ENVIRONMENTAL INFLUENCES ON THE DEVELOPMENT OF THE FEMALE HONEYBEE BRAIN *APIS MELLIFERA*

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Nothing makes sense in biology except in the light of evolution.

Theodosius Dobzhansky

Nothing makes sense in neuroscience except in the light of behavior.

Martin Heisenberg

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ABSTRACT

Olfaction plays an important role in a variety of behaviors throughout the life of the European honeybee. Caste specific, environmentally induced and aging/experience-dependent differences in olfactory behavior represent a promising model to investigate mechanisms and consequences of phenotypic neuronal plasticity within the olfactory pathway of bees. This study focuses on the two different female phenotypes within the honeybee society, queens and workers. In this study, for the first time, structural plasticity in the honeybee brain was investigated at the synaptic level.

Queens develop from fertilized eggs that are genetically not different from those that develop into workers. Adult queens are larger than workers, live much longer, and display different behaviors. Developmental trajectory is mainly determined by nutritional factors during the larval period. Within the subsequent post-capping period, brood incubation is precisely controlled, and pupae are incubated close to 35°C via thermoregulatory activity of adult workers. Behavioral studies suggest that lower rearing temperatures cause deficits in olfactory learning in adult bees. To unravel possible neuronal correlates for thermoregulatory and caste dependent influences on olfactory behavior, I examined structural plasticity of developing as well as mature olfactory synaptic neuropils.

Brood cells were reared in incubators and pupal as well as adult brains were dissected for immunofluorescent staining. To label synaptic neuropils, I used an antibody to synapsin and fluophore-conjugated phalloidin which binds to filamentous (F-) actin. During development, neuronal F-actin is expressed in growing neurons, and in the mature nervous system, F-actin is most abundant in presynaptic terminals and dendritic spines. In the adult brains, this double-labeling technique enables the quantification of distinct synaptic complexes (microglomeruli [MG]) within olfactory and visual input regions of the mushroom bodies (MBs) prominent higher sensory integration centers.

Analyses during larval-adult metamorphosis revealed that the ontogenetic plasticity in the female castes is reflected in the development of the brain. Distinct differences among the timing of the formation of primary and secondary olfactory neuropils were also revealed. These differences at different levels of the olfactory pathway in queens and workers correlate with differences in tasks performed by both female castes. In addition to caste specific differences, thermoregulation of sealed brood cells has important consequences on the synaptic organization within the MB calyces of adult workers and queens. Even small differences in rearing temperatures affected the number of MG in the olfactory calyx lip

regions. In queens, the highest number of MG in the olfactory lip developed at 1°C below the temperature where the maximum of MG is found in workers (33.5 vs. 34.5°C). Apart from this developmental neuronal plasticity, this study exhibits a striking age-related plasticity of MG throughout the extended life span of queens. Interestingly, MG numbers in the olfactory lip increased with age, but decreased within the adjacent visual collar of the MB calyx. To conclude, developmental and adult plasticity of the synaptic circuitry in the sensory input regions of the MB calyx may underlie caste- and age-specific adaptations and long-term plasticity in behavior.

INTRODUCTION

Sociality is one of the most successful animal lifestyles. Among insects, social hymenoptera are known for their highly evolved and elaborate colony organization. One of the key features of colony organization is division of labor (Wilson, 1971; Seeley, 1997; Winston, 1987; Hölldobler and Wilson, 1990). Division of labor is fundamental to the efficient functioning of insect colonies and is believed to be a major determinant of their vast ecological and evolutionary success (Wilson, 1987; Hölldobler and Wilson, 1990). The basis for division of labor seems to be the ability to evolve polyphenisms: the occurrence of morphological phenotypes accompanied by physiological and behavioral differences (Oster and Wilson, 1978). Phenotypic plasticity expressed by polyphenic species enables them to match morphology and physiology to environmental conditions (Moran, 1992).

Social insects, like honeybees, express highly plastic phenotypes. In honeybee societies, the queen and the worker phenotype arise from fertilized eggs that are not different from eggs that develop into workers (Shuel and Dixon, 1959). Sharing the same genome, honeybee queens live much longer and differ substantially from workers in anatomy, physiology, and behavior (Wilson, 1971; Michener, 1974; reviewed in Page and Peng, 2001). Plasticity in behavior most strikingly affects division of labor inside the colony and provides a colony performance that transcends the sum of individual contributions (Robinson, 1992; Gordon, 1996). The underlying neuronal plasticity is likely to be caused by environmentally induced differential expression of genes controlling morphogenesis and endocrine status. Honeybees, therefore, offer a favorable model system to investigate phenotypic plasticity of brain and behavior.

Essential for an understanding of the basis of complex behavior is a thorough examination of the nervous system of the given species. The nervous system processes and conducts relevant sensory information, ensuring the functioning and coordination of effectors. The response of the animal to inputs from peripheral sensory organs is produced and modified by the nervous system. However, the nervous system does not act as a simple relay between receptors and effectors. To mediate different types of behavior, the nervous system must integrate an enormous variety of functions. Activities of different parts of the body are coordinated so that appropriate behavioral responses and internal regulatory changes are made. This coordination involves alterations within the neural circuitry, which ultimately must be understood as specific changes in neuronal structures, membrane

properties and synaptic connectivity (Chapman, 1998; Shepherd, 1994; Kandel et al., 2000).

The structural and functional organization of the adult nervous system is shaped by external as well as internal influences during development and adult maturation. In honeybees, distinct phenotypes and environmentally induced neuronal plasticity represent one of the most striking examples of ontogenetic neuroplasticity. High genetic relatedness and the rich behavioral repertoire of honeybees, as in other social insects, have evoked correlations between environmental, age- and status-dependent effects and neuroanatomy (Durst et al., 1994; Fahrbach et al., 1995a; Gronenberg et al., 1996; Farris et al., 2001; Ehmer and Gronenberg, 2002). Detailed neuroanatomy is an essential basis to coincide changes in neuronal structure with brain function (Crick and Koch, 2003). Comparisons between the olfactory pathway in insects and vertebrates exhibit remarkable similarities of functional organization, physiology, and development. This indicates that olfactory information is processed through neuronal mechanisms that apply to vertebrates and invertebrates alike (Mombaerts et al., 1996; reviewed in Hildebrand and Shepherd, 1997; Boekh et al., 1990; reviewed in Hansson and Anton, 2000). Honeybees, as other insects, heavily rely on olfaction for communication inside their societies (e.g., von Frisch, 1967; Seeley, 1985) and are well known for their olfactory learning and memory capabilities (e.g., Menzel, 1993). Therefore, neuroanatomical analyses within the olfactory pathway of honeybees are experimentally favorable to investigate fundamental principles of olfactory information processing.

1 Environmental influences on juvenile development and adult maturation of female castes in the honeybee *Apis mellifera*

In social insects (termites and social hymenoptera), polyphenism refers to the occurrence of distinct morphological phenotypes caused by environmental conditions (Chapman, 1998; Nijhout, 1999; West-Eberhard, 2003). Environmental factors that influence caste determination include physical factors such as temperature, social factors such as behavioral interactions and pheromones released by colony members, and nutrition (Bloch et al., 2002a). Castes in social hymenoptera are the result of adult polyphenism and discrete alternative phenotypes are expressed without intermediate forms after the metamorphic molt (Nijhout, 2003). This caste determination may represent an example of

heterochrony (Wheeler, 1986), in which the reproductive roles are fixed during juvenile developmental stages (Weaver, 1957). Via environmentally induced differential expression of genes controlling morphogenesis, diploid female larvae can develop into either queens or workers (Evans and Wheeler, 1999). Within this reproductive division of labor, workers exhibit low reproductive potential, whereas queens are considerably more fecund (Wilson, 1971; Michener, 1974).

In honeybees, female larvae are decisively influenced by larval environment rather than by genetic predisposition to mature into queens instead of workers (Weaver, 1957; Evans and Wheeler, 1999). The mature queen lays fertilized eggs that develop into diploid female workers and unfertilized eggs that develop into haploid male drones, thereby controlling the sex of her offspring (Kerr, 1969; Michener, 1974; reviewed in Crozier, 1977; Seeley, 1985). The morphology of the queen is highly specialized for reproduction (Fig. 1). She possesses an enlarged abdomen containing 300 or more elongated ovaries, a large spermatheca and reproduces at high rates. Workers, which may number from tens to millions in a colony, are sterile performing all tasks related to colony growth and maintenance (Wilson, 1971). Besides their diverse reproductive status, queens and workers differ in their development time from egg to adult, longevity, physiology and behavior. These differences are trophogenic in origin, a result of differential food composition and feeding rates controlled by nurse bees during larval stages (reviewed in Page and Peng, 2001). In addition to larval feeding conditions, the developing time from egg into adult particularly depends on the high and constant rearing temperature close to 35±0.5°C during prepupal and pupal stages (Himmer, 1927a; Weiss, 1962; reviewed in Jay, 1963).

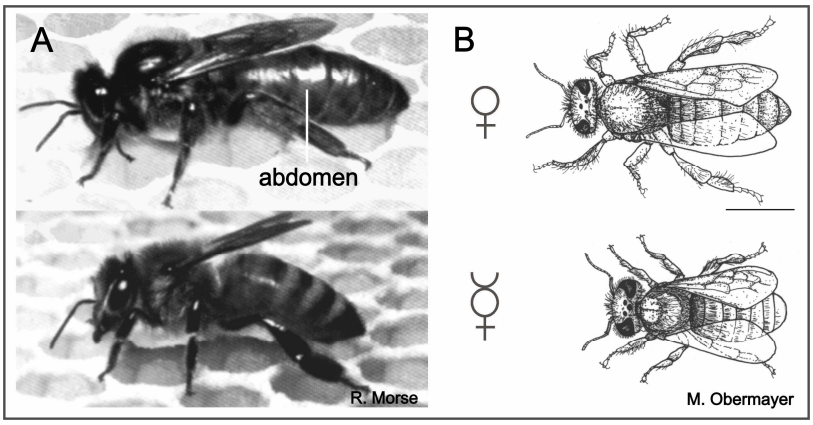

Fig. 1: A) and **B):** Considerable morphological differences exist between the honeybee queen (upper) and the worker (lower) that arise from environmentally induced differential gene expression. The queen is larger and exhibits a proportionately elongated abdomen equipped with numerous ovarioles. Scale bar in B: 5mm. ♀ : queen, ☿ : worker.

1.1 Feeding during larval development

In the honeybee *Apis mellifera*, as in other social insects, larval nutrition is believed to be a major determinant in female caste differentiation (Wheeler, 1986). During larval feeding, the honeybees undergo five molts to facilitate larval growth (Winston, 1987). Newly hatched female larvae have the potential to develop into either a queen or a worker (Fig. 2A). Their developmental trajectories are fated at the end of the third larval instar (Weaver, 1957; Shuel and Dixon, 1959). In particular, the quality and quantity of the food supplied by nurse bees during the residual larval period initiates the caste determination (Haydak, 1943; reviewed in Jay, 1963). At this critical time, queen destined larvae are continuously fed with mandibular gland secretions and show faster weight gains, primarily due to the quality and quantity of larval feeding (Weaver, 1957). In contrast, those larvae that receive large portions of hypopharyngeal gland secretions develop into workers (Liu and Jay, 1976; Chapman, 1998).

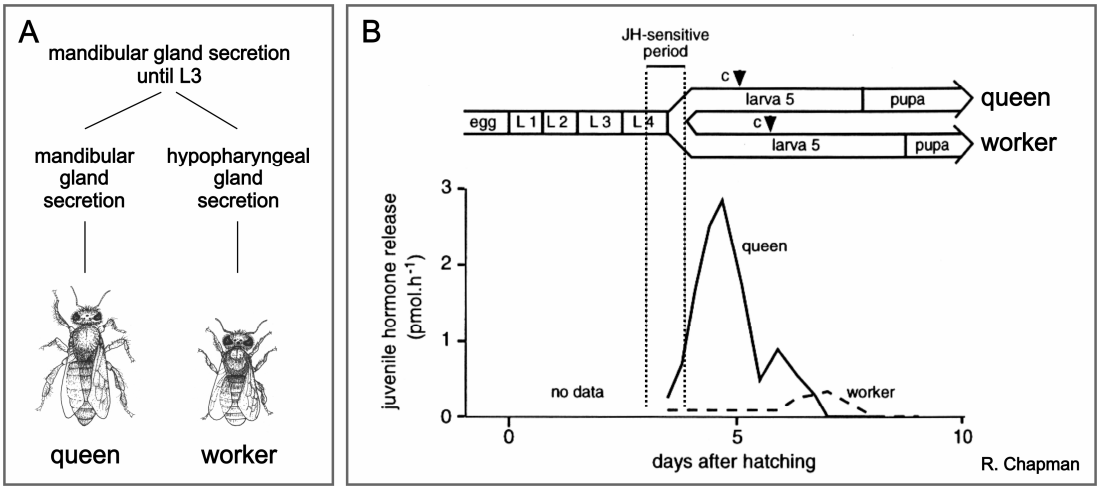

Fig. 2: Control of development of female honeybees. **A)** Female larvae are bipotent until L3 and can develop into either queens or workers depending on feeding conditions. Drawings from M. Obermayer. **B)** Juvenile hormone titers of queens and workers during metamorphosis. The development of larvae diverges in the fifth larval instar that coincides with a JH-sensitive period late in the fourth larval instar. c: cell capping, JH: juvenile hormone, L: larval instar (Chapman, 1998, p.400; after Rachinsky and Hartfelder, 1990).

The endocrine basis for caste determination in Hymenoptera is best understood in honeybees. The development of female larvae diverges after the third larval instar, proceeding under the regulation of juvenile hormone (JH) (Fig. 2B). JH is synthesized and released by the corpora allata, paired endocrine glands that are regulated by neurosecretory cells in the brain. Queen-destined larvae develop enlarged corpora allata (Dogra et al., 1977), have high JH titers (Rachinsky et al., 1990), and the differentiation of their

reproductive organs is well established during the fifth larval instar (Hartfelder and Engels, 1998). Titers of JH in worker-destined larvae are remarkably lower than those of queen-destined larvae (Rembold et al., 1992). Additionally, JH activates the production of ecdysteroids during caste differentiation. An increased ecdysteroids titer immediately after cell capping may act directly at the DNA level, regulating gene transcription that shifts the differentiation toward the queen pathway (Rachinsky and Hartfelder, 1995).

Rearing of queen-destined larvae occurs only in preparation for swarming or in the absence of the mature queen (Allen, 1965). As long as the mature queen is still fertile, her mandibular gland pheromones inhibit queen rearing. Adult workers contribute to the reproductive success of the present queen at the cost of their own reproduction and perform all tasks within the colony (Wilson, 1971). To provide this division of labor within the colony worker brood development must be ensured, especially in springtime, following swarming, or in the wake of heavy mortality from predation or disease (Seeley, 1985).

1.2 Thermoregulatory control during post-capping period

1.2.1 Key to social homeostasis

Honeybees are distributed nearly worldwide in a wide range of climates, despite their strong relatedness of behavior and physiologic functions to ambient temperature (Ruttner, 1978). Their nests serve as incubators for rearing brood and as protection from extreme ambient temperatures (Heinrich, 1993). To survive cold winters, the colony remains devoid of brood and a group of at least 2,000 adult bees assembles in a tight cluster on their combs (Fig. 3A; Southwick, 1984). This clustering is the key to social homeostasis (Gates, 1914; Emerson, 1956). Thermal stability in a winter cluster is based on cluster contraction, endothermic heat production by the core bees and insulation by the mantle bees (Winston, 1987; Stabentheiner et al., 2003). The core is maintained above 18°C and the mantel above 10°C, the vital minimum for honeybees (Free and Spencer-Both, 1960; Esch and Bastian, 1968; Seeley, 1985). These regulatory abilities are processed by their antennal thermal sense, which is sensitive to temperature differences of at least 0.2°C (Heran, 1952). Thermoregulatory control during the winter season exhibits low absolute temperatures and less precision compared with brood rearing during the summer.

1.2.2 Thermoregulatory control of developing prepupae and pupae

In the honeybee colony, the presence of brood is of major impact on high and precise thermoregulation. The developing brood is incapable of regulating its own temperature and is very sensitive to temperature change (Hess, 1926; Himmer, 1927a and 1932). The European honeybee possesses a most advanced system for temperature control within the center of the brood nest. In the summer, the sealed brood area (Fig. 3B), where worker prepupae and pupae develop, is continuously thermoregulated by adult workers. High temperatures within thermo-sensitive sealed brood cells are compensated for by wing fanning (Fig. 3C; Hazelhoff, 1954; Southwick and Moritz, 1987) and by water intake for evaporative cooling (Lindauer, 1954). Low ambient temperatures are raised by brood-heating individuals via endothermic heat production (Esch, 1960; Heinrich, 1981).

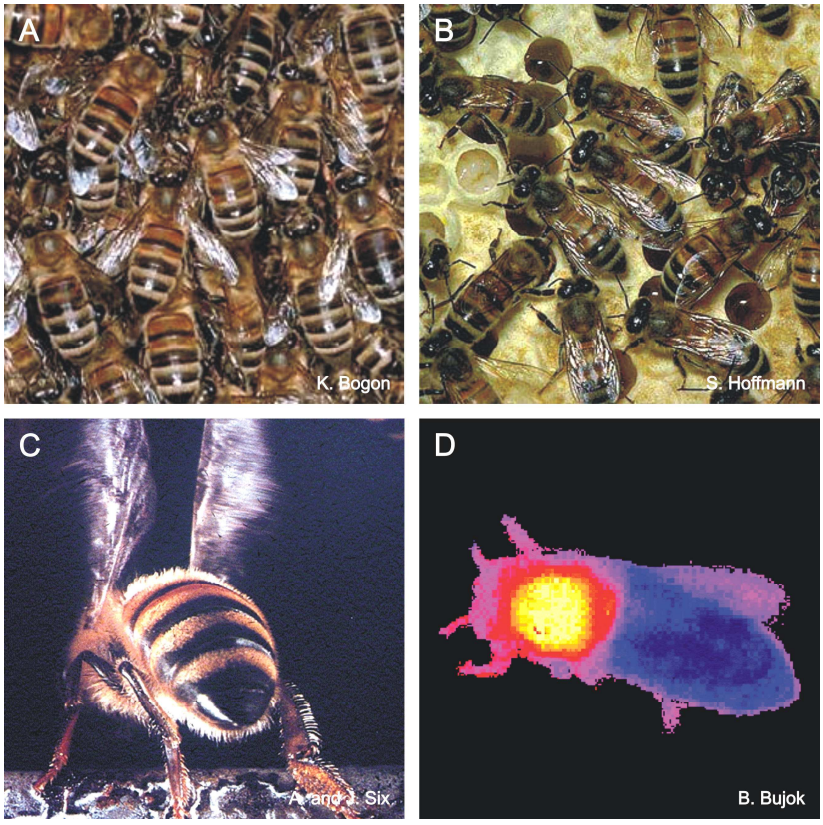

Fig. 3: Different strategies in temperature regulation are employed depending on whether or not the colony is rearing brood. **A)** During the winter season, the colony remains devoid of brood and adult bees crowd on their combs. **B)** In the summer, the capped brood area, where prepupae and pupae develop, is precisely thermoregulated by adult workers via **C)** wing fanning or **D)** endothermic heat production (thermographic recording from B. Bujok).

For queen rearing in queenless colonies or in preparation for swarming elongated cell cups are constructed by adult workers (Allen, 1965). These queen cells are located in the center

of the sealed brood area when ambient temperatures are low and on the periphery of the brood area when ambient temperatures are high (Degrandi-Hoffman et al., 1993).

Adult honeybees, like bumblebees and social wasps, are capable of endothermic heat production (Ishay and Ruttner, 1971; Vogt, 1986). They activate their flight muscles without wing vibration (Esch, 1960; Esch et al., 1991). For brood incubation, heat is transferred via the thorax to the surface of sealed cells and to their sidewalls by entering adjacent empty cells (Bujok et al., 2002; Kleinhenz et al., 2003). High thorax temperatures result from both, heating activities on the comb surface and warm-ups prior to cell visits. During long-duration cell visits, honeybees are able to generate thorax temperatures above 40.6±0.7°C (Fig. 4; Kleinhenz et al., 2003).

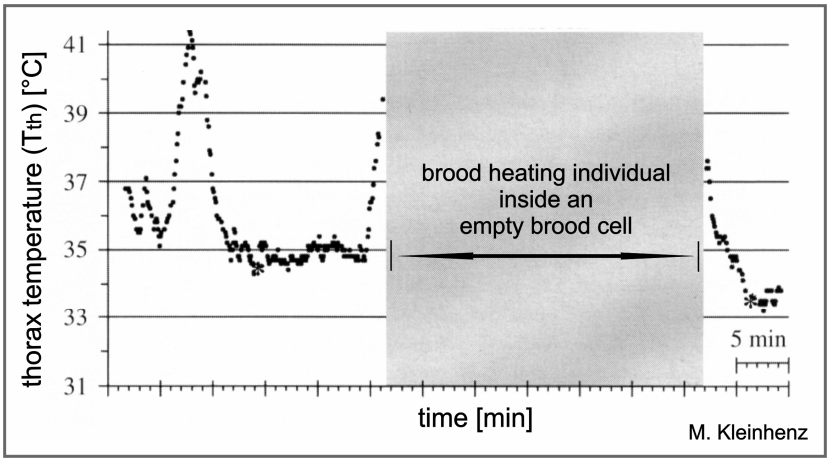

Fig. 4: Thorax temperature of a single brood heating individual on the surface of brood cells before and after visiting an empty brood cell. Thermographic temperature measurement of the thorax inside the observation cell could not be measured in the experiment (grey column). Note the warm-up prior to cell visit (Kleinhenz et al., 2003).

