher wurde das experimentelle Setup zunächst bei Hummeln etabliert, welche sich schon in anderen Studien als zuverlässige und robuste Versuchstiere herausgestellt hatten, beispielsweise während elektrophysiologischer Untersuchungen. Arbeiterinnen und Drohnen der schwarzen Erdhummel, *Bombus terrestris*, waren fähig verschiedene monochromatische Lichtstimuli mit einer Zuckerbelohnung zu assoziieren und schafften es auch, einen unbelohnten von einem belohnten Farbstimulus zu unterscheiden. Auch konnten sie den gelernten Stimulus nach zwei Stunden erneut abrufen und Arbeiterinnen zeigten die Fähigkeit, die gelernte Information erfolgreich in einen neuen Verhaltenskontext zuübertragen. Im nächsten Schritt wurde der Versuchsaufbau für Honigbienen adaptiert, sodass ich diesen in Kapitel 5 bei zwei mittelgroßen Honigbienenarten, der asiatischen Honigbiene, *Apis cerana*, und in der europäischen Honigbiene, *Apis mellifera*, verwenden konnte. Beide Honigbienenarten waren fähig, zwei monochromatische Lichtstimuli, Blau und Grün, mit Absorptionsmaxima von 435 nm und 528 nm, mit einer Belohnung zu assoziieren und zwischen beiden Stimuli zu unterscheiden. Ähnlich den Hummeln, konnten auch die asiatischen und europäischen Honigbienen den gelernten Stimulus erfolgreich nach zwei Stunden erneut abrufen. Die visuellen Konditionierungssetups und -Protokolle in meinen Untersuchungen unterschieden sich von denen vorangegan-

gener Studien um einen entscheidenden Faktor, der von besonderer Bedeutung für eine erfolgreiche visuelle Konditionierung der PER von Bienen zu sein scheint, nämlich die Präsentationsdauer des konditionierten Stimulus. Da in vorangegangenen Studien eine visuelle Konditionierung der PER dazu tendierte nur dann erfolgsversprechend zu sein, wenn der konditionierte Stimulus für mehr als 10 s präsentiert wurde, habe ich in Kapitel 6 verschiedene Längen der Stimuluspräsentation systematisch getestet. Unmanipulierte Honigbienenarbeiterinnen konnten erfolgreich zwischen zwei monochromatischen Stimuli unterscheiden, wenn der Stimulus für 10 s präsentiert wurde, aber scheiterten, wenn die Stimuluspräsentation kürzer als 4 s war. Um ein vergleichbareres Konditionieren der Bienen zu ermöglichen, entwickelte ich ein neues Setup, welches einen Shutter beinhaltete, der durch ein PC basiertes Softwareprogramm gesteuert wurde. Das überarbeitete Setup ermöglicht eine präzisiere und automatisierte visuelle PER Konditionierung und bietet nun für zukünftige Studien eine exzellente Methode, visuelle Wahrnehmung und Kognition von Bienen unter konstanten und kontrollierten Bedingungen zu untersuchen.

1

General Introduction

1.1 The importance of bee's color perception for plant pollination

The superfamily of bees comprises more than 20,000 species and is essential for the pollination of numerous crops and wild plant species (Batra, 1995; Potts et al., 2010). Hence, for entomophilous plants it is of paramount importance to be recognized by potential pollinators like bees. During foraging flights, bees encounter a variety of flowers, differing in their quality and quantity of nectar, as well as in their appearance (Chittka and Raine, 2006; Giurfa et al., 1995; Kleber, 1935). Although flowers are detected from bees by different cues like size, shape or odor, especially the coloration of flowers and the development of distinct floral color signals are considered to be crucial factors for plants to be identified by bees (Chittka, 1996; Chittka and Menzel, 1992; Dyer and Chittka, 2004a; Gumbert, 2000). Moreover, the basis of trichromatic color vision, e.g. the presence of different photoreceptor types, was suggested to be evolved in hymenopterans before flowering plants arose (Chittka, 1996). Whether color vision has been already used in ancestoral insects, and a fine-tuning of the color vision system in response to flower exploitation took place, is still under debate (Chittka, 1996; Chittka and Menzel, 1992). However, the relationship between pollinators and plants is suggested to be mutual, since all bees highly depend on nectar and pollen.

Hence, to identify and discriminate between rewarding and unrewarding flowers, bees need a specialized and well-adapted visual system and cognitive capabilities that enable them to learn and retrieve the occurrence of suitable food sources.

1.2 The visual system of bees

The capability of light detection and the conversion of photon energy into electric signals is one of the major senses in many animals since almost every living organism and many biological processes depend on light energy. Bees possess various forms of vision e.g. color and achromatic vision, polarized vision and motion detection in order to orientate in space, recognize mating partners and suitable nest sites and find rewarding food sources. Besides vision, also non-visual functions like the entrainment of the endogenous clock are mediated by light, which has been shown to be the major Zeitgeber in honeybees (Moore and Rankin, 1993). Bees are equipped with two light sensitive organs: three single eyes, called ocelli, arranged in a triangular pattern at the vertex which are e.g. used for navigation and flight stabilization; and a pair of lateral compound eyes mediating a wide range of visual functions like color vision (Winston, 1987). The compound eyes consist of thousands of single units, the ommatidia, and each ommatidium in turn consists of a dioptric system containing the refractive cornea and the light focusing crystalline cones which together form the lens system. Below, eight large photoreceptor cells (PR 1-8) together with one short basal cell (PR 9) form the light sensitive system (Eisen and Youssef, 1980). The rhabdomeres of the photoreceptors form a fused light-guiding structure in the center, the rhabdom, which contains the visual pigments, a protein of the Gq-coupled opsin family and its conjugated light absorbing chromophore, the retinal (Gärtner, 2000; Fig.1.1). Although only the retinal is capable to absorb photons, the spectral sensitivity of a photoreceptor is determined by the interaction between the chromophore and particular amino acid residues of its respective opsin protein (Briscoe and Chittka, 2001; Wittstock et al., 1993; Zhukovsky and Oprian, 1989). By means of electrophysiological recordings of single photoreceptor cells and by molecular studies, three distinct photoreceptor types have been identified in honeybees, which are most sensitive in the ultraviolet (UV, ca. 340 nm), blue (ca. 430 nm) and green (ca. 540 nm) part of the light spectrum (Chang et al., 1996; Menzel and Blakers, 1976; Peitsch et al., 1992; Townson et al., 1998; Velarde et al., 2005). Similar photoreceptors have also been found

in bumblebees (Peitsch et al., 1992; Spaethe and Briscoe, 2005). Most retinae of insects, vertebrates and even humans possess a distinct arrangement of the different photoreceptors, forming a species- or sex-specific spatial mosaics. In honeybees as well as in bumblebees the three photoreceptors give rise to three distinct ommatidial types in worker eyes, comprising different sets of photoreceptors. Each ommatidial type contains six green photoreceptor cells and additionally either one UV and one blue photoreceptor (type I), two UV photoreceptors (type II), or two blue photoreceptors (type III); the sensitivity of the short distal cell is unknown (Spaethe and Briscoe, 2005; Wakakuwa et al., 2005). Honeybees show a sex-specific distribution of ommatidial types within the retina. The different ommatidial types are more or less evenly distributed within the main retina of workers; in contrast, the compound eyes of drones are divided into a male-specific dorsal part, which consists mainly of UV and blue photoreceptor cells, and a small remaining ventral part, which shows similar composition comparable to the compound eye of workers (Menzel et al., 1991; Peitsch et al., 1992; Velarde et al., 2005).The uppermost part of the compound eye, called dorsal rim area (DRA), is characterized by specialized ommatidia consisting of UV-sensitive photoreceptors which enables polarized vision (Labhart, 1980; Wehner and Strasser, 1985). In contrast to the DRA, which is anatomically characterized by 9 long, non-twisted photoreceptors, the photoreceptors of the remainder eye show a twisted architecture to prevent photoreceptors from being sensitive to polarized light (Wehner and Bernard, 1993).

The development of the retina in the honeybee eyes starts already in the late larval stage and reaches their final form in the late pupal phase (Eichmüller and Schäfer, 1995; Eisen and Youssef, 1980; Phillips, 1905). During pupal development, ommatidia elongate and cellular elements become differentiated until all ommatidia reach their final adult shape (Phillips, 1905). It has been shown, that honeybee optic lobes gradually start to develop their adult form when the optic nerve projections from the photoreceptors reach the lamina during the late pupal phase (Marco Antonio and Hartfelder, 2016). Photoreceptor axons can be divided into long (lvf) and short visual fibres (svf), depending on their length and termination within the optical lobes (Ribi, 1975). The lvf, comprising the axons of the six long-wavelength

sensitive photoreceptors, terminate in the first optical neuropil, the lamina, and are mainly involved in processing of achromatic vision. In contrast, the svf comprising axons of the short- and medium-wavelength sensitive photoreceptors, run through the lamina and terminate in the second optical neuropil, the medulla. The medulla contains distinct neurons which further separate visual information: broad-band neurons that respond to all three photoreceptor types (Kien and Menzel, 1976; Paulk et al., 2009), narrow-band neurons that receive input from only one photoreceptor (Kien and Menzel, 1977; Paulk et al., 2009), and color opponent neurons that are excited by one photoreceptor class and inhibited by another class (Kien and Menzel, 1977; Yang et al., 2004). Achromatic and chromatic information is then further processed via pathways connecting the medulla and the third optical neuropil, the lobula, with central brain structures, like mushroom bodies and the lateral protocerebrum (Avarguès-Weber et al., 2012; Dyer et al., 2011). However, to reliably proof the capability of color perception, behavioral experiments are necessary, e.g. testing color discrimination independently of brightness differences (Kelber et al., 2003; Menzel, 1987).

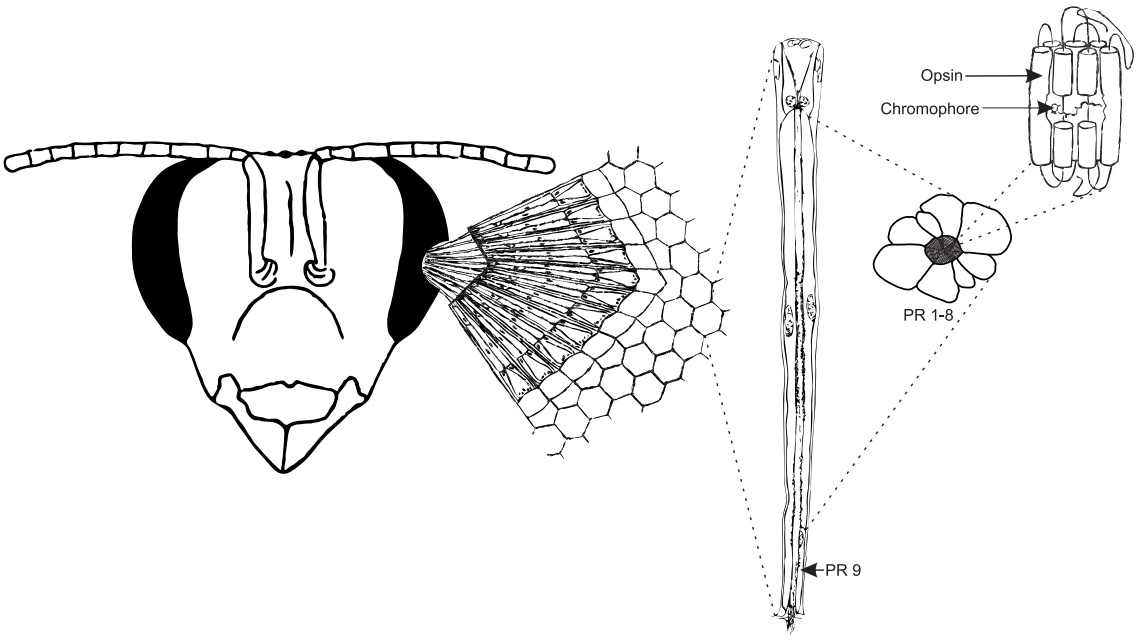

Figure 1.1: Overview of the bee's visual system. The honeybee possesses paired compound eyes and three ocelli at the vertex. Each compound eye contains thousands of single ommatida. Each ommatidium comprises eight large (PR 1-8) and one short basal photoreceptor cell (PR9). The photoreceptor cells form a fused rhabdom containing the visual pigments, an opsin protein and its conjugated chromophore.

1.3 Visual learning in bees

Since Karl v. Frisch firstly demonstrated color vision in honeybees more than 100 years ago (v. Frisch, 1914), numerous studies have been performed to investigate the molecular, morphological and physiological basis of the bee's visual system. A perquisite for color vision are the existence of photoreceptors which are sensitive for different wavelengths, and a neuronal machinery allowing to compare the excitations of these photoreceptors (Kelber, 2016; Menzel and Blakers, 1976). Whereas chromatic perception is mediated by excitatory and inhibitory interactions of color-opponent neurons (Dyer et al., 2011; Kien and Menzel, 1977; Yang et al., 2004), achromatic vision is based on visual input from the green-sensitive photoreceptors only. In contrast to achromatic vision, which is mainly responsible for movement-related visual tasks (Srinivasan and Lehrer, 1984), chromatic vision is essential for the detection and discrimination of colored objects like flowers (Giurfa et al., 1997; Giurfa et al., 1996).

Honeybees and bumblebees provide excellent invertebrate model organisms to investigate visual learning capabilities and limitations of the visual system, since both species are central place forager, collect food not only for themselves but for the whole colony, and are easily trainable to search for food on an artificial feeder. During the last decades, numerous studies on operant conditioning in free-flying bees in the context of foodsearching has been performed which revealed astonishing cognitive capabilities, e.g. the categorization of face-like stimuli (Avarguès-Weber et al., 2010), recognition of human faces (Dyer et al., 2005) or discrimination between Monet and Picasso paintings (Wu et al., 2013), disproving the assumption of bees as simple reflex machines (Avarguès-Weber et al., 2010; Srinivasan, 2010). Besides operant conditioning which originates from learning consequences of behavioral decisions, classical conditioning using the PER (Proboscis Extension Reaction) paradigm, is a promising assay for studying various forms of appetitive associative learning and memory (Abramson, 1994). The ability of bees to learn, retrieve and appreciate contexts of different environmental cues, play an essential part in contributing to a successful adaption to a complex and changing environment. If a

foraging bee gets into contact with nectar through its antennae, tarsi or proboscis, it reflexively extends its proboscis (PER) to collect the sugar solution (Menzel and Muller, 1996). By transferring this PER into the laboratory, bees can be conditioned to associate a certain conditioned stimulus (CS; e.g. an odor or color) with an unconditioned stimulus (US; e.g. a sugar reward). After several conditioning trials, during which the CS and US is presented at the same time, the conditioning lead to a successful association between both stimuli; as a consequence, the CS alone is then able to evoke the PER (Avarguès-Weber and Mota, 2016; Giurfa and Sandoz, 2012; Matsumoto et al., 2012; Menzel and Muller, 1996). The PER paradigm has been successfully applied in bees for olfactory conditioning for more than 50 years (Giurfa and Sandoz, 2012) and has recently also successfully established for visual conditioning in bumblebees (Lichtenstein et al., 2015; Riveros and Gronenberg, 2012) and Africanized honeybees (Jernigan et al., 2014), but is considered to be difficult to use in the Western honeybee (Avarguès-Weber and Mota, 2016). However, the PER assay provides an excellent method to test and understand sensory perception and cognition of bees under constant and controlled laboratory conditions.

1.4 Thesis outline

Since bees uses a variety of visual cues e.g. during foraging bouts, mating flights and orientation in space, vision has been proved to be one of the major senses in bees. During the last decades much effort has been devoted to gain insight into the molecular, morphological and physiological basis of the (sex-specific) compound eyes of bees and their corresponding photoreceptors. However, almost nothing is known about the underlying mechanisms of retinal patterning and photoreceptor determination during pupal development, the function of specific photoreceptor types for color perception, and the visual input for entraining the circadian clock.

Hence, in the first part of my dissertation, I aimed to provide first insight for understanding the molecular mechanisms of retinal patterning in honeybees (Chapter 2 + 3). In Chapter 2, I used qPCR to investigate the intrinsic and temporal expression patterns of visual pigments that give rise to the ultraviolet, blue and green sensitive photoreceptors during pupal and adolescent development of workers and drones in the honeybee, *Apis mellifera*. I also tested whether the opsin expression in young adult bees is under circadian control, as it has been shown for other insects. Confocal microscopy was used to characterize the morphological development of the retina and to screen for possible interactions between morphological and molecular alterations.

In Chapter 3, I focused on the identification of transcription factors which might be involved in the expression of different opsins, resulting in different photoreceptor types. By means of NCBI BLAST and gene specific primers, two promising transcription factors, *spalt* and *spineless*, were identified. Again, qPCR was used to evaluate the relative expression levels of both transcription factors during the pupal stage of honeybee workers and expression patterns were subsequently compared with expression levels of opsins to check for possible correlations. Finally, a custom-made antibody against spineless was generated and used to investigate the protein expression levels during pupation.

The second part of my thesis focuses on the establishment of a suitable behavioral method for evaluating color perception, learning and memory capabilities of bees

under standardized and controlled conditions (Chapter 4, 5 + 6).

Since, PER color vision has been considered to be difficult in honeybees, I aimed to establish the visual PER setup in bumblebees first (Chapter 4). Bumblebees have previously shown to be very robust and reliable during electrophysiology experiments. I thus used the visual PER paradigm to train bumblebee workers and drones in an absolute and differential task and tested their learning and memory capabilities for monochromatic lights under different parameters. Moreover, I tested whether bumblebees were able to transfer the learned chromatic information into a new context under free-moving conditions in a dual choice test.

After successful establishment of the visual PER setup in bumblebees, I adapted the experimental setup for honeybees (Chapter 5). I tested and compared color discrimination and learning in the Asian honeybee, *Apis cerana*, and the Western honeybee, *Apis mellifera*, by means of absolute and differential color conditioning.

In Chapter 6, I investigated the fact that visual PER conditioning seems to be more difficult than olfactory PER conditioning. Moreover, I developed a modified the PER conditioning setup and a computer based training protocol to automatize the conditioning of restrained bees.

2

Temporal and circadian plasticity of opsin expression during pupal and adolescent compound eye development in the honeybee, *Apis mellifera*

2.1 Abstract

Insects possess compound eyes which allow them to catch photons and convert the energy into electric signals. All compound eyes show a repetitive architecture and consist of single ommatidia comprising a fixed number of photoreceptors. Different photoreceptor types, characterized by a specific opsin protein and spectral sensitivity, often form distinct retinal mosaics. The underlying mechanism of retinal patterning, which has been studied in flies and butterflies, consists of a complex temporal interplay of different transcription factors and expression of visual components. In honeybees, males and females possess different photoreceptor types forming distinct retinal mosaics, but data about the mechanisms which pattern the eyes are lacking. The temporal and circadian expression patterns of visual pigments that give rise to the ultraviolet, blue and green sensitive photoreceptors during pupal and adolescent development of honeybees were investigated. By means of immunostainings and confocal microscopy it was found that temporal opsin expression is accompanied by rhabdom maturation and differs between sexes. In contrast to Drosophila*, opsin expression levels increase up to 25 fold during early adult life. Moreover, it was shown that opsin expression in adult bees is under control of the endogenous clock.*

2.2 Introduction

The ability of light detection and processing is one of the major characteristics in most animals, since almost every living organism and many biological processes depend on light energy. Aside from the regulation of biological processes and various forms of vision, the perception of light is also important for entraining the endogenous clock. Insects represent one of the most diverse groups of the animal kingdom and have colonize almost all habitats; they possess a pair of compound eyes that convert photon energy into an electric signal for further processing (Land, 1999; Nilsson, 1990). All compound eyes are composed of repetitive functional units, called ommatidia. Each ommatidium consists of a dioptric system which includes the cornea and the crystalline cone cells forming the lens system, and a specialized receptor system comprising a fixed number of photoreceptor cells which contain the photopigments that are essential for photoreception (Bitsch and Bitsch, 2005). Different photoreceptor types, which are characterized by different spectral sensitivities and expressed opsins, are distributed in the retina and form random mosaics, localized zones or bands (Land, 1999). In the last decade, much effort has been devoted to understanding the molecular and developmental mechanisms giving rise to different photoreceptor types, species and sex-specific patterns of retinal mosaics (reviewed in: Rister and Desplan, 2011; and Wernet et al., 2015). Retinal patterning in insect compound eyes is considered to be formed during retinal development and has been intensely studied in *Drosophila melanogaster*. The main retina of the fly contains two distinct ommatidial types, termed ”pale” and ”yellow”. Both types comprise two distinct pairs of central photoreceptor cells (R7+8) which differ in their spectral sensitivity and are stochastically distributed in the retina. Interestingly, the fate of a pale or yellow ommatidium emerges from a complex temporal interplay of various transcription factors during the second half of pupil development (reviewed in: Morante et al., 2007; and Wernet and Desplan, 2004; Wernet et al., 2006; Wernet et al., 2015). A recent study found surprising similarities in the regulation and determination of photoreceptors during retinal development between flies and butterflies (Perry et al., 2016). Moreover, the presence of homolog transcription factors

in butterflies, which are essential for patterning the retinal mosaic in *Drosophila*, suggests evolutionarily conserved regulatory mechanisms that determine different photoreceptor types (Perry et al., 2016).

Species- and sex-specific retinal mosaics are also prominent in the honeybee, an insect model for (color) visual perception, as well as learning and memory (reviewed in: Srinivasan, 2010). Honeybees possess a pair of compound eyes which comprise different ommatidial types forming a random retinal mosaic (Wakakuwa et al., 2005). Each ommatidium is composed of a set of nine photoreceptor cells (eight large and one short basal photoreceptor), and each cell expresses a specific visual pigment, a protein of the Gq-coupled opsin family, and its conjugated chromophore, the retinal (Gärtner, 2000). Although only the retinal can absorb photons, the spectral sensitivity of a photoreceptor is defined by the interaction between the chromophore and its respective opsin protein (Briscoe and Chittka, 2001; Wittstock et al., 1993; Zhukovsky and Oprian, 1989). By means of electrophysiological recordings of single photoreceptor cells and by molecular studies, three spectral photoreceptor types have been identified in honeybees, which are most sensitive in the ultraviolet (UV, ca. 340 nm), blue (ca. 430 nm) and green (ca. 540 nm) part of the light spectrum (Chang et al., 1996; Menzel and Blakers, 1976; Peitsch et al., 1992; Townson et al., 1998). Moreover, these three photoreceptor types give rise to at least three different ommatidial types in worker eyes, comprising different sets of photoreceptors. Each type contains six green photoreceptor cells, and additionally either one UV and one blue photoreceptor (type I), two UV photoreceptors (type II), or two blue photoreceptors (type III), and the three types are more or less randomly distributed in the main retina (the sensitivity of the short distal cell is unknown) (Wakakuwa et al., 2005). In contrast, compound eyes of drones are divided into a male-specific dorsal part, which mainly consists of UV and blue photoreceptor cells, and a small remaining ventral part, which shows similar photoreceptor composition comparable to the compound eye of workers (Menzel et al., 1991; Peitsch et al., 1992; Velarde et al., 2005). The development of the retina in the honeybee compound eyes begins already in the late larval stage and precursor ommatidia are not completely arranged by the end of the pre-pupal phase, but reach their final form at the late

pupal phase (Eichmüller and Schäfer, 1995; Eisen and Youssef, 1980; Phillips, 1905). Surprisingly, despite the existence of various spectral ommatidial types and a sex-specific ommatidial distribution in the retina, almost nothing is known about the developmental mechanisms that pattern the retinal mosaic or about the onset of opsin expression during retinal development in the honeybee.

Hence, I aim to better understand retinal development and determination of photoreceptor types in honeybees. As a first step, the intrinsic temporal expression pattern of the opsins during pupal and adolescent development of the honeybee worker and drone compound eye was investigated by quantifying opsin mRNA expression in the retina. Second, confocal microscopy was used to characterize the morphological development of the retina with special focus on the light-guiding fused rhabdoms in which the visual pigments are embedded to screen for possible interactions between morphological and molecular alterations. Third, it was also tested whether the opsin expression in young adult bees is under circadian control as has been shown in other animals, e.g. *Noctuidae* (Yan et al., 2014) and vertebrates (Korenbrot and Russel, 1989).

2.3 Material and Methods

2.3.1 Bee keeping and collecting

Honeycombs containing brood of *Apis mellifera carnica* were transferred from the hive, located at the bee facility of the University of Würzburg, to the laboratory and were kept in an incubator at 34°C and 60% humidity at constant darkness. Eight pupal stages in workers (P1-P8; Fig.2.1A) and nine stages in drones (P1-P9; Fig.2.1B) were identifyed, following the classification by Dietermann (2013) and Tofilski (2012). At least 15 pupae from each stage were collected always at the same time of the day (12am – 2 pm) to account for possible diurnal variation of opsin expression. Additionally, newly emerged workers and drones (NE, 0-24 h old), as well as 7 day (A7) and 14 day old bees (A14, workers only) were sampled and kept under constant darkness. For this, newly emerged workers were transferred to small wooden cages and fed with Apiinvert (a liquid bee food, containing fructose and

glucose; Suedzucker, Mannheim, Germany) and a mixture of pollen and honey until they reached the respective age.

To evaluate if opsin expression in adult bees changes during the day and if it is under the control of the circadian clock, newly emerged bees were entrained to a light-dark rhythm (8:00h – 20:00h, CET) for 13 days and were kept in constant darkness on day 14. From these bees, individuals were collected at four hour intervals over a time span of 24 h at day 14, adding up to six individuals per sampled time point (CT2, CT6, CT10, CT14, CT18, CT22). All collected pupae and adolescent bees were immediately transferred to liquid nitrogen and stored at -80°C until further processing.

2.3.2 Dissection, RNA extraction and cDNA synthesis

For further processing, the entire eyes (retina and a small part of the optic lobe) of workers were removed since the different photoreceptor types seem to be approximately evenly distributed in the compound eye (Wakakuwa et al., 2005). In contrast, drones lack green photoreceptor cells in the dorsal part of their eye (Menzel et al., 1991; Peitsch et al., 1992; Velarde et al., 2005), and I thus separated the dorsal and ventral part. However, the ventral worker-like part of the retina covers only ca. 20% of the entire eye (Menzel et al., 1991; Velarde et al., 2005) and I was not able to clearly dissect this part of the eye without contamination and thus used only the dorsal part in drones for further analysis. The eyes were dissected on ice and remained frozen until they were transferred into PCR tubes for further RNA extraction. To isolate mRNA, I used TRIzol Reagent (5 Prime, Hilden, Germany) and the Gold HP total RNA Kit (Peqlab Biotechnologie, Erlangen, Germany) following the manufacturer's protocol. Quantity and quality of mRNA was estimated by spectrophotometric measurement and gel-electrophorese (1% agarose gel, stained with Midori Green Direct) (Nippon Genetics Europe, Dueren, Germany). cDNA synthesis was performed by means of QuantiTec Reverse Transcription Kit (Quiagen, Hilden, Germany) according to the manufacturer's protocol. All cDNAs were stored at -20°C until used for quantitative real-time PCR (qPCR).

Table 2.1: Primers for qPCR of opsins

Gene	Forward primer	Reverse primer	Product length	Primer efficiency
UVop	5'-TAACTGGAATCGGTGCTGCG-3'	5'-CCCCATACTCCCATCACAGG-3'	172 bp	0.97
BLop	5'-AAGACTCTCGCCGGTAAAGC-3'	5'-GATGATCGCGAGTCCGATGT-3'	174 bp	0.97
Lop1	5'-CAAAAAGTCTTCGCACGCCA-3'	5'-AGCCACATCCGAACAAGGAG-3'	177 bp	0.96
Rp49	5'-CGTCATATGTTGCCAACTGGT-3'	5'-TTGAGCACGTTGAACAATGG-3'	150 bp	0.91

Opsin primers and qPCR

qPCR was used to evaluate specific mRNA expression levels of the honeybee opsins. Four different opsin genes have been identified in the honeybee, so far: UV-sensitive opsin, *UVop*, blue-sensitive opsin, *BLop*, (Townson et al., 1998) and two long-wave sensitive opsins, *Lop1* and *Lop2* (Chang et al., 1996; Leboulle et al., 2013; Velarde et al., 2005). Since it has been reported that *Lop2* expression is restricted to the ocelli (Velarde et al., 2005), this chapter focuses on the expression of *Lop1* in the honeybee compound eye. As an internal control the *Ribosomal protein 49* (*Rp49*) which has been previously shown to be a stable housekeeping gene in *Apis mellifera* (Lourenço et al., 2008) was used. Amplification efficiency for each primer pair was determined by means of a serial 1:10 dilution ranging from 10^1 to 10^6 copies (Tab.2.1).

Primers were designed based on the reported sequences of the three opsin genes (Chang et al., 1996; Leboulle et al., 2013; Schulte et al., 2014) using PrimerBLAST (NCBI, Bethesda, USA) and ordered from Metabion (Planegg, Germany) (Table 2.1). All qPCR runs were performed with a Mastercycler ep realplex (Eppendorf, Hamburg, Germany). For all qPCR reactions the Kapa SYBR Fast qPCR Kit (Peqlab Biotechnologie) was used and each qPCR master mix was prepared according to the manufacturer's protocol. Furthermore, experimental conditions were evaluated in technical triplicates for each sample.

Immunostaining of the pupal retina

Pupal retinae were dissected and fixed overnight at 4°C in 4% formaldehyde in PBS. Fixed retinae were rinsed 3 times in PBS for 10 minutes and subsequently embedded in 5% low-melting-point agarose. 80 µm sections were performed by means of a vibrating microtome (Leica VT 1000S, Wetzlar, Germany) and rinsed once for 10 minutes in PBS with 2% Triton-X 100 and twice in PBS with 0.2% Triton-X 100. Sections were then incubated in Alexa Fluor 488 Phalloidin (2.5 µl Phalloidin from Methanol stock solution in 500 µl PBS; Molecular Probes, Eugene, USA) for one day at 4°C to label f-actin. Sections were rinsed three times in PBS and subsequently incubated in Hoechst 34580 (1:000; Life Technologies GmbH, Darmstadt, Germany) for 15 minutes at room temperature to label cell nuclei. Finally, sections were rinsed in PBS five times, transferred to 60% Glycerol in PBS for 30 minutes at room temperature and mounted in 80% Glycerol in PBS on slides. For visualization of the retinae, a confocal laser-scanning microscope (Leica TCS SP2 AOBS, Leica Microsystems AG, Wetzlar, Germany) equipped with an argon/krypton and a three diode laser was used. Single images of sections were taken at a resolution of 1,024 x 1,024 pixels with a HC PL APO objective lens: 20/0.7 NA imm; with additional digital zoom: 2.0-4.0. Furthermore, scanned images were processed and edited in ImageJ 1.51e (Wayne Rasband National Institutes of Health, Maryland, USA) and CorelDRAW Graphics Suite X7 (Corel Corporation, Ottawa, Canada).

Experimental and statistical analysis

To evaluate the relative opsin expression, the $2^{-\Delta\Delta CT}$- method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) was used. Opsin expression data of the pupal development of workers and drones as well as opsin data from adolescent development were normalized separately for each opsin gene to the housekeeping gene *Rp49* and the expression level of newly emerged bees (NE; set to one). The relative proportions of opsin gene expression were calculated and estimated as followed:

$$\frac{OE_{gene}}{OE_{all\ genes}} = \frac{1/(1+E_{gene})^{CT_{gene}}}{\Sigma 1/(1+E_{gene})^{CT_{gene}}}$$

Whereby OE_{gene} is defined as the expression of each individual opsin gene and E_{gene} as the primer efficiency for the respective gene. CT_{gene} depicts the mean threshold cycle determined during qPCR for each pupal stage (for more details see: Wang et al., 2013). Expression data of the circadian expression experiment were treated as described above and the time point of the lowest expression level of each gene was set to one. A general linear model (GLM) with Poisson's distribution was used to analyze differences in opsin expression during pupal development and between sexes. Expression levels at different circadian time points were evaluated by means of log-linear one-way ANOVA. A Kruskal-Wallis test was applied to evaluate possible differences in rhabdom length and diameter during pupal development of workers. All statistical analyses were performed in R software v. 3.3.1.