The capped brood is incubated close to 35±0.5°C during the entire post-capping period (Hess, 1926; Himmer, 1927a; Heinrich, 1993). The maintenance of this steady state temperature, which is just below the body temperature of humans, is essential for optimal development of honeybee prepupae and pupae (Southwick and Heldmaier, 1987). During pupal development, the pupae do not grow, but the muscles, the sensory and the nervous system undergo drastic changes into the adult form (Winston, 1987). Brood care, by providing energy through elevated and constant incubation, strikingly shortens the time required for total pupal development (reviewed in Jay, 1963). Besides the effect on the developing time, thermoregulatory control decreases the rate of morphological deficits, mortality and chalkbrood disease within the honeybee colony (Himmer, 1927a; Maurizio, 1934; Koeniger, 1978; Seeley, 1985). Interestingly, larvae survive temperatures that

deviate from the normal brood incubation temperature better than pupae (Himmer, 1927a; reviewed in Jay, 1963).

1.3 Behavioral performance in adults is influenced by the temperature experienced during post-capping period

The regulation of the microclimate inside the colony is an essential aspect of the behavior of honeybees. Air temperatures close to the brood combs are always in the range of 32 – 36°C (Hess, 1926; Himmer, 1927a; Southwick and Heldmaier, 1987; Heinrich, 1993; Kleinhenz et al., 2003). To maintain the center of the brood nest stable at 35±0.5°C, brood heating individuals require about 40% of the nectar collected within a colony per year (Tautz et al., 2003).

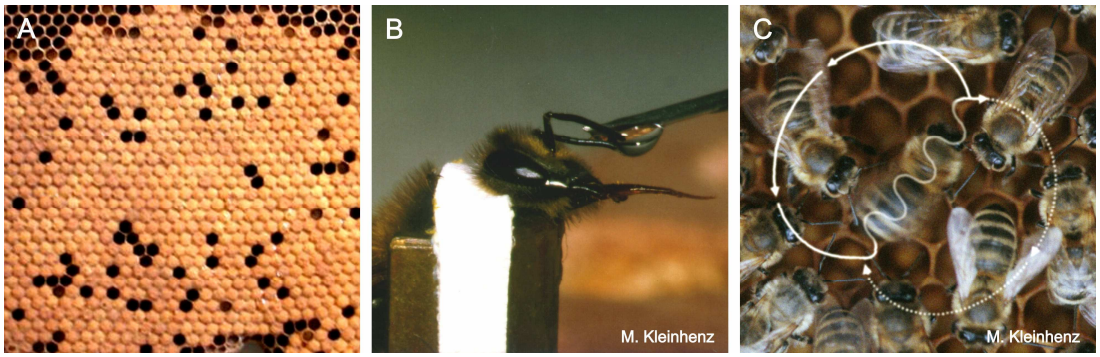

Fig. 5: A) Synchronized sealed worker brood comb. **B)** Bees learn to associate an odor with a reward of sucrose solution delivered to their antennae. They learn to respond to the odor despite the omission sucrose stimulation. **C)** The complex waggle dance of successful foragers communicates information about the distance, direction, and quality of resources.

Worker brood (Fig. 5A), reared within the wide temperature range that naturally occurs inside the hive, emerge with no obvious morphological deficits (Himmer, 1927a; Tautz et al., 2003). However, behavioral studies suggest that brood-temperature control during prepupal and pupal development has important consequences on behavioral performance during adulthood (Tautz et al., 2003). Learning abilities in the honeybee can be commonly tested via olfactory conditioning of the proboscis extension reflex (PER) (Takeda, 1961; reviewed in Menzel, 1993). The PER is elicited by a sucrose stimulation (Fig. 5B) and can be conditioned to odor stimulation. Bees learn to extend their proboscis to the mere presentation of the odor. Worker prepupae and pupae, reared on the lower end of the naturally occurring brood nest temperature range, perform as adults less well in this olfactory learning paradigm. Additionally, thermoregulatory control of capped worker

brood may determine the quality of performing their tasks outside the hive as foragers. Worker bees reared at lower temperatures, within the range of naturally occurring temperatures, showed less accurate waggle dance-performances (Fig. 5C; Tautz et al., 2003). Temperature-mediated effects on anatomical and physiological characters of the developing central nervous system (CNS) may cause these differences in both olfactory learning and dance communication (Groh et al., 2004).

2 Olfactory system of honeybees

2.1 Importance of olfaction related to communication and division of labor

The ability to detect odors is phylogenetically old and serves essential functions in vertebrates and invertebrates. Social insects rely heavily on the detection of odors to regulate the division of labor between the individuals within their colony (Wilson, 1971). Pheromones are subclasses of semiochemicals used for communication between individuals of the same species (Blum, 1985). In the honeybee colony, the queen, the workers, and possibly the drones produce pheromones in a variety of exocrine glands secreting to the external body tegument. These pheromones are important to recognize kin, provide brood care, defend the colony, navigate, locate mating partners, detect food sources and adapt foraging strategies to cope with variable colony demands and food availability (Wilson, 1971; reviewed in Ali and Morgan, 1990). Communication via pheromones exerts either releaser or primer effects on the recipient (Wilson and Bossert, 1963). Releaser pheromones elicit a behavioral response mediated by the nervous system, whereas primer pheromones physiologically alter endocrine and/or reproductive status (Vander Meer et al., 1998).

The honeybee queen secretes a complex blend of pheromones that can affect both the behavior and the physiological state of individual colony members (Free, 1987; Seeley, 1985). These pheromones are distributed throughout the colony as volatile odors or as non-volatiles spread through frequent antennation and food sharing activities of workers (reviewed in Butler, 1967; Free, 1987; Seeley, 1979 and 1985). This dispersal affects, in particular, the maintenance of the colony structure. The queen mandibular gland pheromone, serving a variety of functions, plays a key role in maintaining the colony structure (Fig. 6). Outside the colony, it attracts drones during queen mating flights and workers during swarming (Gary, 1962; Free, 1987; Schmidt et al., 1993). Within the

colony, it attracts a retinue of workers around the queen, prevents workers from queen rearing, inhibits worker ovarian development, and stimulates workers to forage (Jay, 1968; Winston et al., 1989; Schmidt et al., 1993).

Different castes produce different pheromones even from the same glands. While queens synthesize synergistic components, with 9-keto-2(E)-decenoic acid (9ODA) as the major component, in the mandibular glands, workers produce 2-heptanone as an alarm pheromone in the same gland (Winston, 1987; Winston and Slessor, 1992). The alarm pheromone alerts workers to danger and lowers the threshold of sensitivity for attack reactions.

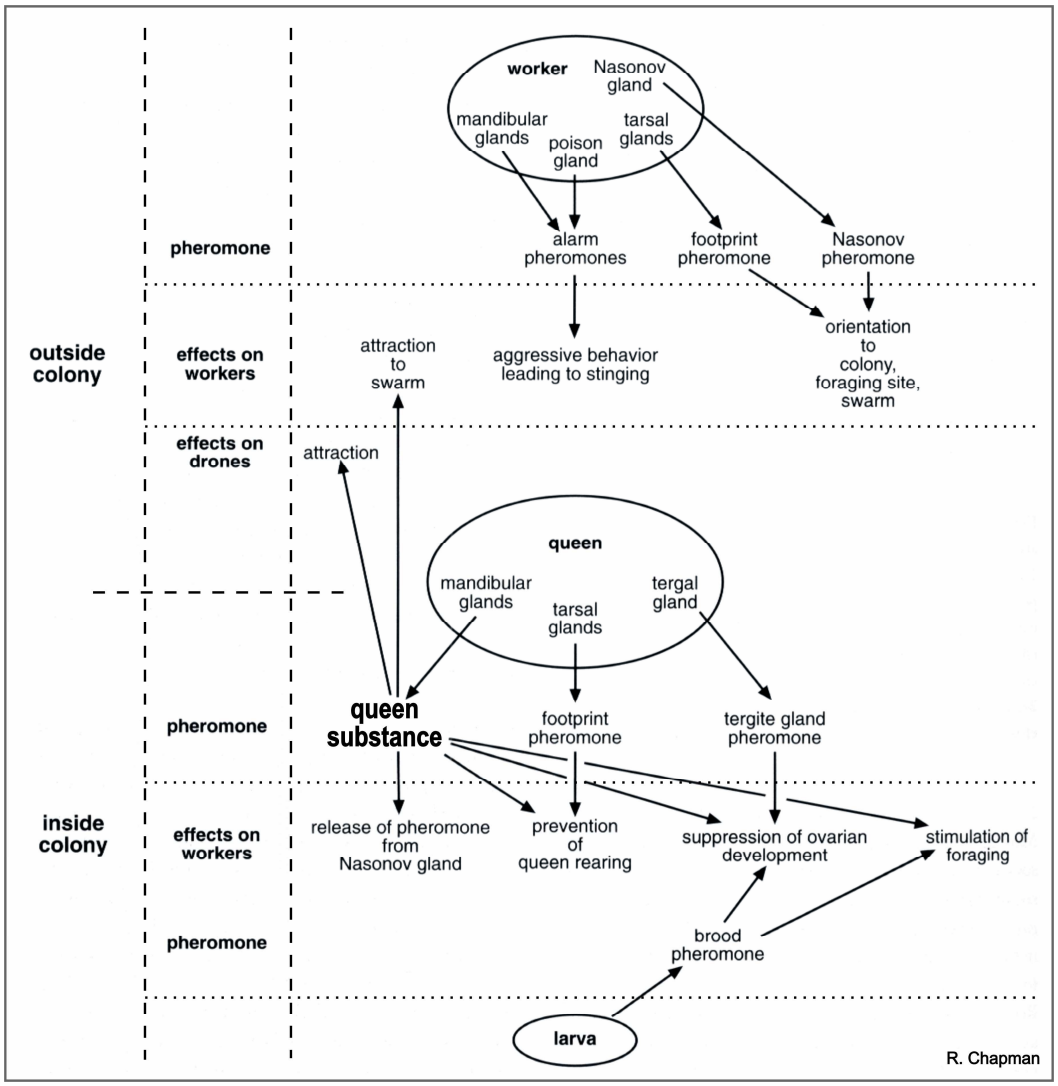

Fig. 6: Honeybees synthesize a variety of pheromones for chemical communication inside and outside their colony. The queen mandibular gland pheromone plays a key role in regulating the organization of social life and constantly advertises the presence of the queen (Chapman, 1998, p.724; after Winston, 1987; Smith and Breed, 1995; Vander Meer et al., 1998).

2.2 Olfactory pathway from the receptive organ to the mushroom body

The sense of smell contributes to generate an internal representation of the external chemical environment and is responsible for the detection of airborne molecules that initiate behavioral responses important for survival and reproductive status. The olfactory system is specialized to recognize small airborne molecules at low concentrations and to discriminate a remarkable number of distinct compounds. An understanding of the mechanisms that underlie this remarkable sensory capacity is a major objective for research in olfaction.

2.2.1 Common organizational and functional features of the olfactory centers in insects and vertebrates

Despite their different evolutionary origins, the olfactory systems of adult insects and vertebrates bear a striking resemblance regarding their morphological organization (Fig. 7; reviewed in Shepherd, 1972; Boekh et al., 1990; Hildebrand, 1995; Hildebrand and Shepherd, 1997). In both systems, the olfactory receptor neurons (ORNs) are primary sensory cells that detect and discriminate odorant molecules and convert the chemical signals into electrical impulses. These ORN axons convey this encoded olfactory information via the antennal/olfactory nerve to the antennal lobe (AL)/olfactory bulb (OB), the primary olfactory centers. Within the AL and OB, ORN axons project an enormous input on a comparatively small number of neurons (Hildebrand and Shepherd, 1997; Schild, 1988). The ORN axons converge on local interneurons (LINs) and projection neurons (PNs) in insects and on periglomerular neurons and mitral/tufted cells in vertebrates. These synaptic interactions are restricted to distinct spheroidal neuropil areas called olfactory glomeruli (e.g., Mombaerts et al., 1996; Hildebrand and Shepherd, 1997; Boekh et al., 1990; reviewed in Hansson and Anton, 2000). Glomerular borders formed by glial cells and/or periglomerular neurons separate these areas of high synaptic density, at least partially, from one another (Tolbert and Oland, 1990; Gonzales et al., 1993; Treolar et al., 1999).

Convergent evidence indicates that olfactory glomeruli represent functional and structural units essential for processing olfactory information (Masson and Mustaparta, 1990; Mombaerts et al., 1996; Hildebrand and Shepherd, 1997; Xu et al., 2000). Each glomerulus receives input from ORN axons that express the same receptor type, and the projection of

the ORN axons to the AL/OB has been visualized (Mombaerts et al., 1996; Vosshall et al., 2000). The emerging olfactory sensory map shows a strict topology of the glomeruli. Their odor specific spatio-temporal excitation patterns appear to be crucial for encoding olfactory information in insects as well as in vertebrates (Joerges et al., 1997; Galizia et al., 2000; Meister and Bonhoeffer, 2001).

To provide further olfactory information processing, the primary olfactory centers of invertebrates and vertebrates are connected with higher order neuropils. PNs and mitral cells extend their axons to, e.g. the protocerebral areas in insects and the olfactory cortex in vertebrates (see also Fig. 7). In the CNS of *Drosophila melanogaster*, changes in odorant-evoked intracellular calcium concentrations in selectively labeled olfactory PNs were visualized both at their postsynaptic site within the AL and at their presynaptic site in the protocerebral MB calyx using optical imaging techniques (Fiala et al., 2002). Fiala et al. showed that calcium imaging with a genetically encoded fluorescence probe is feasible in an insect brain *in vivo*.

The morphological resemblance between olfactory systems in insects and vertebrates mirrors similar capacities for detecting, discriminating and processing olfactory signals, suggesting that general mechanisms may be involved (Boekh et al., 1990; Hildebrand and Shepherd, 1997).

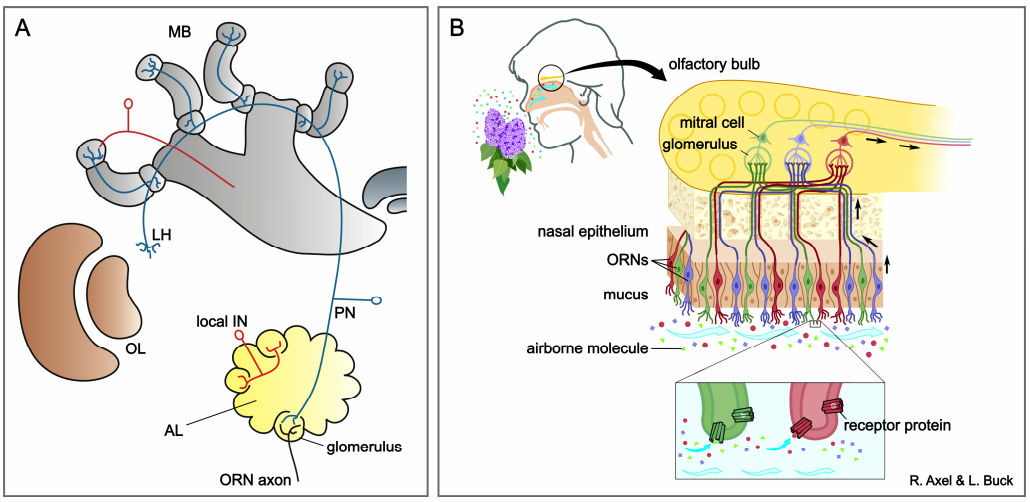

Fig. 7: Diagrammatic representations compare the olfactory pathways of **A)** honeybees and **B)** mammals (not drawn to same scale). In both cases, the first order olfactory neuropil is characterized by an array of glomeruli that receive axonal projections of ORNs. **A)** Within the glomeruli of bees, ORN axons form synapses with central neurons, e.g. local INs and PNs that project the information to the mushroom bodies and the lateral horn. **B)** ORN axons synapse primarily onto olfactory PNs (mitral/tufted cells), and local INs within the glomeruli of vertebrates. Mitral cell axons project via the lateral olfactory tract to higher olfactory centres (olfactory cortex). AL: antennal lobe, IN: interneuron, LH: lateral horn, MB: mushroom body, OL: optical lobes, ORN: olfactory receptor neuron, PN: projection neuron.

2.2.2 Olfactory pathway from the receptor neurons to the mushroom bodies

In the honeybee *Apis mellifera*, the paired antennae are most important multimodal sensory organs. Each antenna consists of a basal scapus, a pedicellus and a distal flagellum that bears a variety of sensory receptors. Beside many other receptor types, the worker flagellum is equipped with 2,600 olfactory sensilla (receptor structures), the sensilla placodea (Esslen and Kaissling, 1976). These sensilla consist of a thin oval cuticular plate perforated by numerous pores and dendrites from 15 – 30 ORNs branch beneath each plate (Schneider und Steinbrecht, 1968). Altogether, 60,000 ORNs reside in the receptor epithelium (Esslen and Kaissling, 1976). Their axons, originating from cell bodies in the antenna, provide olfactory input to the AL via four tracts (T1 – T4) (Suzuki, 1975; Mobbs, 1982; Abel et al., 2001). Single ORN axons target single glomeruli (Fig. 8, left) residing in the ipsilateral AL (reviewed in Homberg et al., 1989; Hansson and Anton, 2000).

The sensory axons synapse mainly on two categories of AL neurons, 4,000 local INs (Witthöft, 1967) and about 800 PNs (Bicker et al., 1993). The local INs exclusively branch within the AL (Flanagan and Mercer, 1989a), whereas the PNs ramify within the AL and send their axons to the mushroom bodies and the lateral protocerebrum (reviewed in Hansson and Anton, 2000). The AL of a worker contains from 156 to 166 glomeruli arranged in a single layer and clusters of cell bodies at the periphery (Arnold et al., 1985; Galizia et al., 1999). According to their size and relative position within the AL arrangement, glomeruli can be individually identified (Flanagan and Mercer, 1989b; Galizia et al., 1999). Axonal fibers of PNs convey the output information from the AL to various target areas in the protocerebrum via three separate tracts (Mobbs, 1982). Uniglomerular PNs of the medial as well as the lateral antenno-cerebral tract (m- and l-ACT; Fig. 8, right) that receive input from one glomerulus project information to the mushroom body (MB) calyces and the lateral protocerebrum (LP). These tracts innervate both target areas in reverse order and differ in their branching patterns within the MB calyces (Menzel et al., 1994; Abel et al., 2001). Multiglomerular axons of the mediolateral (ml) ACT project to the lateral horn and/or the ring neuropil of the alpha-lobe (Fig. 8, left; Abel et al., 2001).

The paired MBs, the most prominent neuropil structures in the dorsal protocerebrum, are involved in higher order computations such as learning and memory (e.g., Erber et al., 1980; Heisenberg et al., 1995; Strausfeld et al., 1998; Liu et al., 1999; Zars et al., 2000; Menzel, 2001). The cellular organization of the MBs, first described for the honeybee, is

similar in all neopteran insects (Strausfeld et al., 1998). Each MB consists of a pair of cup-shaped calyces and peduncles, as well as an α- and β-lobe (Mobbs, 1982). This characteristic architecture results mainly from the parallel arrangement of about 170,000 intrinsic Kenyon cells (KCs) (Kenyon, 1896; Witthöft, 1967). KC dendrites receive different sensory inputs within their respective calyx subdivisions referred to as the lip, collar and basal ring, whereas KC axons project the information via the peduncle and bifurcate into the α- and β-lobe (Mobbs, 1982). Extrinsic neurons supply both input to and output from the KCs. Two prominent groups of MB extrinsic neurons provide information from primary sensory centers. Uniglomerular olfactory PNs from the AL transfer olfactory input via the m- and l-ACT to the calyx lip as well as the basal ring (Fig. 8, right). Axons of these PNs are restricted to the ipsilateral side of the brain (Abel et al., 2001, Gronenberg, 2001). The visual system is represented in the calyx by axonal terminals of visual PNs originating from the ipsilateral lobula and both the ipsi- and contralateral medulla (Gronenberg, 2001). Projections from these optic lobes provide input mainly via the anterior superior optical tract to the basal ring and the collar (Gronenberg, 2001). The calyx also receives processes from other extrinsic neurons considered to have an inhibitory or modulatory function (Schürmann, 1987; Ganeshina and Menzel, 2001).

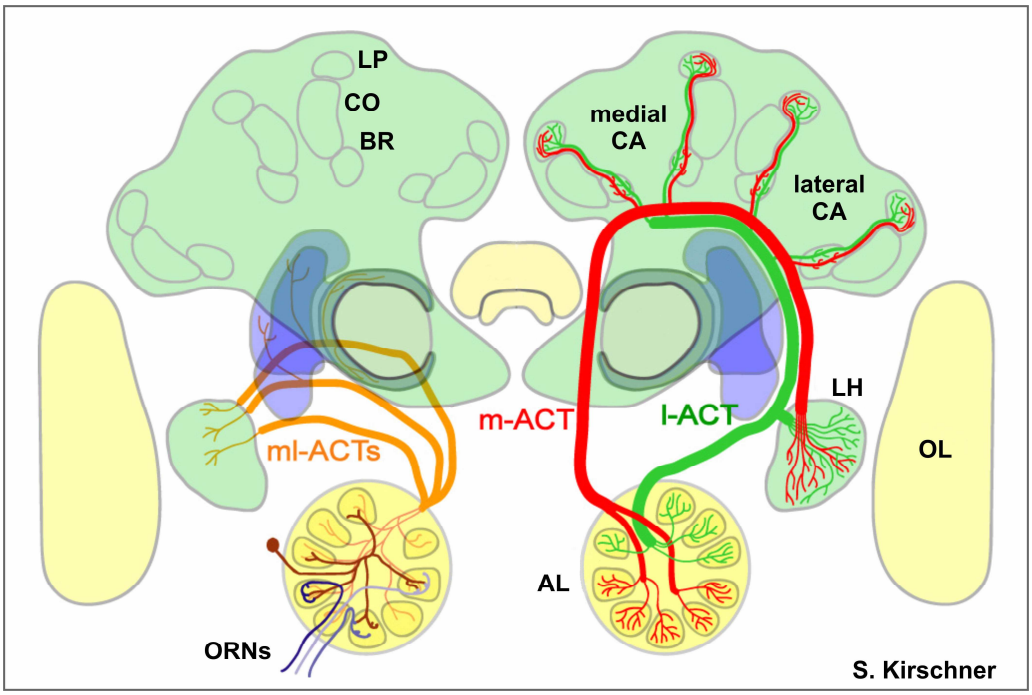

Fig. 8: The diagram shows the innervation pattern of the ml-ACTs (left) and of the m-ACT and l-ACT (right). ACT: antenno-cerebral tract, AL: antennal lobe, BR: basal ring, CA: calyx, CO: collar, LH: lateral horn, LP: lip, OL: optic lobe, ORN: olfactory receptor neuron. After Kirschner, 2005.

2.3 Neuronal plasticity within the antennal lobes and mushroom bodies of adult workers

A striking property of the mature CNS is the ability to adapt structural and functional features to the input received during lifetime. Studies in both vertebrates and invertebrates have contributed toward an understanding of neuronal plasticity (for reviews see Bailey and Kandel, 1993; Kolb and Whishaw, 1998; Meinertzhagen, 2001). In the mammalian brain, postnatal changes in the neuronal complexity correlate with increasing sensory or motor experience. For example, in the rat cerebellar cortex an increase in cortical weight and thickness is associated with exposure to an enriched environment (Diamond et al., 1972). These changes often correspond directly with increased spine density (Knafo et al., 2001) and dendritic length (Greenough and Volkmar, 1973).

In insects, as well as in vertebrates, long-term environmental and behavioral modifications affect the morphology and size of the adult CNS. Honeybee workers provide an excellent model system to investigate structural modifications of the mature brain. They perform a variety of tasks depending on age, a phenomenon referred to as age polyethism (Lindauer, 1953; Seeley, 1978). During their adult life, workers undergo two major phases (first nurses and second foragers) that are vastly different in sensory input and motor activities. Nurses rely extensively on olfactory inputs to fulfill their duties inside the hive, whereas foragers must integrate olfactory and visual stimuli. This age-related behavioral shift is typically highly stereotyped, but also plastic in that workers are able to alter their activity in response to changing colony demands (Winston, 1987; reviewed in Robinson, 1992). Several studies have addressed age- and experience-related changes in the worker brain. Volume changes in the ALs (Withers et al., 1993; Winnigton et al., 1996; Sigg et al., 1997) and most notably in the MBs (Withers et al., 1993; Durst et al., 1994; Farris et al., 2001) correlate with the transition from nursing to foraging behavior. Evidence obtained from observations in bees (e.g., Erber et al., 1980; Menzel, 1993) and *Drosophila melanogaster* (reviewed in Heisenberg, 1994) indicate that the MBs play an essential role in the neuronal control of complex behavioral adaptations.

3 Formation of the antennal lobes and the mushroom body calyces during larval-adult metamorphosis in honeybees

The function of the mature nervous system relies on the formation of precise neuronal connections among its constituents during larval-adult metamorphosis. Neurometamorphosis includes modifications of the central and peripheral nervous system, at the neuromuscular junctions and in the muscles themselves (Truman, 1992) and involves extensive reorganization of neuronal processes and synapses, neurogenesis, and cell death (Truman, 1990; Levine and Weeks, 1990; Fahrbach and Weeks, 2002).

In honeybees, as in other holometabolous insects, the fundamental structure of the brain ganglia differentiates into proto- and deutocerebrum during embryogenesis (reviewed in Reichert and Boyan, 1997). The larval CNS undergoes extensive reorganization during the post-capping period to provide structural and functional modifications at all levels of adult behavioral control (Farris et al., 1999; Schröter and Malun, 2000). In workers, the post-capping period proceeds over two prepupal and 9 pupal stages (P1 – P9), each lasting approximately one day. This metamorphic adult development is faster in queens and proceeds over 7 days, consisting of one prepupal and 6 pupal stages.

3.1 Development of olfactory glomeruli in workers

ORN axons invade the AL from late larval and prepupal stages onward (reviewed in Masson and Mustaparta, 1990; Hähnlein and Bicker, 1997). They are bundled in three tracts projecting to different peripheral areas. At P3, the axonal terminals aggregate first into glomerular precursor structures named preglomeruli (in *Manduca*: protoglomeruli). These neuropil areas might neither overlap with processes of central neurons nor contain any synaptic input (Gascuel and Masson, 1991). From P7 onward, the ORN axonal terminals concentrate in the cortical layer of the mature glomeruli. Deafferentiation experiments in insect species revealed that ORN axons play a crucial role in glomerulus formation (e.g., *Manduca*: Oland and Tolbert, 1987; *Drosophila*: Rodrigues and Pinto, 1989; *Apis*: Gascuel and Masson, 1991). In the absence of ORN axons, the AL exhibits a reduced total volume and lacks the characteristic compartmentalization into glomeruli.

Dendritic arborizations of olfactory PNs already contribute to the homogeneously structured AL neuropil at P1. They are predominantly restricted to a peripheral layer of the AL in which they occupy several lobed regions (Schröter and Malun, 2000). These regions

may represent precursor structures of the developing glomeruli, because glomeruli will arise there in subsequent stages (Hähnlein and Bicker, 1997; Schröter and Malun, 2000). The center of the AL neuropil contains PN processes and local INs without fine arborizations and, therefore, appears darker than the peripheral layer. At P3, the olfactory PNs bear distinct dendritic tufts, coincident with the first appearance of preglomeruli (Hähnlein and Bicker, 1997). These tufts of PNs appear to branch where adult glomeruli are located and gaps between them indicate the borders between the emerging glomeruli. From P4 onward, the dendritic tufts exhibit a glomerular innervation pattern similar to that of olfactory PNs in the adult AL. The formation of the AL neuropil is adult-like at P7 (Schröter and Malun, 2000).

3.2 Formation of mushroom body calyces in workers

In the prepupa, the MB calyx is detectable as the calyx-developing-zone that exhibits no subdivisions (Menzel et al., 1994). From P1 onward, olfactory and visual PNs innervate strictly separated synaptic target areas within the calyx-developing-zone. The arrangement of the dorsal area, innervated by olfactory PN fibers, and the neighboring area, innervated by visual PNs, resembles the arrangement of the mature MB calyx. At P2, the lip and the collar are clearly visible and the PN terminals start to form an inwardly directed knob pointed toward the center of the calyx (Schröter and Malun, 2000). The basal ring arises at P4 (Farris et al., 1999). During the subsequent stages, the MB subunits continuously increase in size and adopt their mature shape. Taken together, olfactory PN terminals arrange their adult arborization pattern within the lip, their main target area, earlier than their dendritic processes form dense glomerular tufts within the AL (Schröter and Malun, 2000).

Chemical ablation of MB neuroblasts performed in *Apis* workers (Malun, 1998) and in *Drosophila* (de Belle and Heisenberg, 1994; Stocker et al., 1997) indicates that KC dendrites might be essential for the proper target finding of ingrowing extrinsic neuron terminals. In the absence of KCs, olfactory PNs omit the outgrowth of axon collaterals into the MB calyces. During normal postembryonic development, neurogenesis of KCs lasts from the first larval instar (Malun, 1998) to P4 (Ganeshina et al., 2000). Subpopulations of KCs emerge from distinct neuroblast clusters at different periods of larval-adult metamorphosis. The non-compact KCs forming the lip and the collar arise during the larval stage. They migrate to the periphery from P2 onward when the inner compact KCs that

form the basal ring emerge. Neurons that are born first are also most likely the first to extend dendrites and form their particular calyx compartment (Farris et al., 1999).

3.3 Role of glial cells for brain development

In the mature CNS, neuronal branching patterns that characterize the organization of neuropils also extend to the arrangement of glial cells within the nervous tissue. Beside their ability to support mature neurons, glial cells play an active role during the development of the CNS. Glia-axon interactions have bee shown to be a key factor in neuronal pathfinding and the formation of neuropil structures during insect brain development (e.g., Bastiani and Goodman, 1986; Jacobs and Goodman, 1989; Tolbert and Oland, 1989; Rössler et al., 1999).

In the CNS of the honeybee, multiple layers of glial cell bodies surround the differentiating neuropils from mid-larval instars onward. After pupal ecdysis, the AL as well as the MB calyx neuropil, both still homogenously textured, start to differentiate in the absence of intrinsic glial somata. During subsequent neuropil expansions and involved refinement of their architecture, glial cells penetrate along neuronal fibers from the periphery into defined locations of their target neuropil. As shown in *Manduca,* it is proposed for honeybees that glial cells can prepattern the borders of developing neuropils (Hähnlein and Bicker, 1997).

The analysis of glial cell development in deafferented developing ALs provides insight into whether ORN axons provide signals to the glial cells, triggering their characteristic changes during glomerulus formation. In *Manduca*, glial cells develop along the normal time course, independent from the arrival of ORN axons (Oland and Tolbert, 1987). However, they are unable to send processes into the AL neuropil and remain in a smooth rind around its perimeter. In the bee, glial cells no longer extend processes into the AL neuropil if they are deprived of access to ingrowing ORN axons (Gascuel and Masson, 1991). Additionally, experiments with glia-deficient ALs, most notably established in *Manduca*, revealed that glial cells play an active role in the process of glomerulus development. ORN axons still coalesce into protoglomeruli (preglomeruli in *Apis*), but they subsequently disintegrate if glial cells are lacking to stabilize their borders (Tolbert and Oland, 1990, Baumann et al., 1996). In *Apis*, so far, it is technically not possible to remove glia cells in the ALs during embryogenesis.

3.4 Temperature effects neuropil formation during metamorphosis in other insects

In contrast to general effects of temperature on embryonic growth or the duration of postembryonic development, more specific effects on the maturation of the metamorphosing nervous system rarely have been investigated (Chapmann, 1998). Recently, a study conducted by Zhong and Wu (2004) in *Drosophila melanogaster* demonstrated that terminal arborizations of motor neurons at larval neuromuscular junctions exhibit a temperature-dependent developmental plasticity of axonal outgrowth. In this case, structural changes in neuronal processes were mediated via changes in neuronal activity linked to the cAMP pathway (Zhong and Wu, 2004). In developing pupae of *Manduca sexta*, temperature gradients influence proper AL formation by affecting neuron-glia interactions and axon pathfinding (Rössler et al., 2000). Temperature effects could also be mediated via changes in neurosecretory systems. Electrophysiological recordings in the locust's *pars intercerebralis* neurosecretory cells showed that the firing probability of these cells was affected by temperature, which could affect the release of neuropeptides in the neurohemal organs (Rössler and Bickmeyer, 1993). In lower vertebrates, temperature manipulations during embryonic development affect sex determination and adult aggressive behavior, most likely mediated by changes in sexually dimorphic brain nuclei (Rhen and Crews, 1999; Crews, 2003). In mammals, slight temperature increases during embryonic development were shown to have profound effects on the nervous system (Edwards et al., 2003).

4 Postsynaptic actin and neuronal plasticity

Neuronal networks need to balance steady state and dynamic conditions. Established synaptic connections within the network must be stable enough to support reliable signal transmission, but the system must be constantly motile to accommodate changes in synaptic connectivity that are essential for behavioral adaptations to sensory experience. Research over the last decades suggests that the neuronal cytoskeleton organizes morphological plasticity in developing and mature neuronal circuits. Microfilaments and microtubules guide and support the growth, as well as the differentiation process, of neurons. Whereas motile actin filaments arrange the exploratory activity of growth cones as they respond to external guidance cues, microtubules provide morphological stability to newly established processes (reviewed in e.g., Mitchison and Kirschner, 1988; Avila et al.,

1994; Heidemann, 1996). In the mature brain, a parallel between both cytoskeletal filament systems may persist in neuronal dendrites. The dendritic cytoskeleton consists of separate microtubule and microfilament domains associated with dendritic shafts and spines, respectively. While microtubules are dominant in dendritic shafts (Peters et al., 1976; Matus et al., 1983) they are absent from spines that contain a meshwork of microfilaments (Peters et al., 1976; Landis and Reese, 1983).

4.1 Rapid actin-based plasticity in dendritic spines

At the end of the 19th century, Ramón y Cajal showed that tiny spines ("espinas") protrude from the main shaft of Purkinje cells' dendrites (Ramón y Cajal 1891 and 1899). In 1959, electron microscopy revealed that dendritic spines are the site of synaptic contacts (Gray, 1959). Due to their strategic location as bridges between axons and dendrites (Fig. 9A), they can serve as basic functional units in neuronal integration (Harris and Kater, 1994; Yuste and Denk, 1995; Shepherd, 1997).

Dendritic spines are micromillimeter-sized specializations that are extremely rich in actin (Fifkova and Delay, 1982; Matus et al., 1982; Cohen et al., 1985; Kaech et al., 2001). High actin concentrations at cell margins often correlate with dynamic changes in cell shape (e.g., reviewed in Cooper, 1991; Bray, 1992). In the mammalian hippocampus, time-laps imaging of living neurons expressing actin tagged with green fluorescent protein (GFP-actin) visualized spontaneous, actin-based shape changes in dendritic spines (Fischer et al., 1998). Rapid spine motility in acute and cultured brain slices corroborates this observation (Dunaevsky et al., 1999). Activity-induced modifications to spine anatomy most likely involve cytoskeletal reorganization. In dissociated neurons, stimulation of glutamate receptors and increasing afferent activity alters the actin network in spines (Fischer et al., 1998; reviewed in Matus, 2000; Ackermann and Matus, 2003). Recent work showed that polymerization of actin in synaptic spines after high frequency stimulation can drive changes in their morphology (Lin et al., 2005).

Postsynaptic actin is highly motile, with an average turnover time of less than one minute. This constant turnover is based on the dynamic equilibrium between the globular (G-) and the filamentous (F-) form of actin in spine heads, and on rapid diffusion between the dendritic shaft and spine heads (Star et al., 2002). Reagents that influence the F-/G-actin equilibrium affect shape, number and motility of dendritic spines, as well as synaptic transmission and plasticity (Fischer et al., 1998; Halpain et al., 1998; Kim and Lisman,

1999; Bonhoeffer and Yuste, 2002). Changes in the shape, number or density of dendritic spines following behavioral alterations occur in a variety of animal species (e.g., Fifkova and van Harreveld, 1977; Coss and Globus, 1978; Brandon and Coss, 1982; Withers et al., 1993; Comery et al., 1996).

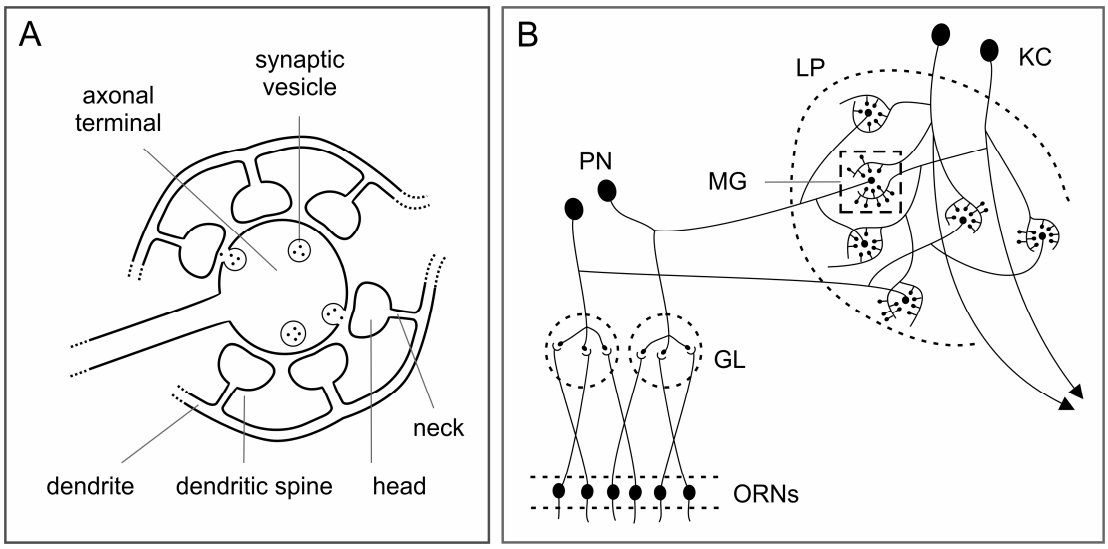

Fig. 9: A) Dendritic spines are motile structures that contain high concentrations of actin, serving as both cytoskeleton and scaffold for synapses. **B)** In *Apis*, as in other insect species, the MB calyx are organized into so-called microglomeruli (MG; see black box). Each MG comprises a presynaptic central bouton from PN terminals surrounded by KC dendritic spines. GL: glomerulus; KC: Kenyon cell; LP: lip; ORN: olfactory receptor axon; PN: projection neuron.

4.2 F-actin in the mushroom body calyces of the insect brain

In the insect nervous systems, as in vertebrates, F-actin is strongly associated with synaptic neuropils (de Camilli et al., 2001; Rössler et al., 2002). This has been demonstrated for the primary olfactory neuropils (Rössler et al., 2002) as well as for the MB neuropils (Frambach et al., 2004) of the insect brain. In the MBs, intense F-actin accumulation appears in distinct synaptic complexes, so-called microglomeruli (MG; Fig. 9B) (Frambach et al., 2004). The spheroidal MG comprise a central bouton from PN axons surrounded by many KC dendritic spines and processes from other extrinsic neurons (GABAergic neurons) (honeybee: Ganeshina and Menzel, 2001; fruitfly: Yusuyama et al., 2002; cricket: Frambach et al., 2004). Within the MG of the calyces, presynaptic boutons appear to be mainly devoid of F-actin (Frambach et al., 2004), whereas F-actin is strictly concentrated

in the postsynaptic dendritic spines, similar to the vertebrate hippocampus and cerebellum (Matus, 2000; Capani et al., 2001).

Synaptic plasticity very likely occurs in the MBs of insects, as they are assumed to be essential for adaptations to a changing environment as well as for adaptations to variations in individual tasks (e.g., Gronenberg et al., 1996). Studies in *Drosophila melanogaster* and *Apis mellifera* showed experience- and age-related outgrowth in KC dendritic trees in adult MBs (Technau, 1984; Farris et al., 2001). The fact that F-actin is aggregated in dendritic spines of KCs in the MB calyx may indicate that calyx MG constitute sites where synaptic and structural plasticity might occur.

5 Objectives

The research on the neuronal mechanisms of behavioral plasticity and adaptation constitutes a particularly fascinating field in neurobiology. In many animal species, crucial behaviors for survival are largely dependent on olfactory cues spread over the lifespan. These provide interesting possibilities for studying neurobehavioral plasticity. Olfaction plays a key role in the behavior of the honeybee, *Apis mellifera*. Caste specific and environmentally induced differences in olfactory behavior of adult bees offer the unique opportunity to examine mechanisms and possible consequences of neuronal plasticity within the olfactory pathway. So far, relatively little is known about specific effects of temperature on the maturation of the metamorphosing nervous system. In this study, I present, in particular, whether small changes in the temperature normally maintained during prepupal and pupal development may influence the synaptic organization in the developing as well as the adult nervous system in female honeybees.

To study the ontogenetic plasticity of the brain and to begin to unravel possible neuronal correlates for the differences in behavior, I comparatively analyzed the postembryonic differentiation of synaptic neuropils in the brain of queens and workers. Quantitative neuroanatomical analyzes focused on higher order neuropils using synaptic and neuronal markers.

I chose the MB calyces to quantify the influence of brood temperature control during post-capping period on the synaptic organization in the adult brain for two reasons. First, previous research approaches suggest that the MBs are involved in higher order computations such as learning and memory (e.g., Menzel, 2001; Heisenberg et al., 1985), which is important for complex behavioral tasks performed by bees. Second, MG in the

MB calyx represent distinct synaptic units (Ganeshina and Menzel, 2001; Yusuyama et al., 2002; Frambach et al., 2004), which I was able to visualize and quantify at excellent resolution.

Long-term environmental changes and behavioral modifications also alter morphology and size of the brain. In particular the adult MBs express age- and experience-dependent volume changes (e.g., Farris et al., 2001), indicating that structural changes in the MBs are related to behavioral plasticity. I quantified age-dependent changes in the structure and the synaptic organization within the mature MB calyx of old, egg-laying queens.

MATERIALS AND METHODS

1 Experimental animals

The experiments were conducted during the field seasons in 2002 – 2004. They were performed on the European honeybee (*Apis mellifera carnica*) obtained from colonies reared at the apiary of the University of Würzburg, Germany. These colonies, each headed by a naturally mated queen, were maintained according to standard commercial beekeeping practice. To minimize genetic variability in the rate of behavioral development and brain structure, all bees observed in this study were derived from four selected colonies during the entire observation period. These selected colonies contained all stages of brood and were particularly strong in nurse bees.