2.3.3 Results

Temporal opsin expression patterns of honeybee workers and drones

In both sexes *UVop* mRNA was found to be expressed during the first pupal stage, whereas *BLop* and *Lop1* mRNA expression commenced in drones much later than in workers (Fig.2.1). Overall, the mRNA expression levels of *UVop*, *BLop* and *Lop1* mRNA significantly increased during pupal development (GLM, poison family, $P < 0.001$; *UVop*: $\chi^2_8 = 2361600$; *BLop*: $\chi^2_8 = 2117777$; *Lop1*: $\chi^2_8 = 4084108$) and differed significantly between workers and drones (GLM, poisson family, $P < 0.001$; *UVop*: $\chi^2_1 = 1042426$; *BLop*: $\chi^2_1 = 1798472$; *Lop1*: $\chi^2_1 = 1320809$; Fig.2.1A,C). Furthermore, a significant interaction between pupal stage and sex was found (GLM, poisson family, $P < 0.001$; *UVop*: $\chi^2_7 = 20020$; *BLop*: $\chi^2_7 = 8449$; *Lop1*: $\chi^2_7 = 73288$), suggesting different mechanisms underlying drone and worker retinal development. Comparing the relative proportions of opsin expression between sexes shows that in workers and drones *UVop* mRNA is expressed during the first pupal stage. In the second half of the pupal development, *Lop1* mRNA expression strongly increases in workers, whereas in the dorsal part of the drone eye *Lop1* mRNA increases only slightly compared to *UVop* and *BLop* mRNA (Fig2.1; Fig.2.2). In workers and drones, opsin expression levels strongly increase after eclosion up to 25 fold, even though all bees were kept in constant darkness (Fig.2.2). In workers the relative pro-

portions of opsin expression levels remain constant after eclosion, however, young drones show a strong increase in *UVop* and *BLop* mRNA expression levels, while relative *Lop1* mRNA expression level drops during adolescent development (Fig.2.2D).

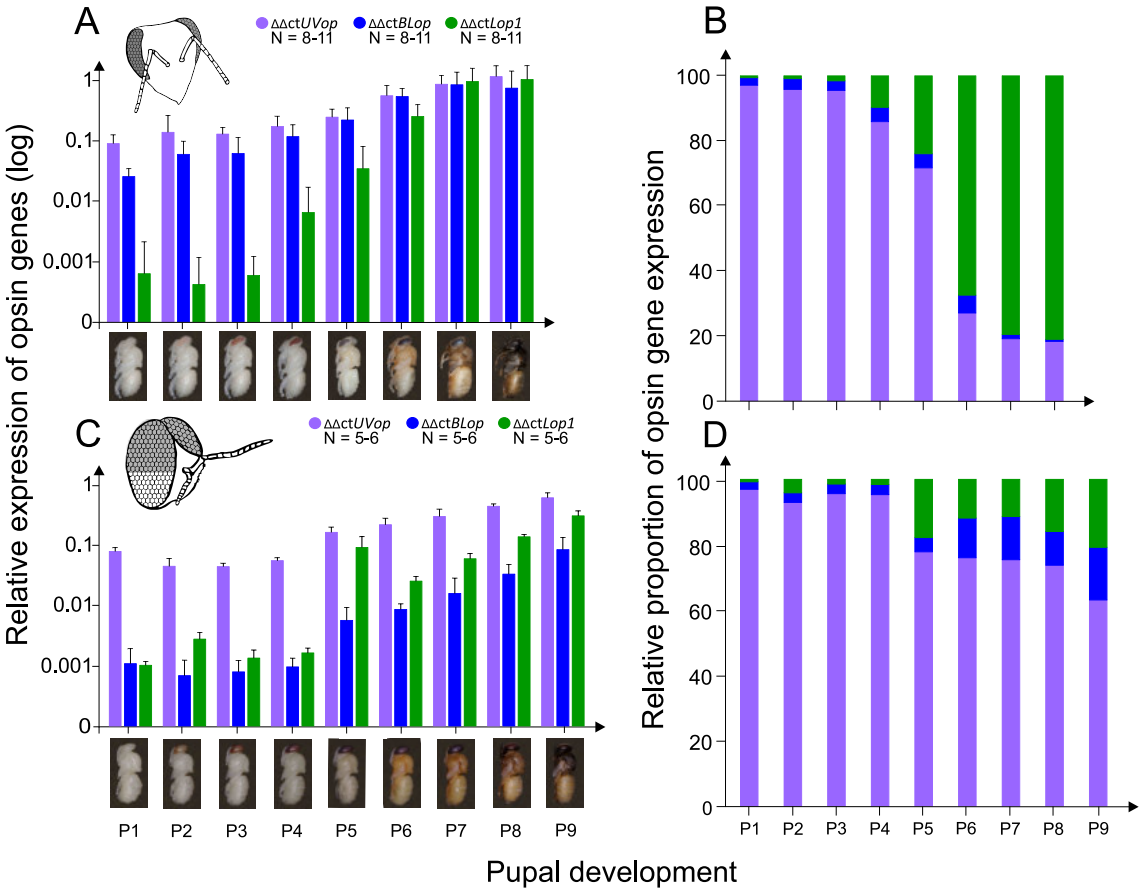

Figure 2.1: Temporal opsin expression during pupal development of workers and drones. A,**B**: Relative mRNA expression levels of ultraviolet (*UVop*), blue (*BLop*) and green (*Lop1*) opsins in the compound eyes during pupal development of workers (pupal stages: P1-P8) and drones (P1-P9; only the dorsal part of the compound eye; grey marking of the retina) by means of qPCR. Expression level for each opsin gene was normalized to the reference gene *Rp49*, and the level of newly emerged bees was set to one (NE; see Fig.2.2). **C**,**D**: Relative proportion of opsin mRNA expression in workers and drones at different pupal stages. Error bars indicate standard deviation.

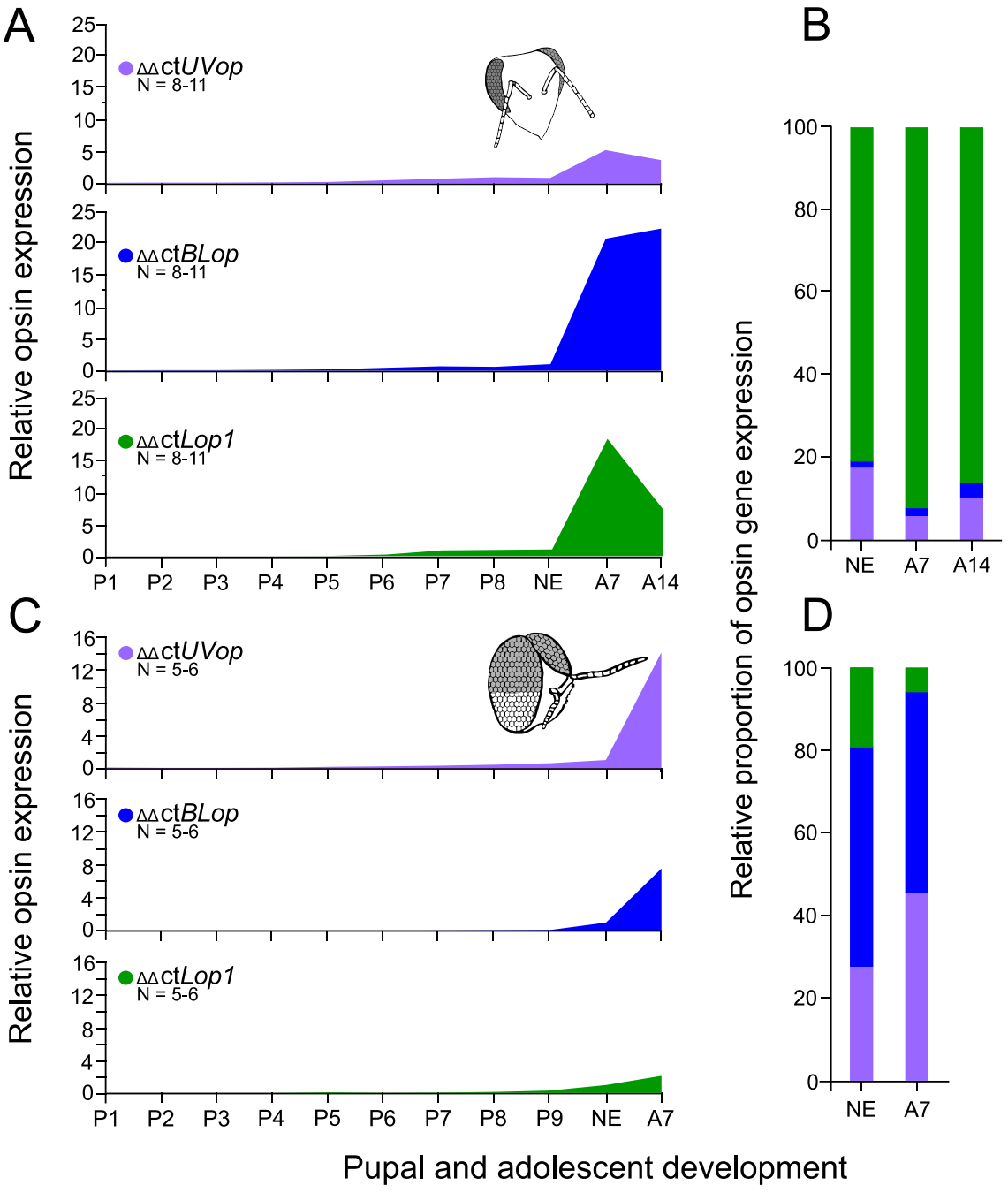

Figure 2.2: Overview of opsin expression during pupal and adolescent development of workers and drones. Relative mRNA expression levels of ultraviolet (*UVop*), blue (*BLop*) and green (*Lop1*) opsin genes in the compound eyes were evaluated during pupal and early adult development of workers (upper half) and drones (lower half; only the dorsal part of the compound eye) by means of qPCR. Opsin expression was normalized separately for each opsin gene to the expression level of newly emerged bees. **A**,**C**: Opsin mRNA levels in both sexes strongly increased after eclosion up to 25 fold. **B**,**D**: In contrast to the pupal phase, the relative proportions of opsin expression levels remained constant after eclosion. Error bars indicate standard deviation. NE, newly emerged; A7, 7 day old; A14, 14 day old.

In 14 day old bees which were kept 24h under constant darkness, it was found that *UVop* (*ANOVA*: $P < 0.01$; $F_{6,14} = 3.5239$), *BLop* (*ANOVA*: $P = 0.05$; $F_{6,14} = 2.3239$) and *Lop1* (*ANOVA*: $P < 0.05$; $F_{6,14} = 2.5316$) exhibit a similar oscillation pattern during 24 hours (Fig. 2.3).

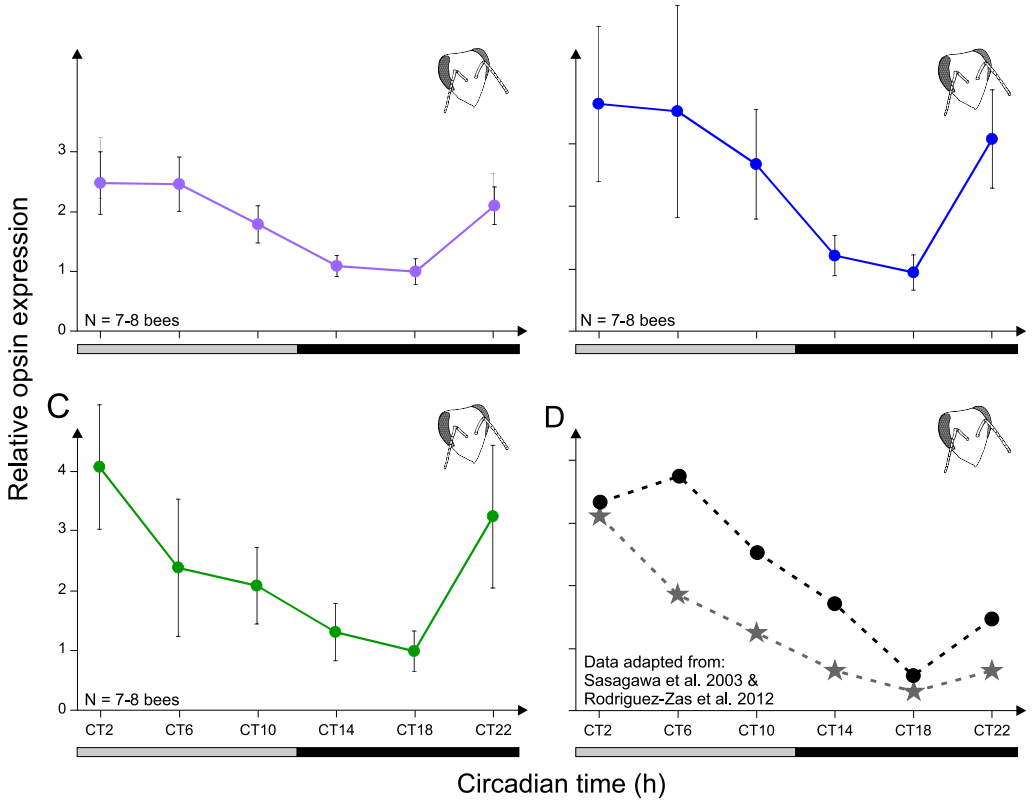

Figure 2.3: Circadian opsin expression patterns of workers. **A**,**B**,**C**: Relative mRNA expression levels of ultraviolet (**UVop**; **A**), blue (*BLop*; *B*) and green (*Lop1*; *C*) opsin genes in the compound eyes of 14 day old workers at six different circadian time points (CT2-CT22). Expression level for each opsin gene was normalized to the reference gene Rp49 and the lowest expression level was set to one. Expression levels were highest shortly after the expected light onset (CT2-CT6) and lowest in the middle of the expected night (CT18). **D**: Expression data of Lop1 compiled from earlier studies for comparison (asterisk: 42, black dots: 58) (Rodriguez-Zas et al., 2012; Sasagawa et al., 2003). Error bars indicate standard deviation.

For all opsins, mRNA expression is highest shortly after the expected light onset at CT2 – CT6 and lowest in the middle of the expected night at CT18 (Fig.2.3). This pattern matches the circadian expression pattern of the *Lop1* in workers described in earlier studies by means of northern-blotting (Fig.2.3D; Rodriguez-Zas et al., 2012; Sasagawa et al., 2003).

Retinal development in the honeybee worker

To evaluate modification of the retina and rhabdoms during pupal development, immunostainings of the retina in all pupal stages and in adult worker bees were performed, and rhabdom length and diameter was quantified. Rhabdom length and diameter significantly increase during pupal development from ca. 13 µm length in 1 day old pupae to ca. 300 µm in adult bees, and from ca. 2 µm in diameter in 1 day old pupae to ca. 5 µm in adult bees (Kruskal-Wallis-Test, length: $P<0.001$; $\chi^2_8 = 68.658$; diameter: $P<0.001$;$\chi^2_{10} = 81,679$. During the first pupal stage (Fig.2.4A), the retina consists only of a thin hypodermis comprising four crystalline cone cells, and several pigment and photoreceptor cells, which extend their axons through the basement membrane towards the first optical ganglion, the lamina (see also: Phillips, 1905; Ribi, 1975). However, except for the four crystalline cone nuclei, all other nuclei of the photoreceptor and pigment cells are clustered in the same level. At this early phase (Fig.2.4A; P1), rhabdoms are short (13 µm) and occur only in the apical part of photoreceptors, just beneath the crystalline cones. During the next few days, rhabdom length increases significantly, with a large step in the middle of the pupal phase between P5 to P6, where the length almost quadruples from ca. 60 µm to 232 µm (Fig.2.4E,F). Shortly before eclosion, the rhabdoms stretch through the whole photoreceptors. Aside from rhabdom growth, photoreceptors increase in size, and nuclei of photoreceptors and pigment cells become separated from each other into two distinct layers (Fig.2.4D). In a later stage, the nuclei of photoreceptors and of pigment cells further segregate from each other (Fig.2.4D-I), and the cell nucleus of the ninth' photoreceptor cell becomes visible (Fig.2.4E,F). During the final phase of pupation, rhabdoms become more compacted which thus leads to a slight reduction in total rhabdom length (Fig.2.5A). In addition to an increase in length, rhabdom diameter significantly increases about threefold between P3 and adult stage (Fig.2.5B).

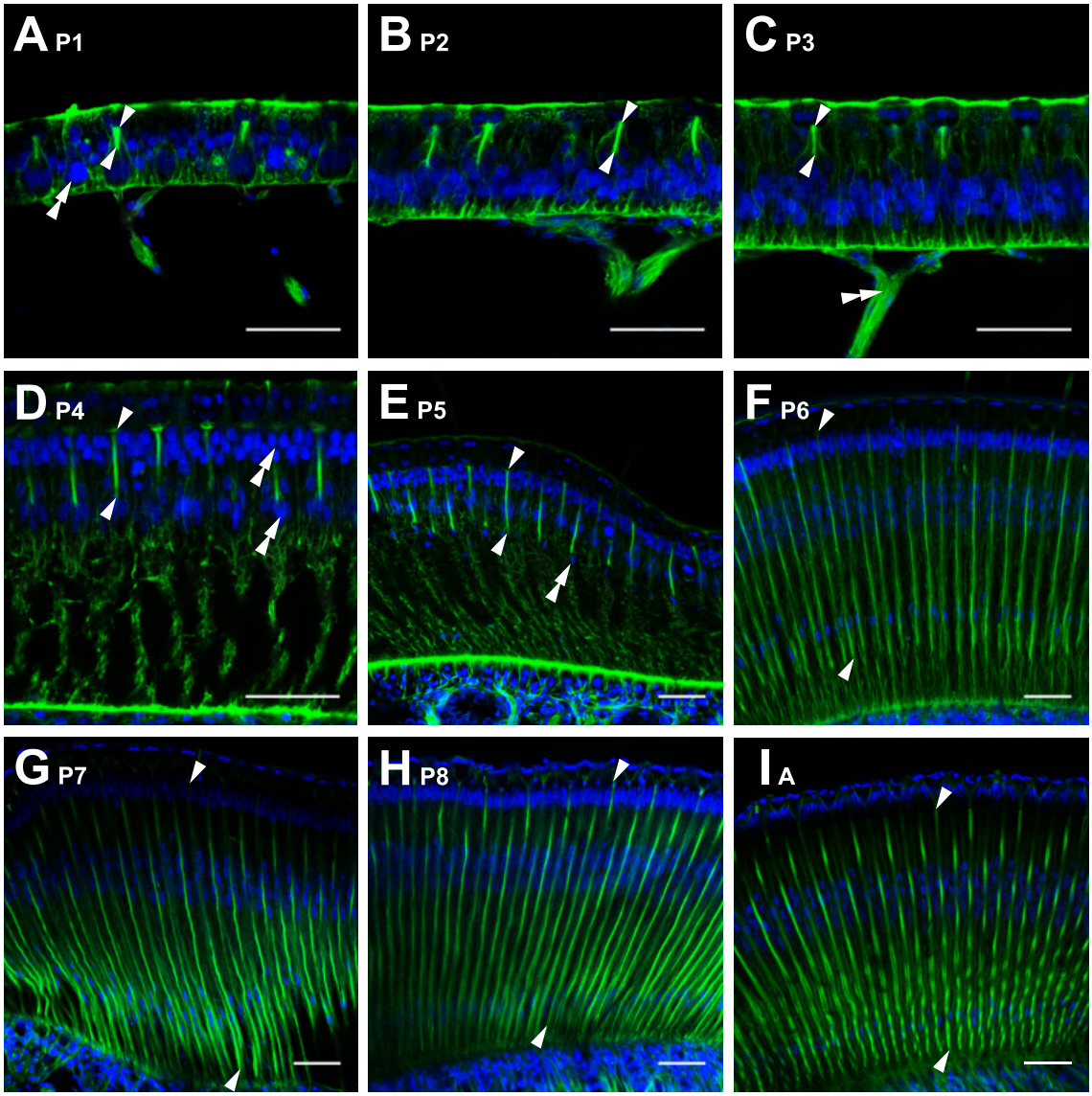

Figure 2.4: Retinal development in the worker pupae. Immunostainings with phalloidin (green) and Hoechst (blue) to label f-actin and nuclei, respectively, were performed to visualize the retinal development. **A**-**C**: At the beginning of the pupal phase the retina consists only of a thin hypodermis comprising crystalline cone cells, several pigment cells, hair cells (double arrow head in A) and photoreceptor cells which contain the fused rhabdoms (distal and proximal boundary of the rhabdom is marked by arrow heads) and extend their axons (double arrow head in C) through the basement membrane. At this early pupal phase rhabdoms are short and all nuclei are clustered in the same level. **D**-**H**: During the next days of pupal development rhabdoms and photoreceptor cells significantly increase in size, and nuclei of photoreceptor and pigment cells become separated from each other into distinct layers (double arrow head in D). The cell nuclei of the ninth photoreceptor cell become first visible in pupal stage P5 and form a distinct layer in P6 (double arrow head in E). **I**: The retina reached its final stage in freshly eclosed bees (A). Scale bar in all figures: 50 µm.

2.3.4 Discussion

The present chapter showed that honeybees express three different opsin genes, *UVop*, *BLop* and *Lop1* during pupal development. Opsin expression patterns differ between sexes, and onset of UV, blue and green opsin mRNA occurs at different time points in development (Fig.2.1; 2.2). At early pupal stages, worker and drone (dorsal) compound eyes exhibit similar expression levels with relatively high levels of *UVop* and only marginal levels of *BLop* and *Lop1* mRNA. This expression pattern significantly changes at about mid-pupation (Fig.2.1). Opsin expression in workers becomes dominated by *Lop1* mRNA (Fig.2.1A,B), which corresponds to the high number of green photoreceptor cells (six out of eight large PR cells) in the worker eye, as revealed by in situ hybridization (Wakakuwa et al., 2005). In contrast, the dorsal drone eye shows high expression levels of *UVop* and *BLop* mRNA, whereas *Lop1* mRNA level decreases (Fig. 2.1C,D), which approximates the proportion of UV and blue-sensitive photoreceptors that are found in the dorsal part of the drone eye (Menzel et al., 1991; Peitsch et al., 1992; Velarde et al., 2005; Fig.2.1C,D). Surprisingly, in both workers and drones *UVop* mRNA is already expressed during the first pupal stage (P1; Fig.1.1) and at least from workers it was confirmed that *UVop* mRNA is also present in late larval stages (unpublished data). Larval and pupal development of the Western honeybee takes place inside the nest under constant darkness (Winston, 1987) and thus the functional significance of an early (UV) opsin expression is unclear. UV opsin expression with an unknown function has already been described in the antennal lobes of adult bumblebees (Spaethe and Briscoe, 2005). Furthermore, *arrestin*, which interacts with rhodopsin and is essential in visual processing, has also been detected in antennal lobes of flies where it is required for normal olfactory physiology in a non-visual process (Merrill et al., 2002). Interestingly, *Rh1* rhodopsin gene expression in *Drosophila* is essential for correct rhabdomere development (Kumar and Ready, 1995), and similar mechanisms might also exist in the honeybee.

BLop mRNA expression also commences early in workers (P1-2) but much later in drones (P5). In contrast, *Lop1* mRNA expression starts late, approximately at

mid-pupation (P4-5) in both sexes. Interestingly, physiological studies have found no green-sensitive photoreceptors in the dorsal eye of drones (Menzel et al., 1991; Peitsch et al., 1992; Velarde et al., 2005), whereas this study demonstrates that up to 20% of *Lop1* mRNA is expressed by the end of the pupal phase. A possible explanation for the discrepancy between physiology and mRNA expression is that the mRNA and protein levels may not necessarily correlate (reviewed in: Vogel and Marcotte, 2012). Regulatory post-transcriptional processes could lead to a degeneration of the *Lop1* mRNA or protein, which may explain the lack of green photoreceptor cells in the dorsal part of the drone eye. Moreover, intracellular recording from individual photoreceptors is difficult, and this approach might have missed very rare photoreceptor types (but see Velarde et al., 2005). Since this study observed only low *Lop1* mRNA expression levels, and the relative proportion of *Lop1* mRNA expression further decreases during adolescent development, the presence of a rare green-sensitive photoreceptor in the dorsal eye of drones might have been overlooked so far. An alternative explanation might be the co-expression of the Lop1 opsin with other opsins, resulting in potential broadband photoreceptors as it has already been shown for other invertebrates (Arikawa et al., 2003; Hu et al., 2014; Hu et al., 2011). Future studies using specific antibodies against the Lop1 opsin are needed to verify the presence of (rare) green-sensitive or broadband photoreceptors in the dorsal part of the drone compound eye.

Visual pigments are located in the fused rhabdom of the bees' photoreceptor cells, which functions as a light guide to convey the photons from the periphery to the light-sensitive molecules (Gärtner, 2000; Snyder, 1975). During pupal development, a slight but steady increase of opsin expression levels, which goes hand in hand with the differentiation of the rhabdoms, was found (Fig.2.4; 2.5; A.1). Since Phillips (1905) first published details on the development of the honeybee retina in the early 20th century, surprisingly few studies investigated the development of the photoreceptors in the bee compound eye. The formation of the retina starts during the larval phase, and the photoreceptors in the ommatidia are fully differentiated and arranged by the end of the pre-pupal phase (Phillips, 1905). In addition, the honeybee optic lobes gradually start to develop their adult form during the last larval

stage when optic nerve projections reach the lamina (Marco Antonio and Hartfelder, 2016). During pupal development the ommatidia lengthen, and cellular elements achieve differentiation by the time all ommatidia have reached their final adult shape (Phillips, 1905). Interestingly, rhabdom development starts during the end of the larval phase and is characterized by formation of a cavity along the ommatidial axis to which rhabdomeres could extend by infolding of the plasma membrane (Eisen and Youssef, 1980). At the beginning of the pupal phase, and in congruence with the observations of current study, rhabdoms are very short and therefore are only present in the apical part just underneath the crystalline cone cells (Fig.2.4A). However, rhabdoms lengthen rapidly during pupal development until they reach the distal part of the basement membrane (Eisen and Youssef, 1980; Phillips, 1905). The most obvious leap in rhabdom extension and photoreceptor cell differentiation was observed at mid-pupation (P5), which coincides with the first distinct increase of opsin expression levels of all three investigated opsins (Fig.2.1; 2.5). Overall, the current results clearly show that ommatidial development and opsin expression during the pupal phase differs between the honeybee and *Drosophila*. Whereas in honeybees, rhabdom maturation develops in conjunction with photoreceptor determination, opsin expression in flies is initiated much later near the end of the retina completion (Earl and Britt, 2006; Kumar and Ready, 1995).

Based on results of this study, a two-step process in the development and differentiation of the honeybee compound eyes was suggested. During the first step, which takes place during mid to end pupation, the photoreceptor cell meets its fate and the corresponding opsin will be expressed. At this time point, the relative proportion of opsin and thus photoreceptor types becomes fixed and remains constant until eclosion. In addition, the rhabdoms increase in length and diameter and absolute opsin expression rises. In a second step which takes place after eclosion, when the determination of photoreceptors has already been completed, the quantity of opsin expression levels in workers and drones increases drastically.

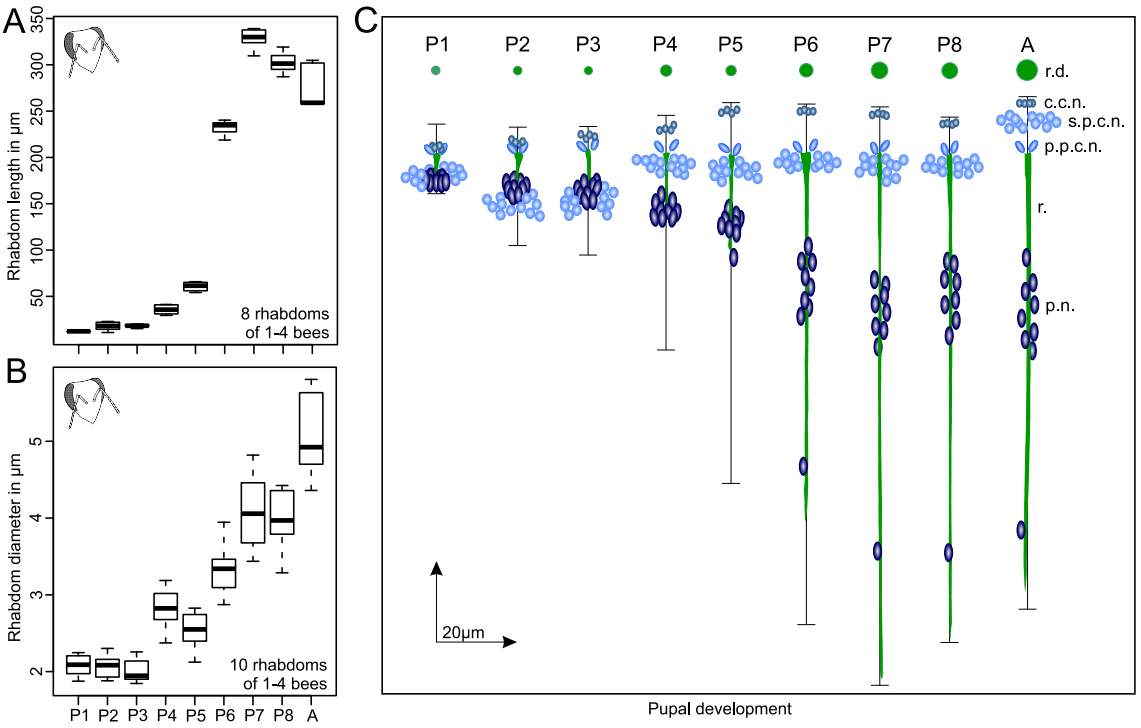

Figure 2.5: Rhabdom and photoreceptor development in the worker. **A**: Rhabdom length and **B**: rhabdom diameter shown for all pupal stages and freshly eclosed bees. **C**: Overview of rhabdom (green bar) and photoreceptor cell elongation (black line), increase in rhabdom diameter (r.d.) and segregation of nuclei from photoreceptor, pigment and crystalline cone cells. c.c.n.: crystalline cone nuclei; s.p.c.n.: secondary pigment cell nuclei; p.p.c.n.: primary pigment cell nuclei; r.: rhabdom; p.n.: photoreceptor nuclei.

This upregulation in opsin expression suggests an early adult maturation phase which takes place during the first days after eclosion, when workers and drones remain in the dark hive and only gradually come into contact with light during the transition from nurses to foragers (workers: Seeley, 1982) or during their first orientation flights (drones: Howell and Usinger, 1933). This delayed transition from indoor to outdoor activities, when the visual system is needed, may have allowed the bees to postpone part of their eye development into the early adult phase in the course of evolution. Similar results have also been found in ants, where expression levels of all three opsins significantly increase during the first few days of adult life (Yilmaz et al., 2016). Such advancement in development might turn out to be a common mechanism in eusocial insects that fulfill an age dependent division of labor including a transition from indoor to outdoor tasks. A regulation of visual components dependent on age, light environment and circadian clock in honeybees has been discussed in an earlier study that found lower expression levels of the *Lop1*

and *arrestin* mRNA in young in-hive bees compared to foragers (Sasagawa et al., 2003). Moreover, adult maturation has also been found in honeybees at the neuronal level. Visual and olfactory boutons of projection neurons in the mushroom bodies significantly increase in size between 1-day-old nurse bees and foragers (Groh et al., 2012). Also, honeybee mushroom body calyxes show a distinct volume increase during the first week after eclosion (Muenz et al., 2015). In contrast to results of the current study in honeybees, opsin mRNA levels in *Drosophila* are higher during the late pupal phase compared to adult flies (Earl and Britt, 2006), which would support the hypothesis that adult retinal (and neuronal) maturation is restricted to animals which undergo an age dependent transition from indoor to outdoor activities.