2 Breeding of worker and queen larvae

To synchronize worker brood, egg-laying queens were confined to single empty brood combs with wire mesh cages that permitted only the passage of workers. Within 24 hours these brood combs were filled up with eggs. After oviposition, the queens were transferred to new, empty brood combs. The combs containing the freshly laid eggs remained in the colony until the brood cells were sealed (capping). Shortly before one brood cell is capped, the larva begins to move within the cell (Bertholf, 1925; Winston, 1987). Cell capping characterizes the beginning of the spinning larval stage and the larva starts to construct a cocoon (reviewed in Jay, 1963). Using this breeding method, the numerous worker brood cells of one brood comb were almost simultaneously capped by adult workers.

For queen breeding under controlled conditions, the breeding queen and the cell builders were housed in two separate hives. Within the foster colony, the queen was removed from the hive box (queenless colony), and the colony was reduced to 10 combs. A wooden bar holding 20 queen cell cups that provide a matrix for queen cells was placed in the centre of the hive box surrounded by three brood combs on both sides. To ensure a stable fostering of queen destined larvae by a large number of worker bees, these surrounding combs containing emerging brood were exchanged at least once a week. The outer spaces of the hive were filled up with honey combs to provide large enough quantities of food. One day after removing the queen, individual larvae (≤ 36h) from the breeding colony were transferred with fine needles into each queen cell cup on the wooden bar (larval grafting). To exclude intercolony differences, all queen destined larvae were taken from the same

breeder colony. Four days following grafting, nurse bees completed larval feeding and covered the queen cell cups with a thin layer of wax (cell capping). As the development of prepupae can be disrupted by vibration (reviewed in Jay, 1963), the freshly capped queen cells were transferred carefully with padded polystyrene boxes from the cell builder colony into incubators in the laboratory.

3 Temperature treatment during post-capping period

3.1 Morphological features of developing female pupae under normal incubation conditions

Immediately after capping, the synchronized sealed worker and queen cells (Fig. 10) were placed into incubators (Bachofer 400 HY-E, Reutlingen, Germany; Mini Oven MK II, Teddington, United Kingdom; Rumed 1000-72039, Laatzen, Germany; Sanyo, MIR-153, Bad Nenndorf, Germany). These cells were reared at 34.5°C, the temperature precisely maintained within natural brood cells (Himmer, 1927a; Heinrich, 1993). The preset temperature within the capped brood cells was recorded automatically in 10 minute intervals using fine thermocoupled wires connected with a digital logger (Almemo 2290-8 V5, Holzkirchen, Germany). These thermoprobes remained in their position throughout the pupal growth until the day of eclosion. Deviations from adjusted temperatures were less than ±0.2°C.

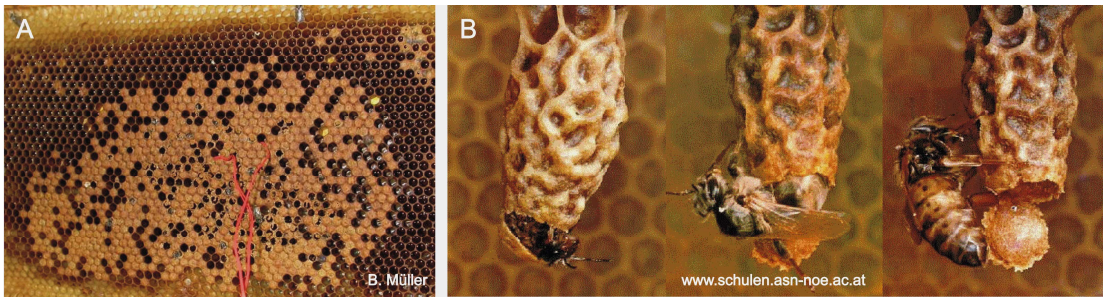

Fig. 10: Capped brood cells of developing workers and queens. Temperature inside brood cells was monitored continuously with thermocoupled wires. **A)** Synchronized sealed worker brood comb. **B)** Emergence of a virgin queen from her brood cell. She bites through the brood cell wall above the sick pad of silk at the capped cell end.

To view external morphological features in developing female pupae and to correlate them with neuroanatomy, pupae were photographed after prepupal ecdysis at each day of pupal development (Nikon Coolpix 4500, Tokyo, Japan) before dissecting their brains. For

worker pupae, a detailed description of morphological features from Eichmüller (1994) was consulted as reference (Tab. 1). In addition to the known age, the pigmentations of eyes, legs, thorax and abdomen were used as criteria for identifying developmental stages. To examine the differentiation processes of olfactory neuropils in developing pupae, the data obtained from pupal stages P1 to P4 were compared with data from Schröter and Malun (2000). For developing queen pupae, morphological and neuroanatomical features during successive pupal stages have not been described in detail.

Tab. 1: Age determination of worker prepupae and pupae based on morphological features. Each developmental stage lasts approximately one day. ANT: antennae, MD: mandibles, OC: ocelli, P: pupa, PP: prepupa, RET: retina. After Eichmüller, 1994.

stage	morphological features
PP	• wormlike larva, completely white
PP	• wormlike larva, completely white
P1	• pupa, completely white
P2	• OC and RET light pink
P3	• OC and RET pink
P4	• OC dark pink, RET red
P5	• OC and RET red • tibiotarsal joints and wing basis light brown
P6	• OC and RET dark brown • tibiotarsal joints, wing basis and mesonotum joint brown • MD, ANT, coxa and claws light brown • abdomen and legs light yellow
P7	• RET black • OC and claws dark brown • MD, ANT (flagellum), tibiotarsal joints, wing basis, and mesonotum joint brown • head and thorax light brown • abdomen, legs, glossa and ANT (scapus and pedicellus) dark yellow • wings folded and light grey
P8	• RET, ANT (flagellum) and OC black • claws, leg joints, wing basis and mesonotum joint dark brown • ANT (scapus and pedicellus), glossa, abdomen and legs light brown • head and thorax dark grey • wings folded and grey
P9	• RET, ANT (flagellum) and OC black • claws, leg joints, wing basis and mesonotum joint dark brown • ANT (scapus and pedicellus), glossa, abdomen and legs dark brown • head and thorax dark • wings stretched and grey • worker emerges

3.2 Temperature treatment

3.2.1 Rearing of capped worker and queen brood under constant temperatures

To examine thermoregulatory influences during post-capping period on the synaptic organization in the adult brain, synchronized worker and queen brood cells were reared in incubators during the entire prepupal and pupal development. The temperature within these cells was set to constant temperatures (Tab. 2) and monitored continuously by using fine thermoprobes. Before starting temperature manipulations in queens, each cell had to be enclosed in an individual hair curler, as freshly emerged virgin queens have a pronounced instinct to sting their competitors to death (Allen, 1965).

Tab. 2: Incubation temperature of capped brood under constant temperature regimes. Worker brood combs with 400 – 1,000 capped brood cells (see also Fig. 10A).

	constant rearing temperature [°C]										
worker	28.0	29.0	30.0	31.0	32.0	33.5	34.5	35.0	36.0	37.0	38.0
combs [n]	1	3	3	3	3	4	6	4	3	3	1
queen	28.0	29.0	30.5	31.5	32.5	33.5	34.5	35.5	36.5	37.0	38.0
cells [n]	10	10	11	27	30	34	37	30	17	22	10

For every temperature treatment, the duration of the post-capping period and the number of emerged bees were recorded in both female castes. Immediately after eclosion, antennae of workers and queens were prepared for scanning electron microscopy and their brains were dissected for immunofluorescence staining.

Additionally, I investigated if thermoregulatory effects on the synaptic organization in the adult worker brain persist during the first week of adult life. Workers reared constantly during the entire post-capping development at 32°C, 34°C and 36°C, the natural temperature variance in the hive, were color marked and introduced into foster colonies. After one week, immunofluorescence staining was performed on brain tissue.

3.2.2 Rearing of capped worker brood at two-step temperature regimes

In honeybees (*Apis mellifera carnica*), prepupae are said to be very sensitive to temperature changes and to vibration (Gontarski, 1957). Additionally, for workers it is known that chilling young pupae produces a higher proportion of malformed adults than chilling older pupae (Weiss, 1962). To quantify the effect of the incubation temperature

during the first half of the post-capping period on the synaptic organization in the adult brain, synchronized worker brood cells were reared at two-step temperature regimes. In bees reared under normal conditions, the subdivisions of the MB calyx are clearly distinguishable at pupal stage 4 (Farris et al., 1999; Schröter and Malun, 2000). Therefore, freshly capped brood cells were first temperature treated up to P4 and subsequently reared at 34.5°C until their emergence (Tab. 3). At P4 and one day after emergence, bees were photographed before dissecting their brains for immunofluorescence staining.

Tab. 3: Incubation temperature of capped worker brood at two-step temperature regimes. Worker brood comb with 400 – 1,000 sealed brood cells. A: adult stage, P: pupa, PP: prepupa.

rearing temperature [°C]	
PP – P4	**P5 – A**
29.0	34.5
31.0	34.5
32.0	34.5
34.5 (control)	
36.0	34.5
37.0	34.5

3.3 Temperature measurement in queen cells inside a colony

To determine the incubation temperature within natural sealed queen cells, a colony of *Apis mellifera carnica* in May 2003 was forced to prepare for swarming by adding adult workers into the hive. In preparation for swarming, this colony started to construct queen cell cups located at the periphery of the brood area. During the entire post-capping period, the incubation temperature within three capped queen cells was recorded automatically every hour using humid resistant thermoprobes (Testostor 175-T2, Lenzkirch, Germany).

4 Temperature effects on antennal olfactory sensilla

4.1 Preparation of adult antennae for scanning electron microscopy

For quantitative analysis of temperature mediated effects on olfactory sensilla, the antennae of workers (Fig. 11A) and queens were prepared for scanning electron microscopy (SEM). After emergence, both antennae were cut off from the head capsule at the distal scapus (Fig. 11B). Each antenna was fixed in dental wax (Dentsply Ltd., Weybridge, UK) to section the antenna longitudinally from the scapus to the flagellum

(Fig. 11C) using a razor blade. To remove the wax, the antenna halves were washed with Rotihistol (Roth, No. 6640.1, Karlsruhe, Germany) overnight. The antenna halves were air dried in order to withstand the vacuum inside the microscope and then mounted with their inner side on a SEM table. All antenna halves were coated with an electrical conductive layer of gold-palladium using a sputter coater (Balzers sputter coater SCD 005, Liechtenstein).

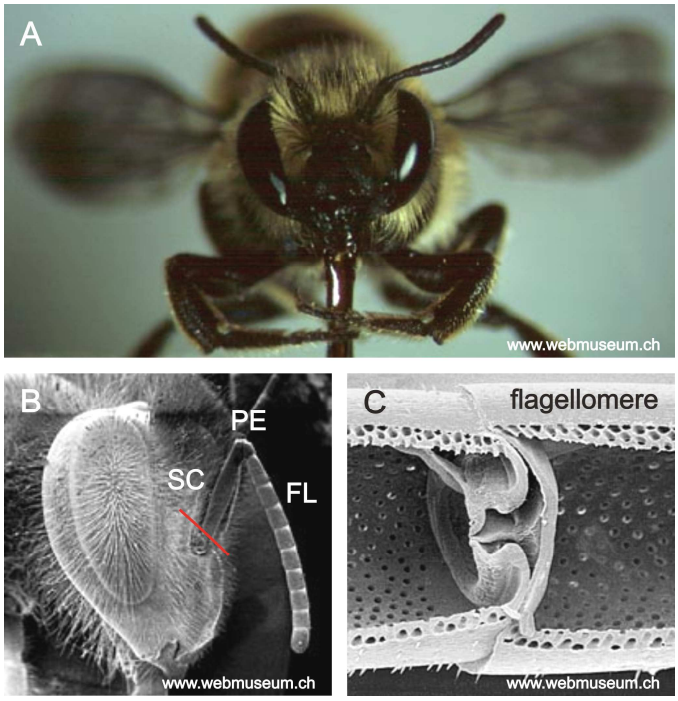

Fig. 11: A) Frontal view of a worker honeybee. **B)** Scanning electron micrograph of the worker head. The antenna is subdivided into scapus, pedicellus and flagellum. The terminal flagellum bears the olfactory sensilla. The red bar indicates the cutting site. **D)** Scanning electron micrograph from two longitudinal sectioned flagellomeres (viewed from interior). FL: flagellum, PE: pedicellus, SC: scapus.

4.2 Scanning electron microscopy (SEM)

Antennae halves were placed inside the vacuum column of a scanning electron microscope (SEM). This SEM generates a beam of electrons inside the vacuum that is collimated by electromagnetic condensor lenses (Fig. 12). The electron beam is focused to a fine spot on the sample with an objective lense and scanned across its surface by electromagnetic deflection coils. As the electron beam hits each spot on the sample, secondary electrons are dissociated from the surface. These electrons are collected by a detector, converted to a voltage and amplified. The final image, rendered in black and white, is built up from the

number of electrons emitted from each spot on the sample (http:www.mos.org/sln/SEM; http://www.mse.iastate.edu/microscopy/path.html).

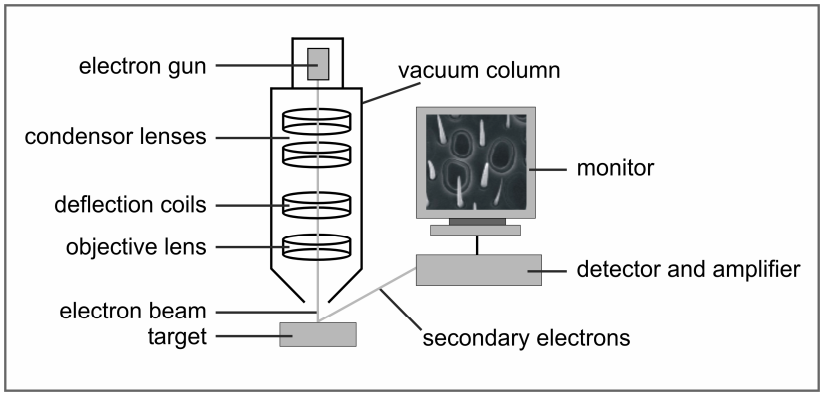

Fig. 12: Schematic diagram of a scanning electron microscope. The images created without light waves are rendered in black and white (see inset of honeybee antennal sensilla).

4.3 Image processing, data acquisition and statistical analysis

In both female castes, the antennal flagellum consists of 10 flagellomeres (Fig. 13). The majority of poreplates are distributed on the medial segments (Esslen and Kaissling, 1976). To investigate the effect of temperature on olfactory sensilla, high resolution scanning electron micrographs of the medial 5th and 6th antennal segments were acquired using a SEM (Zeiss DSM 962, Germany). Corel DRAW was used to aligne images from adjacent antennal regions and to enhance contrast where needed (Corel Corporation, Ottawa, Ontario, Canada). The number of poreplates were counted within a defined rectangle (150 x 250µm) on the 5th and 6th flagellomere. For statistical analysis, data were evaluated by using the Mann-Whitney U-test and the results were corrected after Bonferroni. Statistical tests were performed with SPSS software (SPSS Inc., Chicago, Illinois, USA).

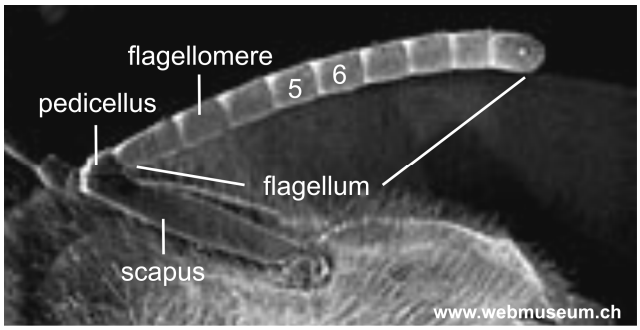

Fig. 13: Honeybee female antenna. The number of antennal olfactory sensilla, located on the flagellum, were counted on the 5th and 6th flagellomere.

5 Neuroanatomical techniques

5.1 Immunocytochemistry

5.1.1 Brain preparation

Developing pupae (n=48), freshly emerged adults (n=134) and one-week-old nurses (n=18) reared in incubators under controlled temperature regimes were anaesthetized with CO_2 and decapitated. For quantification of age-related changes in the synaptic organization of the MBs, we additionally dissected egg-laying queens of different ages: 11-day (n=8), 6-month (n=10), 12-month (n=7), and 36-months-old (n=1). All egg-laying queens were taken from non-experimental colonies from our apiary.

For brain preparation, heads of pupae were fixed without any impact of heat using minutien pins and heads of adults were embedded in dental wax (Fig. 14A). All brains were dissected in cold bee ringer solution. After opening the head capsule, glands and tracheae were removed (Fig. 14B).

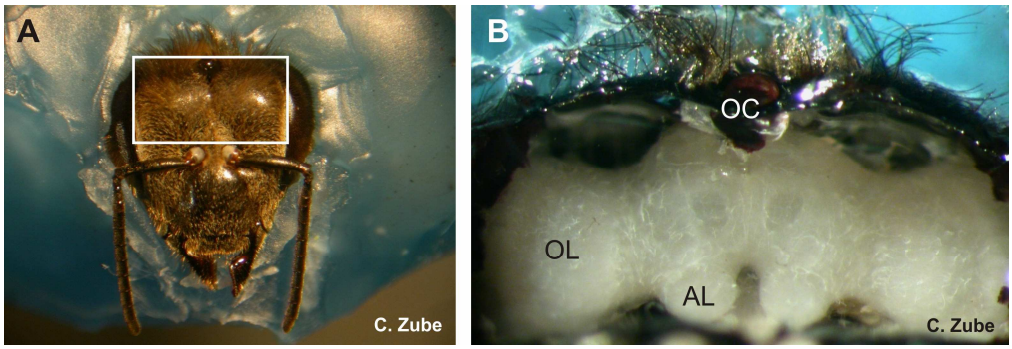

Fig. 14: Worker head in frontal view. **A)** Embedded head in wax for brain preparation. The white box indicates the cutting sites. **B)** After opening the head capsule, the antennal and optical lobes become clearly visible. AL: antennal lobe, OC: ocellus, OL: optical lobe.

Each brain was removed from the head capsule, immersed in cold 4% formaldehyde in phosphate-buffered saline (PBS, pH 7.2) overnight at 4°C and then washed three times in PBS (3 x 10 minutes). After embedding in 5% low melting point agarose, brains were sectioned in the frontal plane at 100µm thickness with a vibratome (Leica VT 1000S, Nussloch, Germany). Free-floating agarose sections were pre-incubated in PBS containing 0.2% Triton X-100 (PBST) and 2% normal goat serum (NGS; ICN Biomedicals, No. 191356, Orsay, France) for one hour at room temperature. During fixation, pre-incubation, incubation and rinses (see below) the sections were placed on a shaker for agitation (Type LS10, Gerhardt, Bonn, Germany).

5.1.2 Immunofluorescence stainings

Double-labeling with fluophore-conjugated phalloidin and anti-synapsin

To label neuronal filamentous (F-) actin, sections were incubated in 0.2 units of Alexa Fluor 488 phalloidin (Fig, 15A; Molecular Probes, A-12379, Leiden, The Netherlands) in 500µl PBST with 2% NGS initially for two hours at room temperature, and subsequently for two nights at 4°C (Rössler et al., 2002; Groh et al., 2004). Brain-sections were double-labeled with a monoclonal antibody against the *Drosophila* synaptic-vesicle-associated protein synapsin I (Fig. 15B; 1:50; SYNORF1; kindly provided by Dr. E. Buchner, University of Würzburg, Germany) (Klagges et al., 1996).

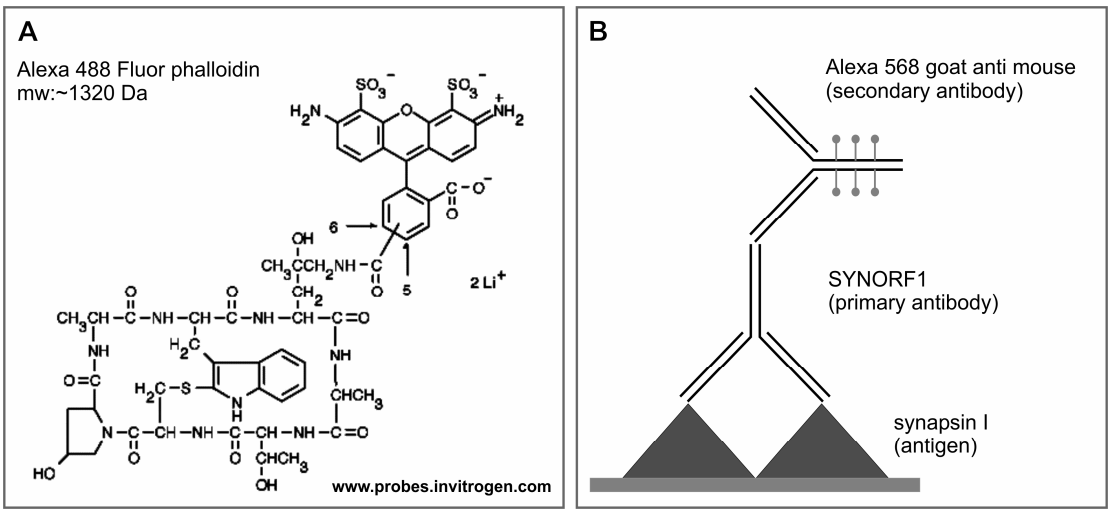

Fig. 15: A) Phallotoxins, isolated from the mushroom *Amanita phalloides*, are bicyclic peptides and bind the variety of isoforms of filamentous (F) actin with high affinity. Phallotoxin conjugates exhibit a small size and low molecular weight and penetrate easily into the tissue. For data acquisition, phalloidin conjugated with the photostable, green-fluorescent Alexa Fluor 488 dye was used. **B)** Schematic diagramm of indirect immunocytochemistry with a primary antibody to synapsin 1 (monoclonal, derived from mouse) and Alexa 568-conjugated goat anti mouse as secondary antibody. Da: daltons, mw: molecular weight.