All samples of this chapter were collected at the same time of the day, since it has been shown that opsin expression in insects (*Noctuidae*: Yan et al., 2014) and even vertebrates (Korenbrot and Russel, 1989) might be regulated by the circadian clock to synchronize their endogenous rhythm with the environment. In flies and ants, for example, electroretinography of the compound eyes revealed a daily cycling of light sensitivity under constant darkness (Chen et al., 1992; López-Riquelme and Fanjul Moles, 2006). Therefore, this chapter investigated whether, in addition to the discovered developmental changes, opsin expression also possesses diurnal plasticity in adult bee eyes. Expression levels of all opsins are found to vary significantly during 24 hours even under constant darkness, suggesting that their expression is under control of the endogenous clock (Fig. 2.3). Similar expression patterns have been shown for the *Lop1* gene by means of qPCR and northern blot analyses (Rodriguez-Zas et al., 2012; Sasagawa et al., 2003). The highest expression levels were found during late night and putative early morning for all opsins, matching the regular activity pattern of foraging bees. Foragers usually show a distinct locomotor activity during the day (Moore and Rankin, 1993) and rest during the night (Klein et al., 2008), matching the necessity of a well-equipped and sensitive visual system for diurnal activities outside the hive. Furthermore, the activity peak also correlates with highest availability of pollen and nectar during the morning (Kleber, 1935). As previously stated, the processing of visual components, such as opsins, in the honeybee seems to be regulated and influenced by a variety of factors e.g. retinal

development, age, light environment, availability of food sources and the endogenous clock.

The current results clearly show a sex-specific and spectral type-specific onset of opsin expression during pupal development in the honeybee. Developmental and molecular timing mechanisms during the formation of the compound eyes have been intensively studied in *Drosophila melanogaster*, and a complex network of interacting and regulating transcription factors during the formation and determination of photoreceptors has been identified (Morante et al., 2007). Interestingly, it has recently been shown that the transcription factor spineless plays a critical role in patterning the retinal mosaic of compound eyes in several insects (Perry et al., 2016; Wernet et al., 2006). Whether spineless is also involved in the retinal patterning of the honeybee compound eye is unknown; however, I found two splice variants of the spineless homolog in the honeybee, and the expression pattern of one isoform shows a prominent peak at about 50% of pupal development (Chapter 3). Hence, spineless might be a promising factor for future studies to unravel the molecular mechanisms underlying retinal patterning in the honeybee.

3

spalt and *spineless* as potential transcription factors involved in retinal patterning during pupal development of the honeybee, *Apis mellifera*

3.1 Abstract

During the last decade much effort has been devoted to unravel the molecular mechanisms that give rise to the spatial organization of the retinal mosaic in insects. Most work has been performed in Drosophila melanogaster *and revealed a complex interplay of various transcription factors during pupal eye development. In particular, two transcription factors, spalt and spineless, have been shown to be essential for the correct retinal patterning of the fly compound eye. The honeybee,* Apis mellifera*, possesses three spectral photoreceptor types forming a sex-specific retinal mosaic, but almost nothing is known about the molecular mechanisms underlying this retinal patterning. In this chapter I present the homolog genes of spalt and spineless in the honeybee, and investigate the temporal expression patterns of both genes during pupal development of honeybee workers. I also test for possible correlations between spalt and spineless expression patterns and the onset of the three opsin genes expressed in the bee's compound eye.*

3.2 Introduction

Most insects, vertebrates and also humans arrange their different photoreceptor types, characterized by different spectral sensitivities, in distinct arrays within their retina to ensure a regular patterning of different photoreceptor types across the retina or to develop specialized areas within the eye, e.g. the dorsal rim area which is important for polarized vision in many insects. During the last decade much effort has been devoted to unravel the developmental and molecular mechanisms that give rise to a spatial retinal mosaic in insects. Most work has been dedicated to the invertebrate model *Drosophila melanogaster* (Wernet and Desplan, 2004; Wernet et al., 2015). In flies, each ommatidium comprises six outer photoreceptor cells which are present in almost all ommatidia and are involved in motion detection; and two inner photoreceptor cells which are involved in color vision. The main retina of the fly comprises two distinct ommatidial types, called "pale" and "yellow" (Franceschini et al., 1981). Both types contain two distinct pairs of central photoreceptor cells which differ in their expression of rhodopsins (Rh). Whereas "pale" ommatidia express *Rh3* (ultraviolet-sensitive) and *Rh5* (blue-sensitive) in their inner photoreceptor cells (R7 + R8), the "yellow" ommatidia are characterized by the expression of *Rh4* (ultraviolet-sensitive) and *Rh6* (green-sensitive) in R7 + R8 (reviewed in: Rister and Desplan, 2011). Both ommatidial types are stochastically distributed within the retina (Wernet et al., 2006). During the second half of the pupal phase, a complex interplay of a set of transcription factors determines the fate of different photoreceptors in the fly retina (Morante et al., 2007). A major step during ommatidial maturation is the expression of the transcription factors of the *spalt* gene complex, which is necessary to distinguish the outer from inner photoreceptor cells (Domingos et al., 2004; Mollereau et al., 2001). Another transcription factor that mediates key functions during retinal development in *Drosophila* is called *spineless*. It was shown that *spineless* is necessary and sufficient to define the ommatidial type "yellow" at about 50% of pupation. At this stage, *spineless* induces the expression of *Rh4* in R7 cells which then triggers the expression of *Rh6* in R8 cells by default (Wernet et al., 2006). Interestingly, a recent study on retinal development in butter-

flies found high similarities in the regulation and determination of photoreceptors, suggesting a conserved pattern of retinal development between flies and butterflies (Perry et al., 2016). In congruent with spalt expression in *Drosophila*, it has been shown that the butterfly homolog of the transcription factor spalt is also expressed in the long visual fibers of photoreceptors (lvf); and also the butterfly homolog of spineless is crucial to define the ommatidial types during the pupal phase (Perry et al., 2016). Despite the progress in disclosing the molecular mechanisms which give rise to different photoreceptor types resulting in a spatial retinal mosaic in different animals, almost nothing is known about retinal patterning in honeybees. Retinal mosaics exist also in honeybees and are characterized by at least three spectral ommatidial types which show a sex-specific distribution within the compound eyes (Peitsch et al., 1992; Velarde et al., 2005; Wakakuwa et al., 2005). Moreover, honeybee photoreceptors differ in their specifc onset of the three main opsins, the ultraviolet (*UVop*), blue (*BLop*) and green-light sensitive opsin (*Lop1*) during retinal development (see Chapter 2). However, whether homolog transcription factors like *spalt* or *spineless* are also involved in honeybee photoreceptor determination is still unknown.

As a first step, this chapter identifies homologs of *spalt* and *spineless* including specific isoforms by homology search in the honeybee genome. Secondly, qPCR was used to evaluate the expression levels of *spalt* and *spineless* (isoforms *SSX1* and *SSX2*) during pupal development in honeybee workers, and expression patterns were subsequently compared with expression patterns of opsins to test for a possible correlation between onset of expression of transcription factors and opsins. Additionally, a custom-made antibody against spineless was used to investigate the expression patterns during pupation at the protein level.

3.3 Material and Methods

3.3.1 General information

All experiments were performed with pupae and adult workers from colonies of the honeybee, *Apis mellifera* which were located at the bee facility of the University of Würzburg.

3.3.2 Primers, PCR and qPCR

To compare expression levels of transcription factors and opsins during pupal development, cDNAs of bee pupae were generated (see Chapter 2 for further details). PCR and gel-electrophorese (1% Aggarose in TBE) were used to test the respective primers. qPCR was used to evaluate specific mRNA expression levels of *spalt* and *spineless* during pupal development of honeybee workers. Based on annotated nucleotide sequences of *spalt* and *spineless* in *Drosophila*, NCBI BLAST (NCBI, Bethesda, USA) was used to identify the respective homolog genes including splice variants in the honeybee, *Apis mellifera*. Respective primers were subsequently designed by means of PrimerBLAST (NCBI, Bethesda, USA) and based on predicted gene sequences from the NCBI GenBank. The *ribosomal protein 49* (*Rp49*), which has been shown to be a stable housekeeping gene in *Apis mellifera* (Lourenço et al., 2008), was used as an internal control.

All qPCR runs were performed with a Mastercycler ep realplex (Eppendorf, Hamburg, Germany). PeqGold PCR Mastermix S (Peqlab Biotechnologie, Erlangen, Germany) was used for PCR reactions. qPCR reactions were performed with the Kapa SYBR Fast qPCR Kit (Peqlab Biotechnologie). Each PCR and qPCR master mix was prepared according to the manufacturer's protocol (Appendix A and Chapter 2) and experimental conditions were evaluated in technical triplicates for each sample. The $2^{-\Delta\Delta CT}$- method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) was used and data of the *spalt* and *spineless* splice variants were normalized separately for each transcription factor to the housekeeping gene *Rp49* and the highest expression level for each gene was set to one.

Table 3.1: Primers for qPCR of transcription factors

Gene	Forward primer	Reverse primer	Product length	Primer efficiency
spalt	5'-GCCTGTCTATCCTGCCATCC-3'	5'-CGAGCAAACACTTGTGCCAG-3'	415 bp	0.96
SSX1	5'-ACGTCTCAGTATCAAGCGGC-3'	5'-GGTGTTGCACTCCTTGCCAT-3'	126 bp	0.96
SSX2	5'-ACGTCTCAGTATCAAGCGGC-3'	5'-GGGGGCGTGCTCGGA-3'	139 bp	1.02
Rp49	5'-CGTCATATGTTGCCAACTGGT-3'	5'-TTGAGCACGTTGAACAATGG-3'	150 bp	0.91

3.3.3 Western blot analysis

To investigate spineless at the protein level two isoform specific polyclonal antibodies were generated by injecting two isoform specific peptides (SSX1: CPSDSKPDTGSP, SSX2: AAGMGPTVPQIPNRTPG; Fig.A.2) in rabbits to elicit an immune response (immunoGlobe, Himmelstadt, Germany). Since the generation of an antibody for SSX1 failed (Fig.A.3), only an antibody for SSX2 was used in further experiments. For investigation of the spineless antibody specificity by means of western blots, forager bees were caught directly from the hive entrance and immobilized on ice. Retinae of compound eyes were subsequently dissected and the two retinae from each bee were pooled and dissolved in 100 µl Laemmli buffer, homogenized with a micro pestle and stored at -80°C until further processing. Homogenized eyes were defrosted and incubated at 95°C for 5 minutes to denaturate proteins. Proteins were than separated during electrophorese by loading 20 µl of probes on a 10% acrylamide gel. After blotting proteins on a PVDF membrane for 90 minutes at 54 mA, blots were blocked for 1 hour with 5% (w/v) BSA in TBST at room temperature to block unspecific binding-sites. Blots were then incubated with the primary antibody against SSX2 (immunoGlobe, Himmelstadt, Germany; pruified from rabbit; SSX2: 0.1 µg/ml in TBST) at 4°C over night. As a control, parts of the proteins were incubated with the SSX2 peptide (immunoGlobe, Himmelstadt, Germany) to prevent SSX2 antibody from binding and to identify unspecific binding. Membranes were subsequently washed 3x in TBST for 5 minutes, once in Urea-Triton-Glycerin buffer for 3 minutes and again once in TBST for 5 minutes. After washing, blots were incubated with the secondary antibody (Jackson ImmunoReseach, Baltimore,

USA; Peroxidase-conjugatedAffiniPure goat anti-rabbit IgG (H+L): 1:20000) for 1.5 hours. Following several washing steps (3x in TBST for 5 minutes and briefly in H2O), blots were incubated with an ECL solution for 5 minutes and visualized with a ChemoCam Imager (INTAS, Göttingen, Germany).

3.3.4 Immunostainings of pupal retina

Pupal retinae were dissected and fixed overnight at 4°C in 4% formaldehyde in PBS. Fixed retinae were rinsed 3 times in PBS for 10 minutes and subsequently embedded in 5% low-melting-point agarose. 80 µm sections were performed by means of a vibrating microtome (Leica VT 1000S, Wetzlar, Germany). Sections were rinsed once for 10 minutes in PBS with 2% Triton-X 100 and twice in PBS with 0.2% Triton-X 100. Following a pre-incubation in PBS with 0.2% Triton-X 100 and 2% normal goat serum (NGS) for 1 hour at room temperature on a shaker, sections were incubated with an antibody against SSX2 (immunoGlobe, Himmelstadt, Germany; pruified from rabbit; 3 µg/ml in PBS with 0.2% Triton-X 100 and 2% NGS) for two days at 4°C. Sections were washed 5 times in PBS for 10 minutes. They were then incubated in Alexa Fluor 568 (goat anti rabbit; 1:250; Molecular Probes, Eugene, USA) in PBS with 1% NGS and Alexa Fluor 488 Phalloidin (2.5 µl Phalloidin from Methanol stock solution in 500 µl PBS; Molecular Probes, Eugene, USA) for one day at 4°C. To label cell bodies, sections were rinsed three times in PBS and subsequently incubated in Hoechst 34580 (1:000; Life Technologies GmbH, Darmstadt, Germany) for 15 minutes at room temperature. Following 5 further washing steps in PBS, sections were transferred to 60% Glycerol in PBS for 30 minutes at room temperature and mounted afterwards in 80% Glycerol in PBS on slides. A confocal laser-scanning microscope (Leica TCS SP2 AOBS, Leica Microsystems AG, Wetzlar, Germany) equipped with an argon/krypton and a three diode laser was used for visualization. Single images of sections were taken at a resolution of 1,024 x 1,024 pixels with a HC PL APO objective lens: 63/1.4 NA imm; with additional digital zoom: 3.0. Scanned images of immunostainings and western blots were processed and edited in ImageJ 1.51e (Wayne Rasband National Institutes of Health, Maryland, USA) and CorelDRAW Graphics Suite X7 (Corel Corporation, Ottawa, Canada).

3.4 Results

3.4.1 Homolog genes of *spalt* and *spineless* in the honeybee

By means of NCBI BLAST, one predicted homolog gene of *spalt* (NCBI reference sequence: XM_006568249.1; Fig.3.2) and two predicted splice variants of the *spineless* homolog *SSX1* (NCBI reference sequence: XM_003250988.2; Fig.3.2) and *SSX2* (NCBI reference sequence: XR_409137.1; Fig.3.2) have been identified. In more detail, *spalt* is characterized by 7 exons and a coding sequence of 5499 bp on chromosome 6. The putative protein comprises several zinc-finger domains, 1602 amino acids and a molecular weight of about 176 kDa. *spineless* is located on chromosome 8, consists of 10 different exons and encodes for two different splice variants *SSX1* (cds: 2814 bp) and *SSX2* (cds: 2900 bp). Both splice variants differ in the last 86 bp of exon 9 which results in a frame shift of two base pairs in the open reading frame and leads to two different spineless proteins (SSX1: 103 kDa; SSX2: 105 kDa) which are both characterized by a helix-loop-helix-motif and two domains of the PAS superfamily. PCRs with isoform-specific primers as well as sequencing (LGC Genomics, Berlin, Germany) of the respective isoform-specific sequence confirmed the putative difference in *spineless* exon 9 (Fig.3.1).

SSX1

ACGTCTCAGTATCAAGCGGCGGCGGCTGCAGCGGCCGCGGCCGCGGCAG
CGGCGGCCAGCACTATCGGATACGCGCACTCGCATCCAGGCCACGGGCA
CGGGGACACGGGCTCCGA**GCACGCCCCC**

SSX2

ACGTCTCAGTATCAAGCGGCGGCGGCTGCAGCGGCCGCGGCCGCGGCAG
CGGCGGCCAGCACTATCGGATACGCGCACTCGCATCCAGGCCACGGGCA
CGGGGACACGGGCTCCGAG**GTATGGCAAGGAGTGCAACAC**

Figure 3.1: Sequencing of *SSX1* and *SSX2* Differences of splice variant *SSX1* and *SSX2* in in the last 86bp of exon 9 were confirmed by means of isoform specific primers and subsequently sequencing of cDNA products. Forward and reverse primers are marked in gray. The red nucleobase guanine marks the last identical base of both splice variants. Blue bases in *SSX1* indicates the beginning of exon 10. Green bases indicates the isoform specific prolongation of 86 bp in exon 9 of *SSX2*.

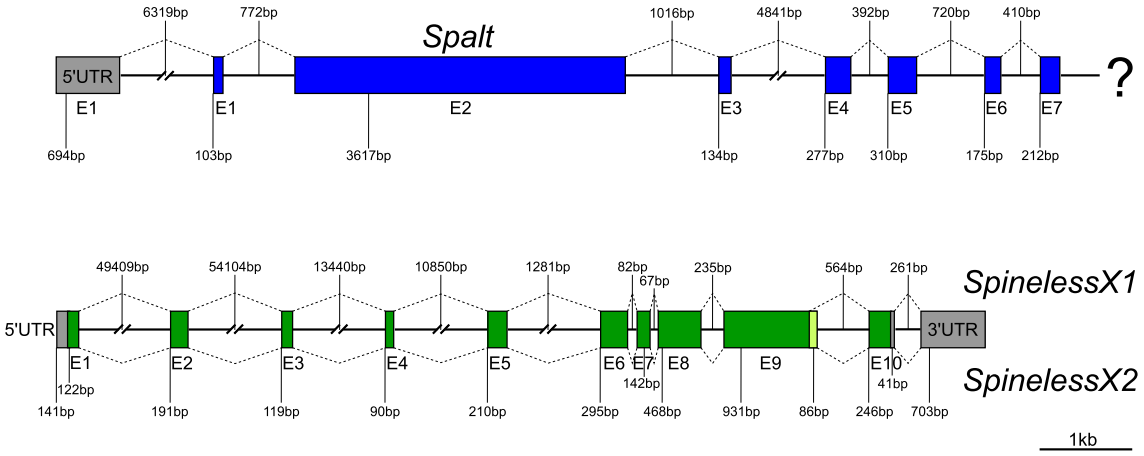

Figure 3.2: Schematic overview of investigated transcription factors. Predicted spalt is located on chromosome 6 and consists of 7 putative exons with a coding sequence of 5499 bp. Based on data from NCBI (reference sequence: XM_006568249.1) the existence of further exons or a 3'UTR at the c-terminus have not yet identified. Putative transcription factor spineless is located on chromosome 8, comprises 10 exons and encodes two isoforms *SSX1* (cds: 2814 bp; NCBI reference sequence: XM_003250988.2) and *SSX2* (cds: 2900; NCBI reference sequence: XR_409137.1). Both splice variants differ in the last 86 bp of exon 9.

3.4.2 mRNA expression levels of transcription factors during pupal development

qPCR was used to evaluate *spalt* and *spineless* expression levels in compound eyes of honeybee workers during the pupal phase. *spalt* was found to be highest expressed mainly during the first three pupal stages when *BLop* mRNA expression starts (Fig.3.3; see also Chapter 2) and showed the highest expression peak at P2 (Fig.3.3). *SSX1* was highest expressed in the first and the last pupal stage (Fig.3.3). *SSX2* showed a similar expression pattern, but additionally showed a prominent peak at mid of pupation (P4) which coincides with the onset of *Lop1* mRNA expression (Fig.3.3; see also Chapter 2).

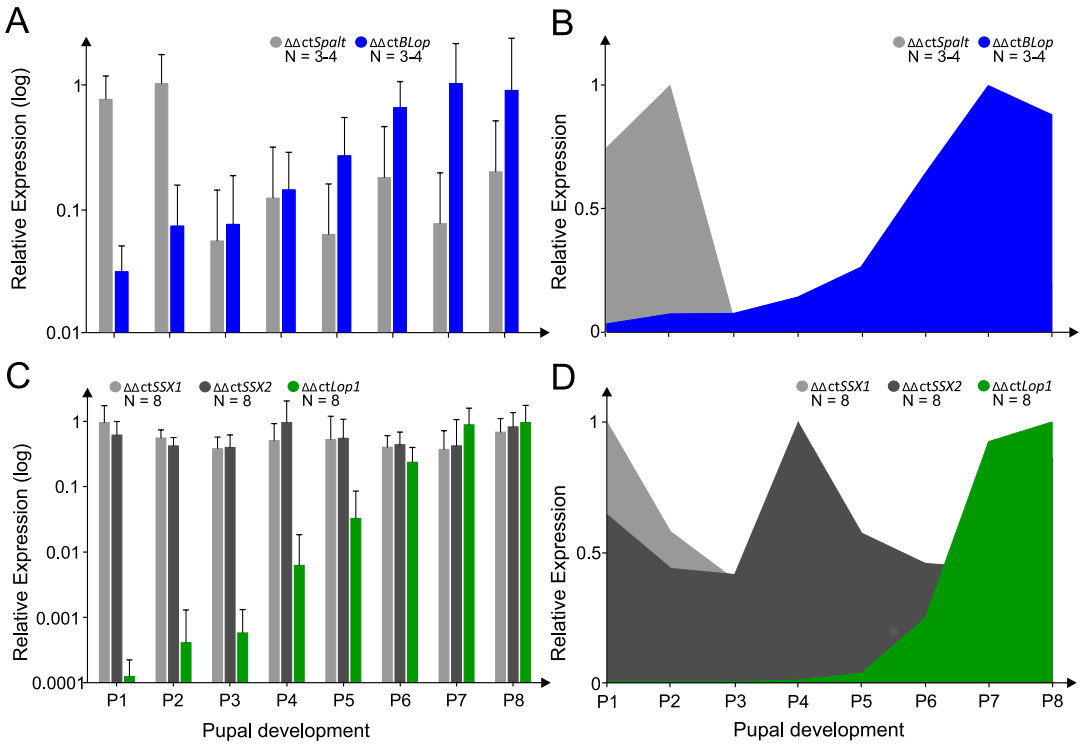

Figure 3.3: Temporal expression patterns of transcription factors and opsins. Relative mRNA expression levels of *spalt*, *spineless* (*SSX1* and *SSX2*), *BLop* and *Lop1* in the retina during pupal development of workers by means of qPCR. Expression level for each gene was normalized to the housekeeping gene *Rp49* and the highest expression level during pupal development for each gene was set to one. Error bars indicate standard deviation.

3.4.3 Western blot and immunostaining of spineless

Since the generation of a specific custom-made antibody against SSX1 failed (Fig.A.3), only a custom-made polyclonal antibody against SSX2 could be tested by means of western blot analysis for specificity, and was used afterwards to investigate spineless expression at the protein level during pupal development in honeybee worker. Western blot analysis showed a specific band with the predicted molecular weight of about 105 kDa. A peptide competition assay was used to confirm the specificity of SSX2 (Fig.3.4A). By means of confocal microscopy SSX2 expression was detected in all pupal stages. However, whereas in some pupal stages (e.g. P2 and P7) SSX2 was mainly expressed in the cytosol and outside of cell nuclei, other stages (e.g. P5) showed a completely different expression pattern where SSX2 was almost exclusively expressed within the cell bodies (Fig.3.4B; A.4).

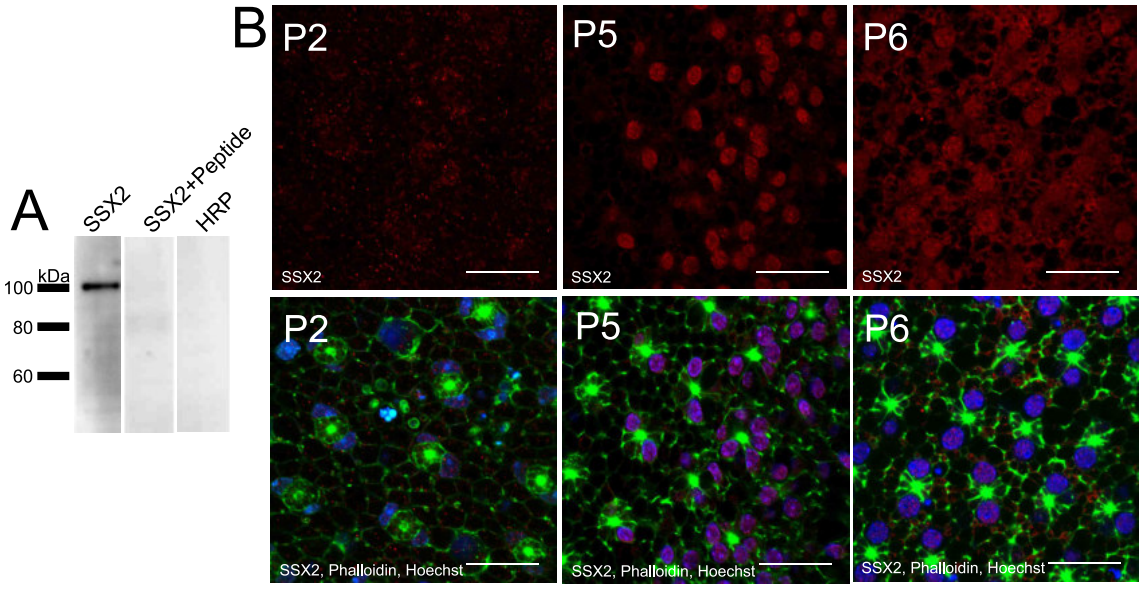

Figure 3.4: Spatial spineless expression patterns during pupal development. Predicted spalt is located on chromosome 6 and consists of 7 putative exons with a SSX2 immunoreactivity was tested on lysates of adult forager retinae by using western blots. First lane shows the protein maker (Roti-mark 10-150; Carl Roth, Saarbrücken, Germany), second lane, anti-SSX2 antibody without peptide, third lane, anti-SSX2 antibody with peptide, and fourth lane, secondary antibody only (HRP). Immunostainings with SSX2 antibody (red), Phalloidin (green; f-actin) and Hoechst (blue; cell nuclei) were performed to visualize spineless expression patterns during the pupal phase of workers. Three examples of expression patterns of different pupal stages (P2, P5 and P6) are shown. Scale bar in all figures: 100 µm

3.5 Discussion

In the current chapter it has been shown that the homolog transcription factors *spalt* and *spineless*, which are necessary in the retinal patterning of flies (Morante et al., 2007), are also found in the honeybee. In 2014, when the experiments were designed and performed, only one predicted homolog gene of *spalt* und and two predicted isoforms of *spineless*, *SSX1* and *SSX2*, were identified by means of NCBI BLAST. However, in summer 2015, new predictions of *spalt* and *spinless* homologs appeared in the NCBI GenBank (Tab.3.1). Meanwhile, four different splice variants of *spalt* are predicted by new bioinformatics programs which mainly differed in their n-terminus. Based on new predictions, previous designed *spalt* primers yet detect all four isoforms and the presented findings thus illustrate total *spalt* expression and do not distinguished between the different *spalt* splice variants. In contrast to *spalt*, *spineless* completely lost its isoform *SSX1*. Interestingly, *SSX1* and *SSX2* has been confirmed by sequencing of the isoform specific segments and expression patterns

Table 3.2: NCBI predicted isoforms of *spalt* and *spineless*

Predicted isoforms before May 2015		**Former NCBI Accession**	**Predicted isoforms after May 2015**		**Current NCBI Accession**
spalt	—	XM_006568249.1	*spalt*	*X1*	XM_016912780.1
spineless	*X1*	XM_003250988.2		*X2*	XM_006568249.2
	X2	XR_409137.1		*X3*	XM_016912781.1
				X4	XM_016912782.1
			spineless	—	XM_016913769.1

in honeybee retinae of both isoforms show obvious differences in expression during pupal development (Fig.3.2; 3.3C,D). However, since sequences of both genes are still based on computational algorithms, changes in algorithms and updates of the gene bank might led to a loss of already predicted sequences.

In *Drosophila*, the *spalt* gene complex includes two paralogue genes, *spalt-major* (*salm*) and *spalt-related* (*salr*) which are suggested to originate from a former gene duplication event and are characterized by several sets of zinc-finger motifs (Barrio et al., 1996; Kühnlein et al., 1994). Since both genes have almost identical expression patterns in most tissues, similar or overlapping functions have been suggested (Barrio et al., 1996). It is evident that *spalt* genes are involved in many developmental processes (de Celis and Barrio, 2009), and interestingly, it has been shown that the *spalt* complex is also essential for retinal patterning in flies. *spalt* is expressed in a subset of fly photoreceptor cells throughout photoreceptor development and determination. In the absence of the *spalt* complex, inner photoreceptor cells are not correctly determined and show clear characteristics of outer photoreceptor cells, like the expression of *Rh1* in all photoreceptors and rhabdomeres which stretch throughout the entire ommatidium (Domingos et al., 2004; Mollereau et al., 2001). Even in mammals, the *spalt* homolog *Sall3* is necessary for a correct regulation of photoreceptor development suggesting conserved functions in insects and mammals (de Melo et al., 2011). The current study shows that *spalt* is also expressed in the honeybee retina during pupation (Fig.3.3A,B). Whereas mRNA levels of spalt

are highest during the first two pupal stages of workers (P1; P2), *spalt* expression levels strongly decreases during P2 and P3 and remained rather low during the remaining of pupal development (Fig.3.3A,B). Interestingly, *BLop* mRNA expression, which is considered to start during early pupal development of honeybees (Chapter 2), coincides with the highest *spalt* expression level and strongly increases during the following stages (Fig.3.3A,B). This pattern suggests a possible regulation of the honeybee *BLop* expression by *spalt*, which has been already shown in the blue-light sensitive opsins in *Drosophila* (*Rh5*: Sprecher et al., 2007) and mice (blue-sensitive cone opsin: de Melo et al., 2011).

The transcription factor *spineless* is indispensable for retinal patterning in *Drosophila* where it determines the two main photoreceptor classes "yellow" and "pale" and initiates the expression of *Rh4* in R7 cells (Wernet et al., 2006). Recently, it has been shown that the *spineless* homolog in butterflies is crucial in retinal patterning, and in vertebrates, the absence of *AHRR*, a transcriptional repressor of aryl hydrocarbon receptor (*Ahr*), the vertebrate homolog of *spineless*, leads to a down-regulation of photoreceptor-specific genes like short, medium and long-wave opsins in zebrafishes (Aluru et al., 2014). In this chapter two different splice variants of the *spineless* homolog have been identified and confirmed by isoform specific primers in a PCR and subsequently sequencing (Fig.3.1). Expression levels of both splice variants revealed obvious differences in expression patterns during pupal development of honeybee workers. Whereas *SSX1* showed highest mRNA expression levels at the beginning and end of the pupal phase, *SSX2* showed its highest expression in a prominent peak at P4 which corresponds to the temporal expression pattern found in *Drosophila* (Wernet et al., 2006). Comparing *spineless* expression patterns with that of opsins, a correlation between the peak level of *SSX2* and the onset of *Lop1* expression might be assumed (Fig.3.3C,D).

By using a custom-made specific antibody against SSX2, spineless has been also detected at the protein level (Fig.3.4). SSX2 showed different protein expression patterns depending on different pupal stages. Whereas immunostainings of some pupal stages showed that SSX2 is mainly expressed in the cytosol of photoreceptor and pigment cells, in other pupal stages SSX2 is predominantly expressed within

the cell nuclei of photoreceptor cells (Fig.3.4B), suggesting a more active state of spineless. In vertebrates it has been shown that Ahr is also cytoplasmic, but after binding to a hydrocarbon ligand it translocates to the nucleus where it dimerizes with its partner protein Arnt. Even in *Drosophila*, spineless is cytoplasmic and, in contrast to vertebrates, is able to translocate and act alone, or forms a complex with its Arnt homlog, Tango, already before entering the nucleus (Crews and Fan, 1999). Due to the observed changes in spatial expression patterns there might be similar regulatory mechanisms in the honeybee. Moreover, separating nuclei from cytosol during pupal development of honeybees and subsequently quantifying the protein expression levels by means of western blots will allow to distinguish active spineless states from rather inactive forms and will further clarify a possible correlation between SSX2 and Lop1 expression. Although, the current findings might support the assumption of conserved functions of *spalt* and *spineless* during photoreceptor determination in different animals, further effort e.g. RNAi knockdown of spalt and SSX2 during P2 and P4, respectively, is necessary to unravel whether these genes also involved in the retinal patterning of the compound eye in the honeybee.

4

Dumb and lazy? A comparison of color learning and memory retrieval in drones and workers of the buff-tailed bumblebee, *Bombus terrestris*, by means of PER conditioning

4.1 Abstract

More than 100 years ago, Karl von Frisch showed that honeybee workers learn and discriminate colors. Since then, many studies confirmed the color learning capabilities of females from various hymenopteran species. Yet, little is known about visual learning and memory in males despite the fact that in most bee species males must take care of their own needs and must find rewarding flowers to obtain food. Here the proboscis extension response (PER) paradigm was used to study the color learning capacities of workers and drones of the bumblebee, Bombus terrestris*. Light stimuli were paired with sucrose rewards delivered to the insects' antennae and inducing a reflexive extension of the proboscis. Color learning and mid-term memory retention abilities of bumblebees were evaluated by means of absolute and differential conditioning protocols. Different monochromatic light stimuli in combination with neutral density filters were used to ensure that the bumblebees could only use chromatic and not achromatic (e.g. brightness) information. Furthermore, it was tested if bees were able to transfer the learned information from the PER conditioning to a novel discrimination task in a Y-maze. Both workers and drones were capable of learning and discriminating between monochromatic light stimuli and retrieved the learned stimulus after two hours. Drones performed as well as workers during conditioning and in the memory test, but failed in the transfer test in contrast to workers. This chapter clearly shows that bumblebees can learn to associate a color stimulus with a sugar reward in PER conditioning and that both workers and drones reach similar acquisition and mid-term retention performances. Additionally, this thesis provide evidence that only workers transfer the learned information from a Pavlovian to an operant situation.*

The following chapter is largely based on the peer-reviewed publication: Lichtenstein L, Sommerlandt FMJ, Spaethe J. (2015). *Dumb and lazy? A comparison of color learning and memory retrieval in drones and workers of the buff-tailed bumblebee, Bombus terrestris, by means of PER conditioning.* PLoS ONE 10(7):e0134248

4.2 Introduction

> "Long before starting to build their first queen cells, the worker-bees have constructed some drone cells, from which the first drones are due to emerge about the beginning of May — 'lazy, stupid, fat, and greedy', according to the German poet Wilhelm Busch. Indeed they do not attempt to take any part in the collection of food, an activity for which they are not properly equipped by nature, anyhow. Most of them are too indolent even to help themselves to their own share of the hive's food stores, leaving it to the worker-bees to feed them. The brain of the drone is smaller than that of both worker and queen—we are not left in any doubt as to the intellectual inferiority of the male in this case." [v. Frisch, 1953]

Research about sensory and cognitive capabilities in eusocial bees, such as bumblebees and honeybees, has almost exclusively focused on workers, since they possess a wide repertory of colony-related behaviors like brood caring, cell building and cleaning, and foraging for pollen and nectar. In contrast, the role of drones within the colony as well as their cognitive capabilities have for centuries been only of minor scientific interest. Drones were often assumed to be dumb and lazy (v. Frisch, 1953), a sentiment that dates back to Aristotle who noted that they are "devoid of sting, and lazy" (Hist. Anim. V, English translation by Thompson, 1907). The majority of studies on drones have focused on mating tasks such as searching for and mating with queens (Paxton, 2005). In the honeybee, *Apis mellifera*, newly emerged drones are fed by workers for the first few days until they are able to feed from honey combs within the hive (Nixon and Ribbands, 1952; Free, 1957). After about seven days drones start with orientation and mating flights (Rowell et al., 1986; Winston, 1987). If they do not mate during their mating flight with a fertile young queen, they return to their colony or drift into neighboring hives to find shelter and to feed (Winston, 1987; Moritz and Neumann, 2010). However, in most of the ca. 20,000 different bee species (Michener, 2007), mature males are not provided with food by the females, but have to forage by themselves (male traits in social insects are reviewed in Boomsma et al., 2005). In bumblebees, for example, drones stay after eclosion

only for the first few days within the colony, before they fly out and never return. Thus, during most of their life they must find food and shelter by their own and thus must learn to recognize and discriminate rewarding flowers in order to collect pollen and nectar for their own needs (Goulson, 2009). Recently, it has been shown that males significantly contribute to the pollination of several plant species (Ostevik et al., 2010; Wolf and Moritz, 2014; Ogilvie and Thomson, 2015), and possess longer flower handling times and transfer larger amounts of pollen than females (Ostevik et al., 2010). However, to what extent bumblebee drones can learn and memorize visual flower characteristics, has until present not been investigated. Color conditioning experiments are well established with free-flying honeybee (v. Frisch, 1965; Schubert et al., 2002; Dyer et al., 2008; Giurfa, 2007; Avarguès-Weber et al., 2011) and bumblebee foragers (Dyer and Chittka, 2004b; Morawetz and Spaethe, 2012), where individuals are rewarded with sucrose solution if they land on the intended color targets. However, this setup does not allow to completely control the environmental experience and stimulus perception of an individual. A more promising method for a quantitative evaluation of learning and memory in bees under controlled environmental conditions is the proboscis extension response (PER) assay (Kuwabara, 1957; for review see Matsumoto et al., 2012). According to this method, bees are harnessed individually and learn to associate a conditioned stimulus (CS; e.g. novel odor or color) with an unconditioned stimulus (US; i.e. food taste. Naive bees, for example, show the PER after the presentation of sucrose solution (US) to their antennae. After a few paired presentations of the CS and US, the CS alone provokes a conditioned response, the extension of the proboscis. PER conditioning is well established in honeybees (Matsumoto et al., 2012; Bitterman et al., 1983) and bumblebees (Menzel et al., 2001; Laloi et al., 1999; Riveros and Gronenberg, 2009) for which olfactory stimuli act as efficient CS. However, for several decades the PER assay failed in honeybees when visual stimuli were used as CS, except when the antennal flagellae were removed (Kuwabara, 1957; Hori et al., 2006; Niggebrugge et al., 2009; Mota et al., 2011). Only recently, some groups successfully applied the PER assay using light as conditioned stimulus in intact workers of honeybees (Dobrin and Fahrbach, 2012; Jernigan et al., 2014) and bumblebees (Riveros and

Gronenberg, 2012). This might be explained by the fact, that in recent protocols the CS (color stimulus) was presented for a longer time (up to 15 s) than in earlier studies or studies using olfactory stimuli as CS (see also discussion).

In this chapter visual PER conditioning in bumblebee drones and workers was performed, and their acquisition and mid-term visual retention was compared. Thus far, drones have been largely neglected in studies about learning and memory in bees (but see Nagaraja and Brückner, 2013), even though drones of most social bee species undergo a different life history (mainly solitary and self-sustaining) than highly social and central place foraging workers. To address this issue, the PER of intact bumblebee workers and drones was conditioned by using different monochromatic light stimuli (435, 455, 488, and 528 nm) in combination with neutral density (ND) filters to vary light intensities. In this way, only chromatic cues were available as CS. Individuals were subjected to absolute (A+) and differential (A+ vs. B-) conditioning tasks (with A and B being the stimuli conditioned) and memory retention was tested two hours after the end of conditioning (mid-term memory; reviewed in Menzel, 2001). Additionally, it was studied if memories, established in the Pavlovian context of PER conditioning can be transferred to the operant free-moving context of a Y-maze in which bees were confronted to the formerly trained light stimuli. Since this transfer is possible after olfactory appetitive and aversive learning (Carcaud et al., 2009; de Brito Sanchez et al., 2015), the current chapter investigated whether it is also possible in the visual domain.

4.3 Material and Methods

4.3.1 Preparing and pre-testing of bumblebees

For all experiments workers and drones from *Bombus terrestris* colonies (obtained from Koppert Biological Systems; Berkel en Rodenrijs, The Netherlands) were used. The colonies were kept in a two chambered nest box (240 x 210 x 110 mm each chamber) at 25°C, 70% relative humidity and 12 h/12 h light/dark photoperiod. Each colony was provided with commercially available Apiinvert (a mixture of sucrose, fructose and glucose; Südzucker AG, Mannheim, Germany) and dried pollen ad libi-

tum. Bees were randomly collected from their colony one day prior to conditioning. Individuals were chilled on ice and fixed in plastic tubes by means of paper clips and adhesive tape (as previously described by Sommerlandt et al., 2013). In this setup harnessed bumblebees could only move their head and the first pair of legs to facilitate perception of the US. Restrained bees were fed to saturation with a 30% sucrose solution (w/v) and placed over night in a dark climate cabinet (temperature: 23°C; relative humidity: 75%). Before the onset of the conditioning experiment, all bees were pre-tested for an intact PER by carefully touching the antennae with a toothpick soaked with 50% sucrose solution (w/v). For the conditioning experiments, only individuals that exhibited an intact PER during the pre-test were used.

4.3.2 Stimuli qualities and experimental setup

For absolute and differential PER conditioning I used four different monochromatic light stimuli provided by different monochromatic filters (Schott & Gen, Jena, Germany) with absorption maxima at 435 nm, 455 nm, 488 nm and 528 nm and half band width of ca. 10 nm (Fig.4.1A). To prevent the bees from learning achromatic information such as brightness, I additionally used two ND filters with 13% and 51% transmission, respectively. Thus, each monochromatic light stimulus was presented at three different intensities (transmission 100%, 51% and 13%; Fig.4.1B). The conditioning setup consisted of a non-reflective gray acrylic movable sleigh with nine individual chambers (50 mm x 60 mm x 50 mm), a filter holder, which housed the color and ND filters, and a cold light lamp (Fig.4.1C). The filter holder could be placed above each chamber in which an individual bee was placed. Bees trained with differential conditioning were subsequently tested for a possible information transfer in a Y-maze made from plywood (Fig.4.1D). The Y-maze consisted of an entrance chamber (100 mm x 50 mm), in which the bees were released, followed by a decision chamber, in which the bees could choose to enter either one of the illuminated arms. The arms of the Y-maze were 200 mm long and 50 mm high, and arranged perpendicularly. Each arm was divided into a test chamber (tc; 100x50x50 mm; Fig.4.1D) and a filter chamber (fc; 80x50x50 mm; Fig.4.1D), and both chambers were connected via a circular opening, where the color filters were attached to.

A bifurcated light guide attached to a cold-light lamp illuminated each of the filter chambers from the back side in each arm. A color filter was attached to one side of the circular opening (f; Fig.4.1D), and a diffusor (parchment paper, d) to the other side to scatter the light which entered the test chamber. The light intensities for all tested colors were leveled by means of ND-filters. The setup was placed on a rectangular black cardboard, which was regularly replaced to exclude olfactory cues left by the walking bees, and covered with a Perspex plate. All experiments were conducted under red light conditions.

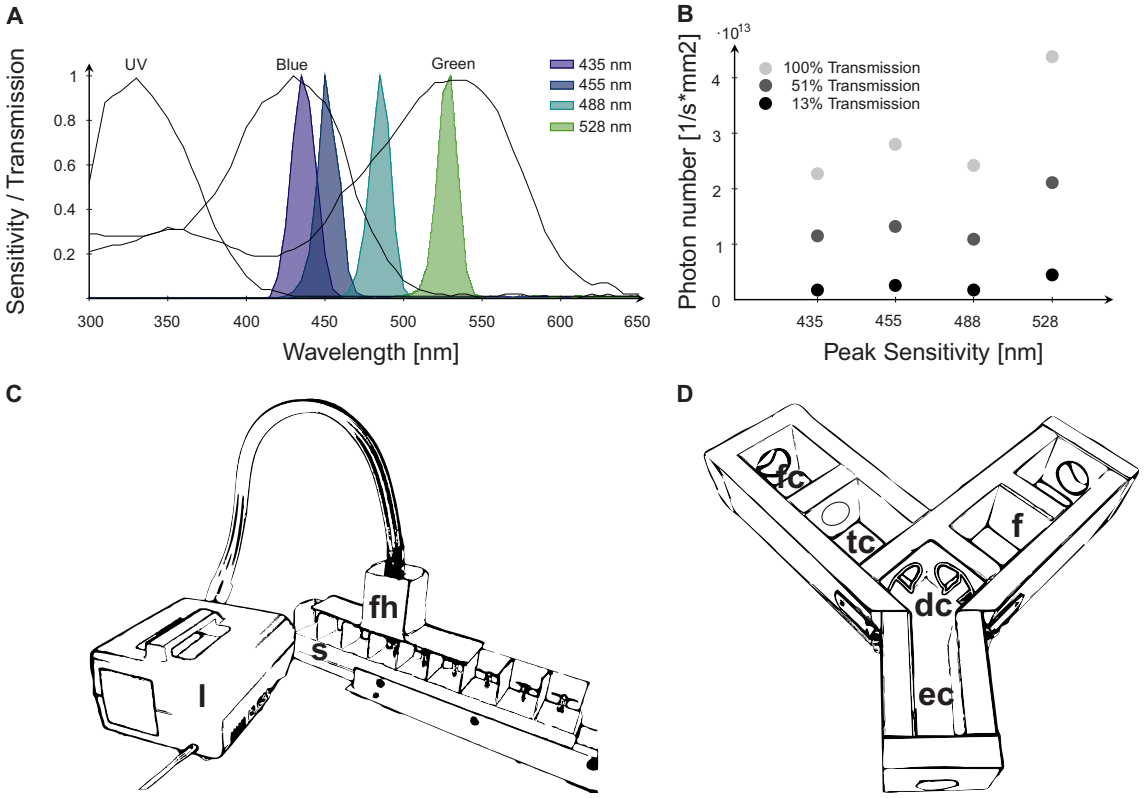

Figure 4.1: Stimuli qualities and experimental setup. A: Spectral sensitivity of the three photoreceptor types in *Bombus terrestris* (data obtained from Skorupski and Chittka, 2010), overlaid by transmission of the four tested color filters (435 nm, 455 nm, 488 nm, and 528 nm). B: Intensities (photons per second and mm2) of the monochromatic light stimuli generated by means of different ND filters (13%, 51% and 100% transmission). C: Illustration of the set-up for visual PER conditioning. See text for detailed description. fh, filter holder; s, movable sleigh. D: Y-maze set-up used for the transfer test after differential PER conditioning. The diffusor is omitted in the left arm to make the color filter (f) and the opening between the two chambers visible. See text for description. d, diffusor (parchment paper); fc, filter chamber; f, color filter; tc, testing chamber; ec, entrance chamber; dc, decision chamber.

4.3.3 PER conditioning protocol

The conditioning protocol was adapted from Riveros and Gronenberg (2012) who showed for the first time that restrained bumblebees with intact antennae can be conditioned with light as CS using the PER paradigm. The training procedure started when the filter holder was placed on top of the chamber containing the first harnessed bee. Each individual was allowed to become accustomed for 10 s to the given situation. Afterwards the light stimulus was switched on for 12 s. 6 s after stimulus onset the bee was rewarded with 50% sugar solution presented on a tooth pick for 3 s. Following the offset of the light stimulus each bee had another 10 s rest before the sleigh was moved and the next bee was positioned under the filter holder. Although Riveros and Gronenberg (2012) obtained high learning levels with their conditioning protocol, the 3 s overhang of the CS after the US, has added a backward component to the conditioning procedure, which might have generated an inhibitory learning effect (Schneider and Lewis, 2004). To test whether the prolonged CS affects the forward relationship between the light stimulus and the sucrose solution, I performed a control experiment (Fig.A.5) with 528 nm as CS+ where I compared male and worker bees trained with the protocol mentioned above and a slightly modified protocol with no backward component, so that the CS and US ended together at the same time (and thus comprised only a forward component, the procedure which is commonly used in Pavlovian conditioning).

In all conditioning experiments an inter-trial interval (ITI) of 8 min was used. A bee that responded with extending its proboscis during the first 6 s of stimulus exposure was scored as 1, whereas a bee that responded only to the sucrose reward or did not respond at all was scored as 0. Bees that showed no response to sucrose in more than four US presentations were excluded from further analysis.

4.3.4 Absolute color conditioning

During absolute PER conditioning the bumblebees had to associate only one monochromatic light stimulus with a sugar reward. I trained two groups of bees: a test group and a control group. Within the test group each bee was trained over 10 trials, and

the light stimulus (CS) and the sugar reward (US) were always presented simultaneously (paired group). Within the control group the light stimulus and the sugar reward were presented separately in different trials (unpaired group). Hence, each bee of the unpaired group had to complete 20 trials: 10 trials only with the light stimulus and 10 trials only with the sugar reward, in a randomized order. Using this protocol, the unpaired group received twice as many trials as the paired group (10 vs. 20 trials) which might led to a fatigue of animals in the control group (for a detailed discussion see Matsumoto et al., 2012).

4.3.5 Differential color conditioning

During differential PER conditioning the bees had to discriminate between a rewarded (CS+) and an unrewarded (CS-) light stimulus. Each bumblebee was trained over 18 trials (9 CS+ and 9 CS-) in a randomized order. To prevent bees from learning achromatic cues, I presented each light stimulus at three different intensities (transmission: 13%, 51% and 100%; Fig.4.1B).

4.3.6 Mid-term memory test

To assess mid-term memory retention of conditioned bumblebees, all individuals trained in absolute and differential conditioning were tested for memory retention two hours after the end of conditioning. To reactivate the bees and exclude individuals that do not react at all, bumblebees were tested for an intact PER just before re-testing. To exclude that the application of the US prior to the CS leads to an unspecific sensitization, bees that underwent absolute conditioning were confronted with the conditioned stimulus (CS) and with a novel color (NCol, as control) to test their response specificity. During stimulus presentation individuals were not rewarded and the succession of CS and NCol was randomized. When bees were trained to 435 nm, 455 nm and 488 nm, in each case the 528 nm light was used as NCol. For bees that were previously trained to 528 nm, NCol was 435 nm. In case of differential conditioning, I presented first the CS- and afterwards the CS+. At the end of the training protocol the CS+ was rewarded to avoid extinction learning prior to the subsequent Y-maze experiments.

4.3.7 Y-maze transfer test

To test whether the bumblebees can transfer the learned Pavlovian association from the PER conditioning to a novel operant free-moving context, bees were tested after differential conditioning and the two hour memory test in a Y-maze. All bees were chilled on ice until they calmed down (bees still showed slow movements of their antennae and first leg pair) and carefully released from their holders. They were then individually placed in the Y-maze and observed for 180 s following a protocol modified after Carcaud et al. (2009). A decision was recorded when the bee entered one of the illuminated test chambers within 180 s after release. Since chilling can have amnestic effects on olfactory memory (Erber et al., 1980), control experiments were performed (Fig.A.6) Here, bees were released from the holder and transferred to the Y-maze without chilling.

A custom-made computer program (YMaze, version 1.1, programmed by Matthias Lichtenstein) was used to document the first decision and the length of stay in any of the arms for each bee.

4.3.8 Statistical analysis

Statistics for the acquisition curves were done on the basis of an individual's number of responses towards the light stimulus (depending on the number of trials between 0 and 9 in absolute, and between 0 and 8 in differential conditioning). In absolute conditioning, learning performance of paired and unpaired groups was compared using Mann-Whitney-U test. Kruskal-Wallis test was used to compare the learning performance of all four monochromatic light stimuli. Memory retrieval was calculated with χ^2-test statistics (fourfold table). In differential conditioning, learning performance within treatment groups was compared using Wilcoxon test and among groups using Mann-Whitney U test. Mann-Whitney-U test was also applied to compare the discrimination index as a measure of performance (Pelz et al., 1997) between sexes and control experiments. Memory retrieval was calculated using χ^2-test. First choice performance in the Y-maze transfer test was compared to random choice (50%) using Pearson's χ^2-goodness-of-fit test and the duration of stay in each arm

was compared using Wilcoxon test. All statistics were calculated with IBM SPSS Statistics (Version 20.0.0) software.

4.4 Results

4.4.1 Absolute color conditioning

When bees were trained to associate a sucrose reward with a monochromatic light stimulus (absolute conditioning), both workers and drones were able to build an association between CS and US after a paired presentation. For all colors, the paired groups in workers (435 nm: $P < 0.001$, Z = -4.940; 455 nm: $P < 0.001$, Z = 3.757; 488 nm: $P < 0.001$, Z = -4.731; 528 nm: $P < 0.001$, Z = -4.436; Fig.4.2) and drones (435 nm: $P < 0.001$, Z = -3.636; 455 nm: $P < 0.001$, Z = 3.514; 488 nm: $P < 0.001$, Z = -3.702; 528 nm: $P < 0.001$, Z = -3.305; Fig.4.3) performed significantly better than individuals trained with an unpaired presentation of the stimuli. No differences were found among color stimuli (workers: n.s., χ^2= 4,465; drones: n.s., χ^2= 0,673) or between sexes (n.s., Z = -1.798). In the memory test two hours after conditioning, workers (435 nm: P<0.001, χ^2= -19.412; 455 nm: $P < 0.001$, χ^2= 15.086; 488 nm: $P < 0.001$, χ^2= -7.400; 528 nm: $P < 0.001$, χ^2= -10.185; Fig.4.2) and drones (435 nm: $P < 0.001$, χ^2= -19.342; 455 nm: $P < 0.001$, χ^2= 19.556; 488 nm: $P = 0.008$, χ^2= -6.988; 528 nm: $P < 0.001$, χ^2= -13.537; Fig.4.3) responded significantly more often to the conditioned color stimulus than to a novel test color, which also indicates that activating the bees with sucrose solution before presenting the CS did not affect choice specificity.

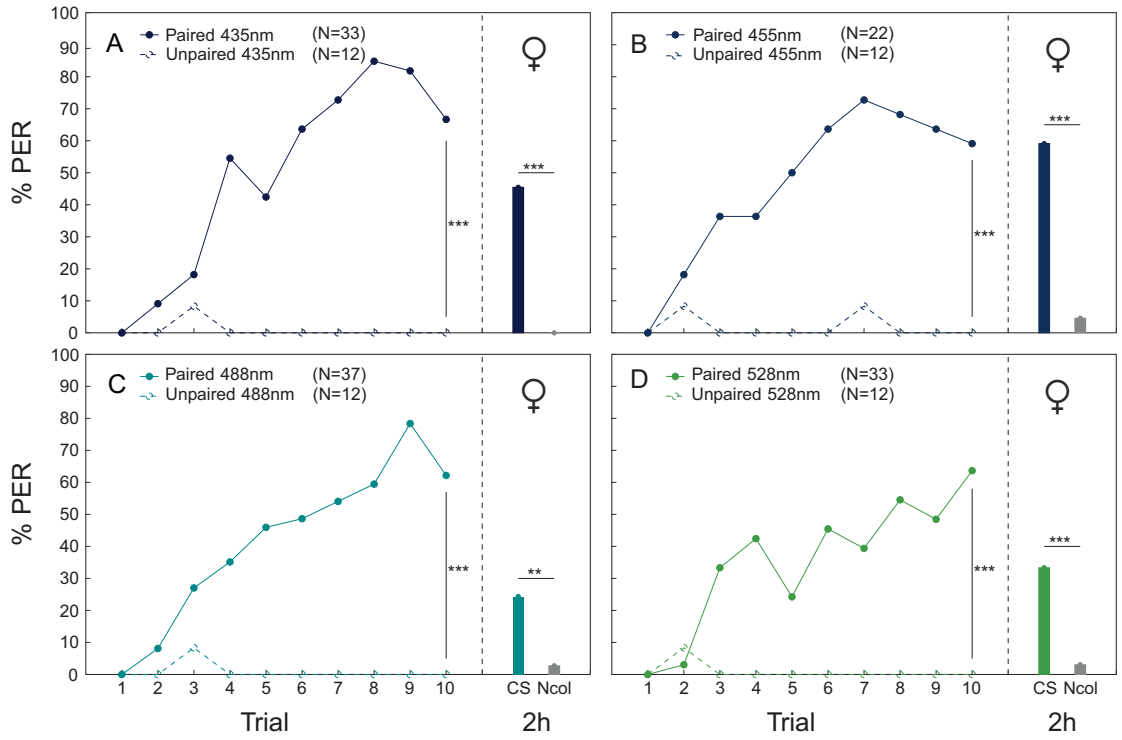

Figure 4.2: Absolute color conditioning and memory retrieval in workers. Acquisition curves (proportion of bees that responded to the tested color stimulus extending the proboscis [% PER]) of workers during absolute conditioning of four different color stimuli (A: 435 nm, B: 455 nm, C: 488 nm and D: 528 nm). Workers were trained either with a paired (filled circles) or an unpaired (empty circles) presentation of CS and US. Memory retrieval was tested by presenting the CS (colored bar) and a novel color stimulus (NCol: gray bar) to the bees 2 h after conditioning. *** $P < 0.001$; ** $P < 0.01$

Since the used protocol might have induced an inhibitory effect on learning performance due to the 3 s overhang of the CS after the end of the US presentation, I also tested an additional group of males and workers presenting the 528 nm light as CS but omitted the 3 s overhang. No significant difference was found in the learning performance and mid-term retention between both protocols and both sexes (for statistics see supporting information, Fig.A.5), indicating that the prolonged CS presentation did not induce any inhibitory effect on learning.

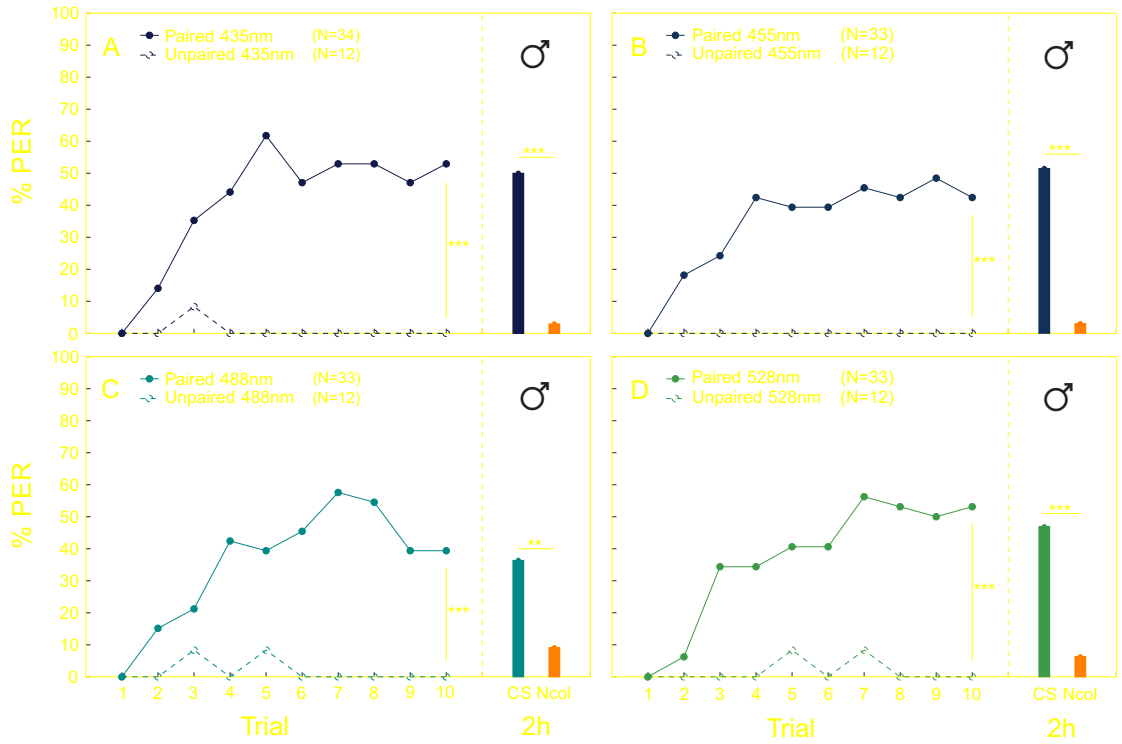

Figure 4.3: Absolute color conditioning and memory retrieval in drones. Acquisition curves (in % PER) of drones during absolute conditioning of four different color stimuli (A: 435 nm, B: 455 nm, C: 488 nm and D: 528 nm). Drones were trained either to a paired (filled circles) or an unpaired (empty circles) presentation of CS and US. Memory retrieval was tested by presenting the CS (colored bar) and a novel color stimulus (NCol: gray bar) to the bees 2 h after conditioning. *** $P < 0.001$; ** $P < 0.01$

4.5 Differential color conditioning

Bumblebees were able to discriminate different monochromatic light stimuli with large wavelength differences (Fig.4.4; 4.5). Workers (435 nm vs. 528 nm: $P < 0.001$, Z=-6.318; 435 nm vs. 488 nm: $P = 0.001$, Z = -3.306; Fig.4.4; Fig.4.5B) and drones (435 nm vs. 528 nm: $P < 0.001$, Z = -6.092; 435 nm vs. 488 nm: $P < 0.001$, Z = -4.175; Fig.4.4A,C) could significantly discriminate between CS+ (conditioned stimulus) and CS- (unconditioned stimulus) when the wavelength difference of the stimuli was 93 nm and 53 nm, respectively, irrespective of which wavelength was the rewarded or unrewarded stimulus. However, for the largest color distance, workers performed significantly better when 435 nm was the rewarded stimulus, compared to the case when 528 nm was rewarded ($P < 0.001$, Z = -3.302; Fig.4.4).

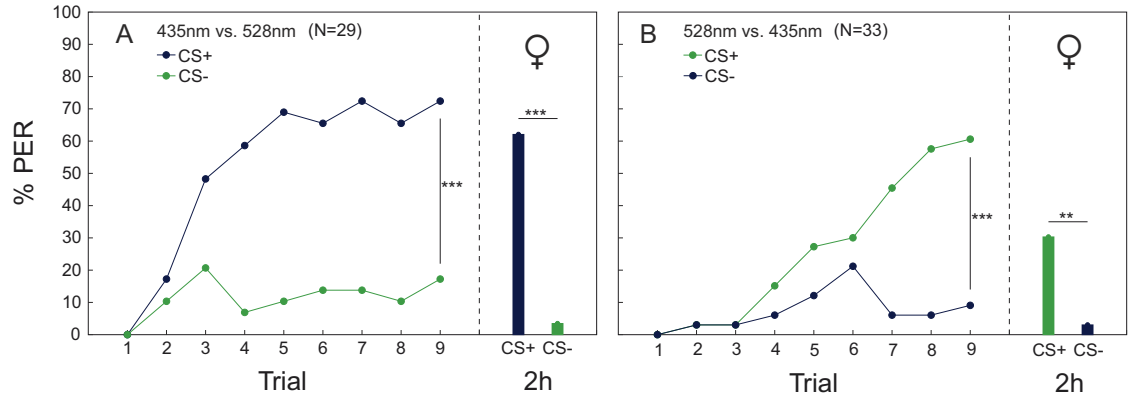

Figure 4.4: Differential color conditioning and memory retrieval in workers. Workers were trained by differential conditioning to discriminate between the color stimulus pair 435 nm and 528 nm ($\Delta\lambda$=93 nm). (A) 435 nm was used as the rewarded color stimulus (CS+) and 528 nm as the unrewarded color stimulus (CS), or (B) vice versa. Memory retrieval was tested by presenting the CS+ and the CS- to the bees 2h after end of conditioning. *** $P < 0.001$; ** $P < 0.01$

No such asymmetry was found in workers for the other two combinations (435 nm vs. 488 nm/455 nm; Fig.4.5B,D) or in any combination tested in drones (Fig.4.5A, C,E), so that data of counter experiments were pooled for statistical analysis (Fig.4.5). However, both workers (435 nm vs. 455 nm: n.s., Z = -1.837) and drones (435 nm vs. 455 nm: n.s., Z=-0.044) failed to discriminate stimuli with a relatively small wavelength difference of 20 nm (Fig.4.5D,E). The memory test revealed that workers (CS+435 vs. CS-528: $P < 0.001$, $\chi^2 = 22.621$; CS+528 vs. CS-435: $P = 0.003$, $\chi^2 = 8.836$; Fig.4.4; 435 nm vs. 488 nm: $P = 0.004$, $\chi^2 = 8.428$; Fig.4.5B) and drones (435 nm vs. 528 nm: $P < 0.001$, χ^2=36.219; 435 nm vs. 488 nm: $P = 0.001$, $\chi^2 = 11.168$; Fig.4.5A,C) were able to recall the learned information two hours after conditioning. Both sexes failed the memory test for the smallest wavelength difference between 435 nm and 455 nm after two hours (workers: n.s., $\chi^2 = 1.667$; drones: n.s., $\chi^2 = 3.048$; Fig.4.5D,E). For the combinations 435 nm vs. 488 nm and 435 nm vs. 455 nm I also compared the discrimination index (Pelz et al., 1997) between males and workers. No significant differences were found (435nm/488nm: n.s., Z = -1.315; 435 nm/455 nm: n.s., Z = -1.357), indicating that both sexes could discriminate the light stimuli equally well.