After five rinses in PBS (5 x 10 minutes) to remove unbound phalloidin and anti-synapsin, double-labeled preparations were incubated in Alexa 568-conjugated goat anti-mouse secondary antibody (Fig. 15B; 1:250; Molecular Probes, A-21124) in 1% NGS/PBS for two hours at room temperature. Sections were finally washed in at least five changes of PBS (5 x 10 minutes), transferred into 60% glycerol/PBS for 30 minutes and then mounted on slides for confocal microscopy in 80% glycerol/PBS.

Double-labeling with fluophore-conjugated phalloidin and propidium iodide

After removal of unbound phalloidin (see previous page), sections were incubated for 15 minutes in 25 µg/ml propidium iodide (Molecular Probes, A-11003) in 500 µl PBST at room temperature to stain glial and neuronal cell nuclei. The preparations were rinsed in PBS five times (5 x 10 minutes), transferred into 60% glycerol/PBS for 30 minutes and mounted on slides in 80% glycerol/PBS.

Double-labeling with fluophore-conjugated phalloidin and anti-serotonin

Simultaneous labeling of F-actin and serotonergic neurons was performed in some preparations. After pre-incubation (see above), the brain tissue was incubated for two nights at 4°C in a rabbit antiserum against serotonin (1:4,000; DiaSorin, Stillwater, MN, Cat. No. 20080, Lot No. 051007) diluted in 500µl PBST with 2% NGS, together with 0.2 units of Alexa Fluor 568 phalloidin (Molecular Probes, A-12374). Sections were subsequently washed six times in PBS (6 x 10 minutes), and Alexa 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, A-11008) was applied at a dilution of 1:250 in 1% NGS/PBS for two hours at room temperature. Unbound secondary antibody was washed in six changes of PBS (6 x 15 minutes), transferred in 60% glycerol/PBS for at least one hour, and finally mounted on slides in 80% glycerol/PBS.

Labeling with rhodamine dextran

Axonal projections of antennal lobe PNs and dendrites of KCs were labeled with rhodamine dexran (Microruby, Molecular Probes, D-7162; Schröter and Malun, 2000). Tips of glass electrodes were coated with the dye and inserted into the AL lobe neuropil or the KC soma cluster in freshly dissected brains. The dye was allowed to diffuse for ~3h. Brains were fixed and processed as described under 5.1.1.

5.2 Golgi technique

The Golgi technique (for details see Strausfeld, 1980) randomly impregnates single neurons or glial cells with silver nitrate. Using this technique, it is possible to obtain thick brain sections in which cell processes can be traced for long distances through surrounding unstained tissue. Two modifications of this technique were applied to observe temperature-mediated effects on the morphology of individual Kenyon cells in the MB calyx (n=42).

5.2.1 Golgi impregnation

The bees were decapitated and the mouthparts and antennae were removed. A window was cut across the back of the head capsule approximately at the height of the MBs. The heads were fixed in a solution containing 4 volumes 2.5% potassium dichromate (Fluka, No. 60200, Buchs, Switzerland) diluted in distilled water, 1 volume 25% glutaraldehyde (Serva, No. 23114, Heidelberg, Germany) and 4% sucrose for 3 days at room temperature in the dark. After several rinses in 0.1% $AgNO_3$ (Fluka, No. 85228), the heads were impregnated in 0.75% $AgNO_3$ for 3 days at room temperature in the dark. The tissue was treated twice by the chromating-impregnation routine (double impregnation). After several washes in distilled water, the heads were dehydrated in increasing grades of ethanol for 10 min each and soaked twice in propylene oxide (Fluka, No. 82320) for 20 min. For polymerization at 65°C, the heads were embedded in BEEM caps (SCI Science Services, No. 70000, München, Germany) in soft Araldite (epoxy embedding medium: Durcupan ACM [Fluka, 44613]). The heads were serially sectioned in the frontal plane at 30µm thickness with a sliding microtome (Jung, Heidelberg, Germany) and mounted in Entellan (Merck, No. 1.07961.0100, Darmstadt, Germany). Sections were viewed using a camera (Spotinsight Color, Visitron Systems) attached to Zeiss Axiophot microscope.

5.2.2 Golgi-Colonnier

The heads were pre-fixed in 2.5% glutaraldehyde in PBS and 4% sucrose overnight at 4°C and the further processed following the Golgi impregnation protocol as described above.

6 Confocal laser scanning microscopy, image processing and data acquisition

6.1 Confocal principle

The key feature of confocal microscopy is the possibility to obtain fluorescence images with high resolution due to effective suppression of light scattered from outside the focal plane. The laser scanning microscope scans the object point by point, line by line so that very thin, sharp, blur-free optical sections can be recorded. By moving the focus plane, optical sections at various depths can be obtained to record a three dimensional stack that can be digitally processed afterward.

In a confocal laser scanning microscope (Fig. 16) the laser beam passes through a light source aperture. It is reflected by a dichroic beamsplitter that reflects light of short wavelengths and transmits light of longer wavelengths. Via the microscope objective, the laser beam is then focused to a spot within a fluorescent specimen. Emitted fluorescent light and reflected laser light from the illuminated spot are then re-collected by the objective lens. The beam splitter reflects the laser light and only the fluorescent light passes into the detector aperture, usually taken from the camera port of the microscope. After passing a pinhole, the fluorescent light is converted to an electrical signal by the detector (photomultiplier).

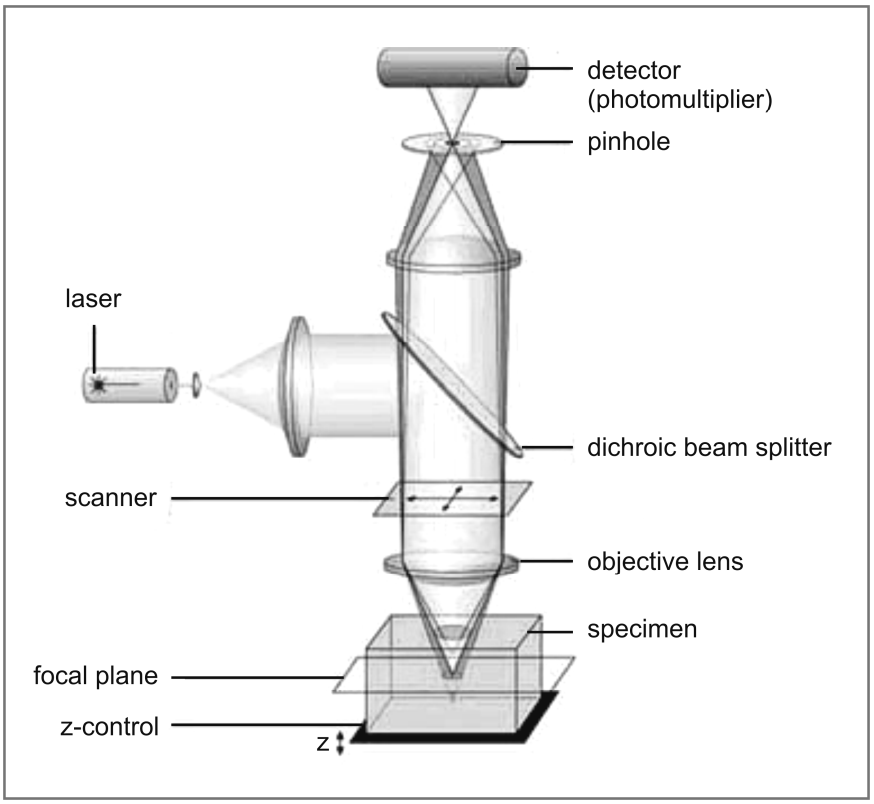

Fig. 16: Schematized diagram of a confocal laser scanning microscope (CLSM). After www.zeiss.com.

The detector aperture blocks light rays not descending from the focal plane of the objective lens. Fluorescent light originating from below and above the focal plane is focused before and after reaching the detector pinhole. Only the in-focus portion of fluorescent light is focused at the pinhole and passes through the detector. Information from out-of-focus fluorescence is eliminated from the final image, resulting in sharper images compared to conventional microscopy techniques (http://en.wikipedia.org/wiki/Confocal_microscope; http://www.neuro.ki.se/neuro/confocal/manual/confprinc.html; http://www.zeiss.com).

6.2 Image processing

Brain preparations were viewed using a laser scanning microscope (Leica TCS SP, Leica Microsystems AG, Wetzlar, Gemany) equipped with an argon-crypton laser as light source. Single optical sections and series of sections were imaged at intervals of 0.5 – 2.0µm through the depth of the sections. Images were acquired using three different types of Leica objectives (PL FLUOTAR 10x/0.30, HC PL APO 20x/0.70 and PL APO 63x/1.20). To quantify distinct MG within the MB calyces of both female castes, single optical sections were imaged as described in Tab. 4.

Tab. 4: Settings of the scanning software for data acquisition. For quantitative analysis of synaptic complexes in the MB calyces, brain sections double-labeled with Alexa 488 Fluor phalloidin and anti-synapsin were viewed.

filter 1	FITC
	excitation: 488nm
	emission: 490 – 550nm
filter 2	TRITC
	excitation: 568nm
	emission: 575 – 680nm
lens	PL APO 63x/1.20
scale	1024x1024
mode	xy
speed	4 (medium)
zoom	0.7x – 2.0x
pinhole	1.0
accumulation	2

In cases where image stacks were taken, two-dimensional projections were generated for each channel. Zeiss imaging software was used to enhance contrast where needed (LSM 5 Image Browser, Carl Zeiss, Göttingen, Germany). The two-dimensional projections of each channel were merged using Corel PHOTO-PAINT (Corel Corporation, Ottawa, Ontario, Canada). Brain schemata were drawn with Corel DRAW (Corel Corporation) and 3D reconstructions were performed with AMIRA software (Mercury Computer Systems Inc., Berlin).

6.3 Data acquisition

For data analysis, a defined plane in the adult brain was chosen where the MB calyces and several landmarks (the fan-shaped and ellipsoid body of the central complex and the pedunculi of the MBs) were clearly identifiable. At this plane, the MB lip and collar were sectioned transversally, and the inner branch of the marginal calyx and the outer branch of the medial calyx of both hemispheres were analyzed (Fig. 17A and B).

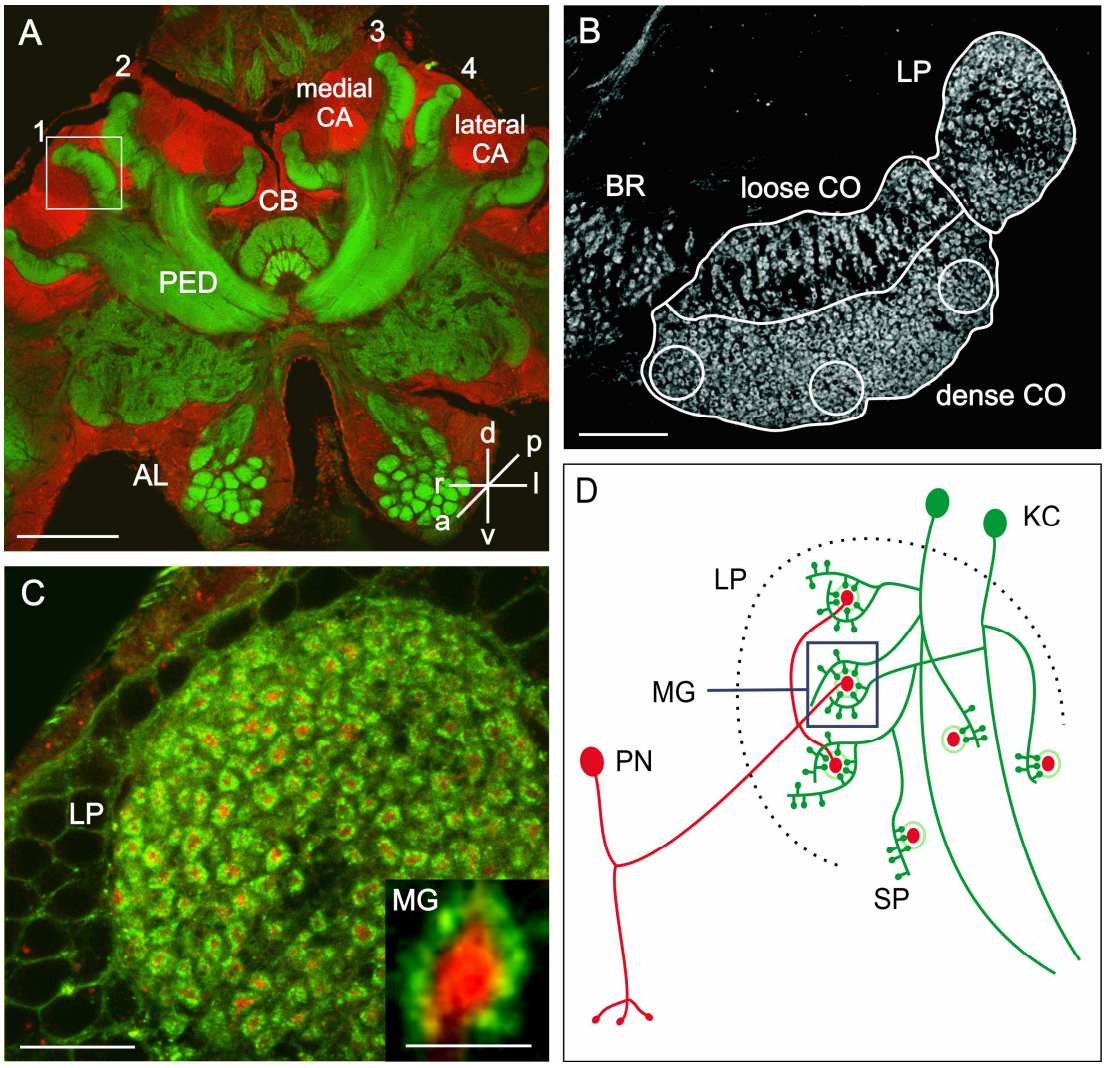

Fig. 17: Quantification of synaptic complexes in the adult MB calyx. **A)** Overview of the central worker brain labeled with phalloidin (green) and propidium iodide (red). **B)** Magnification of one calycal branch labeled with phalloidin (box in A). The measured areas are indicated. **C)** Pre- and postsynaptic labeling of microglomeruli (MG) in the lip using phalloidin (green) and anti-synapsin (red). Each MG (inset) comprises a presynaptic central bouton from antennal lobe PN terminals surrounded by KC dendritic spines. **D)** Schematic olfactory pathway from the antenna to the MB lip. For simplification, further intrinsic neurons within the calyx are not included. a: anterior, AL: antennal lobe, BR: basal ring, CA: calyx, CB: central body, CO: collar, d: dorsal, KC: Kenyon cell, l: left, LP: lip, p: posterior, PED: pedunculus, PN: projection neuron, r: right, SP: (dendritic) spine, v: ventral. Scale bars: A: 200µm, B: 50µm, C: 15µm (inset: 3µm).

To examine temperature-mediated and age-related changes on the formation of the MB input regions, synaptic contacts between extrinsic and intrinsic neurons of the MB calyces were analyzed. Double-labeling with fluorophore-conjugated phalloidin and anti-synapsin (Fig. 17C) revealed these sites of calycal synaptic contact referred to as microglomeruli (Frambach et al., 2004). Each microglomerulus (MG) consists of a central core occupied by projection neuron axonal terminals, encircled by Kenyon cell dendritic spines (Fig. 17D) and processes from other extrinsic neurons (Ganeshina and Menzel, 2001; Yusuyama et al., 2002). Phalloidin binds to F-actin that is most abundant in dendritic spines (Wieland, 1987; Lee and Cleveland, 1996; Matus, 1999) and, therefore, labels the postsynaptic surrounding area of the calycal MG. As synapsin is associated with synaptic vesicles in presynaptic terminals, anti-synapsin labels the large bouton of the calycal MG.

For data acquisition in adult MB calyces, phalloidin-labeled MG profiles were counted in the olfactory lip and the visual collar region in the calyces marked in Fig. 17A. In the collar, MG arrangement exhibited a more loosely and a densely arranged subdivision. Within the dense portion, MG numbers were estimated for three circular areas (20µm in diameter each) and extrapolated to the total dense area (Fig. 17B). In the same optical section, the cross sectional area and the density of the lip and the adjacent collar region were determined. For each brain, the number of MG profiles, the cross-sectional areas and the densitiy of the lip and the dense collar portion were averaged and a mean value was calculated from several brains of each temperature treatment.

6.4 Statistical analysis

To compare mean counts of MG in the lip and in the dense region of the collar between temperature treated groups, a one-way ANOVA was used (Kruskal-Wallis one-way Analysis of Variance on Ranks, $p<0.05$). When significant differences between temperature treated groups were identified, pairwise *post hoc* comparisons were performed using the Mann-Whitney U-test ($p<0.05$). Where necessary, the results were corrected after Bonferroni ($p<0.0125$).

In adult egg-laying queens, I used Spearman's Rank Correlation to test if ongoing age correlated with three parameters in the lip and the collar: the number of MG profiles, the cross-sectional area and the density of MG within the cross-sectional area (Spearman's Rank correlation coefficient [Rho], $p<0.05$).

Statistical analyses were performed with SPSS software.

7 Solutions for immunocytochemistry

Agarose

2,5g low-melting-point agarose (Agarose type II, No. 210-815, Amresco, Solon, OH) diluted in 50ml distilled water.

Bee ringer

	molar mass [mM]	**gram/liter [g/l]**
NaCl	130	7.6
KCl	5	0.375
$MgCl_2$	4	0.815
$CaCl_2$	5	0.55
Hepes	15	3.55
Glucose	25	4.5
Sucrose	160	54.5

Diluted in distilled water and adjusted to pH 7.2 with NaOH and HCl.

Fixative

10 ml 16% formaldehyde (EM Grade, No. 15710, Electron Microscopy Sciences, Washington) diluted in 30ml PBS (pH 7.2).

Glycerol

60% and accordingly 80% glycerol (A3552-1000, AppliChem GmbH, Darmstadt, Germany) diluted in PBS (pH 7.2).

Phosphate buffer (PB)

	molar mass [mM]	**gram/liter [g/l]**
Na_2HPO_4	162	22.94
NaH_2PO_4	38	5.25

0.2M (stock solution) diluted with distilled water to 0.1M and adjusted to pH 7.2.

Phosphate buffered saline (PBS)

	molar mass [mM]	**gram/liter [g/l]**
NaCl	130	40.03
KCl	2.7	1.0
Na_2HPO_4	8	7.2
KH_2PO_4	1.4	0.95

One volume and 4 volumes distilled water adjusted to pH 7.2 with NaOH and HCl.

Phosphate buffered saline with Triton X-100 (PBST)

0.2% Triton X-100 diluted in PBS (pH 7.2).

RESULTS

1 Development of adult morphological features during pupal development

In honeybees, the post-capping period includes the prepupal period followed by a molt revealing the pupa and several subsequent pupal stages (reviewed in Jay, 1963). During pupal development, the pupae do not grow, but their muscles, organ system and nervous system undergo massive alterations into the mature form (Winston, 1987). The time course of metamorphic adult development (sealed or capped brood) differs tremendously between workers and queens (reviewed in Jay, 1963). To view these differences in developing pupae of both female castes, I photographed worker and queen pupae at each day of pupal development.

1.1 Morphological features of female pupae under normal incubation conditions

1.1.1 Workers

After two prepupal stages (PP, PP) that are followed by the prepupal ecdysis, the head, eyes, antennae, mouthparts, thorax, legs, and abdomen of the worker pupae all showed adult characteristics. The successive developmental stages of the pupa (P1 – P9) exhibited morphological changes of the cuticle as well as changes visible through the cuticle (Fig. 18).

During the first four pupal stages (P1 – P4) only the pigmentation of the eyes that gradually altered from white to red could be used as criteria to differentiate these pupal stages, as the head, antennae, thorax, legs and abdomen remained completely white. First signs of coloring occurred in the tibitorsal joints of the first two leg pairs at pupal stage 5. Additionally, the head and the thorax became slightly yellowish. At P6, all leg pairs showed distinctly colored tibitorsal joints and brown claws. The cuticle of the abdomen and the head as well as the thorax became yellowish and yellow, respectively. From P7 onward, the legs exhibited a pointed pattern and dark brown claws. The coloring of the abdomen and the head as well as the thorax appeared yellow and light brown. In addition, the antennal flagella showed brown pigmentation and the wings were grey and folded. At P8, the pointed pattern of the legs became more obvious. Additionally, the head, the antennae and the thorax exhibited a dark grey cuticle. Moreover, the wings were still grey

and unfolded. One day before emergence (P9), the morphology of the pupae appeared adult-like exhibiting straightened wings. These results largely corroborate descriptions from Eichmüller (1994). The series of photographs served for the determination and comparison of pupal stages in subsequent experiments.