Figure 4.5: Differential color conditioning and memory retrieval in workers and drones. Three different monochromatic color stimuli combinations (435/528 nm; 435/488 nm and 435/455 nm) with different wavelength distances (93 nm; 53 nm and 20 nm) between stimuli were tested. Bumblebees were trained to discriminate the rewarded (CS+) and the unrewarded color stimulus (CS-). Each color stimulus combination was tested reciprocally. For the memory retrieval test the rewarded color stimulus (CS+: black bar) and the unrewarded color stimulus (CS-: gray bar) were presented to the bees 2 h after end of conditioning. Since no effects of asymmetrical discrimination between the two colors of each combination was found (except for 435/528 nm in workers, see Fig.4.4), data were pooled. *** $P < 0.001$; n.s.: not significant

4.6 Transfer test to Y-maze

Individuals were tested for a transfer of the learned Pavlovian association to a novel operant free-moving situation in a Y maze. Immediately after testing for memory retrieval, bees were confronted in the Y-maze with the same set of stimuli (CS+ and CS-) as they had experienced during differential PER conditioning. I recorded the first choice of a bee's movement towards one of the two presented monochromatic light stimuli (Fig.4.6A,B). Additionally, the time an individual spent in the respective arms during the first three minutes was measured (Fig.4.6C,D). Workers chose significantly more often the arm with the previously learned color when the wavelength difference was largest (435 nm vs. 528 nm: $P < 0.001$, $\chi^2 = 10.756$), but showed no preference when the wavelength differences were smaller (435 nm vs. 488 nm: n.s, $\chi^2 = 0.290$; 435 nm vs. 455 nm: n.s., $\chi^2 = 1.286$; Fig.4.6A). In contrast, drones showed no preference in their first decision when confronted with the previously rewarded color in the PER experiment, regardless of the combination of stimuli (435 nm vs. 528 nm: n.s., $\chi^2 = 0.381$; 435 nm vs. 488 nm: n.s, $\chi^2 = 0.732$; 435 nm vs. 455 nm: n.s., $\chi^2 < 0.001$; Fig.4.5B). When comparing the time the bees spent in both arms of the Y-maze, workers ($P = 0.021$, Z = -2.313) and drones ($P = 0.018$, Z = -2.357) stayed significantly longer in the arm in which the previously rewarded color was presented when the wavelength difference was largest (435 nm vs. 528 nm: 92 nm; Fig.4.6C,D). For smaller wavelength differences, no preferences were observed for workers (435 nm vs. 488 nm: n.s., Z = -0.813; 435 nm vs. 455 nm: n.s., Z = -0.49; Fig.4.6C) or drones (435 nm vs. 488 nm: n.s., Z = -0.103; 435 nm vs. 455 nm: n.s., Z = -0.393; Fig.4.6D). To exclude that chilling the bees before they were transferred to the y-maze interact with memory retrieval, an additional group of workers was tested. This group was not cooled when released from the fixation. No significant differences were found for the first choice and the time spend in each arm between groups (for statistics see supporting information, Fig.A.6).

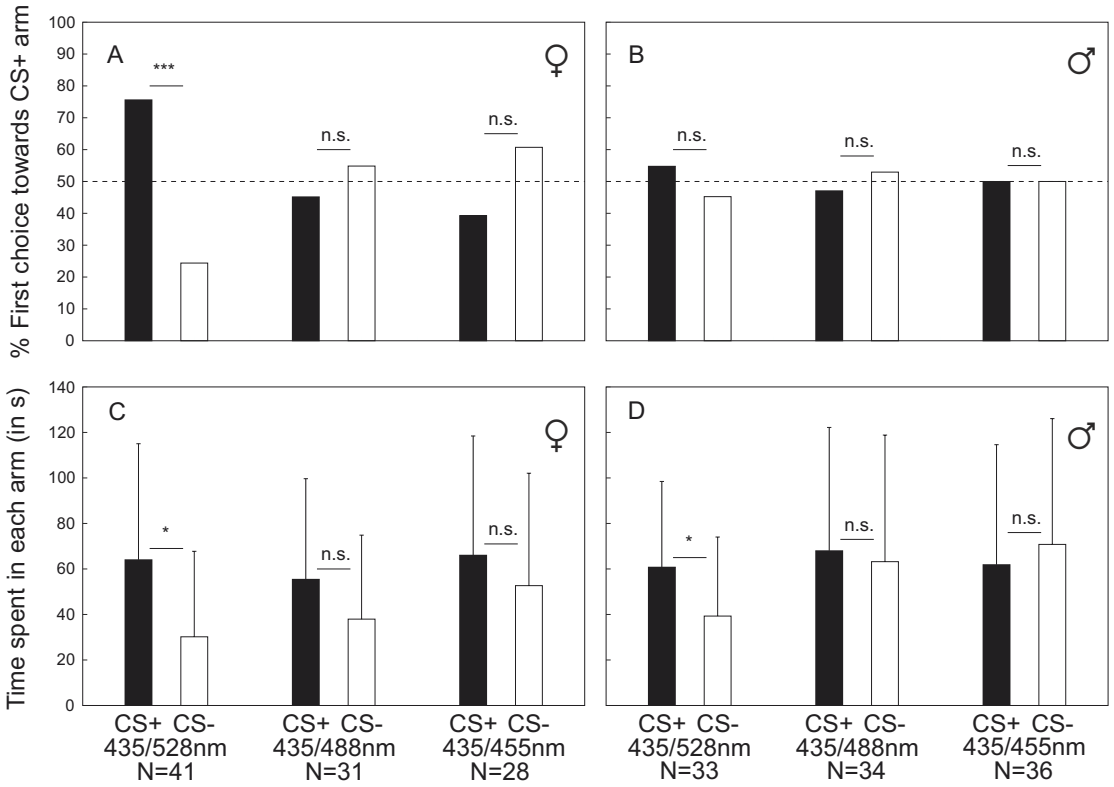

Figure 4.6: Information transfer after PER conditioning to Y-maze. Workers (A; C) and drones (B; D) were tested 2h after end of conditioning for transfer of the learned color information to a novel operant context. Proportion of first choice of the bumblebees towards the CS+ arm (A: workers; B: drones) for three different color combinations (435/528 nm; 435/488 nm and 435/455 nm) and time (C: workers; D: drones) spent in each arm (in seconds). Since there were no significant differences within the respective color combination regarding the rewarded stimulus during the first choice towards the CS+ arm and time spent in each arm, all data were pooled for each tested color combination. *** $P < 0.001$; * $P < 0.05$; n.s.: not significant

4.7 Discussion

The current chapter provide evidence that (i) intact drones and workers of *Bombus terrestris* perform equally well in learning and discriminating monochromatic lights based only on chromatic information, (ii) workers but not drones show an asymmetric learning performance when they must discriminate between blue and green light, and (iii) workers and drones differ in their capability to transfer conditioned chromatic information acquired in a Pavlovian context to a novel operant free-moving situation.

The present data add to the recent findings that harnessed bees can be conditioned to monochromatic light stimuli in absolute and differential conditioning tasks without removing their antennae which is in contrast to some earlier studies. A possible explanation for the discrepancy is the prolonged presentation of the light stimulus, which was between 12 and 15 s in the studies where conditioning was successful (Jernigan et al., 2014; Riveros and Gronenberg, 2012, present study) but only 7 s where intact bees could not learn (Hori et al., 2006; Niggebrugge et al., 2009).

In absolute conditioning, bumblebee workers as well as drones were able to learn all tested monochromatic lights (435, 455, 488, 528 nm). This is partially in line with findings from Africanized honeybees (AHB), where bees showed comparable acquisition curves when using light stimuli in the human-blue range of the chromatic spectrum (Jernigan et al., 2014). In contrast, AHB performed much poorer compared to our bumblebees when they were conditioned to green light (520 nm) (Jernigan et al., 2014). In this chapter, the learning performances of the bumblebees did not differ among the different tested stimuli-wavelengths. Another difference between both species refers the memory test, where bumblebees were able to retrieve the learned color and successfully discriminate it from a novel color. AHB, in contrast, exhibited an overall poor memory retrieval (Jernigan et al., 2014). However, this statement should be treated with caution since I tested mid-term memory (2h), whereas AHB workers were tested after 24h (early long-term memory; Jernigan et al., 2014). Neither of these memories rely on protein synthesis (reviewed by Menzel, 2001) and, as reported for olfactory conditioning, memory performance on popula-

tion level does not differ between mid-term and early long-term memory (Grünbaum and Müller, 1998).

To assess wavelength discrimination capabilities, three pairs of monochromatic lights with decreasing wavelength differences (93 nm, 53 nm and 20 nm, respectively) were tested in a differential conditioning paradigm. The current data showed that workers and drones were able to discriminate between different monochromatic light stimuli and retrieve the learned stimulus after two hours. These results coincide with findings by Riveros and Gronenberg (2012) who reported that *Bombus impatiens* workers were able to discriminate color stimuli with a difference of 53 nm using differential conditioning. The minimal color distance that can be distinguished and learned by workers and drones in the current chapter was between 20 and 53 nm, thus indicating a poorer color discrimination capability compared to free flying honeybees. In the early 1970's, von Helversen first demonstrated that free flying honeybees were able to discriminate different monochromatic colors and found a discrimination threshold of 15 nm in the used wavelength area (v. Helversen, 1972). The poorer discrimination abilities in this study might be attributed to the fixation of the bees, since not only harnessed bumblebees but also harnessed honeybees showed a worse discrimination performance (Niggebrugge et al., 2009). Further explanation for poorer discrimination might be the type of training. In the PER setup, stimuli are presented successively, while in experiments with freely moving bees, target and distractor stimuli can be perceived simultaneously. It has been shown that the manner of stimuli presentation is essential for discrimination performance (Dyer and Chittka, 2004b). In contrast to successive presentation of the color stimuli, simultaneous presentation enables bees to choose the features which allow an easy discrimination between rewarded and non-rewarded stimulus. In short, honeybees (Grünbaum and Müller, 1998) and bumblebees (Dyer and Chittka, 2004b) perform significantly better when the test stimuli are presented at the same time.

Surprisingly, the behavior of males and workers differed when they had to discriminate between blue (435 nm) and green (528 nm) light. While workers and drones performed equally well when the blue light was presented as CS+ and green light as CS-, workers performed significantly poorer compared to drones in the reversed

situation, i.e. when discriminating 528 nm as CS+ from 435 nm as CS-. This phenomenon might be explained by an intrinsic preference of workers for blue color stimuli (v. Helversen, 1972; Giurfa, 2004) that may bias performance and affect visual learning, as recently shown for honeybees (Morawetz et al., 2013). For workers it might be of advantage to possess a color preference when leaving the hive on their first foraging flight. Previous studies have shown that bumblebee workers possess an innate preference for violet and blue (Raine and Chittka, 2005; Gumbert, 2000). This innate preference coincides with findings that violet and blue (i.e. bee-blue and bee-UV-blue) flowers provide on average more nectar than flowers of any other color category (Giurfa et al., 1995). Furthermore, bumblebee workers showed faster and higher learning acquisition rates for stimuli of shorter wavelengths (Riveros and Gronenberg, 2012; Gumbert, 2000). Bumblebee drones, in contrast, are not subjected to evolutionary pressure for efficient foraging, since they only need to obtain food for themselves and do not forage for the colony, i.e. they have no impact on the colony's fitness. Thus, an innate color preference might not contribute to the drones' fitness. However, the proximate reasons for the sex-dependent differences which were observed in the current chapter need to be investigated.

In all previous studies testing color discrimination by means of PER, chromatic LED lights were used and adjusted to equal brightness (measured as photon numbers) (Dobrin and Fahrbach, 2012; Jernigan et al., 2014; Riveros and Gronenberg, 2012) to prevent the bees from learning brightness differences between stimuli. However, photoreceptors adapt to the background intensity and hence the sensitivity of the photoreceptors can significantly differ (v. Helversen, 1972; Chittka, 1992), causing different receptor excitations despite identical stimulus intensities.

In his seminal work, v. Helversen (1972) demonstrated that honeybees are >10 times more sensitive for UV than for green light (v. Helversen, 1972). This implies that a standardized brightness (adjusted to equal number of photons) might still lead to an unequal excitation of different photoreceptors and hence a different perception level. To address this issue and hence to provide a "reliable method of demonstrating color vision" (Menzel, 1979), monochromatic filters in combination with different ND filters were used to prevent bees from learning receptor-specific excitation differences

instead of chromatic differences.

Under natural conditions, foraging bees may profit from capabilities which allow them to transfer information gained in one context to a novel situation. Specific flower characteristics, for example, can be learned and associated with a nectar reward, although flowers never appear twice with exactly identical properties and under the same environmental conditions. Nevertheless, foragers must recognize flower types and thus generalize to a certain degree in order to optimize foraging rates. Moreover, information transfer is also necessary when information (e.g. flower specific odors) about profitable food sources is communicated inside the nest of social bees. Newly recruited foragers can learn the odor of recently collected nectar (provided by nestmates) and use this information on their own foraging flights (bumblebees: Dornhaus and Chittka, 1999; honeybees: Farina et al., 2005). When honeybees are exposed to odors during early adult stages or foraging flights, they later respond in PER conditioning more frequently to the experienced odor, but have difficulties associating a new odor with a sugar reward (Gerber et al., 1996; Sandoz et al., 2000). Mc Cabe and Farina (2009) reported a transfer of olfactory information in stingless bees, where learning performance of *Melipona quadrifasciata* in olfactory PER conditioning was positively influenced by previous in-hive experiences of the learned odor. Moreover, honeybees were shown to transfer olfactory information acquired during PER conditioning to a novel operant choice situation in a Y-maze (Carcaud et al., 2009) or orientation flight (Chaffiol et al., 2005). In the present chapter I trained bumblebees to discriminate between pairs of color stimuli in the PER paradigm, before I tested them two hours later for information transfer in a Y-maze choice experiment. Bumblebee workers chose significantly more often the arm of the Y-maze where the previously rewarded color was presented and also spend more time in that arm. This was true for the stimuli pair with the largest wavelength difference (425 nm vs. 528 nm), but not for smaller color differences. Drones, in contrast, showed no preference towards the reinforced color target in their first decision, even when presenting the largest color difference. However, when testing the 425 nm / 528 nm pair, drones spent more time in the chamber illuminated by the previously rewarded color, compared to the unrewarded color. Drones in gen-

eral were significantly faster in making their first decision compared to workers (34 seconds vs. 49 seconds; $P < 0.001$, Z = 3.241, MWU test; data not shown). These differences could be based on a sex-specific difference in speed and accuracy trade-off, as shown by Chittka et al. (2003) on the level of individual bumblebee workers. In general, bumblebee workers may need to transfer information about rewarding flowers (e.g. the floral scent) acquired in the hive from returning foragers to direct their own foraging behavior towards the advertised flower type (Dornhaus and Chittka, 1999, for review see Dornhaus and Chittka, 2004). In contrast, drones do not rely on such information transfer abilities, since they do not communicate with other conspecifics (but see Gruter and Leadbeater, 2014 for potential information gain via social information).

The fact, that males possess similar learning skills compared to workers might be surprising at first glance, since at least in honeybees, drones have been believed to perform only simple, reflex-like behavior, such as feeding and mating (v. Frisch, 1927). These behaviors might be facilitated by a simple response to olfactory, visual or tactile key stimuli (Shorey, 1973). Workers, in contrast, show more complex social behaviors. As central place foragers, they must not only recognize, but also memorize locations and landmarks, as well as shapes, colors and odors of profitable food sources. However, honeybee drones are reported to successfully associate odor stimuli with food reward in PER conditioning (Nagaraja and Brückner, 2013; Becker et al., 2015) and colored light stimuli with electric shocks in a free-moving avoidance assay (Dinges et al., 2013). Moreover, drones of *Bombus terrestris* have been successfully trained to learn olfactory stimuli using the PER paradigm and performed equally well in absolute and differential conditioning compared to workers (Sommerlandt et al., 2012). In both, drones and workers, learning and memory formation is the product of the (central) nervous system and there is no reason to assume that the neuroanatomical structure is completely different between sexes and casts of the same species. Although sex-specific modifications may appear in the brain, and particular at the peripheral sensory level, which enables the bearer to become particular sensitive to distinct stimuli (Streinzer et al., 2013b; Roselino et al., 2015), the neuronal processes connecting a stimulus (CS+) to the reward system (US cir-

cuit; Hammer, 1993) probably constitute a basic feature of the nervous system of all bees and even insects.

In conclusion, the present chapter show that intact bumblebees are able to associate a sugar reward with a monochromatic light stimulus in an absolute and differential Pavlovian conditioning paradigm. In contrast to honeybees, bumblebees can be easily trained without antennal deprivation. I was able to establish a visual PER conditioning setup for bumblebee workers and drones, and found similar learning and memory performance in both sexes. Workers were also capable of transferring learned information to a new behavioral context. Due to their phylogenetic relationship to honeybees, their experimental robustness and their learning abilities, bumblebees provide a suitable model to study the neurobiological and molecular mechanisms underlying visual learning and memory formation by means of classical PER conditioning.

5

Learning of monochromatic stimuli in *Apis cerana* and *Apis mellifera* by means of PER conditioning

5.1 Abstract

Honeybees are globally distributed and have received increased attention due to their high economic and ecological value for pollination, their exceptional eusocial lifestyle and complex behavioral repertoire. In addition to the well-studied Western honeybee, Apis mellifera*, several related species of the genus* Apis *exist in Asia, but most research on learning and memory in honeybees have been performed in* A. mellifera *and other honeybee species were largely neglected. In the current chapter the Pavlovian PER paradigm was used to train workers of* A. mellifera *and* A. cerana *(the Eastern honeybee) to two monochromatic light stimuli, with maximum wavelengths at 435 and 528 nm. Workers of both honeybee species were able to form an association between the color stimulus and a sugar reward and significantly discriminated between both color stimuli in a differential discrimination test. However, besides similar performance levels during visual PER conditioning,* A. cerana *showed a reduced mid-term memory compared to* A. mellifera*. Finally, performance of the visual PER conditioning in the current chapter reached similar levels as found in olfactory PER conditioning. I thus recommend the visual PER conditioning approach as a useful tool for studying species-specific visual learning and memory capabilities in honeybees under controlled laboratory conditions.*

5.2 Introduction

Honeybees are not only important pollinators of crop and wild plant species worldwide (Batra, 1995; Klein et al., 2007; Potts et al., 2010), but due to their complex behavioral repertoire, they are considered as a valuable invertebrate model for studying basic mechanisms of cognition, e.g. visual perception, learning and memory (Srinivasan, 2010). To understand the mechanisms underlying learning and memory in bees, different conditioning procedures have been developed. The well-established proboscis extension reaction (PER) paradigm was successfully used in studying olfactory learning and memory in the Western and Eastern honeybees (Bitterman et al., 1983; Wang and Tan, 2013), bumblebees (Laloi et al., 1999; Riveros and Gronenberg, 2009; Sommerlandt et al., 2013), stingless bees (Mc Cabe et al., 2007), ants (Guerrieri and d'Ettorre, 2010) and even in flies (Chabaud et al., 2006). In contrast, the PER paradigm was only rarely applied in vision research, because it was assumed to be too difficult and not applicable in honeybees. However, very recently visual PER conditioning was successfully performed in bumblebees (Riveros and Gronenberg, 2012; Lichtenstein et al., 2015) and Africanized honeybees (Jernigan et al., 2014). In contrast, in the Western honeybee, *A. mellifera*, visual PER conditioning was only successful after ablating the bee's antennae (Kuwabara, 1957; Hori et al., 2006; Niggebrugge et al., 2009) or when presenting the color stimuli in combination with other cues (e.g. in combination with motion: Balamurali et al., 2015 or with odor: Mota et al., 2011). Apart from that, the overall learning performance during visual PER conditioning was low in workers with intact antennae (Dobrin and Fahrbach, 2012).

During (visual) PER conditioning, a bee has to learn to associate, e.g. a color stimulus (conditioned stimulus, CS) with a sugar reward (unconditioned stimulus, US). If the association is established after a few conditioning trials, the bee will extend its proboscis when presenting the color stimulus alone (Matsumoto et al., 2012; Avarguès-Weber and Mota, 2016). The majority of studies on learning and memory have been performed in the Western honeybee, *A. mellifera*, which is distributed in Europe, Africa, and Middle East (Ruttner, 1988; Engel, 1999), and only

little attention was given to other members of the genus which are all restricted to Asia (Ruttner, 1988; Engel, 1999; Radloff et al., 2010); e.g. the widely distributed Eastern honeybee, *A. cerana*. To the best of my knowledge, no attempt has been made to investigate visual learning of *A. cerana* using classical PER conditioning. The present chapter pursued two aims: first, establishing the visual PER paradigm in the Eastern honeybee allowing a comparative approach of visual learning and memory capabilities in the genus *Apis*. Second, the PER paradigm was used to compare visual learning and mid-term memory in *A. cerana* and *A. mellifera* in an absolute (color A+; color B+) and differential (color A+ vs. color B-) conditioning experiment.

5.3 Material and Methods

5.3.1 General information

The experiments were performed with workers of the Western honeybee, *Apis mellifera carnica* and the Eastern honeybee, *Apis cerana indica.* Workers of the Western honeybee were collected from managed colonies at the bee station of the University of Würzburg. Learning experiments with *Apis cerana* were conducted with workers caught from a wild colony at the National Centre of Biological Sciences in Bangalore, India. At both locations, foragers were caught in the morning directly from the hive entrance and were subsequently immobilized on ice. Honeybees were restrained in small metal holders and were only able to freely move antennae and mouthparts (reviewed in: Matsumoto et al. 2012). Restrained bees were fed to saturation (30% w/v sugar solution) and placed for three hours in a dark box (temperature: 25°C, relative humidity: 50%). They were then pre-tested for a proper PER by carefully touching their antennae with a toothpick soaked with sugar solution (50% w/v). For PER conditioning, only individuals that showed an intact PER were used.

5.3.2 Experimental setup

For visual conditioning, I used two monochromatic filters (Schott & Gen, Jena, Germany) with absorption maxima at 435 nm (blue) and 528 nm (green) and a half bandwidth of ca. 10 nm. A neutral density (ND) filter was used to vary stimulus intensity (51% transmission with the ND filter added) to prevent honeybees from learning brightness cues during conditioning; both intensities were presented in a random order (for more details see also: Lichtenstein et al. 2015). The experimental setup consisted of a non-reflective conditioning chamber (5 mm x 60 mm x 50mm) in which an individual bee was placed. The corresponding monochromatic filter was positioned on top of the box and was illuminated by a cold-light lamp (for detailed description of the setup see Lichtenstein et al., 2015). All experiments were performed in the dark under red light conditions.

5.3.3 Conditioning protocol

For both species identical PER conditioning protocols were used. During absolute conditioning, bees had to associate a color stimulus (CS: 435 nm or 528 nm) with a sugar reward (US). In the test group the CS and US presentation overlapped within one trial (= paired group); in the control group the CS and US were presented in separate trials in a random order (= unpaired group). Thus, animals of the control group perceived the same number of CS and US presentation in twice as much trials than the paired group. During differential conditioning, bees had to discriminate between a rewarded and an unrewarded color stimulus (CS+ 435 nm vs. CS- 528 nm and vice versa). Rewarded and unrewarded stimuli were presented in a pseudo randomized order. An individual honeybee was placed in the conditioning box for 10 s prior to stimulus onset to become accustomed to the experimental set up. Then, the color stimulus was presented for 13 s and the bee was allowed to lick from a toothpick soaked in sucrose solution (50%, w/v) during the last 3 s. Subsequently, the honeybee had another 10 s rest before it was replaced by the next individual. All bees were tested using an inter-trial interval of 10 min. The conditioning protocol was adapted from Jernigan et al. (2014). Mid-term memory of the bees were tested

2 hours after conditioning by presenting the CS+ and the CS- in a random order without reward. Afterwards, bees were tested for a proper PER and only bees that still reacted were chosen for further analysis.

5.3.4 Statistics

The learning performance was estimated by counting individual PER responses towards the respective color stimulus. A reaction was scored as "1", when the bee extended its proboscis towards the color stimulus before the reward was offered. Honeybees which did not react at all or extended its proboscis only in response to the sucrose reward were assessed as "0". I excluded all honeybees from further analysis which showed a PER towards the sucrose reward in less than 50% of the trials. Mann-Whitney-U test was used for analyzing learning performance of absolute PER conditioning, and Wilcoxon test was applied in the differential conditioning experiment. χ^2-test was used to evaluate memory retention. To compare learning performances in the differential conditioning experiment between both species, a discrimination index (DI) for each individual honeybee was calculated as the difference of the sum of PERs towards the CS+ and the sum of PERs towards the CS- for all conditioning trials (Pelz et al., 1997 for DI calculation; see also Arenas et al., 2009). Differences of DIs between species were evaluated by means of Mann-Whitney-U test. All statistical tests were performed in IMB SPSS v. 20.

5.4 Results

Both *Apis mellifera* and *A. cerana* workers successfully associated any of the tested wavelengths with a sugar reward during absolute PER conditioning (Fig.5.1). Learning performances of the paired group (P) differed significantly from those of the unpaired group (UP) in *A. mellifera* and *A. cerana* (*Am*: 435 nm P vs. UP: $P <$ 0.001, Z = -4.096; 528 nm P vs. UP: $P < 0.001$, Z = -5,552; *Ac*: 435 nm P vs. UP: $P < 0.001$, Z = -3.940; 528 nm P vs. UP: $P < 0.001$, Z = -3.938). Moreover, both species reached similar performance levels of up to 70% correct choices during absolute conditioning (Fig.5.1). During differential conditioning, *A. mellifera* and *A. cerana* were also able to distinguish significantly between both stimuli and

reached similar learning performance levels (*Am*: 435 nm CS+ vs. 528 nm CS-: $P < 0.001$, Z = -3.640; 435 nm CS- vs. 528 nm CS+: $P < 0.001$, Z = -3.591; *Ac*: 435 nm CS+ vs. 528 nm CS-: $P < 0.001$, Z = -3.951; 435 nm CS- vs. 528 nm CS+: $P < 0.001$, Z = -3.610). Interestingly, *A. cerana* workers seemed to need more trials to reach the maximum performance level (Fig.5.2), but DIs of both species did not significantly differ (435 nm *A. mellifera* vs. *A. cerana*: $P = 0.440$, Z = -0.773; 528 nm *A. mellifera* vs. *A. cerana*: $P = 0.960$, Z = -0.051; Fig.5.3).

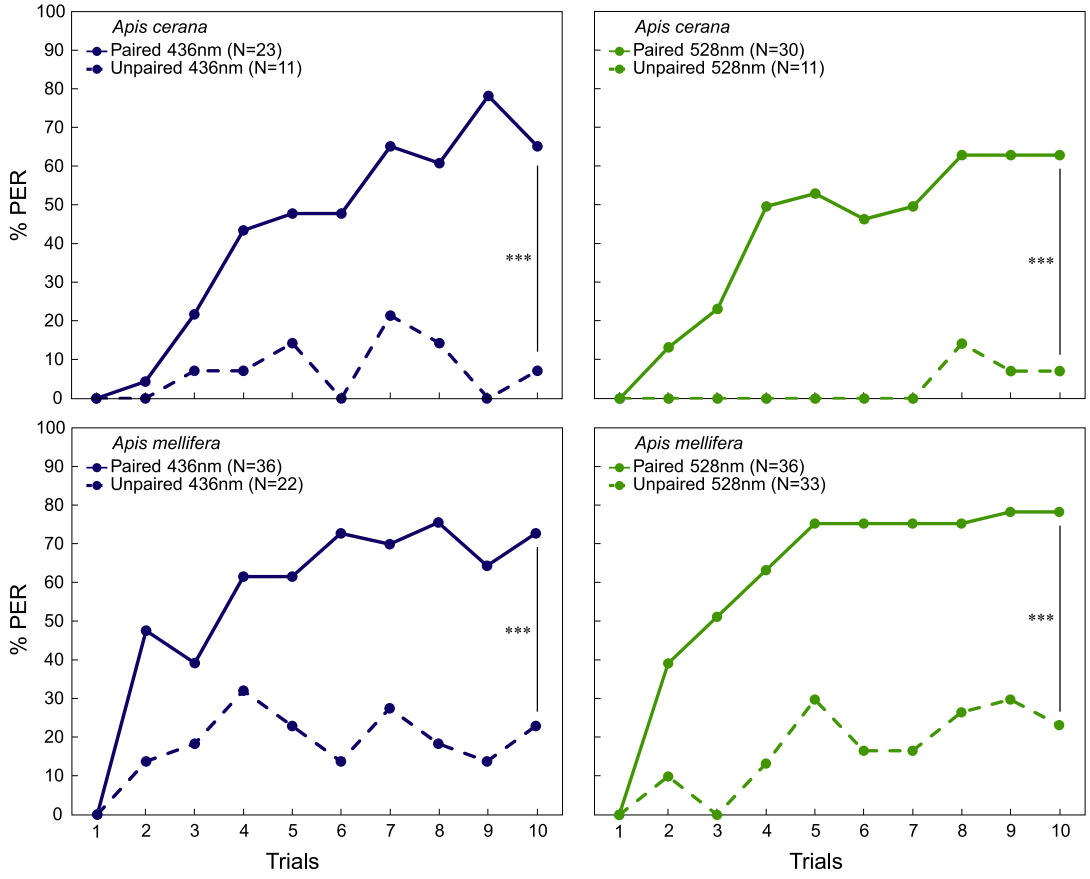

Figure 5.1: Absolute color conditioning in *A. cerana* and *A. mellifera* workers. Bees were trained either paired (solid line) or unpaired (dashed line) with two monochromatic lights (435nm and 528nm; for details see material and methods). *** $P < 0.0001$

Both species were also able to retrieve and discriminate the CS+ from the CS- after 2 hours (*A.m.*: 435 nm CS+ vs. 528 nm CS-: $P < 0.0001$, $\chi^2 = 16.2$; 528 nm CS+ vs. 435 nm CS-: $P = 0.005$, $\chi^2 = 7.714$; *A.c.*: 435 nm CS+ vs. 528 nm CS-: $P < 0.039$, $\chi^2 = 4.167$; 528 nm CS+ vs. 435 nm CS-: $P = 0.001$, $\chi^2 = 10.421$).

However, memory retrieval in *A. mellifera* was significantly better than in *A. cerana* (*A.m.* vs. *A.c.*: 435 nm CS+ vs. 528 nm CS-: $P = 0.007$, $\chi^2 = 7.125$; 528 nm CS+ vs. 435 nm CS-: $P = 0.001$, $\chi^2 = 10.134$; Fig.5.2).