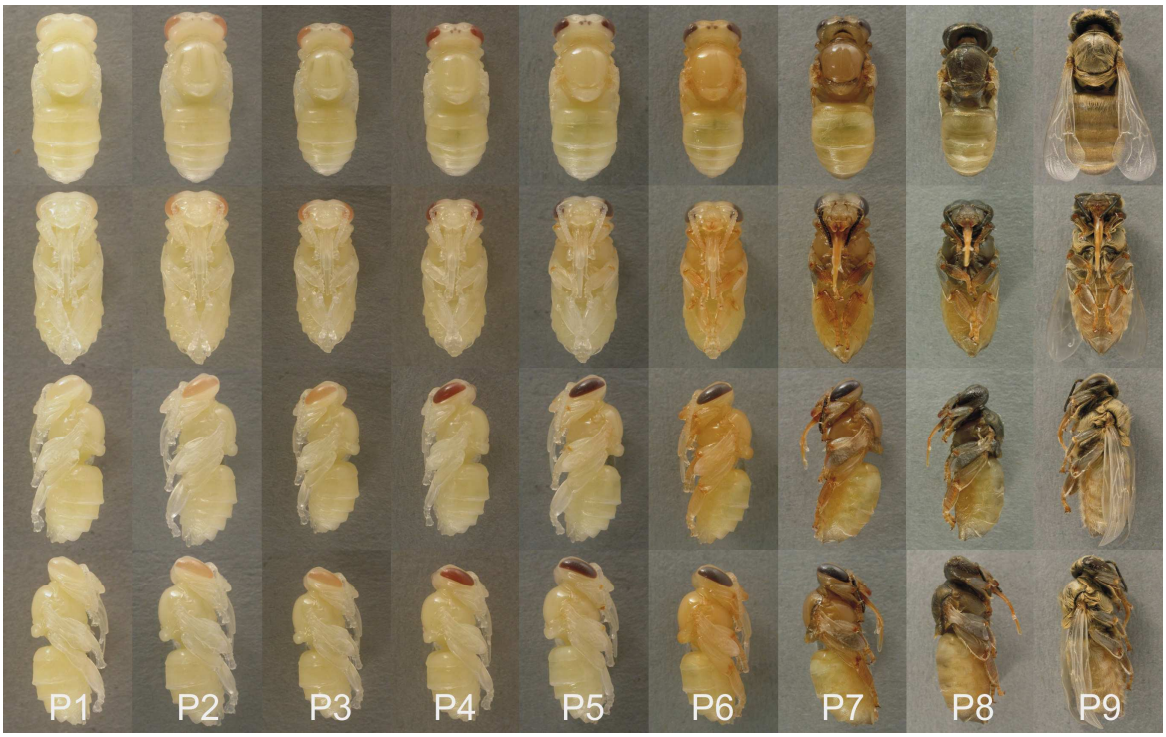

Fig. 18: Development of adult morphological features in worker pupae (P1 – P9) constantly reared at 34.5°C, the naturally occurring temperature in the central brood area. Each developmental stage lasts approximately one day. P: pupal stage.

1.1.2 Queens

Queens develop from fertilized eggs that are genetically not different from eggs that develop into workers. However, from the last larval stage (L5) onward, queen larvae develop significantly faster into adults and exhibit only one prepupal and six pupal stages (reviewed in Jay, 1963). Fig. 19 most strikingly reveals the faster development of adult morphological features in queens compared to workers (see Fig. 18 for comparison).

Freshly hatched queen pupae (P1) exhibited no pigmentation of the eyes, body, legs, claws or tibitorsal joints (Fig. 19). Within the following two pupal stages (P2 – P3) the pigmentation of the eyes changed from red into light brown. At P4, all leg pairs showed distinctly colored tibitorsal joints. Additionally, the head and the thorax became light brown. At P5, the cuticle of the head, the antennae, the thorax and the claws of all leg pairs

altered into brown. In addition, the cuticle of the abdomen was colored in yellow. One day before emergence (P6), the morphological features of the queen pupae appeared adult-like exhibiting grey and stretched wings.

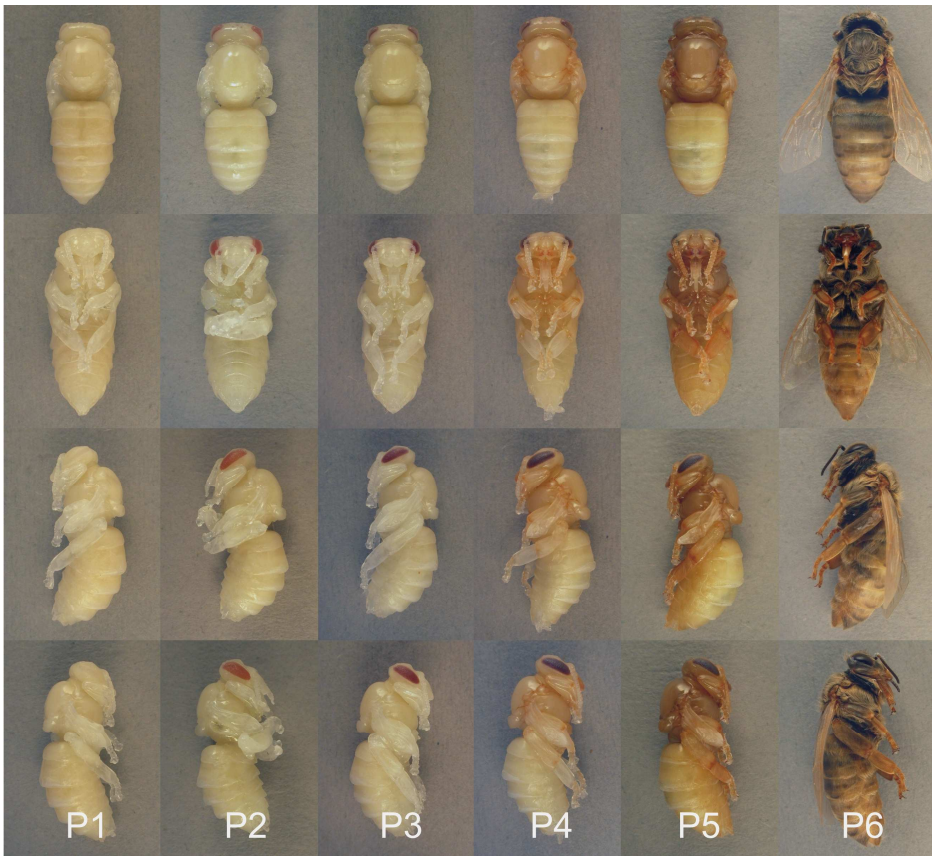

Fig. 19: Development of morphological features in queen pupae also constantly reared at 34.5°C. Photos were taken at each day of pupal metamorphosis. Within this period of pupal development, queens develop significantly faster than workers (see Fig. 18, previous page, for comparison). P: pupal stage.

1.2 Temperature effects on morphological features in workers

Within the period of postembryonic development, honeybee larvae survive temperatures lower than naturally maintained in the central brood nest (35°C±0.5) better than pupae (Himmer, 1927b). Cooling experiments with capped worker brood revealed that chilling prepupae and young pupae produces a higher proportion of crippled adults than chilling older pupae (Gontarski, 1957; Weiss, 1962). To gain insight into the temperature effect on the development of morphological features, I raised freshly capped brood combs in incubators at different constant temperatures. Photos were taken six days after cell sealing.

This point of time marks the end of the first half of the post-capping period under normal breeding conditions.

Temperature deviations that occur after the cell capping considerably affected the development of morphological features in workers (Fig. 20). Pupae reared constantly at the normal incubation temperature (34.5°C; control) exhibited red pigmented eyes and a completely white body (Fig. 20, middle; see also Fig. 18). In contrast, pupae at the same age , but incubated at 31°C, which is lower than the tolerated temperature range within the hive (32 – 36°C; Himmer, 1927a; Seeley, 1985) delayed the development of morphological features. At this temperature regime, the cuticle showed no pigmentation of the eyes. This usually is characteristic for P1 under normal incubation temperatures (Fig. 20, left; see Fig. 18 for comparison). Pupal rearing at 37°C – just below the lethal maximum of honeybees (Himmer, 1927a) – revealed a remarkable increase in the development of morphological features (Fig. 20, right). The abdomen and the head as well as the thorax were colored in yellow and light brown, respectively. Additionally, the pupae exhibited brown-pigmented eyes and folded grey wings. These features are typical for P6/P7 under normal rearing conditions (compare with Fig. 18).

Fig. 20: Temperatures deviating from the temperature naturally maintained in the capped brood area affect morphological features of worker pupae. Photos were taken six days after cell capping (corresponding to P4 at normal incubation temperature, middle).

2 Temperature dependence of the duration of the post-capping period and the emergence rate

2.1 Workers

In honeybee workers, the post-capping period proceeds over two prepupal and 9 pupal stages under normal breeding conditions (reviewed in Jay, 1963; Eichmüller, 1994). To quantify precisely the effect of temperature on the duration of the post-capping period and the emergence rate, I placed synchronized sealed worker brood combs in incubators. These combs were exposed to different constant temperatures between 28 and 38°C.

Post-capping period was shortest between 34.5 and 37°C (10 – 11 days; Tab. 5), increased at lower temperatures and went up to almost twice the normal duration at 29°C (19 – 22 days). Emergence rates were highest between 31 and 36°C (89 – 100%), drastically dropped above and below these temperatures, and came down to zero at 28°C and 38°C, basically confirming earlier studies (Himmer, 1927a; Koeniger, 1978).

Upon emergence, bees reared between 32 and 36°C exhibited no obvious morphological deficits. Some of the bees reared below 32°C and above 36°C, however, showed malformations of their wings, sting, proboscis or legs whereas others had no morphological defects. From these groups (29, 30, 31, and 37°C), only bees without any obvious morphological defect were used for neuroanatomical analyses.

Tab. 5: Rearing temperature affects the duration of the post-capping period and the emergence rates in honeybee workers. Data presented as minima and maxima from observations on three to four brood combs for each temperature.

constant rearing temperature [°C]	post-capping period [days]	emergence rate [%]
28	–	0
29	19 – 22	8 – 12
30	17 – 19	66 – 73
31	14 – 15	89 – 100
32	12 – 15	98 – 100
33.5	11 – 12	97 – 100
34.5	10 – 11	94 – 100
35	10 – 11	96 – 98
36	10 – 11	93 – 99
37	10 – 11	36 – 42
38	–	0

2.2 Queens

2.2.1 Temperature measurement in natural queen cells

In order to estimate the range of natural incubation temperatures I performed measurements in queen cells during the entire post-capping period. In a colony that was prepared for swarming, adult workers constructed elongated, conical cells for queen rearing. Queen cells were located at the periphery of the central brood area, extending downward from the edge of the brood comb. After completion of larval development, temperature measurements within sealed queen cells (n=3) revealed average temperatures of 34.7 ± 0.3°C, 34.8 ± 0.3°C and 35.0 ± 0.3°C throughout the entire pupal development. Despite the peripheral localization, fluctuations of incubation temperatures in queen cells were in a similar range as those described for capped worker brood cells (Hess, 1926; Himmer, 1927a; Heinrich, 1993; Kleinhenz et al., 2003).

2.2.2 Rearing of capped queen brood under different constant temperatures

From the last larval stage onward, queens go through a faster postembryonic development compared to workers (reviewed in Jay, 1963; see also Figs. 18 and 19). To investigate the effect of temperature on the duration of the post-capping period and the number of emerging queens, I reared freshly capped queen cells under different temperature regimes.

Tab. 6: Relation between the incubation temperature in queens and the duration of post-capping period and emergence rates. Data presented as minima and maxima are based on three observations at incubation temperatures from 32.5 – 37°C and a single observations at 28 – 31.5°C and 38°C, respectively.

constant rearing temperature [°C]	reared pupae [n]	post-capping period [days]	emergence rate [%]
28	10	–	0
29	10	–	0
30.5	11	8	91
31.5	27	8 – 9	100
32.5	30	7 – 9	80 – 100
33.5	34	6 – 7	67 – 75
34.5	37	6 – 7	55 – 100
35.5	30	6 – 7	70 – 80
36.5	17	5 – 6	57 – 80
37	22	6 – 7	50 – 60
38	10	–	0

The post-capping period in queens is significantly shorter and appears less sensitive to changes in incubation temperature compared to workers, especially at lower temperatures. Within the range of 33.5 – 37°C the duration of the post-capping period remained almost constant between 5 – 7 days (Tab. 6). At lower temperatures (30.5 – 32.5°C), pupae took 7 – 9 days to complete metamorphosis. All pupae that completed metamorphosis emerged without any external morphological defects.

The average emergence rate in queens was high (71 – 100%) within a relatively broad temperature range (30.5 – 36.5°C) and declined to 50 – 60% at 37°C. However, mortality was 100% at temperatures below 30°C and above 37°C, and metamorphosis at these temperatures stopped at early pupal stages.

3 Influence of temperature on the development of antennal olfactory sensilla in adults

An early study in honeybee workers revealed that the majority of olfactory sensilla (*Sensilla placodea,* poreplates) are distributed on the medial segments of the antennal flagellum (Esslen and Kaissling, 1976; Fig. 21A). To quantify the number of *Sensilla placodea* on medial segments in queens and to compare an effect of temperature on the number of olfactory sensilla between both female castes, I counted the number of *Sensilla placodea* within a defined rectangle (150 x 250µm) on the 5th and 6th medial flagellomere.

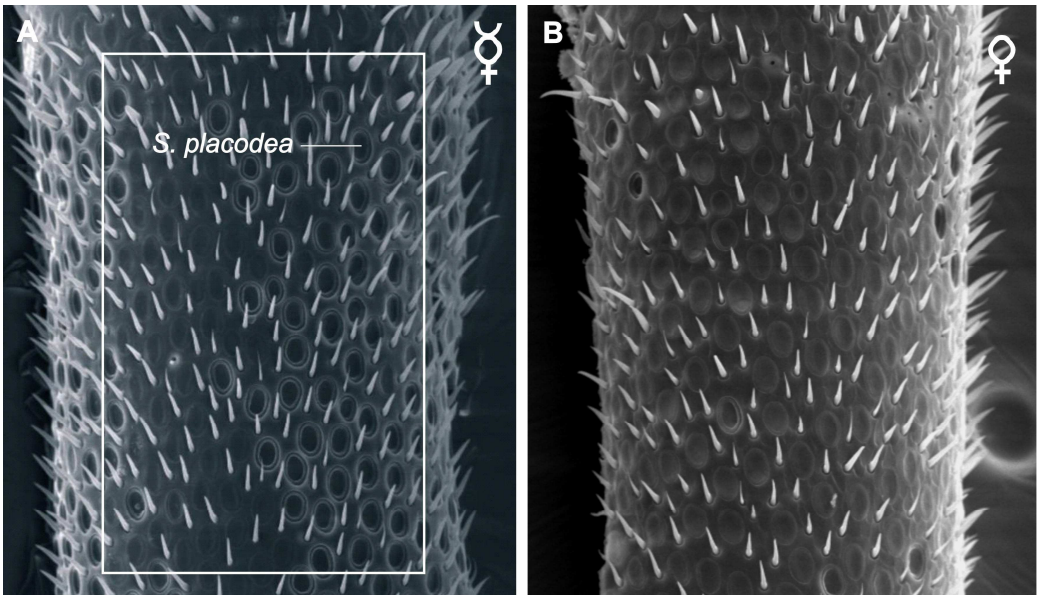

Fig. 21: Scanning electron micrographs of the antennal surface. The 5th flagellar segment bears numerous *Sensilla placodea* in mature **A)** workers and **B)** queens.

The antennae of both female castes were covered with many olfactory sensilla (Fig.21). Based on their distinct cuticular structure, these olfactory receptors were clearly distinguishable from any other antennal receptor type. Compared to workers (Fig. 22, left), queens – regardless if reared under normal or extreme breeding conditions – possessed, on average, a lower number of *Sensilla placodea* on the observed 5th and 6th antennal segment (Fig. 22, right). In both female castes, no significant temperature-mediated effects were found in the number of poreplates on medial flagellar segments, even at extreme temperatures (Mann-Whitney U-test, $p \geq 0.05$).

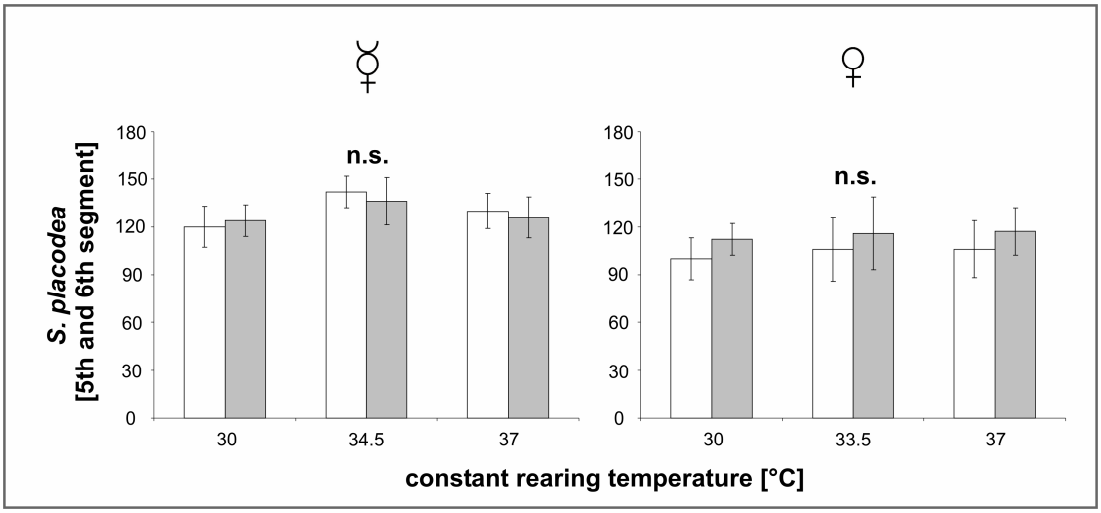

Fig. 22: Temperature mediated effects on the number of *Sensilla placodea* on medial antennal segments (n=6 each). In both female castes, the estimated number of *S. placodea* is not affected by the temperature experienced during post-capping period. White column: 5th segment, grey column: 6th segment.

4 Developmental plasticity in the formation of primary and secondary olfactory neuropils during post-capping period

In honeybee colonies, queens develop from fertilized eggs that are genetically not different from eggs that develop into workers (Weaver, 1957). Within the period of postembryonic pupal metamorphosis, queens develop much faster than workers. Nevertheless they are, as adults, larger than workers, live much longer, and display a very different behavior. To study the ontogenetic plasticity of the brain and to begin to unravel possible neuronal correlates for the differences in behavior, I comparatively analyzed the postembryonic differentiation of olfactory neuropils in the brain of workers and queens (constantly reared at 34.5°C) using two synaptic markers.

4.1 Development of the antennal lobe and the mushroom body calyx in workers

In adult bees, the AL neuropil consists of spherical glomeruli where receptor neuron terminals synapse with central neuron processes. In the mature AL, phalloidin labeling indicates high concentration of F-actin in presynaptic axonal compartments within the glomeruli (Rössler et al., 2002). To visualize glomerulus formation during the post-capping period, I performed immunofluorescent stainings of growing neurons using fluophore-conjugated phalloidin. To look more closely into the compartmentalization of the synaptic neuropil within glomeruli, the preparations were double-labeled with an antibody to synapsin that is restricted to presynaptic compartments (Klagges et al., 1996).

Following the time course of prepupal and pupal development, bright phalloidin labeling was observed within the area where glomeruli arise, within preglomeruli and within glomeruli, respectively (Fig. 23). In addition, phalloidin labeling was also prominent along the olfactory receptor axons within the antennal nerve during all developmental stages. At the prepupal stage (PP), the AL neuropil was homogeneously structured. At this time, ORN axons already invade the AL projecting to different peripheral AL regions (Hähnlein and Bicker, 1997). During the next two days (P1 – P2), the still homogenous AL neuropil increased in size. From late P2/early P3 onward, spherical structures, that were more irregular shaped than mature glomeruli, appeared in the periphery of the AL neuropil. This corresponds to a study from Gascuel and Masson (1991) describing that axonal terminals aggregate first into glomerular precursor structures – named preglomeruli – at P3. At pupal stage 6, glomeruli had formed and the Al neuropil showed an adult-like arrangement. Phalloidin labeling was concentrated within all glomeruli and absent from dendritic branches and processes of neurons as well as glial cells within the center of the AL. During the residual pupal stages (P7 – P9), glomeruli increased enormously in volume.

During the entire post-capping period, synapsin-IR was prominent within the developing AL neuropil and lacking along the ORN axons. From P3 onward, synapsin was concentrated exclusively within the AL neuropil. By pupal stage 5, synapsin-IR and phalloidin labeling showed a little overlap which increased during the following two subsequent days (P6 – P7). From that time on, the distribution of synapsin-IR resembled the distribution of phalloidin. This coincides with the observation that ORN axonal terminals concentrate in the cortical layer of the mature glomeruli (Gascuel and Masson, 1991).

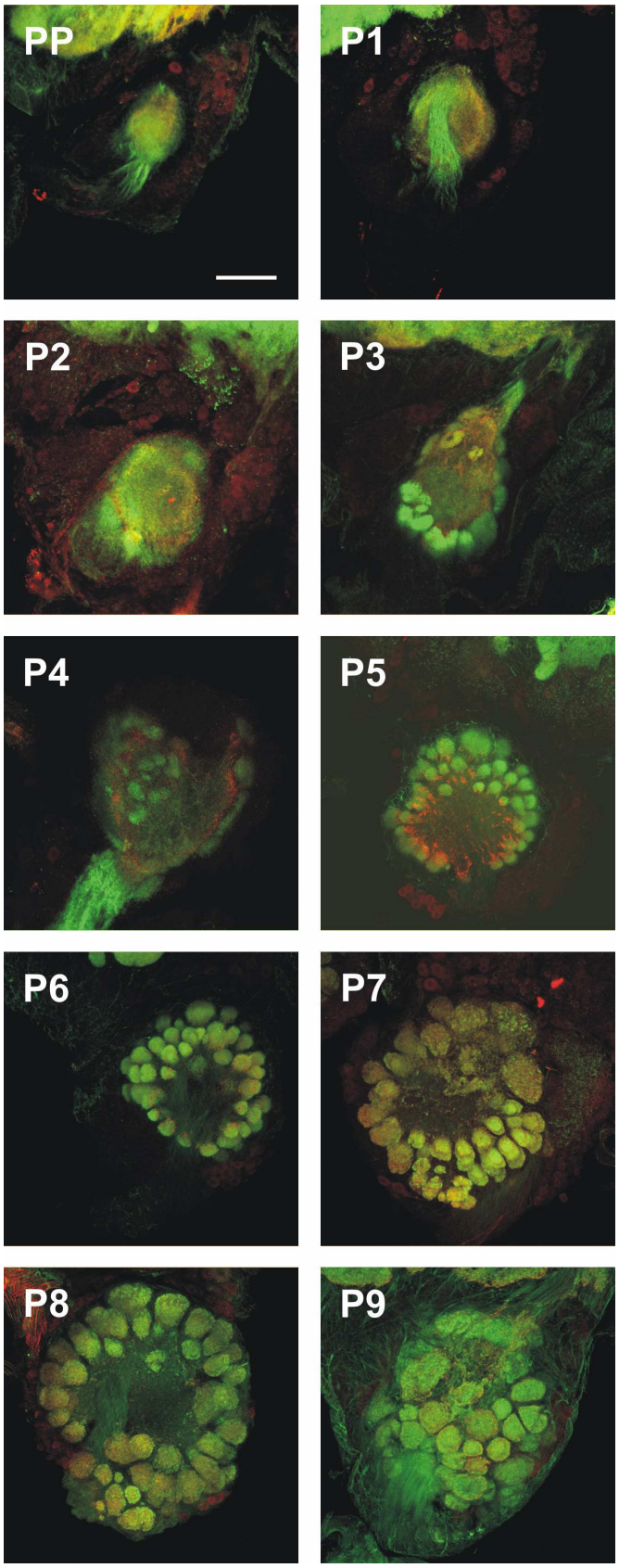

Fig. 23: Differentiation of the AL neuropil in worker prepupae (PP) and pupae (P1 – P9). Brains were labeled using two synaptic neuropil markers: an antibody to synapsin (red) and fluophore-conjugated phalloidin (green). Note the overlap between both stainings (yellow areas). Stack size (thickness) PP – P9: 35 – 40µm. Scale bar: 100µm.

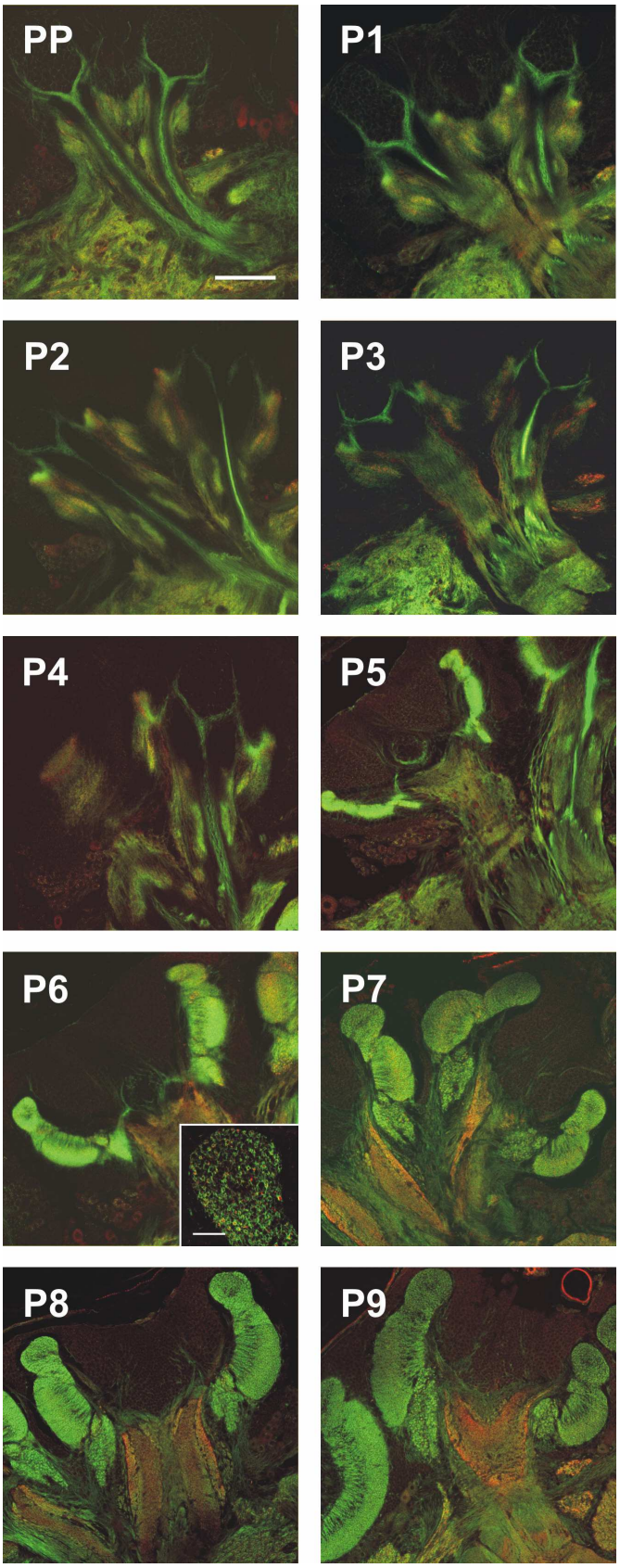

Fig.24: Confocal micrographs of the MB calyx at successive prepupal and pupal stages of workers showing the formation of synaptic complexes (microglomeruli). Stack size (thickness) PP – P9: single optical images. Scale bar: 100µm, inset at P6: 20µm.

In addition to the differentiation process of the AL, phalloidin is a useful marker to visualize the compartmentalization of the calyx subdivisions. Using phalloidin labeling, the MB calyx is detectable in the prepupa (PP) as homogenously textured neuropil, the so-called "calyx-developing-zone" (Fig. 24; Menzel et al., 1994). After prepupal ecdysis, the "calyx-developing-zone" began to divide into the olfactory lip and the visual collar. From P1 to P4, phalloidin labeling was highly concentrated in the calyx lip region. During this time, non-compact KCs forming the lip and the collar migrated to the periphery of the calyx neuropil (Schröter and Malun, 2000). At P5, the basal ring emerged and the calyx exhibited, for the first time, an adult-like architecture. From pupal stage 6 on, characteristic synaptic complexes (microglomeruli [MG]) that appeared as ring-shaped structures in single optical sections became clearly visible (see inset in Fig. 24). Within the MG of the calyces, similar as in adult crickets, intense phalloidin labeling was strictly accumulated in the postsynaptic dendritic spines surrounding a central core that appeared to be devoid of fluorescent phalloidin (for crickets compare Frambach et al., 2004).

Double-labeling with anti-synapsin visualized the presynaptic site of synaptic complexes within the MB calyx in honeybees. Within the first half of pupal development (PP – P5), a few synapsin-IR boutons were visible within the developing calyx neuropil (Fig. 24). As the homogenously structured calyx neuropil divides into lip and collar, olfactory and visual PNs begin to innervate their non-overlapping synaptic target areas (Schröter and Malun, 2000). At P6, anti-synapsin labeled boutons of PNs were, for the first time, encircled by KC dendritic spines (formation of MG). The number of phalloidin-surrounded presynaptic boutons increased during residual pupal stages (P7 – P9).

4.2 Development of the antennal lobe and the mushroom body calyx in queens

For honeybee queens, the development of primary and secondary olfactory neuropils during metamorphosis has not been described previously. This may particularly be due to costly rearing procedures. Twice a day, I dissected brains of metamorphosing queens to detect the formation of olfactory neuropils during their fast post-capping period. To visualize the development of this system, I used fluophore-conjugated phalloidin to label F-actin microfilaments.

From PP to early P2, the AL neuropil exhibited no subcompartments (Fig. 25, upper). At late P2, irregularly shaped preglomeruli were visible at the periphery of the developing AL neuropil. Within the next pupal stage (P3), glomeruli had formed, and the structure of AL

neuropil appeared adult-like. During the residual pupal stages (P4 – P6), the volume of the AL neuropil increased. Taken together, the formation of the first olfactory glomeruli appeared earlier in queens than in workers. Upon emergence, it was shown that the AL overall size and the size of individual glomeruli were smaller in queens compared to workers (Müller, 2005).

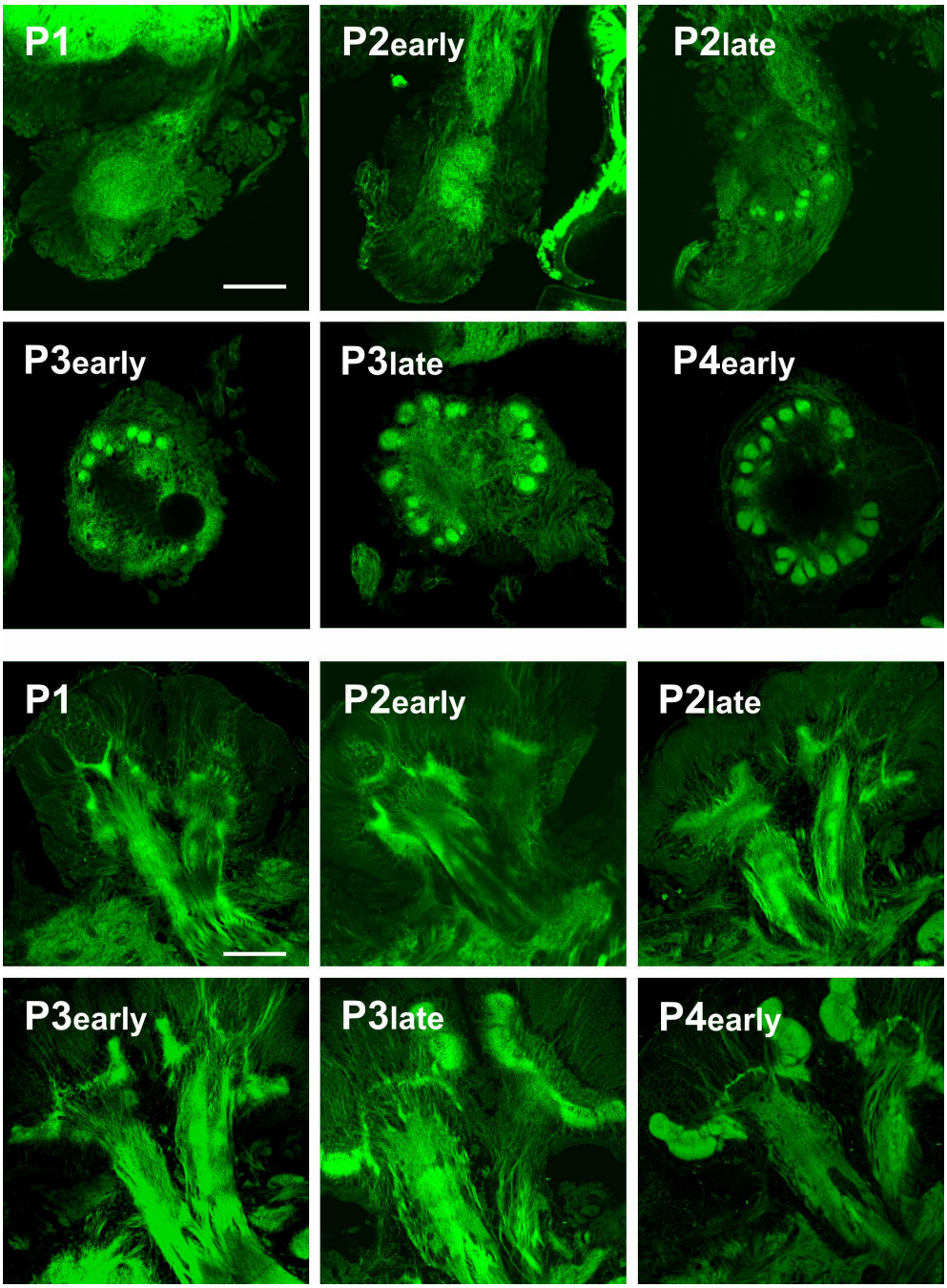

Fig. 25: Confocal micrographs of the AL (upper) and the MB calyces (lower) at successive pupal stages showing phalloidin labeled F-actin. Note the fast formation of the primary and secondary olfactory centers at pupal stage 2/3. Stack size (thickness) P1 – P4: single optical images. P: pupa. Scale bar: 100µm.

In addition, the sequence in the formation of subunits in the MB calyx did differ in queens and workers. Using phalloidin labeling, the lip and the collar were already distinguishable at P1 (Fig. 25, lower). At late P3, the basal ring emerged from the "calyx-developing-zone". From this time on, the architecture of the calyx was adult-like and increased progressively in volume. In addition, distinct phalloidin-labeled postsynaptic profiles appeared within the MB calyx.

Fig. 26 summarizes the main events in the differentiation process of both the AL and MB calyx comparing workers and queens.

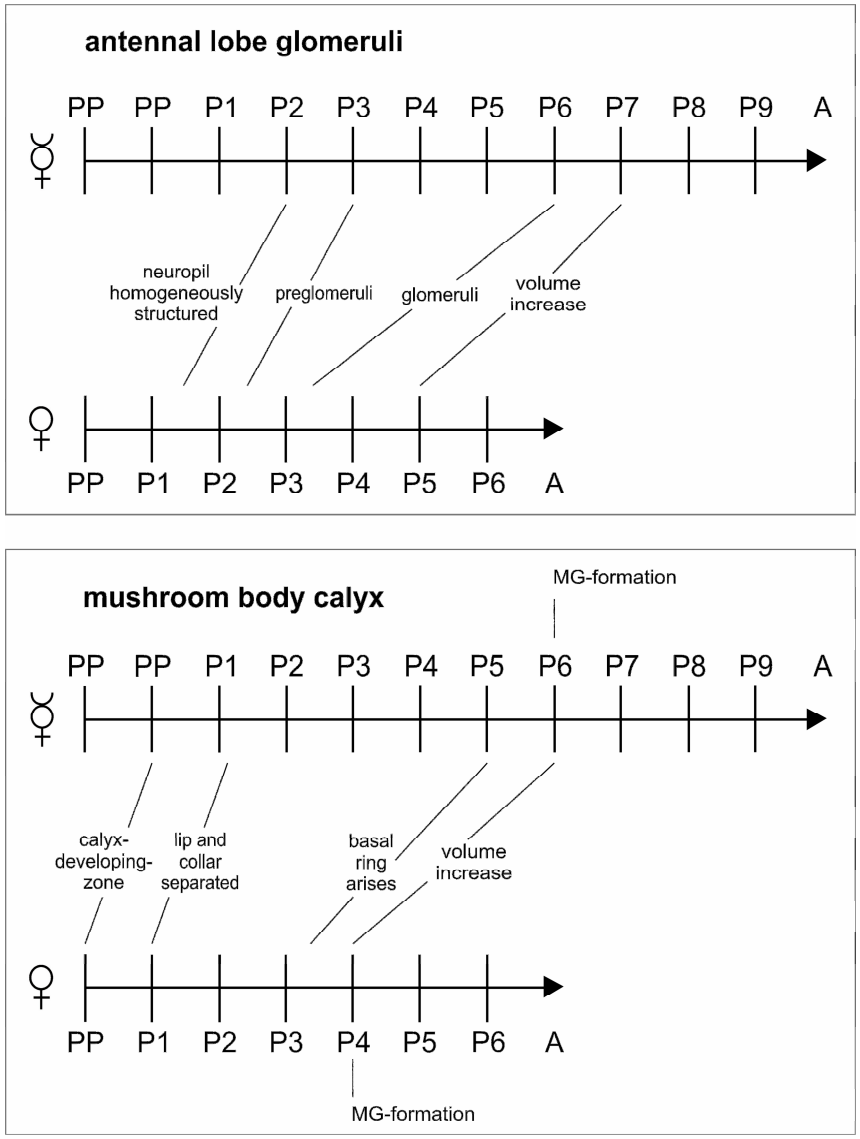

Fig. 26: The ontogenetic plasticity between workers and queens is reflected in the development of their brain. The timetable summarizes the main events in the differentiation process of the AL (upper) and the MB calyces (lower) during larva-adult metamorphosis. A: adult, MG: microglomerulus, P: pupa, PP: prepupa.

4.3 Development of serotonergic neurons in workers

In adult honeybee workers, the nervous system contains high levels of biogenic amines (Mercer et al., 1983; Schürmann and Klemm, 1984) that are influenced by environmental as well as genetic factors and change during maturation (Harris and Woodring, 1992; Taylor et al., 1992). Biogenic amines, in particular serotonin (5-HT), modulate a number of responses in the adult sensory system (Erber et al., 1993). Serotonin depresses neural and behavioral activity (Lopatina and Dolotovskaya, 1984; Lopatina et al., 1985). Learning paradigms demonstrate that the injection of serotonin into the CNS reduces the response to conditioned olfactory stimuli (Mercer, 1982; Mercer and Menzel, 1982). Additionally, serotonin may not only act as neurotransmitter or modulator in mature neuronal circuits. The presence of serotonin in outgrowing neurites at the last larval instar suggests that it may also have developmental functions (Boleli et al., 1995). In *Manduca sexta*, serotonin enhances the growth of AL neurons *in vitro* (Mercer et al., 1996). As serotonin may influence neural outgrowth during insect metamorphosis, I examined the developmental expression of serotonin-immunoreactive (serotonin-IR) fibers in olfactory neuropils in the postembryonic nervous system of the workers.

Earlier immunofluorescence studies described that the adult worker brain contains about 75 serotonin-IR cell bodies grouped into distinct clusters (Schürmann and Klemm, 1984; Rehder et al., 1987). Serotonin-immunoreactivity in the AL arises from a single interneuron, the so-called deutocerebral giant (DCG) that interconnects the antennal and dorsal lobes with the suboesophageal ganglion (Rehder et al., 1987).

This study revealed that serotonin-immunoreactivity in the outgrowing DCG appeared before the neurites have reached their target neuropil, as tiny serotonin-IR fibers were visible within the homogenously textured AL neuropil during the prepupal period (Fig. 27, upper). After pupal ecdysis, the branching patterns of serotonin-IR fibers conspicuously increased within the still unstructured AL neuropil. During preglomeruli formation (P3 – P5), the network of serotonin-IR fibers was present within the center of the AL neuropil. At pupal stage 5 (P5), these fibers began to invade preglomeruli, as there was a little overlap between phalloidin-labeled preglomeruli and serotonin-labeled fibers. From P7 onward, when glomeruli had formed and the adult-like AL increased in volume, serotonin-IR fibers were restricted to the periphery of the AL, where they vastly penetrated glomeruli.

Whereas the DCG was already visible in the developing AL neuropil before prepupal ecdysis, the calyces of the mushroom bodies were devoid of serotonin-IR processes during the entire post-capping period (Fig. 27, lower). The lack of serotonin-immunoreactivity in the MB calyces was not due to an incomplete staining, as tiny serotonin-IR fiber branches were visible in the adjacent peduncle from P2 onward. Even in adult worker brains, no serotonin-IR fibers were identifiable in the MB calyces (Schürmann and Klemm, 1984; reviewed in Bicker, 1999; own data). This corroborates the consideration that serotonin-IR fibers in the MBs are extrinsic in origin and spread mainly into the peduncle and the lobes (Schürmann and Klemm, 1984).