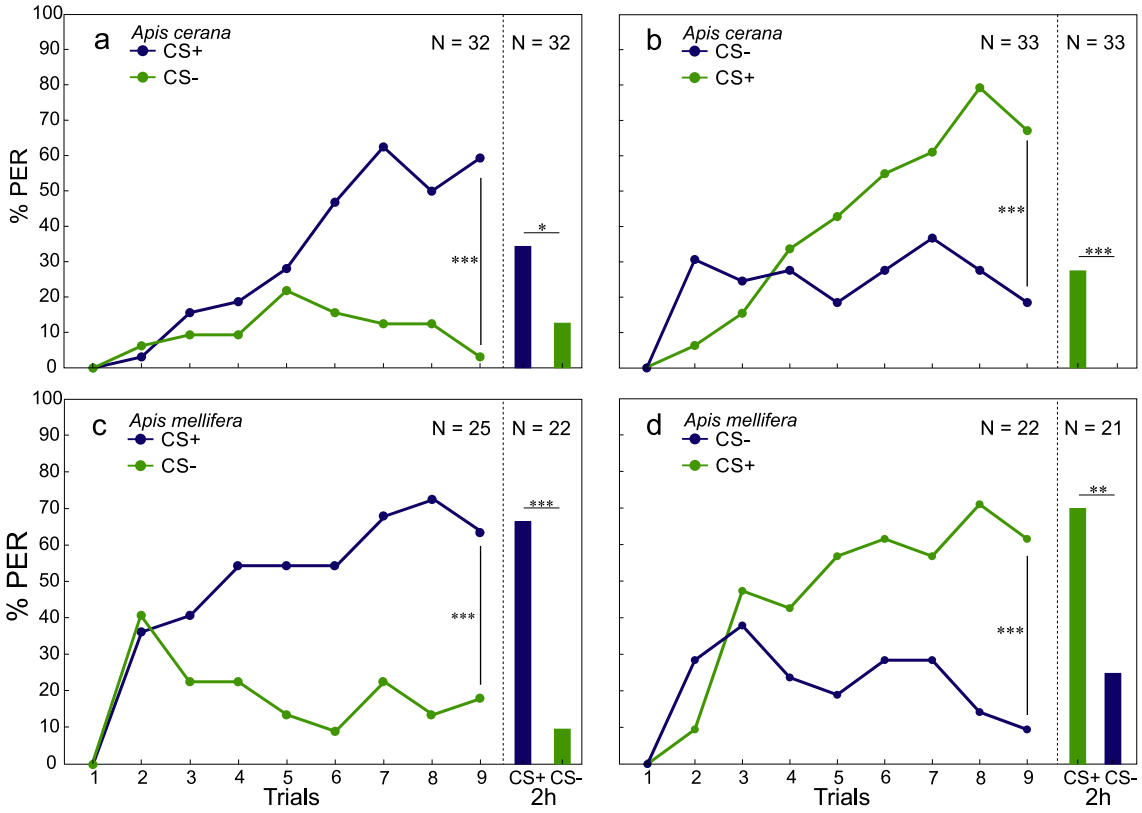

Figure 5.2: Differential color conditioning and 2 h memory test in *A. cerana* and *A. mellifera* workers. Bees had to discriminate between two monochromatic light stimuli (435 nm and 528 nm). Memoryretrieval was tested 2 h after conditioning. *** $P < 0.0001$; ** $P < 0.001$; * $P < 0.01$

5.5 Discussion

In the current chapter the Pavlovian PER paradigm was used for the first time to compare visual learning performance and memory retrieval in *A. cerana* and *A. mellifera* workers. I was able to establish the visual PER setup for workers with intact antennae of the Eastern and Western honeybee, since both species were capable of learning, and distinguishing between two monochromatic light stimuli and successfully retrieving the rewarded stimulus after two hours (Fig.5.1; 5.2). Interestingly, visual PER conditioning in *A. mellifera* has been considered to be difficult for sev-

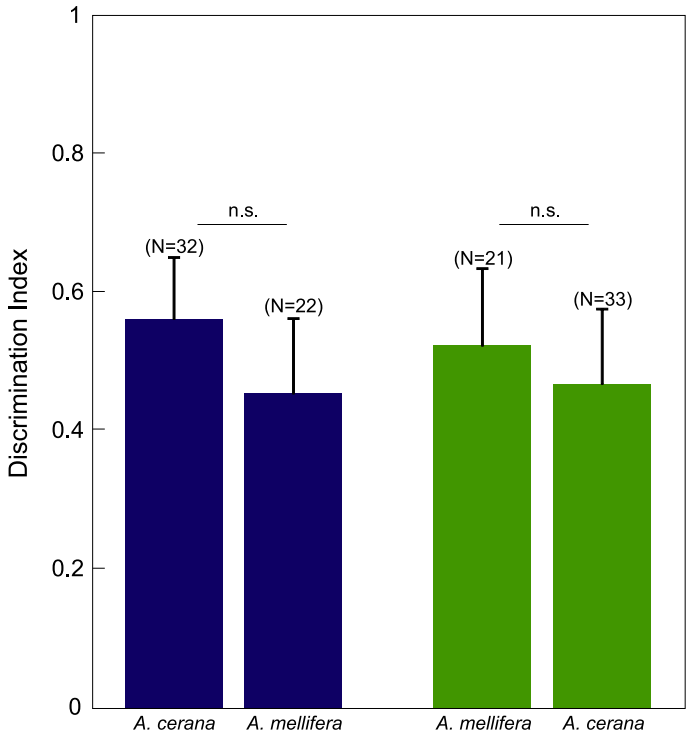

Figure 5.3: Comparison of learning performance in *A. cerana* and *A. mellifera* workers during differential conditioning. Discrimination index (DI) was calculated by the number of correct responses minus the number of incorrect response for each individual bee over all trials (for details see Pelz et al., 1997; Arenas et al., 2009).

eral decades and in earlier studies, visual PER conditioning was found to be only successful after ablation of the antennae or in combination with additional cues like odors or motion (Hori et al., 2006; Niggebrugge et al., 2009; Mota et al., 2011; Balamurali et al., 2015). Short stimulus durations of the visual stimulus might be the reason for the variability and inconsistency in earlier studies (Avarguès-Weber and Mota, 2016). In olfactory conditioning, odor stimuli are usually presented for a few seconds before the sugar reward is given (Giurfa and Sandoz, 2012), but visual PER conditioning seems to be only successful when the CS was presented for more than 10 seconds (Avarguès-Weber and Mota, 2016). Thus, I adapted my protocol from successful conditioning study of Africanized honeybees (Jernigan et al., 2014) and used a stimulus presentation length of thirteen seconds. However, the reason why the CS duration for a successful visual PER conditioning needs to be three to four times longer than for olfactory conditioning is still unknown.

After the successful establishment of the PER setup, visual learning and memory performances of *A. mellifera* and *A. cerana* were compared. *A. cerana* and *A. mel-*

lifera did not differ in their overall learning performance, but memory retrieval in *A. mellifera* was significantly better compared to *A. cerana*, which is in contrast to a study investigating visual learning abilities of free-flying workers of *A. mellifera* and *A. cerana* (Qin et al., 2012). In this study, workers flew 20 meters from the hive to the setup and had to discriminate between color and grating stimuli in a Y-maze. Both species reached acquisition levels of above 80% (similar to the levels found in our PER study), but *A. cerana* showed overall better performances in learning and memory than *A. mellifera* (Qin et al., 2012; Fig.5.3).

In contrast to vision, olfactory PER conditioning has already been established in Asian honeybees (Kaspi and Shafir, 2012; Nagaraja and Brückner, 2013; Wang and Tan, 2013; Tan et al., 2015; Gong et al., 2016; Shakib and Mehdi, 2016). For instance, drones of *A. cerana* showed faster learning and higher memory retrieval than drones of *A. mellifera carnica*, but reached lower performance levels in learning and memory than drones of *A. mellifera meda* (Hunt and Chittka, 2015; Shakib and Mehdi, 2016). In contrast, Wang and Tan (2013) found higher learning performances during olfactory PER conditioning in workers of the Western honeybee compared to the Eastern honeybee, but no difference in memory recall between both species. Additionally, and similar to our findings (Fig.5.2), workers of *A. cerana* tended to need more time for acquisition and showed lower PER scores during the first conditioning trials than *A. mellifera* (Wang and Tan, 2013). In another study, workers of *A. florea* showed higher learning performance levels and increased memory retrieval than workers of *A. mellifera* during olfactory PER conditioning (Kaspi and Shafir, 2012).

Overall, heterogeneous and often contrary results on learning and memory abilities among different honeybee species are common and might originate from divergent experimental protocols in different studies or are the results of local adaptations of respective (sub)species to different environments. Differences at the behavioral and physiological level between Eastern and (introduced) Western honeybees have often been reported and are in line with the observed differences in learning performances among different species.

For instance, despite their smaller body size, *A. cerana* workers seem to be more

active at lower temperatures, tend to start foraging earlier during the day and spend less time on each flower than *A. mellifera* (Verma and Dulta, 1986; Ruttner, 1988). Western honeybees suffer from parasites, e.g. *Varroa* mites, while most Eastern honeybees possess resistant mechanisms (Peng et al., 1987; Oldroyd, 1999). Moreover, Western honeybees compete with other Asian honeybee species for food (Sakagami, 1959) and show worse adaptations in avoiding and defending predators, e.g. to the wasp *Vespa velutina*, than Asian honeybees, suggesting a co-evolution between predators and honeybees (Tan et al., 2007). Interestingly, this chapter found a significantly lower memory acquisition two hours after conditioning in *A. cerana* compared to *A. mellifera*. Since mid-term memory enables bees to retrieve learned flower cues for up to several foraging bouts within a day (Menzel, 1999), the current results fit to the observation that *A. cerana* workers are less flower constant compared to *A. mellifera* (Wells and Rathore, 1994). Moreover, Eastern honeybees are more prone to abscond, admittedly forage at shorter distances but visit a broad variety of flowers, whereas the *A. mellifera* tends to fly larger distances and forage at more homogeneous flower patches (Ruttner, 1988; Dyer and Seeley, 1990; Hepburn, 2011).

Recent studies comparing elemental and more complex, non-elemental learning abilities revealed that, for instance, the Western honeybee and the related bumblebees showed similar performance levels when faced with a simple elemental task, e.g. to associate an odor with a sucrose reward. However, in contrast to honeybees, bumblebees failed in solving complex learning tasks, like negative pattern discrimination (Giurfa, 2003; Sommerlandt et al., 2013). In the future, further studies comparing the performance in more complex or specialized tasks will allow to investigate species-specific differences in visual learning and memory within the genus *Apis*.

To summarize, the visual PER paradigm provides an excellent method to study visual learning and memory under constant and controlled conditions which is a prerequisite to unravel species-specific differences in learning and memory that might have evolved due to different life strategies, parasitic stressors and other environmental factors.

6

Length of conditioned stimulus presentation is critical for efficient visual PER conditioning in restrained honeybees

6.1 Abstract

Learning visual cues is an essential capability of bees for vital behaviors such as orientation in space and recognition of nest sites, food sources and mating partners. To study learning and memory in bees under constant and controlled conditions, the proboscis extension response (PER) provides a well-established behavioral paradigm. While many studies have used PER conditioning for testing olfactory learning in bees due to its robustness and reproducibility, studies on PER conditioning of visual stimuli are rare. More than 50 years ago, the first visual PER conditioning study in honeybees was published by Kuwabara (1957), and since then only a handful studies have been carried out, and results were found to be inconsistent. For example, early studies stated that visual PER conditioning is only successful when the bee's antennae are ablated, but recent studies successfully performed visual PER conditioning with intact bees. An important feature found to significantly differ between visual and olfactory conditioning protocols is the duration of the conditioned stimulus presentation. In this chapter I tested how stimulus length affects visual learning performance during PER conditioning. Intact honeybee workers could successfully discriminate two monochromatic lights when the stimulus was presented 10 s before reward was offered, but failed, when the duration of stimulus presentation was 2 and 4 s, respectively, and thus in the range commonly used in olfactory conditioning. Moreover, an open source computer program "TimingProtocol" was developed which allows a more reliable and reproducible visual PER conditioning, facilitating performance levels comparable to olfactory conditioning experiments. Finally, possible reasons for the difference found between sensory modalities in PER conditioning were discussed.

6.2 Introduction

Learning visual cues constitutes an essential ability of bees for orientation and recognition of nest sites, food sources and mating partners (Barth, 1985; Winston, 1987). During their foraging flights, bees are confronted with a variety of flowers which differ in their appearance, but also in their quantity and quality of food supply (Gumbert, 2000). To efficiently discriminate between different flowers, bees may use specific signals or cues like flower size, color, odor or shape, of which color seems to be the major feature (Chittka and Menzel, 1992; Gumbert, 2000). Since Karl von Frisch provided conclusive evidence that honeybees possess the capability to discriminate between different color stimuli more than a century ago (v. Frisch, 1914; v. Frisch, 1953), many studies on color learning and discrimination in free-flying bees confirmed his findings (honeybees: Avarguès-Weber et al., 2011; Dyer et al., 2008; Giurfa, 2007; Schubert et al., 2002; v. Frisch, 1965; bumblebees: Dyer and Chittka, 2004b; Morawetz and Spaethe, 2012). Although, experiments with free flying bees enables learning under more natural conditions, it is more difficult to control the bee's behavior and the perception of stimulus features. Hence, many studies used the conditioning of the proboscis extension response (PER) to evaluate learning and memory in bees, due to its robustness and reproducibility under constant and controlled environmental conditions. By using the PER paradigm harnessed bees have to learn to associate a conditioned stimulus (CS; e.g. novel color) with an unconditioned stimulus (US; i.e. food taste). After a few paired presentations of the CS and US, the CS alone provokes the extension of the proboscis (for review see Matsumoto et al., 2012). Whereas PER conditioning with odors as CS is well established in honeybees (Bitterman et al., 1983; Hammer and Menzel, 1995; Menzel, 2001) and bumblebees (Laloi et al., 1999; Riveros and Gronenberg, 2009; Sommerlandt et al., 2013), visual conditioning of the PER is considered to be difficult. More than 50 years ago Kuwabara performed the first study of visual PER conditioning to investigate color learning of restrained honeybees and concluded that visual PER conditioning is only successful when the bee's antennae are deprived (Kuwabara, 1957). Several studies confirmed his observation (Hori et al.,

2006; Mota et al., 2011; Niggebrugge et al., 2009), but recently, other studies could successfully condition a visual stimulus with a sucrose reward using the PER in intact honeybees (Balamurali et al., 2015; Dobrin and Fahrbach, 2012; Jernigan et al., 2014) and bumblebees (Lichtenstein et al., 2015; Riveros and Gronenberg, 2012). However, at least for the Western honeybee, the performance level reached during visual conditioning of restrained individuals with intact antennae appears significantly lower compared to olfactory conditioning (Stollhoff et al., 2008); or was only successful in combination with olfactory (Mota et al., 2011) or motion cues (Balamurali et al., 2015). To better understand the causes for this inconsistency among the visual PER studies and to improve the performance level of visual PER conditioning in honeybees, this chapter aimed to compare different conditioning protocols used in previous studies. Since the length of stimulus presentation was the most noticeable factor that differed among all the studies (Tab.6.1, Avarguès-Weber and Mota, 2016), I conditioned honeybee workers to discriminate between two monochromatic lights and systematically varied the length of the CS presentation before US onset. In addition, a new setup was developed which includes a shutter to control CS onset and offset, driven by a PC based software program "TimingProtocol", which allows a more precise and automatized visual PER conditioning, facilitating performance levels comparable to olfactory conditioning.

6.3 Material and Methods

6.3.1 Conditioning of bees

Preparation and pre-testing

For all experiments I used departing bees which were caught in the morning directly at the hive entrance of *Apis mellifera carnica* colonies, which belong to the bee station of the University of Würzburg. Immediately after catching, bees were immobilized on ice and harnessed in metal tubes (for details see Matsumoto et al., 2012). Harnessed bees could only move their antennae and mouthparts to facilitate reception of the US. They were subsequently fed to saturation with a 30% sucrose solution (w/v) and placed for a few hours in a dark box (temperature: 25°C; rela-

Table 6.1: Summary of PER color conditioning studies (in chronological order)

Species	Tested wavelengths [nm]	Stimulus size (viewing angle)	Stimulus position in setup	CS length until onset of US [s]	Total CS length [s]	US length [s]	Type of US	US Application	CS trials	ITI [min]	Conditioning type	Antennae deprived	Reference
Apis mellifera	400-420, 440-460, 480-500, 510-530, 620-640, 360	?	?	?	10	?	60% sucrose solution	Tarsi	?	20	absolute	Yes	Kuwabara, 1957
Apis mellifera	618, 540, 439	diffuse	from above	4	7	3	1.4M sucrose solution	Proboscis	20	20	absolute	Yes	Hori et al., 2006
Apis mellifera	broad-band green, blue, yellow	70°	frontal to the right eye	4	7	3	50% sucrose solution	Proboscis	10	12 +/- 2	absolute & differential	Yes	Niggebrügge et al., 2009
Apis mellifera	350, 439, 540, 618	diffuse	from above	4	7	3	50% sucrose solution	Proboscis	2 x 10	10	absolute	Yes & No	Mota et al., 2011
Apis mellifera	465, 525	?	frontal	2	5	3	50% sucrose solution	Antennae	10	5	differential	No	Dobrin & Fahrbach, 2012
Bombus impatiens	523, 470, 390	diffuse	from below	10	15	3	50% sucrose solution	Antennae and/or proboscis	10	10	differential, CS+ vs. CS++	No	Riveros & Gronenberg 2012
Africanized *Apis mellifera*	520, 462, 406	diffuse	from below	10	13	3	50% sucrose solution	Antennae and proboscis	10 & 7	?	absolute & differential	No	Jernigan et al., 2014
Apis mellifera	469, 593	28°	frontal, mainly to the right eye	4	7	3	50% sucrose solution	Proboscis	10	10	absolute	No	Balamurali et al., 2015
Bombus terrestris	528, 488, 455, 435	70°	from above	6 & 9	12	3	50% sucrose solution	Antennae and Proboscis	10 & 9	8	absolute & differential	No	Lichtenstein et al., 2015

tive humidity: 50%). In the early afternoon, all bees were pre-tested for an intact PER by carefully touching the antennae with a toothpick soaked with 50% sucrose solution (w/v). For the conditioning experiments, only bees that exhibited an intact PER during the pre-test were used.

Stimuli and experimental setup

Bees were conditioned to two different light stimuli provided by different monochromatic filters (Schott & Gen, Jena, Germany) with absorption maxima at 435 nm and 488 nm, a half band width of ca. 10 nm and an aperture angle of ca. 70° (Fig.6.1A). To prevent bees from learning achromatic cues, I presented each color stimulus at three different intensities, which were generated using ND filters (transmission: 13% and 51%; for more details see also Lichtenstein et al., 2015). The experimental setup consisted of a non-reflective gray acrylic movable sleigh with nine individual chambers (50 mm x 60 mm x 50 mm) in which individual bees were placed. A filter holder, which housed the color and ND filters and a parchment paper as diffusor, was positioned on top of the sleigh and attached to a computer-controlled shutter and shutter driver (Uniblitz VCM-D1, Uniblitz, USA; Fig.6.1C). Light was provided by a cold light lamp (Schott KL1500, Germany). Once the shutter opened, the light illuminated the chamber from above and the bee faced directly towards the light at a distance of ca. 5 cm. The shutter was controlled by a custom-made software program "TimingProtocol" (see below).

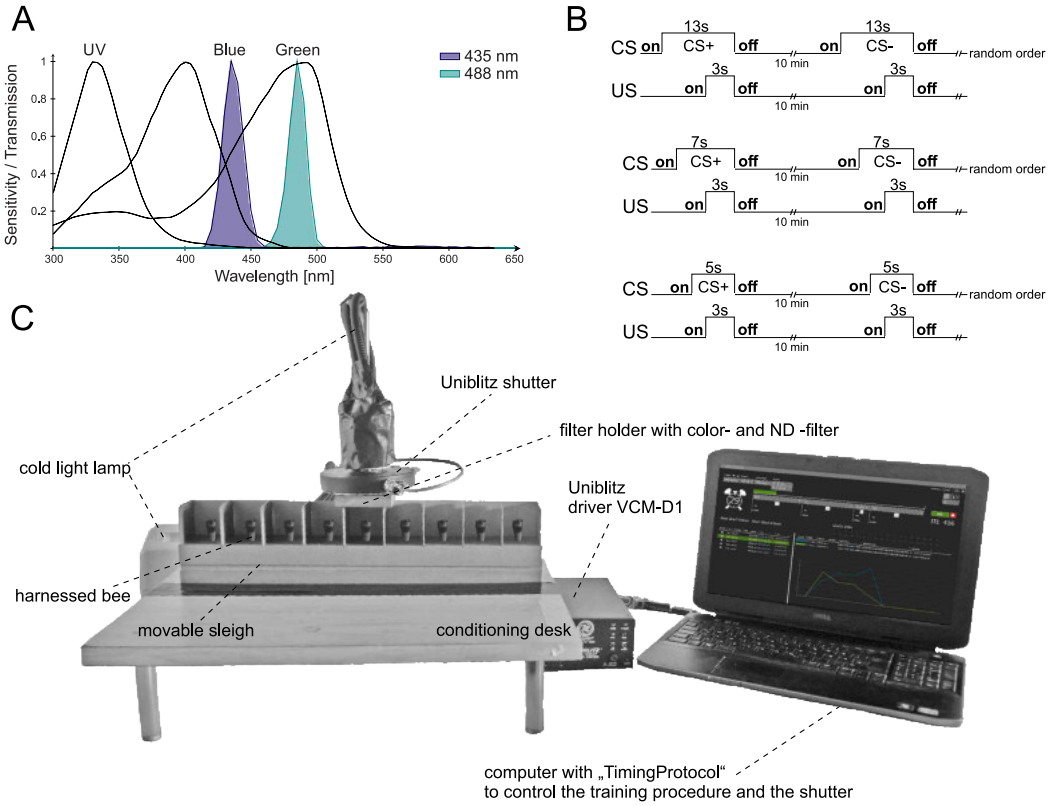

Figure 6.1: Color stimuli and experimental setup with overview of training protocols. **A**: Spectral sensitivity of the three photoreceptor-types of *Apis mellifera* (Peitsch et al., 1992), overlaid by transmition spectra of the two tested monochromatic color filters. **B**: Overview of used training protocols. **C**: Illustration of the setup for visual PER conditioning. Light from a cold-light source passes a shutter controlled by the program TimingProtocol and a color filter, and reaches the harnessed bee, placed on a movable sleigh. See text for detailed description.

6.3.2 Computer-based program "TimingProtocol"

To simplify the PER conditioning of bees, the computer program "TimingProtocol" (TP) was developed which allows a precise control of all training properties, e.g. conditioning type, length of stimulus presentation, inter-trial interval and recording and visual presentation of current results (Fig.6.2; 6.3). TP organizes conditioning protocols in a corresponding project file. Each project can be named and hold a set of different protocols. Further, it is possible to define each training protocol individually. In a first step, the user has to create a new project or open an already existing project. Each further setting will then be made in a second step for the current selected protocol. For safety reasons the user has to turn on the "edit-mode" (pencil symbol) in order to change settings. TP can be utilize for two types of

conditioning: absolute and differential conditioning. Furthermore, it is possible to name the used stimuli and define a pseudo- randomized order of the differential conditioning stimuli (CS+ and CS-) and absolute conditioning stimuli (for the test group it is possible to add blanc trials; for control group: CS and US), respectively, by pressing the "+"-button to add a trial. Stimuli can be changed by a double click on the respective trial.

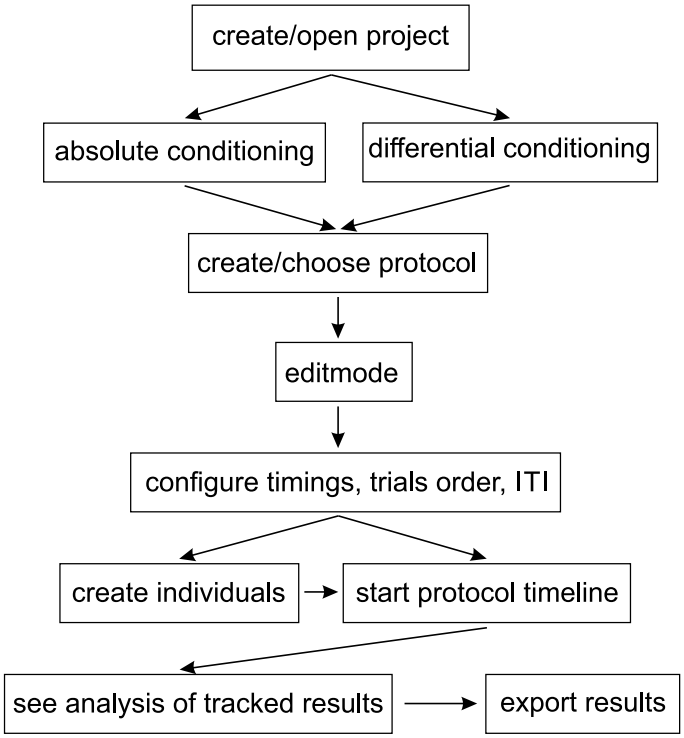

Figure 6.2: Workflow of TimingProtocol (TP). Before starting the program, the user has to create or open a project and to choose between absolute or differential conditioning. Further settings like protocol timings, trials order, ITI length must be made. Once new individuals were created, TP can be started. TP records and visualizes all results automatically. Results can be exported at any time during conditioning.

Further settings can be made by defining the threshold of trials without PER at which an individual will be excluded, and by setting the training protocol. With add and remove buttons, new time spans with name and length in seconds, a shutter action (see below) and a sound signal can be defined. The shutter action as well as the sound will occur at the beginning of the corresponding timespan. The program can also control a shutter (which is optional). If a shutter for precise control of stimulus length is used, the port of the connected shutter driver (e.g. Uniblitz VCM-D1) must be selected before conditioning. The timeline of the training protocol

can be started, paused, stopped and an inter-trial interval can be defined. TP also accepts the space key to start and pause the timeline. In a third step, new individuals can be added by clicking in the empty row at the bottom of the table. To support protocols with different stimulus lengths within the same conditioning, each timing setting is individual specific. To simplify and speed up the creation process, settings from existing individuals can be copied and pasted to the next one, by using the buttons in the first column of the table. After each run, the user is asked for the reaction of the corresponding individual. The result will then be tracked in the table (Fig.6.3D). The program runs in auto save mode, which means that the results are saved in the project file as soon as they were entered. At any time, the user can export the result table to a csv file. TP was programmed by Matthias Lichtenstein in C# and based upon the Microsoft .NET Framework. It runs on all current Windows versions with .NET 4.0.

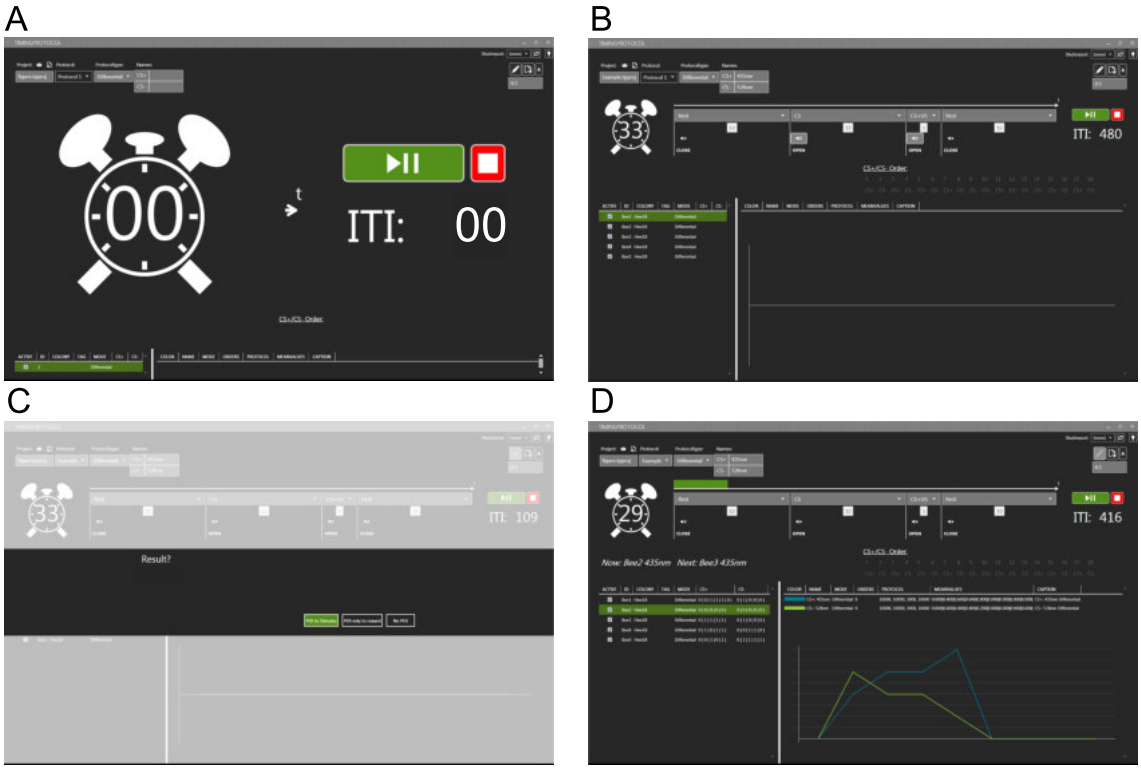

Figure 6.3: Graphical user interface (GUI) of TimingProtocol during PER conditioning Exemplary workflow of a conditioning experiment by means of TP. **A**: Initial overview of TP. **B**: Once all timing settings are justified, bees is trained after a defined protocol. **C**: The behavior of each bee is recorded after each trial, and **D**: current results are automatically visualized.

6.3.3 Differential PER conditioning

All bees were trained in a differential learning task. During conditioning the bees had to discriminate between a rewarded (CS+) and an unrewarded (CS-) stimulus. Both color stimuli combinations were tested (435 nm rewarded, 488 nm unrewarded; and vice versa). Each bee was trained for 18 trials (9 CS+ and 9 CS-) in a randomized order. The conditioning protocol was adapted from (Jernigan et al., 2014), who showed that harnessed Africanized honeybees with intact antennae can be conditioned to different colors by using the PER paradigm. At the beginning of the training procedure, each bee had a rest for 10 s to become familiarized to the given situation. Afterwards the color stimulus was switched on for 5, 7, or 13 s, respectively, and the bee was rewarded with 50% sugar solution presented on a soaked toothpick during the last 3 s, resulting in a CS presentation of 2, 4, or 10 s before the reward was given (Fig.6.1B). These durations were chosen to cover the range of CS presentations used in most of the earlier studies (see Tab.6.1). Following the offset of the color stimulus each bee had another 10 s rest before the sleigh was moved and the next bee was positioned under the shutter. In all conditioning experiments I used an inter-trial interval (ITI) of 10 min.

6.3.4 Statistical Analysis

Statistics for the learning curves were done on the basis of an individual's number of PER responses towards the color stimulus. A bee that showed a PER during the CS+ presentation before the reward was presented was scored as 1, whereas a bee that extended its proboscis only to the sucrose reward or did not respond at all was scored as 0. Bees that showed PER to sucrose reward in less than 50% of CS+ presentations were excluded from further analysis. Learning performance within one treatment group (CS+ vs. CS-) was analyzed using Wilcoxon test and among different groups by means of Mann-Whitney U test. Statistical analyzes were performed in IBM SPSS v. 20.

6.4 Results

6.4.1 PER color conditioning

The PER paradigm was used to test the color learning capabilities of harnessed intact *Apis mellifera carnica* workers. Bees were able to distinguish between two different monochromatic color stimuli (435 nm and 488 nm) and reached a performance level up to 70%, if the presentation of stimulus length was 13 s (10 s before reward onset), irrespective of which color was the rewarded or unrewarded stimulus (435 nm+ vs. 488 nm- 13s: $P = 0.005$, Z = -2.829; 488 nm+ vs. 435 nm- 13s: $P = 0.004$, Z = -2.913). However, if the stimulus length was shorter, bees were not able to significantly discriminate between the CS+ and CS- stimulus (7 s: 435 nm+ vs. 488 nm-, $P = 0.109$, Z = -1.604; 488 nm+ vs. 435 nm-, $P = 0.255$, Z = -1.138; 5s: 435 nm+ vs. 488 nm-, $P = 0.180$, Z = -1.342; 488 nm+ vs. 435 nm-, $P = 0.395$, Z = -0.850). Since no significant difference in discrimination between both color combinations was found for any CS lengths (CS+ vs. CS- 13 s: $P < 0.001$, Z = -4.047; CS+ vs. CS- 7 s: $P = 0.075$, Z = -1.783; CS+ vs. CS- 5 s: $P = 0.176$, Z = -1.354), data were pooled (Fig6.4). To estimate the advantage of the computer controlled PER protocol, an inexperienced student, who never did PER conditioning before was asked, to perform three conditioning runs each with both the program and by simply using a stop watch in an random order. Conditioning levels were found to be significantly better (program CS+ vs. stopwatch CS+: $P = 0.031$, Z = -2.153; program CS- vs. stopwatch CS-: $P = 0.007$, Z = -2,684) when the program was used compared to a stop watch only (Fig.A.7), although overall performance of the unexperienced student was lower compared to an experienced experimenter (compare Figs.A.7 and Fig.6.4).

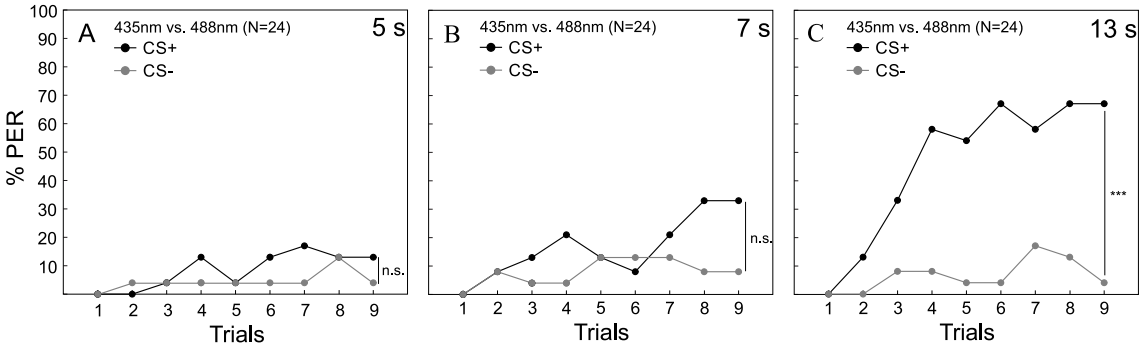

Figure 6.4: Differential color conditioning in honeybee workers Bees were trained with three different training protocols (stimulus presentation length: 5 s (**A**), 7 s (**B**) and 13 s (**C**)) to discriminate between two monochromatic light stimuli (435 nm and 528 nm; $\Delta\lambda = 53$ nm). Both color combinations were tested reciprocally for all three training protocols and data were pooled, since no significant difference in discrimination between both combinations was found. *** $P<0.001$; n.s.: not significant.

6.5 Discussion

The current chapter could show that restrained workers of the Western honeybee *Apis mellifera* with intact antennae were capable to learn and discriminate between two monochromatic lights by using the PER paradigm. Whereas early studies stated that visual PER conditioning is only successful when the bee's antennae were ablated, recent studies revealed controversial results (Balamurali et al., 2015; Dobrin and Fahrbach, 2012; Jernigan et al., 2014; Lichtenstein et al., 2015; Riveros and Gronenberg, 2012). The present study provides further evidence that antennal deprivation is not necessary for a successful color learning in restrained honeybees. In contrast to earlier studies, intact honeybees of the present study reached learning performance levels up to 70% which is much higher compared to all other studies so far. In the following, possible reasons for the distinct variation of learning performance among these studies will be discussed.

Since the first experiments on visual PER conditioning were performed, especially the stimuli presentation lengths changed over the last 50 years. Whereas particularly older studies preferred a shorter stimulus presentation length which was more comparable to most olfactory PER conditioning protocols (for review see Frost et al., 2012), recent studies chose longer stimulus lengths (Tab.6.1). The three stimulus durations tested in this study (5 s, 7 s and 13 s) have already been used in previous studies (Tab.6.1), but only bees confronted with a 10 s light stimulus before US

onset were able to successfully learn the association and reached performance levels comparable to what was found in olfactory PER conditioning (Bitterman et al., 1983; Hammer and Menzel, 1995; Giurfa and Sandoz, 2012; Tan et al., 2015). This observation is in congruence with the few successful studies which already applied stimulus durations of 9, 10, or 12 s before US onset in visual conditioning (Jernigan et al., 2014; Lichtenstein et al., 2015; Riveros and Gronenberg, 2012). Hence, I conclude that the duration of the stimulus presentation seems to be crucial for efficient visual PER conditioning. But why requires visual PER conditioning much longer stimulus lengths compared to olfactory conditioning?

A possible explanation might be that the processing of visual stimuli is more complex and time consuming due to the multitude of light receptive organs present in the bee and the absence of other (visual) cues, compared to the processing of olfactory information which is restricted to the antennae.

During the acquisition phase of olfactory PER conditioning, bees usually extent their proboscis already after less then two seconds after stimulus onset and after less then one second when the association has been learned (Rehder, 1987). This "reaction time" includes the activation of the odor sensitive receptors at the antenna, passing the information via the antennal lobes (Joerges et al., 1997) and the dual olfactory tracts (Rössler and Brill, 2013) to higher order brain centers (e.g. the mushroom bodies, MB; Sandoz, 2011). The output neurons of the MB probably send the processed information into the lateral protocerebrum where the actual information is compared with the (olfactory) memory, and finally the activation of the proboscis muscles is initiated. During the association process, the VUMmx1 neuron and its putative transmitter octopamine mediate the reinforcement (Hammer, 1997). Moreover, octopamine can substitute the US reinforcement (e.g. sucrose solution) and a conditioned stimulus is capable of activating the VUMmx1 neuron, thereby maintaining the function of the reinforcer in absence of the US (Hammer, 1993; Hammer and Menzel, 1998).

In contrast to olfaction, light reception is not restricted to an exclusive input channel but is sensed via several parallel light-sensitive pathways. The compound eyes mediate object and motion detection via chromatic and achromatic vision and

relay the information via the optic lobes and various projection neurons to the MBs, the anterior optic tubercle (AOTU) and the posterior protocerebrum (Gronenberg, 1986; Paulk et al., 2009; Paulk and Gronenberg, 2008). The dorsal rim area of the compound eye is a specialized region, capable of sensing polarized light by means of the UV sensitive photoreceptors, from where the information is projected via the dorsal part of the optic lobes to the AOTU and finally to the central body's lower division (Held et al., 2016). The three ocelli, located at the vertex of the bee's head, possess UV and green sensitive photoreceptors (Goldsmith and Ruck, 1958) and project via the ocellar interneurons to the median posterior protocerebrum (Maronde, 1991). And finally, a putative light sensitive extra ocular photoreceptor, expressing pterosin as light sensitive rhodopsin (Velarde et al., 2005), with unknown sensitivity and function, is located between the lobula and the lateral protocerebrum. By shining light on the bee's head some or even all of these light-sensitive pathways become activated and (so far unknown) higher order processes are needed to integrate all the information and put it in the correct context. Since a restrained bee lacks, in contrast to a freely moving one, other information like motion or spatial cues which are received during flight, processing of visual information that lead to the correct behavioral action might become prolonged. Furthermore, there is no anatomical evidence that the VUMmx1 neuron (or any other VUM neuron) is connected with any of the visual pathways (Schroter et al., 2007), suggesting that the reward system for visual learning might be different (and probably more complex) than in the olfactory system.

Although visual and olfactory stimuli are processed via different neuronal pathways, it remains still unclear from an ecological perspective why bees should need longer for processing visual compared to olfactory cues when associating a stimulus with a reward. One possible explanation for this difference may lay in the nature of visual and olfactory stimuli. Odors are mixtures of chemical compounds which appear in plumes, do not reach far and hit the olfactory system of insects jerkily and unpredictably (Chittka and Menzel, 1992; Galizia and Menzel, 2000; Sandoz, 2011; Touhara and Vosshall, 2009). Therefore, olfactory information needs to be processed fast since availability is short and unreliable. In contrast, flower color,

once detected, stays constant in space and quality during approaching and provides more time for processing (Chittka and Menzel, 1992). This may allow a more reliable and precise information processing. A tradeoff between speed and accuracy of flower discrimination was shown in bumblebees when different colored flowers were presented and needed to de discriminated (Chittka et al., 2003).

Finally, the mode of the visual stimulus might also affect learning. In most of the previous studies on PER color vision, color stimuli were presented using LEDs or monochromatic filters, either by globally illuminating the entire training setup from above (Hori et al., 2006; Mota et al., 2011), or presenting the color stimuli from below (Dobrin and Fahrbach, 2012; Jernigan et al., 2014; Riveros and Gronenberg, 2012) or from the front (Balamurali et al., 2015). In contrast, in the present chapter, each harnessed bee was held in a single small quadratic chamber and all color stimuli were presented directly from the top of the chamber which are thus seen by the bee as a clear defined circular and colored object which might resemble an object-like form of a flower. Moreover, no study has successfully been performed using non-reflecting stimuli, usually applied in studies with free-flying bees (e.g. Dyer et al., 2008). Thus, futures studies need to address how stimulus quality (self-luminous versus reflecting stimuli), spatial extension (subtended visual angle at the bee's eye) and the activation of distinct light sensitive pathways (excitation of the compound eyes, ocelli, extra-retinal photoreceptors and any combination of these) affect visual learning in harnessed bees.

In conclusion, this chapter could show that visual PER conditioning might not only be affected by the conditioning protocol but also by qualities of the conditioning setup and by the individual conditioning skills of the conditioner. Comparing the bee's learning performances recorded by an unexperienced student, with and without the custom-made computer software TP, the application of TP during conditioning lead to a more precise and automated bee conditioning, resulting in higher conditioning levels of the bees. Hence, the current setup provides for the first time a promising PER conditioning setup to test visual learning and memory in restrained bees in future studies.

7

General Discussion

Honeybees and humans live in a close relationship since several thousand years, and we rely on bee products like honey and wax and their outstanding importance for pollination of many crop and wild plants (Potts et al., 2010). To date, honeybees receive increased attention due to their exceptional eusocial lifestyle and complex behavioral repertoire, which makes them an interesting model to study mechanisms of sensory perception, learning and memory.

In the eusocial bees, hundreds to thousands of individuals form a colony and comprise three distinct castes: the reproductive queen, the non-reproductive workers and the drones (Winston, 1987). The three castes differ in morphology, physiology and larvae developmental time (Winston, 1987). Crucial for the formation of the morphological and physiological characteristics of each caste is the timing of specific developmental programs during the bee's juvenile phase. Experiments conducted in the present thesis provide the first step in elucidating the developmental and molecular mechanisms that determine the (sex-)specific composition of the compound eye in bees. The thesis demonstrates that retinal development and cast-specific retinal patterning mainly occurred during pupal development and is followed by an early adult maturation of the visual system. The current thesis further successfully established the visual proboscis extension reaction (PER) paradigm, which has been assumed to be difficult or even impossible in intact honeybees. This behavioral assay now provides an excellent method for future studies to evaluate visual perception and cognitive capabilities of bees, e.g. the function and adaptive value of specific photoreceptor types for color perception.

7.1 Retinal development and adult maturation of the bee's visual system

In the first part of my thesis, I aimed to better understand the retinal development and photoreceptor determination in the compound eye of honeybees. Despite a high variety in eye types found across the animal kingdom, visual systems of most animals comprises related visual pigments to capture photons (Terakita, 2005). Moreover, to enable animals to see colors and extract chromatic information, the comparison of the output of at least two different photoreceptors (characterized by different spectral opsins) is a prerequisite. For instance, the visual system of humans is perfectly adapted to master different visual requirements. The retina of humans comprises two different types of photoreceptors, cones and rods. Three different subclasses of cones (most sensitive to blue, green and red wavelengths) are stochastically distributed within the retina but green and red sensitive cones are notably concentrated in the center, the fovea and rods in the periphery, which enables high spatial resolution and color vision (Roorda and Williams, 1999). Interestingly, the arrangement of different photoreceptor types resulting in retinal mosaics has been also found in insects (Wernet et al., 2015). In analogy to human cones, flies e.g. also arrange their two ommatidia types, called "pale" and "yellow", in a stochastically distributed retinal mosaic (Rister and Desplan, 2011; Wernet et al., 2006). Retinal mosaics are also prominent in the honeybee but only little is known about retinal development and photoreceptor determination in honeybee workers and drones during pupal and adolescent development. As demonstrated in Chapter 2, the current thesis provides evidence that workers and drones express three different opsins (*UVop*, *BLop* and *Lop1*) during pupation. Both sexes already express *UV* opsin mRNA during early pupal stages, but a significant increase in the quantity of UV, blue and green opsin expression levels becomes first visible at about mid of pupation in workers and at end of the pupal phase in drones. This delay in opsin expression onset between both sexes might correlate with the longer pupation phase found in honeybee drones (Dietermann et al., 2013; Tofilski, 2012). Interestingly, compound eyes of drones from various honeybee species do not only differ from workers in their morphological ap-

pearance, e.g. possessing enlarged eyes with large dorsal facets, but also in their photoreceptor distribution within compound eyes (Menzel et al., 1991; Peitsch et al., 1992; Streinzer et al., 2013a; Velarde et al., 2005). During pupal and adolescent development, drones express mainly *UVop* and *BLop* in their dorsal part of the eye (Chapter 2) that fits the hypothesis to be efficiently prepared for detecting a virgin queen against the bright sky (Menzel et al., 1991).

As further demonstrated in Chapter 2, photoreceptor determination in honeybees goes hand in hand with retinal development. At the beginning of pupation the honeybee retina consist only of a thin hypodermis but all retinal structures, e.g. photoreceptors or pigment cells, are already present. In congruent with earlier studies (Eisen and Youssef, 1980; Phillips, 1905), the rhabdoms rapidly lengthen during pupal development. However, I observed the most obvious leap in rhabdom length and diameter extension as well as photoreceptor cell differentiation at mid of pupation, which coincides with the first distinct increase of UV, blue and green opsin expression levels. Based on the current results, this thesis suggests a two-step process in photoreceptor development and determination in the honeybee compound eye. In a first step, when the retina almost reached its final form, the photoreceptor cells meet its fate during the late pupal phase. At this developmental stage, the relative proportion of opsins and thus photoreceptor types become fixed. At the same time, an increase of rhabdom length and diameter probably leads to an increase in absolute opsin expression levels. In a second step, the quantity of opsin expression in each photoreceptor strongly increases up to 25-fold during adolescent development, suggesting an early adult maturation phase of the visual system during the first days after eclosion.

Honeybees undergo an exceptional age dependent division of labor including a transition from inside duties (e.g. cleaning cells or feeding the brood), to foraging activities outside the hive (Seeley, 1982; Winston, 1987) and at least for the cavity nesting honeybee, *Apis mellifera*, it was shown that workers and drones only gradually come into contact with light during the transition from nurses to foragers (workers: Seeley, 1982) or during their first orientation flights (drones: Howell and Usinger, 1933). Within the hive, the task allocation largely rely on pheromonal and

tactile communication (Schneider and Lewis, 2004; Slessor et al., 2005; Trhlin and Rajchard, 2011). However, the transition from the dark hive to the bright outer world requires a well-adapted visual system. An adult maturation of the visual system has not only detected at the molecular level (strong increase in opsin expression levels after eclosion, see Chapter 2) but also at neuronal and behavioral level. For example, honeybee mushroom body calyxes show a distinct volume increase during the first week after eclosion (Muenz et al., 2015) and light exposure significantly triggers the reorganization of microglomeruli in the collar, a visual sub-region of the mushroom body calyx (Scholl et al., 2014). Moreover, 1-day old honeybees show a reduced positive phototactic behavior than 7-day old bees (Becker et al., 2016). Thus, a delayed transition from indoor to outdoor activities, when the visual system is needed, may have allowed the bees to postpone part of their eye development into the early adult phase in the course of evolution.

Besides a high plasticity in opsin expression levels and neuronal changes, the visual system seems also be regulated by the circadian clock to synchronize the bee's endogenous rhythm with the environment. As demonstrated in Chapter 2, the expression levels of all opsins significantly cycle during 24 hours even under constant darkness, suggesting that their expression is under control of the endogenous clock. Highest expression levels were found during late night and putative early morning for all opsins, matching the activity pattern of foraging bees (Klein et al., 2008; Moore and Rankin, 1993). Furthermore, the bee's activity peak also correlates with highest availability of pollen and nectar during the morning (Kleber, 1935). Overall, processing and adaption of the visual system seemed to be influenced and regulated by a variety of factors, e.g. retinal development, age, light environment, availability of food sources and the endogenous clock.

During the last decades, much effort has been devoted to unravel the underlying mechanisms of retinal patterning, leading to different photoreceptor types in insects. Most work on that issue has been conducted in the model organism *Drosophila melanogaster* and to some extend also in butterflies, revealing a complex interplay of different interacting transcription factors during retinal development (Morante et al., 2007; Perry et al., 2016). Interestingly, the regulation and determi-

nation of photoreceptors in flies and butterflies show high similarities, suggesting a conserved code in retinal patterning within insects (Perry et al., 2016; Wernet et al., 2015). As shown in Chapter 3, two transcription factors, *spalt* and *spineless*, which have been shown to play a critical role in photoreceptor determination in flies and butterflies have also been identified in the honeybee. Expression patterns of both transcription factors differ from each other during the different pupal phases of honeybee workers, and highest expression levels of both genes coincides with the time of expression found in *Drosophila* homologs (Morante et al., 2007; Wernet et al., 2006). For instance, *spalt*, which is necessary for the differentiation between outer and inner photoreceptors in flies, is mainly expressed during the first few pupal stages of workers and might correlate with the onset of the blue opsin expression (see also Chapter 2). *Spineless*, in contrast, is necessary and sufficient to determine the photoreceptor class "yellow" in flies and showed a prominent peak at 50% of pupal development, which might initiate the expression of green opsin in bees (Chapter 2+3). However, whether *spalt* and *spineless* are also essential for photoreceptor determination in the honeybee has still to be investigated, e.g. by a knockdown/out of the respective transcription factor during pupal development which would lead to a spectral phenotype, e.g. a dichromatic eye. Such spectral phenotypes can then be tested in behavioral experiments in order to test the function of specific photoreceptors for color perception and the entrainment of the circadian clock. For instance, generating and behaviorally testing different fly mutants lacking different rhodopsins, revealed that motion detection is fully separated from color vision (Yamaguchi et al., 2008). A knockdown (e.g. RNA-interference) or knockout (e.g. CRISPR Cas9) of a target of interest might be an appropriate method for investigating sensory functions in the honeybee. In fact, RNAi injection into the retina has been already applied by inhibiting the green photoreceptor (Leboulle et al., 2013), but the inhibiting effect was weak, strongly affected by the circadian clock, and limited in time (Leboulle et al., 2013). However, since *spalt* and *spineless* have been shown to be involved in many developmental processes in flies and vertebrates (Crews and Fan, 1999; de Celis and Barrio, 2009), a gene knockout of the respective transcription factor by means of the novel CRISPR Cas9- system during embryogenesis of honeybees might

lead to (sub)lethal effects. Moreover, the basement membrane of honeybees which separates the retina from the optic neuropils, has been shown to serve as a kind of blood-chain barrier (Shaw and Varney, 2000). This might benefit investigations of visual components by a knockdown of related genes which are restricted to the retina. Thus, an inhibition of the respective transcription factors at specific time points during pupal development might be sufficient to construct different spectral phenotypes and unravel the molecular pathways underlying photoreceptor determination in the honeybee.

7.2 Visual PER conditioning – A method for studying color perception in restrained bees

In order to evaluate color discrimination capabilities of bees and the quality of color perception, a reliable behavioral essay under controlled and standardized conditions is a prerequisite. Hence, in the second part of my thesis, I aimed to establish the visual PER paradigm as a suitable method for behaviorally testing color vision in bees. During visual PER conditioning, a bee has to associate e.g. a color stimulus (conditioned stimulus, CS) with a sugar reward (unconditioned stimulus, US). If the association is established after a few conditioning trials, the bee extends its proboscis after presenting the color stimulus alone (Avarguès-Weber and Mota, 2016; Matsumoto et al., 2012). For many decades, visual PER conditioning has been considered to be difficult in bees. In contrast to olfactory conditioning (Giurfa and Sandoz, 2012), visual PER conditioning was only successful after ablation of the antennae, presenting the light stimulus together with additional cues like odors or motion (Balamurali et al., 2015; Hori et al., 2006; Mota et al., 2011; Niggebrugge et al., 2009). Only recently, visual PER conditioning in intact bees has been successfully applied in Africanized honeybees (Jernigan et al., 2014) and the closely related bumblebees (Lichtenstein et al., 2015; Riveros and Gronenberg, 2012; Chapter 4). In Chapter 5 and 6, I could demonstrate by means of visual PER conditioning that both Eastern and Western honeybees are able to associate and discriminate between two monochromatic light stimuli. Moreover, both species successfully retrieve the learned stimulus after two hours. A possible explanation for the discrepancy in learning performance of bees found in previous studies, might be the prolonged presentation of the light stimulus. In contrast to olfactory PER conditioning, odor stimuli are usually presented only for a few seconds before the sugar reward is given (Giurfa and Sandoz, 2012), but in visual PER conditioning, it tended to be only successful when the CS is presented for more than 10 s (Avarguès-Weber and Mota, 2016). Further evidence supporting this hypothesis is shown in Chapter 6. By systematically testing different lengths of stimulus presentation, intact honeybee workers could successfully discriminate two monochromatic lights when the stimu-

lus was presented for 10 s before sugar reward was offered, but failed, when the duration of stimulus presentation was shorter. An explanation for the necessity of a prolonged presentation of visual stimuli might originate from different neuronal channels underlying the association process of olfactory and visual stimuli. In olfactory conditioning several studies could show that the VUMmx1 neuron and its putative transmitter octopamine mediate the reinforcement in associative olfactory learning (Hammer, 1997). However, it has also been shown that the visual system is not innervated by VUM neurons (Schroter et al., 2007) and thus, the neuronal substrate which mediates the association between a visual stimulus and an unconditioned stimulus (i.e. the sucrose reward) might be different for visual stimuli. In contrast to olfaction, bees possess several for light-input systems: the compound eyes for chromatic and achromatic information including the dorsal rim area which enables the bee to perceive polarized light (Labhart, 1980; Wehner and Strasser, 1985), three ocelli probably used for navigation and flight stabilization and extra-retinal visual pigments (Winston, 1987), e.g. the protein pteropsin which is suggested to be involved in the regulation of the circadian clock (Velarde et al., 2005). Hence, a comparison and allocation of visual information from different input channels might also cause delay in visual processing of relevant cues. Overall, and as shown in Chapter 4, 5 and 6, the improvement of the visual PER conditioning setup now allows a more precise and automated visual PER conditioning of restrained bees, facilitating performance levels comparable to olfactory conditioning and thus provides a suitable method to evaluate visual perception and cognition of bees, e.g. in combination with electrophysiological recordings or RNAi. Moreover, it now provides the possibility to investigate the differences between visual and olfactory processing under controlled conditions in future studies.

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Appendix

A.1 Supplementary Results

A.1.1 Supplementary Results Chapter 2

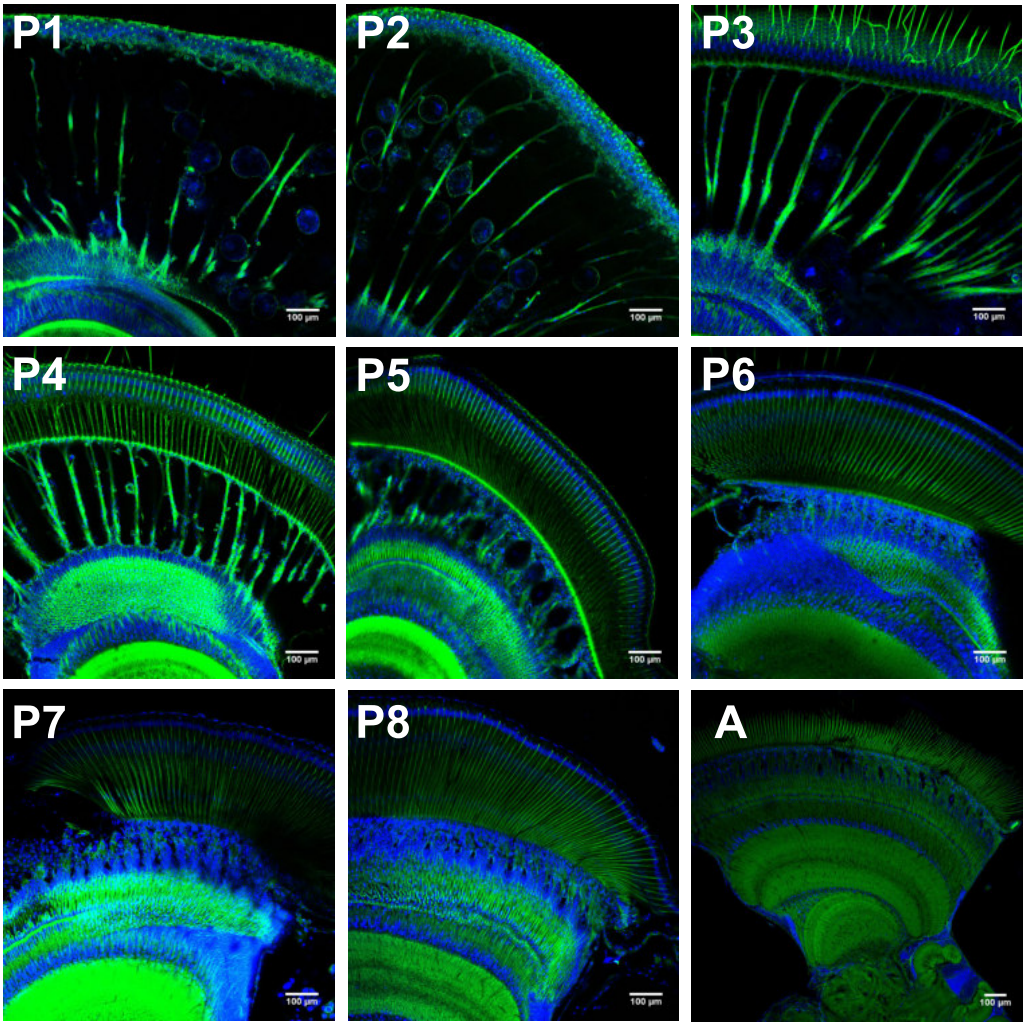

Figure A.1: Overview of retina development during pupal development of honeybee workers. Immunostainings with phalloidin (green) and Hoechst (blue) to label f-actin and nuclei, respectively, were performed to visualize the retinal development of honeybees. P1-P3: At the beginning of the pupal phase the retina consists only of a thin hypodermis and streched photoreceptor axons extend through the basement membrane towards the lamina. At this early pupal phase rhabdoms are short and exist only in the dorsal part of the eye. P4-P7: During the next days of pupal development rhabdoms and photoreceptor cells significantly increase in size, and photoreceptor axons become shortened and twisted. A: The retina reached its final stage after eclosion of the bee.

A.1.2 Supplementary Results Chapter 3

SSX1

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  1 MTPTSAPVPT PTTASGPNNP VAMSQLGTVY ATKRRRRNGK SLKPPQKDGV TKSNPSKRHR
 61 ERLNAELDTL ASLLPFEQNI LSKLDRLSIL RLSVSYLRTK SYFQVVMHKD KEENSHHDSH
121 YRARELAAFA AYDHHHLDGE MFLQALNGFL LILTCDGEVF FATHSIESYL GFHQSDIVHQ
181 SVYELVHSED REELQRQLMW NSFLPSESAS LPLHDALSPQ HSHLLERSFT VRFRCLLDNT
241 SGFLRLDIRG RVKILHGQNR KTEEPPLALF ALCTPFGPPS LLEVPQKDVM FKSKHKLDLA
301 LVSMDQRGKM LLGYSDAELA NLGGYDLVHY DDLAYVASAH QELLKTGASG MIAYRFQKKD
361 GGWQWLQTSS RLVYKNSKPD FVISTHRPLM EEEGRDLLGK RTMDFKVSYL DAGLTNSYFS
421 DSDSLTGSVM TPTLPSQPTS QRVNRRYKTQ LRDFLSTCRN KRTKLSAQSS VSPPATPTVA
481 SVDYLAADTS AAAAVAAAYS NLNTMYPTAY APTAVAATTD PSLTTYIGHT GNYHQTLYPA
541 TALDNRYLTA ATENLFQYRP LGTYYPEYHT STAYNGFIDV PLPTYETHQL ASKAEEKLYC
601 QQLGESPKYS YVETRHASSV SGSPYASSPV AATASTAMQQ QQQQQQQQQH TTDIGIVRAG
661 SRHSLEGGPG SSSNSAGSSP VTGATNGVLT PKIEDVKPEV YGGEAPRQTV LMWGAPPART
721 PPRNNGSYSP PTPHSTHSST HSTNATGGGG DPLKSLAEMN SMNGECKWRQ ASPGEQQGAA
781 PPGSPRAKAQ PQHQQQQQQQ QHHQQQQQQQ HQQYPVTTSQ YQAAAAAAAA AAAAAASTIG
841 YAHSHPGHGH GDTGSEHAPV SCGNNGDRGR HGHRAGQQHQ QQQRLVPPSA PPPPPSAPTR
901 GGIISGGNGA NCPSDSKPDT GSPLLSISEV TNTLLNQ
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SSX2

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1   MTPTSAPVPT PTTASGPNNP VAMSQLGTVY ATKRRRRNGK SLKPPQKDGV TKSNPSKRHR
61  ERLNAELDTL ASLLPFEQNI LSKLDRLSIL RLSVSYLRTK SYFQVVMHKD KEENSHHDSH
121 YRARELAAFA AYDHHHLDGE MFLQALNGFL LILTCDGEVF FATHSIESYL GFHQSDIVHQ
181 SVYELVHSED REELQRQLMW NSFLPSESAS LPLHDALSPQ HSHLLERSFT VRFRCLLDNT
241 SGFLRLDIRG RVKILHGQNR KTEEPPLALF ALCTPFGPPS LLEVPQKDVM FKSKHKLDLA
301 LVSMDQRGKM LLGYSDAELA NLGGYDLVHY DDLAYVASAH QELLKTGASG MIAYRFQKKD
361 GGWQWLQTSS RLVYKNSKPD FVISTHRPLM EEEGRDLLGK RTMDFKVSYL DAGLTNSYFS
421 DSDSLTGSVM TPTLPSQPTS QRVNRRYKTQ LRDFLSTCRN KRTKLSAQSS VSPPATPTVA
481 SVDYLAADTS AAAAVAAAYS NLNTMYPTAY APTAVAATTD PSLTTYIGHT GNYHQTLYPA
541 TALDNRYLTA ATENLFQYRP LGTYYPEYHT STAYNGFIDV PLPTYETHQL ASKAEEKLYC
601 QQLGESPKYS YVETRHASSV SGSPYASSPV AATASTAMQQ QQQQQQQQQH TTDIGIVRAG
661 SRHSLEGGPG SSSNSAGSSP VTGATNGVLT PKIEDVKPEV YGGEAPRQTV LMWGAPPART
721 PPRNNGSYSP PTPHSTHSST HSTNATGGGG DPLKSLAEMN SMNGECKWRQ ASPGEQQGAA
781 PPGSPRAKAQ PQHQQQQQQQ QHHQQQQQQQ HQQYPVTTSQ YQAAAAAAAA AAAAAASTIG
841 YAHSHPGHGH GDTGSEVWQG VQHHHHYQYY PYHHHHPAPP RHAPHTPPSA VGTTGTGVGM
901 GIGQDSSTSN SNVLYHPPHH HHHPQHQHAV GSSAAGMGPT VPQIPNRTPG VLCCPSLR
```

Figure A.2: Protein sequences of spineless isoforms. Isoform specific sequences for isoform SSX1 and isoform SSX2 are shown in red. Peptide sequences used for antibody generation are shown in blue. Both isoforms are characterized by a similar molecular weight of 103 kDA (SSX1) and 105 kDA (SSX2), respectively.

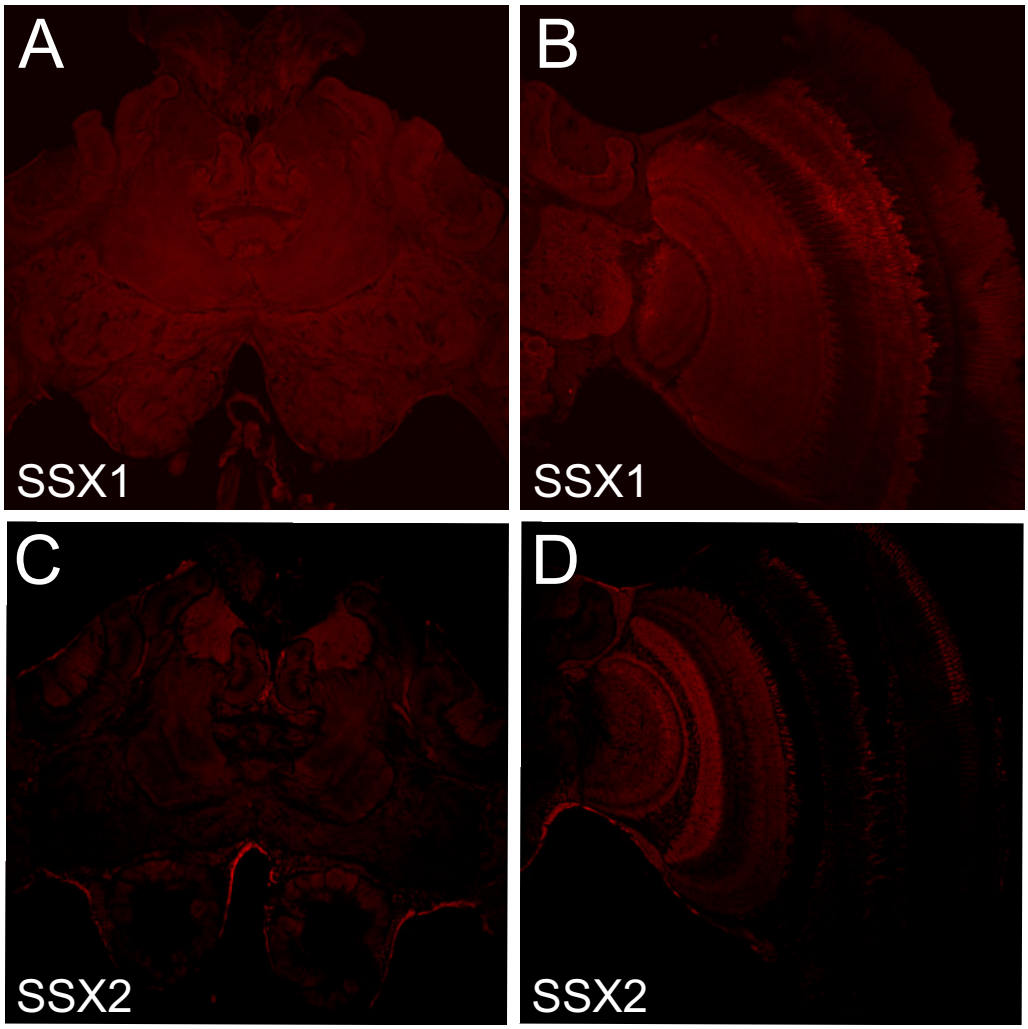

Figure A.3: Spineless isoform antibody test by means of immunostainings. Immunostainings against isoform SSX1 revealed an unspecific antibody staining in the central brain and optic lobes of honeybee workers (A, B) which is in contrast to a specific immunostaining found for isoform SSX2 (C, D).

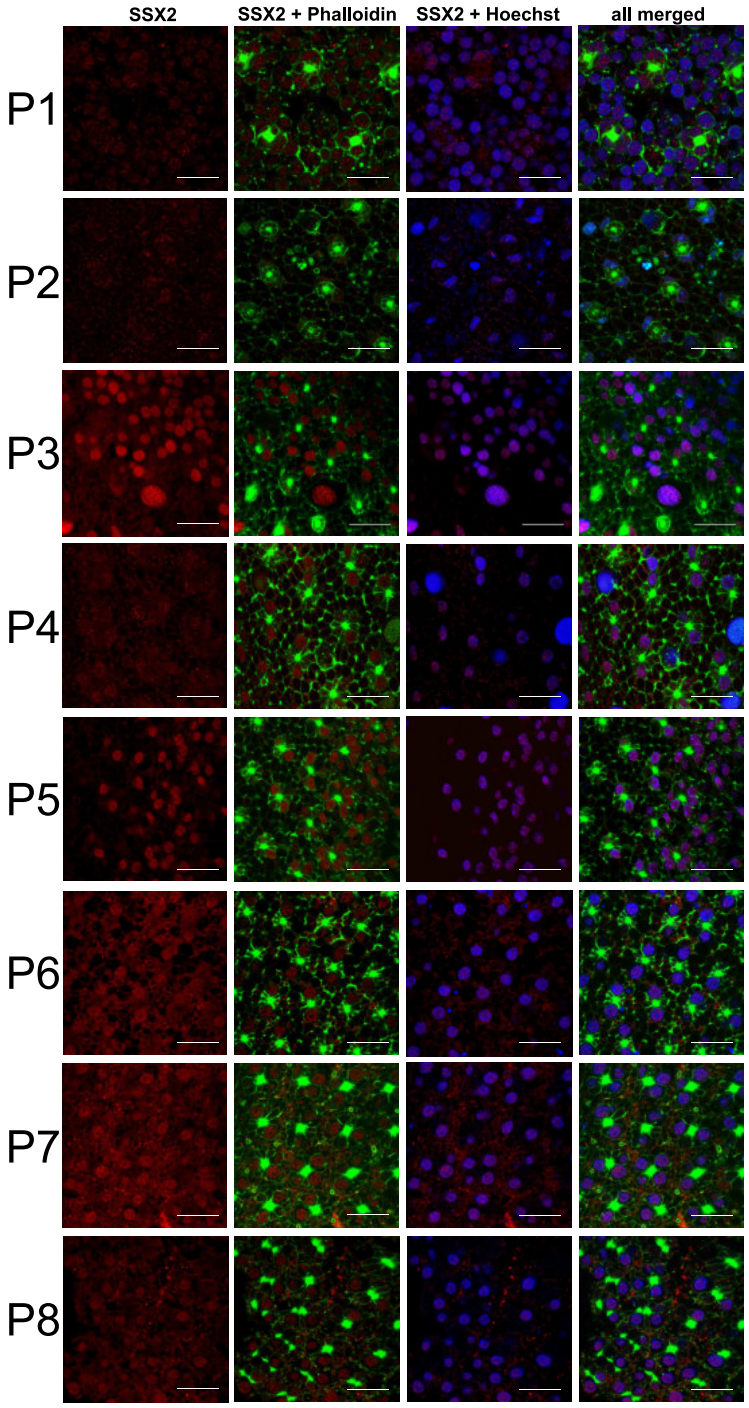

Figure A.4: Spineless expression patterns in the retina (cross section) during pupal development of honeybee workers. Immunostainings with SSX2 antibody (red), Phalloidin (green; f-actin) and Hoechst (blue; cell nuclei) were performed to visualize spineless expression patterns during the pupal phase of workers. Scale bar in all figures: 100 µm.

A.1.3 Supplementary Results Chapter 4

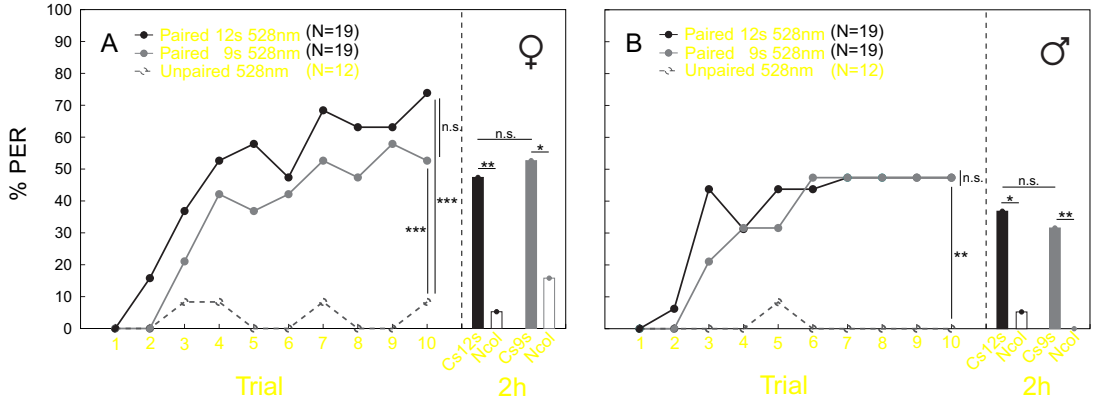

Figure A.5: Impact of the conditioning protocol on performance in absolute color conditioning and memory retrieval. Learning curves of (A) workers and (B) drones during absolute conditioning. Bees were trained either with a paired (filled circles) or an unpaired (open circles) presentation of CS and US. Two groups of bees of each sex were trained with different conditioning protocols of the paired CS-US presentation: one group (Paired 12 s) was presented in each trial with 12 s of CS, and 3 s of US 6 s after CS onset, which led to a 3s CS overhang after end of US; a second group (Paired 9s) received 9 s of CS and 3 s of US 6 s after CS onset. In the latter group, CS and US terminated simultaneously. Memory retrieval was tested by presenting the CS and a novel color stimulus (NCol) to the bees 2h after end of conditioning. A, Paired 12 s/Unpaired: MWU, $P < 0.001$, Z=-3.587; Paired 9 s/Unpaired: MWU, $P < 0.001$, Z= 3.587; CS12 s/Ncol: $P = 0.003$, χ^2=8.686; CS9 s/Ncol: $P = 0.017$, χ^2=5.729). B, Paired 12 s/Unpaired: MWU, $P = 0.009$, Z=-2.612; Paired 9 s/Unpaired: MWU, $P = 0.009$, Z= 2.612; CS12 s/Ncol: $P = 0.017$, χ^2=5.700; CS9 s/Ncol: $P = 0.008$, chi^2=7.125). *** $P < 0.001$; ** $P < 0.01$

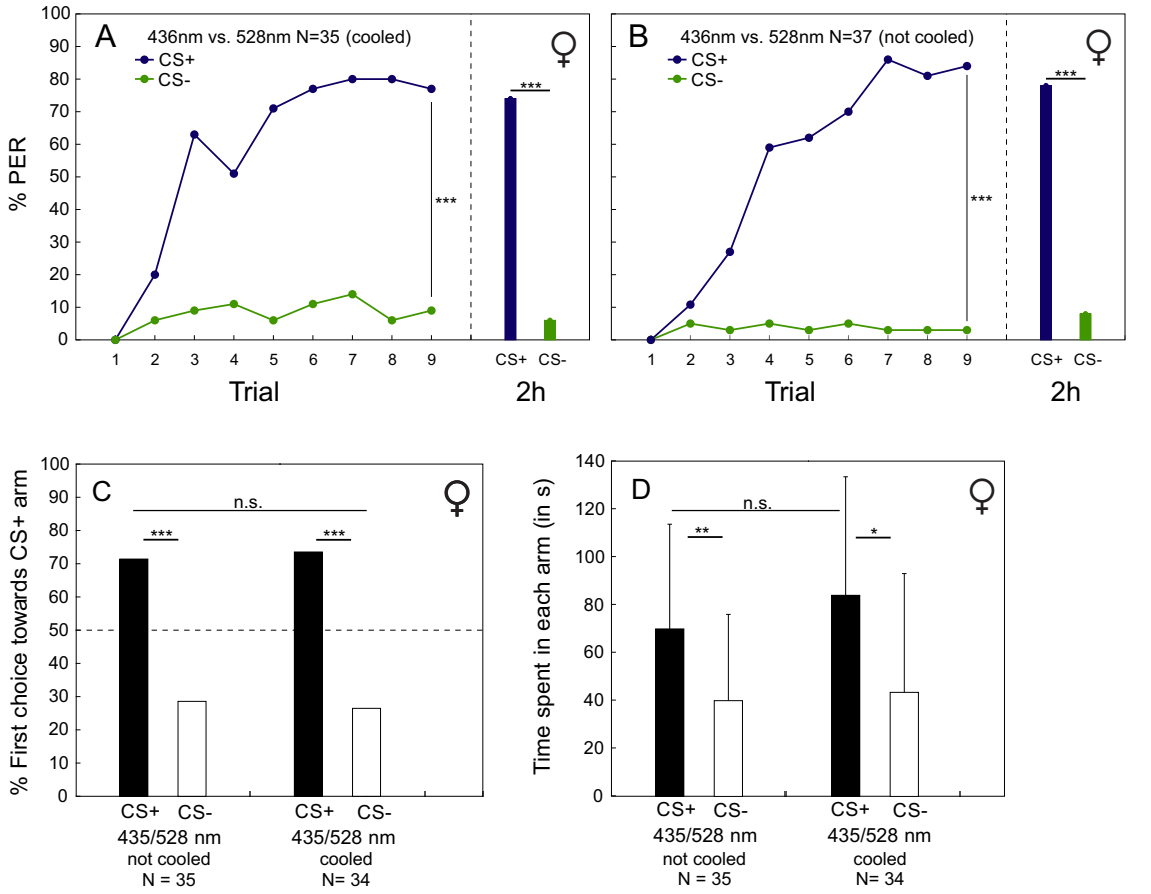

Figure A.6: Effect of cooling on information transfer. Workers were trained in differential PER conditioning (A, B) and 2 h later (after the mid-term memory test), tested for transfer of the learned color information to a novel operant free-moving situation (Y-maze; C, D). Two groups were tested: in one group bees were slightly chilled on ice right before their fixation was removed and they were transferred to the Y-maze, the other group of bees was not cooled at all. Discrimination index (n.s., Z=-0.228) and memory retrieval (response to CS+; n.s.; χ^2=0.167), as well as performance in the Y-maze (first decision: n.s., χ^2=0.038; duration of stay in the CS+ arm: n.s.; Z=-0.456) did not differ between treatment groups. From the results it can be concluded, that slightly cooling on ice before the transfer test had no significant effect on bee's choice behavior in the Y-maze.

A.1.4 Supplementary Results Chapter 6

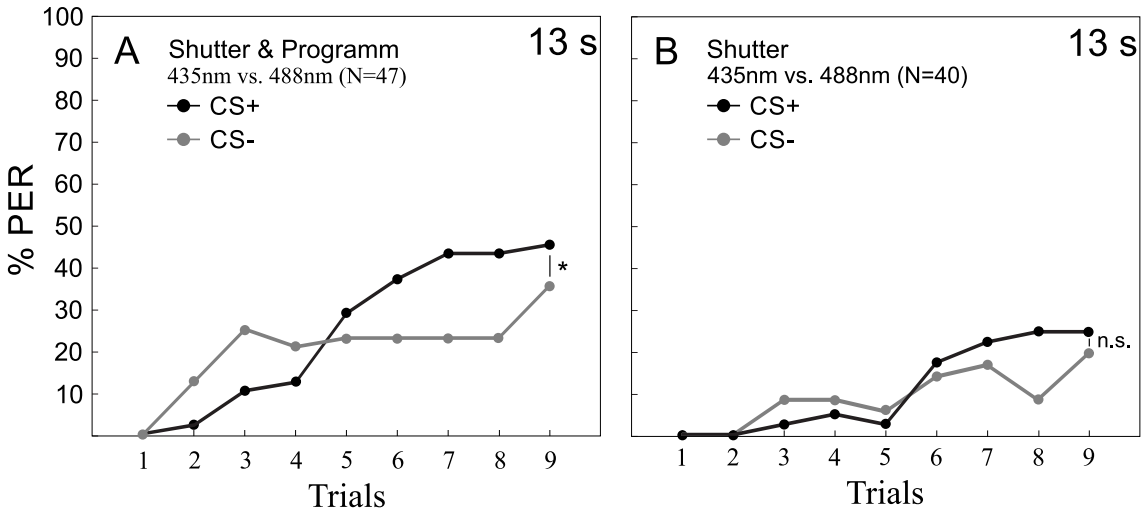

Figure A.7: Effect of the utilisation of the computer program TP on performance levels during differential visual PER conditioning. The learning performanceses of bees were significantly better when the program TP was used compared to a stop watch only. $*P < 0.01$

A.2 Supplementary Material and Methods

A.2.1 Used devices

Thermomixer comfort	Eppendorf, Hamburg, Germany
Mini spin	Eppendorf, Hamburg Germany
Mastercycler	Eppendorf, Hamburg, Germany
Eppendorf Biophotometer plus	Eppendorf, Hamburg, Germany
Centrifuge 5424R	Eppendorf, Hamburg, Germany
Centrifuge 5430	Eppenforf, Hamburg, Germany
Realplex2	Eppenforf, Hamburg, Germany
Vortex Genie 2	Scientific Industries, New York, USA
Tissuelyser LT	Quiagen, Hilden, Germany
Power Pac 300	Bio Rad, Hercules, USA
Gel separation system	Owl Scientific, San Francisco, USA
Gel iX20 imager	Intas Science imaging, Göttingen, Germany
Intas ECL Chemocam imager	Intas Science imaging, Göttingen, Germany
Kombischüttler KL-2	Edmund Bühler, Hechingen, Germany
µCuvette G1.0	Eppendorf, Hamburg, Germany
Leica MZ6	Leica Microsystems, Wetzlar, Germany
Leica VT1000 S microtome	Leica Microsystems, Wetzlar, Germany
Leica EZ 4d	Leica Microsystems, Wetzlar, Germany
Leica SPs AOBS	Leica Microsystems, Wetzlar, Germany
Power Pac 300	Bio Rad, Hercules, USA
Balance Universal U4800p	Sartorius, Darmstadt, Germany

A.2.2 Used devices

Adhesive Masterclear real-time PCR film	Eppendorf, Hamburg, Germany
Polyvinylidene fluoride/PVDF membrane	Merck, Darmstadt, Germany
Twin.tec PCR 96 well plates	Eppendorf, Hamburg, Germany
epT.I.P.S. PipetteTips	Eppendorf, Hamburg, Germany

A.2.3 Software

CorelDraw X7

ImageJ

IBM SPSS Statistics 20

R software v. 3.3.1

Eppendorf Mastercycler ep realplex software version 2.2

A.2.4 Chemicals

Amoniumpersulfat	Sigma-Aldrich, St. Louis, USA
6-Aminocaproic acid	Merck, Darmstadt, Germany
Acrylamide, Rotiphorese Gel40	Carl Roth, Karlsruhe, Germany
Chloroform	Carl Roth, Karlsruhe, Germany
Courmaric Acid	Sigma-Aldrich, St. Louis, USA
Glycin	Ajinomoto, Tokyo, Japan
Glycerin	Carl Roth, Karlsruhe, Germany
H_2O_2	Merck, Darmstadt, Germany
Luminol	Sigma-Aldrich, St. Louis, USA
2-Mercaptoethanol	AppliChem, Darmstadt, Germany
Methanol	AppliChem, Darmstadt, Germany
Milk powder	Carl Roth, Karlsruhe, Germany
Ponceau S solution	Sigma-Aldrich, St. Louis, USA
Roti garose NEEO Ultra-Qualität	Carl Roth, Karlsruhe, Germany
Sodiumdodecylsufate, SDS	Carl Roth, Karlsruhe, Germany
Tetramethylethylenediamine, TEMED	Carl Roth, Karlsruhe, Germany
Tris	Carl Roth, Karlsruhe, Germany
Triton-X 100	Sigma-Aldrich, St. Louis, USA
Urea	VWR, Darmstadt, Germany

A.2.5 Antibodies

spineless SSX2 (IG_P1008_X2_3), anti-rabbit	Immunoglobe, Himmelstadt, Germany
Alexa Fluor 568, anti-rabbit	MoBiTec GmbH, Göttingen, Germany
Horseradish Peroxidase (HRP), anti-rabbit	Jackson ImmunoReseach, Baltimore, USA

A.2.6 Molecular Protocols

RNA Extraction

Frozen tissues were transferred in 500 µl TRIzol Isol-RNA lysis Reagent (5Prime, Düsseldorf, Germany) and subsequently homogenized by means of steel beads and a tissuelyser (Quiagen, Hilden, Germany) for 3 minutes at 35 Hz. After a rest of 5 minutes at room temperature, 100 µl chloroform were added and samples were vortexed for 15 seconds. Following a further resting phase of 3 minutes, samples were centrifuged for 15 minutes at 4°C and 12,000 x g. Then the Gold HP total RNA Kit (Peqlab, Erlangen, Germany) and the manufacturer's protocol was used for further mRNA extraction.

Gel electrophoresis

To separate nucleic acids based on their size and charge, 1% agarose (in TBE) gels were loaded with mixture of 4 µl sample and 1 µl Midori Green (Nippon Genetics Europe, Düren, Germany) and placed in an electrophorese chamber with 130 mV for 30 minutes. Gels were subsequently analyzed by means of a Gel iX20 imager (Intas Science imaging, Göttingen, Germany). As reference marker the GeneRuler 100bp DNA Ladder/Plus (Thermp Fischer Scientific, Waltham, USA) was used.
Tris-borat-EDTA (TBE):

90 mM TRIS base

90 mM Boracic acid

2 mM EDTA-NA_2

Polymerase chain reaction (PCR)

To validate primers PCRs were performed by using the peqGold PCR Mastermix S (Peqlab Biotechnologie, Erlangen, Germany).

Reaction mix:

- 12.5 µl Taq DNA polymerase mix
- 6.5 µl H_2O
- 2 µl forward primer (10 µM)
- 2 µl reverse primer (10 µM)
- 2 µl template cDNA

Temperature cycling protocol:

1. T: 95°C t: 5 min
2. T: 95°C t: 1 min
3. T: 63°C t: 45 sec
4. T: 72°C t: 1min

Go to step 2 and repeat 35x

Hold on T: 4°C

Western blotting

Buffers:

Laemmli buffer:

125 mM Tris
6% Glycerin
2% SDS
10% Mercaptoethanol
0.004% Bromphenol blue, pH 6.8

10x chamber buffer:

250 mM Tris pH 8.3
1.9 M Glycin
1% (w/v) SDS

APS:

10% (w/v) ammonium persulfate

Separating gel buffer:

1.5 M Tris, pH 8.8
0.8% (w/v) SDS

Stacking gel buffer:

0.5 M Tris, pH 6.8
0.8% (w/v) SDS

Anode buffer I:

300 mM Tris base
20% (v/v) methanol, pH 10.4

Anode buffer II:

25 mM Tris-Base
20% (v/v) Methanol, pH 10.4

Cathode buffer:

40 mM 6-aminohexanoic acid
20% (v/v) Methanol, pH 7.6

TBST buffer:

0.1% (v/v) Tween 20
10 mM Tris-base
150 mM NaCl, pH7.5

Urea buffer:

2 M Urea
0.1 Glycin
1% (v/v) Triton-X-100

Polyacrylamide gels:

10% Acrylamide separation gel (for two gels):

3,0 ml Acrylamide

3.0 ml Seperation buffer

6.0 ml H2O

12 µl Temed

70 µl APS

6% Acrylamide stacking gel (for two gels):

0.45 ml Acrylamide

1.0 ml Separation gel buffer

2.55 ml H2O

4 µl Temed

30 µl APS

Structure of western blots:

Bottom:

4 filter papers soaked with anode buffer I

2 filter papers soaked with anode buffer II

PVDF membrane (equilibrated in Methanol for 5 minutes)

Polyacrylamide gel

Top:

6 filter papers soaked with cathode buffer

B

Appendix

B.1 Acknowledgments

Zum Schluss möchte ich mich ganz herzlich bei all den Menschen bedanken, die mich während meiner Promotion unterstützt und zu dem Erfolg dieser Arbeit beigetragen haben:

Lieber Johannes, es hat mich voller Stolz erfüllt, als du mir vor gut dreieinhalb Jahren die Stelle zu diesem sehr spannenden Dissertationsthema angeboten hast. Auch wenn aus fünf Minuten gerne mal eine Stunde wurde, du warst jederzeit bereit, mit mir über meine Ergebnisse zu diskutieren, neue Ideen zu entwickeln und anfallende Probleme zu lösen. Deine uneingeschränkte Begeisterung für Hummeln und andere Bienen hast du eins zu eins auf mich übertragen, ohne dich hätte ich wohl nie erfahren, wie faszinierend Bienen sind und wäre vermutlich nie zur Imkerei gekommen! Ich bewundere deinen Optimismus, sowie deine nie ausgehenden Ideen zu neuen wissenschaftlichen Projekten und bedanke mich ganz herzlich für die schöne Zeit und die gute Betreuung!

Charlotte und Axel danke ich für ihre Zeit und die Co-Betreuung meiner Arbeit. Außerdem danke ich Axel für die Möglichkeit in Indien in eine ganz andere Welt einzutauchen und mit asiatischen Honigbienen arbeiten zu dürfen.

Thomas für die Übernahme des Prüfungsvorsitzes meiner Arbeit und die netten Gespräche, die wir in den Hohen-Tauern während der Suche nach *Bombus gerstaeckeri* führen konnten.

Lieber Frank, danke für all deine Hilfe während der letzten Jahre und die guten Gespräche, die wir über Wissenschaft, Bienen, Gesellschaft und das Leben führen konnten. Mit deiner witzigen Art hast du mich auch an stressigen Tagen immer aufmuntern können!

Stefan, herzlichen Dank, für die Einführung in die Welt der Proteine. Dein molekularbiologisches und biochemisches Wissen ist beeindruckend.

Allen (ehemaligen) Mitgliedern der AG Spaethe für die entspannte Arbeitsatmosphäre und all die schönen und lustigen Abende, die wir zusammen verbracht haben. Auch wenn wir zwischenzeitlich nur zu zweit oder dritt waren, ich hätte mit keiner anderen AG tauschen wollen. Wie sagt man so schön: klein aber fein!

Wolfgang für die Möglichkeit an seinem Lehrstuhl nicht nur meine Masterarbeit, sondern auch meine Doktorarbeit schreiben zu dürfen.

Conny, du warst mir eine riesen Hilfe bei allen Immunfärbungen und all den Stunden, die wir gemeinsam am „Confocal“ verbracht haben, um die schönsten Bilder aufzunehmen und der Entwicklung der Bienenretina nach und nach auf die Spur zu kommen.

Karin und Tina für die Unterstützung bei der RNA Isolation und cDNA-Synthese unzähliger Proben; Sara für statistische Ratschläge und Markus für seine Arbeit als Hiwi.

Dirk, herzlichen Dank für die Einführung in die Imkerei, sowie für die Möglichkeit dem Laboralltag immer mal wieder für ein paar Stunden zu entkommen und dir bei deiner Arbeit mit den Bienen über die Schulter zuschauen zu dürfen.

Susanne, Petra und Irina für die Hilfestellung bei der Bewältigung bürokratischer Formalitäten.

Olli, Anette, Adrienne, Claudia, Markus, Martin, Daniela, ToM, Franzi, Steffi, Anne, Pauline, Flo, Jan, Mira, Tina, Ayse, Maren, Nils, Agustina, Nicole und allen anderen Mitgliedern der Zoo II für große und kleine Hilfestellungen und die netten Gespräche egal ob am Gang, auf gemeinsamen Konferenzen oder beim Mittagessen.

Lieber Matthias, mein Dank an dich lässt sich eigentlich nicht in Worte fassen! Durch die zahlreichen kleinen Programme, die du mir während der letzten Jahre geschrieben hast, hast du mir nicht nur den Laboralltag erleichtert, sondern auch maßgeblich zu dem Erfolg dieser Arbeit beigetragen! Du hast mir stets den Rücken freigehalten und mich immer in all meinen Entscheidungen unterstützt. Ich möchte mir gar nicht vorstellen, was ich all die Jahre meines Studiums und meiner Promotion ohne dich gemacht hätte!

Zu guter Letzt danke ich meinen Eltern, die nie an meinen Fähigkeiten zweifelten und mir entgegen aller Ratschläge anderer, nicht nur zum Abitur und einem universitären Abschluss, sondern nun auch noch zur Promotion verholfen haben. Wer hätte das gedacht?!

B.2 Curriculum Vitae

LEONIE LICHTENSTEIN

Department of Behavioral Physiology and Sociobiology, Zoology II
Biozentrum, University of Würzburg
97074 Würzburg, Germany

Scientific Education

since 10/2013	PhD thesis: "Color vision and retinal development of the compound eye in bees" Zoology II, Julius-Maximilians-University of Würzburg
09/2013	Degree: Master of Science at the Julius-Maximilians-University of Würzburg Master thesis: "Color vision and visual learning in the buff-tailed bumblebee, *Bombus terrestris*" (in german)
08/2011	Degree: Bachelor of Science at the Friedrich-Alexander-University Erlangen-Nürnberg Bachelor thesis: Isoform-specific expression analysis of the organizer protein Pericentrin in various cilia-bearing tissues (in german)

Research stay and Fellowships

12/2015	GSLS travel fellowship: 2. International symposium and workshop on "Frontiers in Insect Behavior" at the Arizona State University in Phoenix, Arizona, USA
07/2015	GSLS travel fellowship: 16th Congress of the" Society for Molecular Biology and Evolution" at the Hofburg, Vienna, Austria
07/2014	GSLS travel fellowship: 17th Congress of the "International Union for the Study of Social Insects" at the Convention Center, Cairns, Australia
01/2014	Research stay at the National Centre for Biological Science, Brockmann lab, Bangalore, India

Place, Date

Signature

B.3 Publications and statement of individual author contributions

Manuscript 1 (Chapter 2): **Lichtenstein L**, Grübel K, Spaethe J. (submitted). Temporal and circadian plasticity of opsin expression during pupal and adolescent compound eye development in the honeybee, *Apis mellifera*.

Contributions: L.L. and J.S. conceived and designed the experiments. L.L. collected the molecular data, L.L. and K.G. collected the confocal data. L.L. analyzed the data. L.L. wrote the original draft of the manuscript. L.L. and J.S. edited and finalized the manuscript. All authors participated in discussing the results and checking the paper.

Manuscript 2 (Chapter 4): **Lichtenstein L**, Sommerlandt FMJ, Spaethe J. (2015). Dumb and lazy? A comparison of color learning and memory retrieval in drones and workers of the buff-tailed bumblebee, *Bombus terrestris*, by means of PER conditioning. *PLoS ONE* 10(7):e0134248

Contributions: L.L. and J.S. conceived and designed the experiments. L.L. performed all experiments. L.L. and FMJS analyzed the data. L.L., FMJS and J.S. wrote the manuscript. All authors participated in discussing the results and checking the paper.

Manuscript 3 (Chapter 5): **Lichtenstein L**, Brockmann A, Spaethe J. (under review). Learning of monochromatic stimuli in *Apis cerana* and *Apis mellifera* by means of PER conditioning.

Contributions: L.L. and J.S. conceived and designed the experiments. L.L. performed all experiments. A.B. and J.S. contributed materials. L.L. wrote the original draft of the manuscript. L.L., A.B. and J.S. edited and finalized the manuscript. All authors participated in discussing the results and checking the paper.

B.4 Affidavit

I hereby confirm that my thesis entitled "Color vision and retinal development of the compound eye in bees" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date	Signature

B.5 Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation "Farbensehen und retinale Entwicklung des Komplexauges bei Bienen" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum	Unterschrift