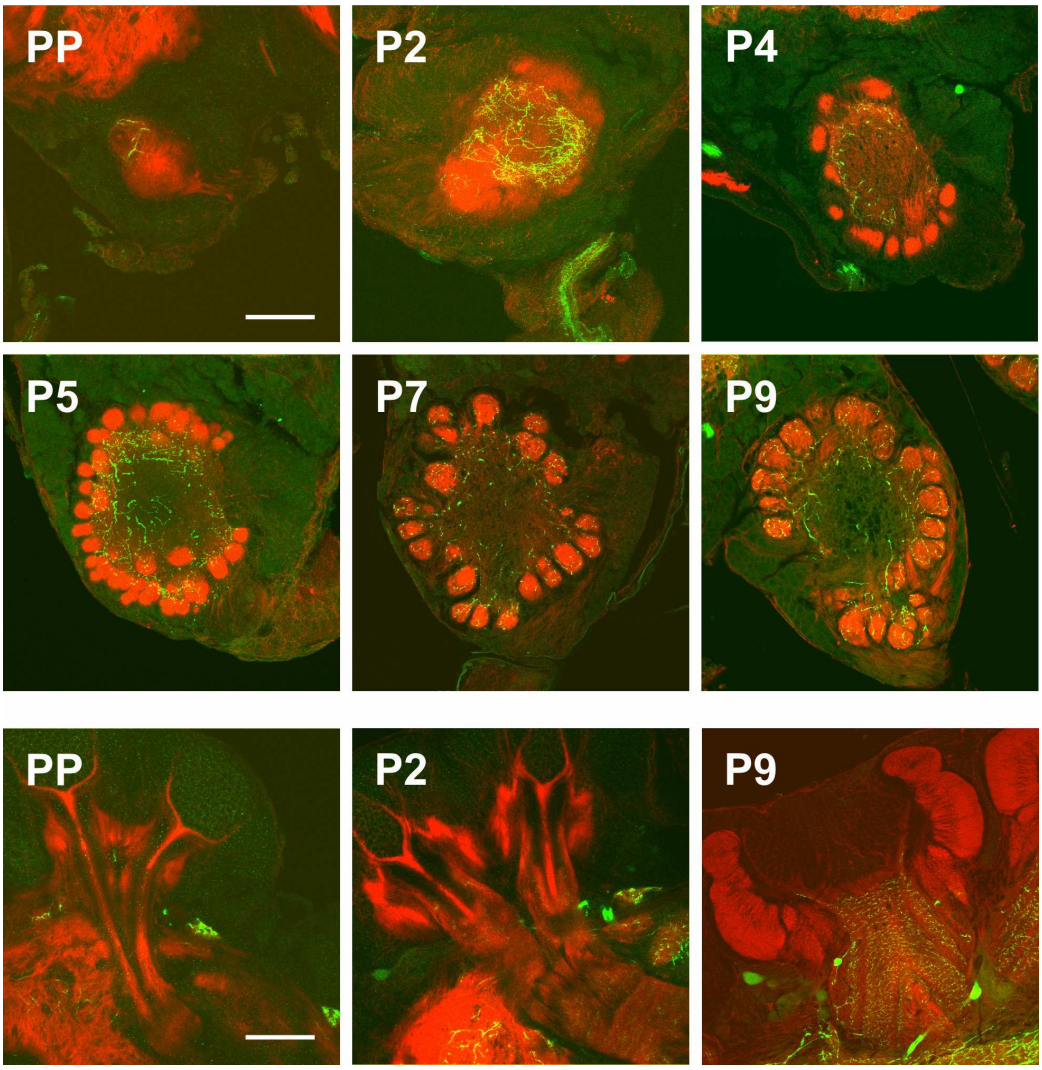

Fig. 27: Serotonin-immunoreactive (serotonin-IR) fibers in the brain of developing worker prepupae and pupae. The brains were double-labeled with phalloidin (red) and an antibody specific for serotonin (green). Serotonin-IR fibers invade the developing AL lobe neuropil from prepupal stage onward (upper). The mushroom body calyces do not express any immunofluorescence (lower). P: pupa. Scale bar: 100µm.

5 Temperature influences during post-capping period on developing olfactory neuropils in workers

The temperature experienced during post-capping period affected the developmental time and the emergence rate of workers (Tab. 5). In contrast to these general effects of temperature, specific effects on the developing brain are more difficult to examine. To gain insight into temperature influences during post-capping period on developing olfactory neuropils, I reared freshly sealed brood under different constant temperature regimes. For immunofluorescence staining, brains were dissected six days after cell capping that marks the end of the first half of post-capping period in bees reared at 34.5°C.

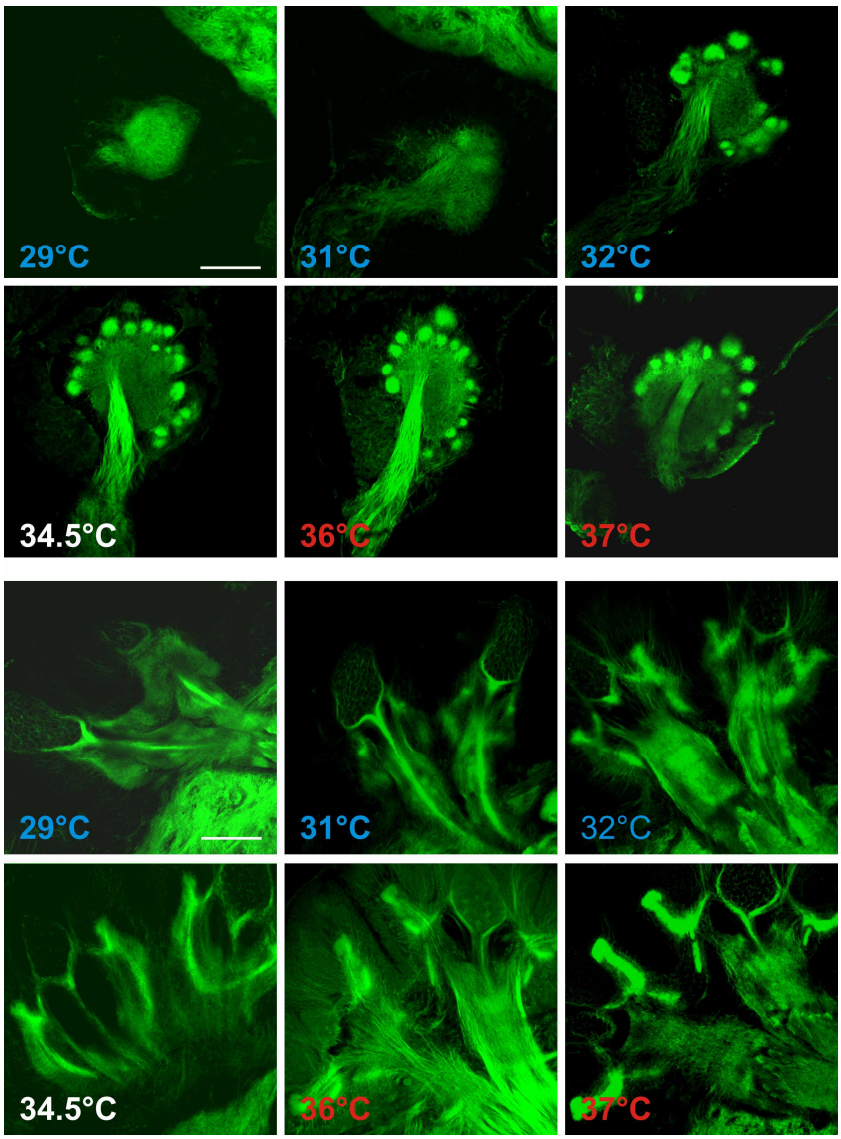

Fig. 28: Temperature influence during the first 6 days of post-capping period on synaptic neuropil development (labeled with phalloidin) in the AL (upper) and MB calyces (lower). Scale bar: 100µm.

After cell capping, temperature deviations obviously influenced not only the development of morphological features (Fig. 18), but also the development of neuropil structures in workers (Fig. 28). Within the AL neuropil, the formation into various preglomeruli was clearly identifiable in brains of pupae reared at 34.5°C, the temperature normally maintained in the brood nest (Fig. 28, upper; see also Fig. 23 for comparison). Rearing pupae at lower temperatures noticeably delayed the synaptic neuropil development within the AL. Pupae exposed to 29°C exhibited a still homogeneously textured AL neuropil. A few irregularly shaped preglomeruli started to arise at the periphery of the AL in pupae incubated at 31°C and were clearly visible in pupae reared at 32°C. Pupal rearing above 34.5°C revealed no obvious differences in the formation of preglomeruli and the size of the AL neuropil.

In addition, the temperature experienced during post-capping period affected the compartmentalization of the MB calyx neuropil (Fig. 28, lower). The lip and the collar of the MB calyx were distinguishable in pupae reared between 31 and 34.5°C, but the size of the calyx decreased at lower temperatures. In brains of pupae incubated at 29°C, the calyx neuropil was visible as small, homogenously structured neuropil that was not divided into subcompartments. Rearing pupae above 34.5°C revealed an increase in the formation of the calyx subdivisions. In pupae exposed to 36 and 37°C, the basal ring already emerged and the calyx exhibited an adult-like architecture.

6 Temperature influences during post-capping period on the synaptic organization of the adult mushroom body neuropil of workers

During pupal metamorphosis of honeybees the temperature is controlled precisely close to 35±0.5°C (Hess, 1926; Himmer, 1927a). Exposure to strong deviations from normal brood temperatures are known to result in increased pupal development time, mortality (Tab. 5) and morphological deficits (e.g., Himmer, 1927a). Behavioral studies also suggest that behavioral performance in adult honeybees is affected by the temperature experienced during the post-capping period, even within the range of natural occurring temperatures. Especially lower rearing temperatures cause deficits in olfactory learning in adult workers (Tautz et al., 2003). Here I explored whether minimal differences in the temperature normally maintained in the capped brood area may influence the synaptic maturation in the developing nervous system. The analyses particularly focused on the synaptic input regions in the MB calyces. These prominent neuropils function as multimodal sensory integration centers (e.g., Menzel, 2001).

6.1 Characterization of microglomeruli in the adult calyx

In the adult honeybee brain, phalloidin, specifically binding to F-actin (Wieland, 1987; Lee and Cleveland, 1996), labeled all synaptic neuropils (Fig. 17A) as shown previously for other insect species (Rössler et al., 2002; Frambach et al., 2004). I found that the MB calyx was among the most intensely labeled structures, and the lip, collar and basal ring were clearly distinguishable (Fig. 17B).

Using high-resolution confocal microscopy and double-labeling with anti-synapsin visualized sites of calycal synaptic contact named microglomeruli (MG; Frambach et al., 2004). Synapsin-IR labeled the central bouton of MG and phalloidin the surrounding area (Fig. 29A – C, and reconstructed in J) which appeared as a ring-like structure in single optical sections. Labeling of antennal lobe PN axons (Fig. 29D – G) and KC dendrites (Fig. 29H and I) using rhodamine dextran combined with phalloidin labeling revealed that PN boutons occupy the central core of MG, and KC dendrites colocalize with phalloidinergic rings (Fig. 29H – J). This indicates that F-actin is located predominantly in the postsynaptic (dendritic) compartments of MG. Similar results were found in cricket MBs (Frambach et al, 2004). Phalloidin labeling, therefore, is a useful method to quantify the distribution of MG within subcompartments of the MB calyx.

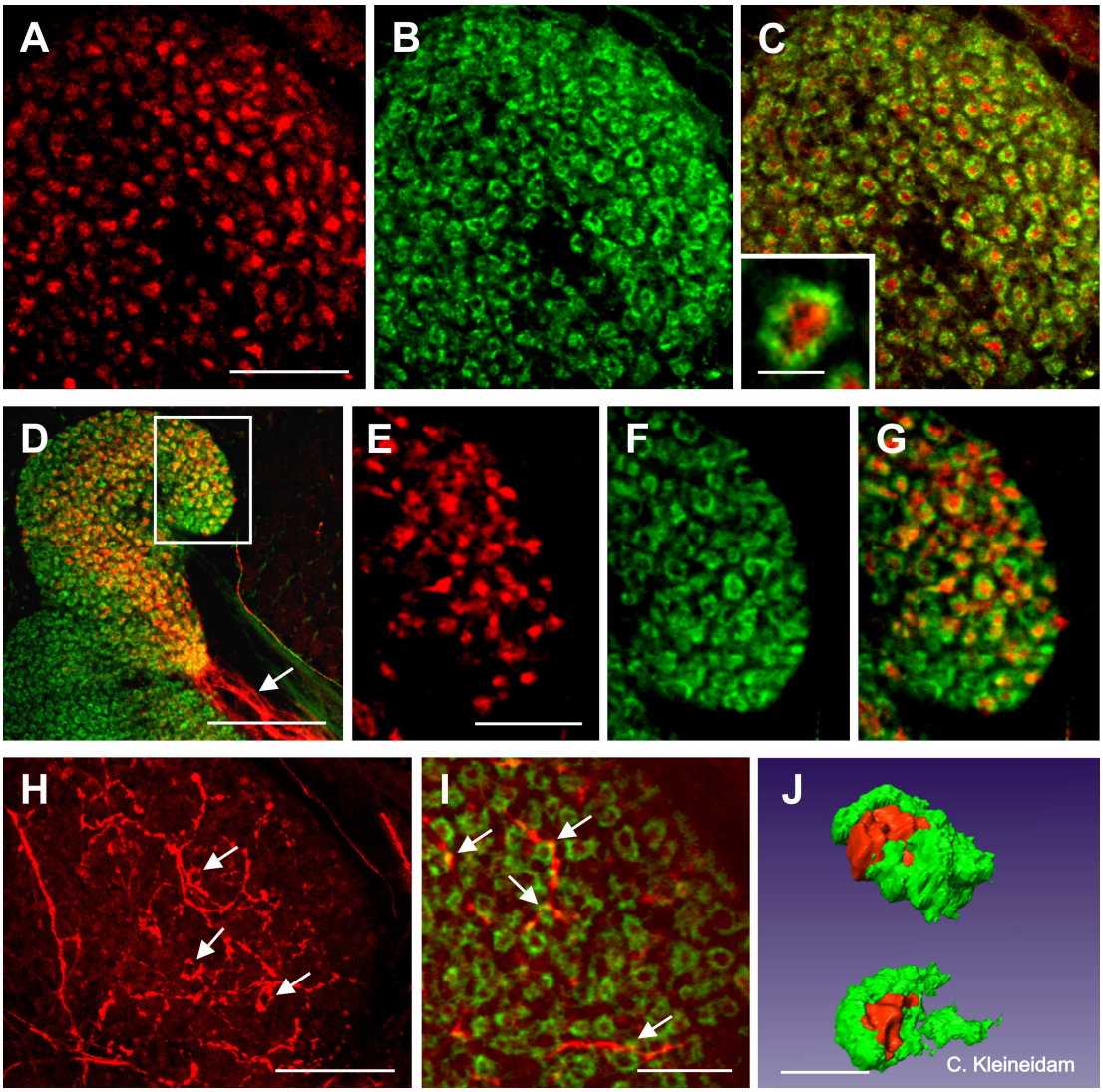

Fig. 29: Immunofluorescence labeling of MG in the MB calyx of the worker brain. **A – C)** Double-labeling of the calyx lip (synapsin-IR, red; phalloidin, green). Merged images in C (inset: high magnification of an individual MG). **D – G)** Antennal lobe PNs (E, red) and phalloidin (F, green). The image is merged in D and G. The arrow in D indicates PN axons. **H and I)** KC dendrites (H, 16.2µm stack of 27 sections) and phalloidin labeling (I, green). Merged images of single optical sections are shown in I. The arrows indicate MG and overlap. **J)** 3D reconstruction of two MG (synapsin-IR, red; phalloidin, green) using AMIRA software. Scale bars: A – C: 20µm (inset: 3µm), D: 50µm, E – H: 20µm, I: 10µm, J: 3µm.

6.2 Temperature-dependent developmental plasticity of synaptic complexes in the calyx of freshly emerged workers

The MBs are strongly suggested to play a key role in the neuronal control of adaptive behavioral modifications in adult insects (*Apis mellifera*: e.g., Erber et al., 1980; *Drosophila melanogaster*: reviewed in Heisenberg, 1994). Therefore, synaptic plasticity very likely occurs in the adult MBs. F-actin is assumed to constitute the structural basis of

synaptic plasticity in vertebrates as well as in insects (e.g., Matus, 1999; Frambach et al., 2004). Thus, labeling of MB synaptic complexes (microglomeruli, MG) using fluophore-conjugated phalloidin and double-labeling with anti-synapsin appears to be useful to investigate thermoregulatory influences during post-capping period and their possible consequences for the synaptic organization in the adult MB calyx.

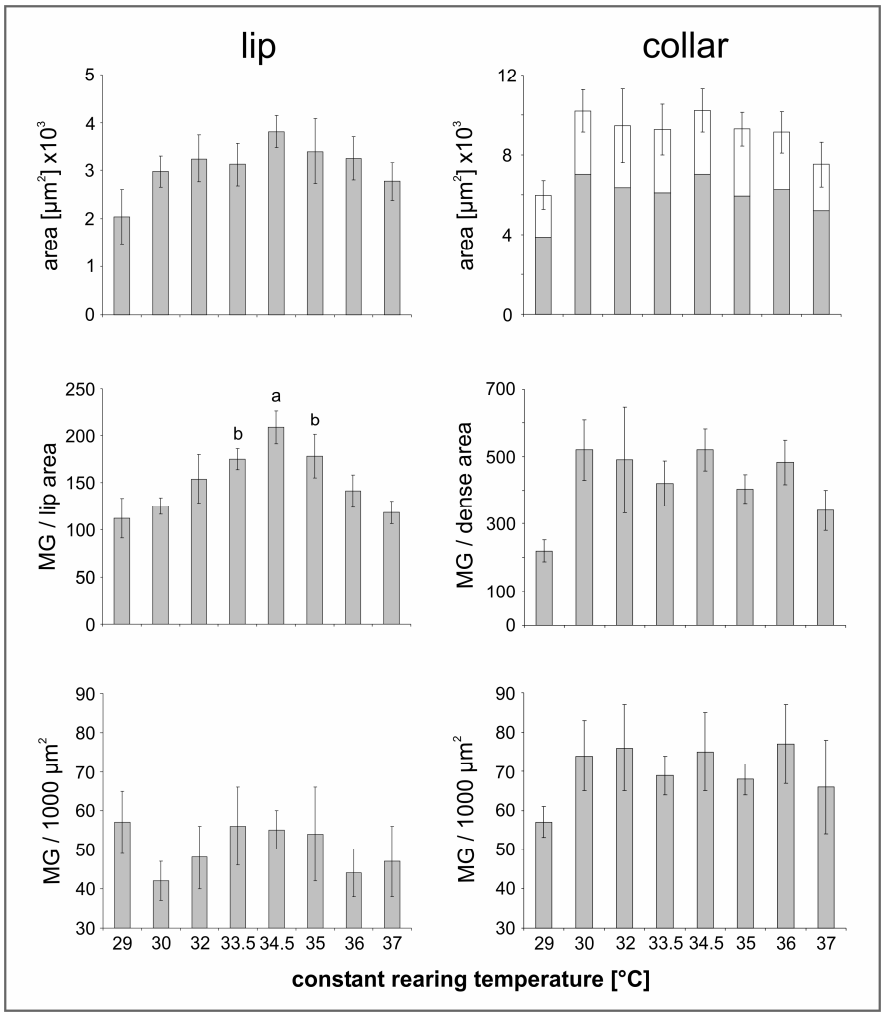

Fig. 30: Changes in MG with different rearing temperatures (1-day-old bees). The histograms at the top show the changes in cross-sectional areas of the olfactory lip (left) and the visual collar (right; white bars: loose region, grey bars: dense region). The histograms in the middle show the number of MG profiles in the lip (left) and the dense portion of the collar (right) (a and b indicate significance). The histograms at the bottom show the estimated density of MG in the lip (left) and the dense region of the collar (right; normalized to 1,000µm^2).

In the olfactory lip of the MB calyx, the dimensions of the cross-sectional areas and the number of MG varied at different rearing temperatures (Fig. 30, left). Significant changes in the number of MG profiles were found at temperature differences of 1°C (e.g., 34.5° vs. 33.5°, n=4, $p<0.001$; 34.5° vs. 35°, n=4, $p<0.004$; 34.5° vs. 36°, n=4, $p<0.001$). The

number of MG profiles in the lip was highest at 34.5°C, decreased above and below, and dropped to almost 50% at 29°C and 37°C. The density of MG was highest at 33.5 – 35.5°C and at 29°C. Comparison with synapsin-IR indicated that the number of presynaptic boutons was also affected (data not shown). In the adjacent visual input region (collar), temperature-mediated effects were less obvious between 30°C and 36°C (Fig. 30, right). A significant decrease in MG profiles, however, was observed at 29° and 37°C (34.5°C vs. 29°, n=4, $p<0.001$; 34.5° vs. 37°, n=4, $p<0.001$). The proportions of the dense (70 – 72%) and loose (28 – 30%) regions remained essentially constant.

The size of individually phalloidin-labeled MG showed a tendency to increase at low and high temperatures. At 34.5°C in the lip, the outer diameter of MG profiles ranged around 3µm (3.09 $\pm$ 0.2 µm; 60 randomly selected MG from 3 bees). At 29° and 37°C, MG with diameters ≥3.5 – 4 µm were observed frequently in the lip region. MG profiles in the collar were smaller (1.89 $\pm$ 0.15 µm; 60 MG from 3 bees), and temperature-dependent changes were not detectable.

To reveal temperature-mediated effects on the morphology of individual KC dendritic spines in the MB calyx lip, two modifications of the Golgi method were used. Applying the Golgi-Colonnier method turned out that less glial cells were stained within the tissue.

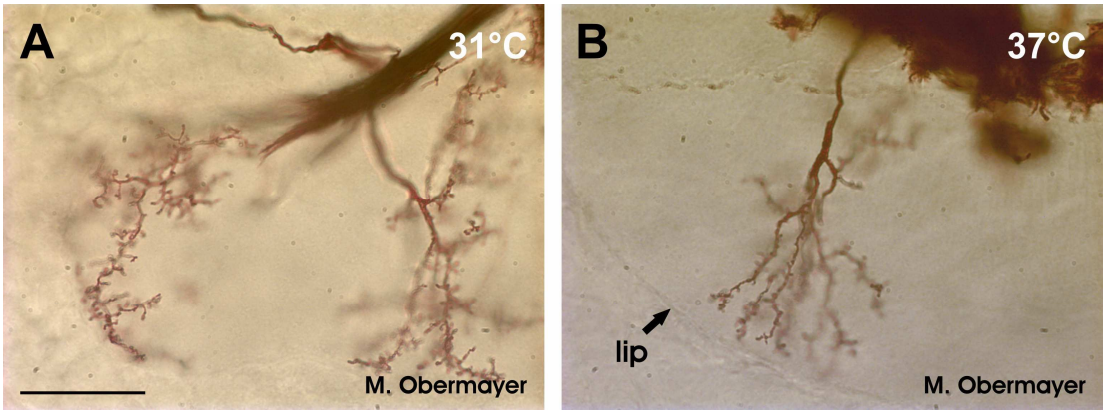

Fig. 31: Golgi-impregnated Kenyon cells of the olfactory calyx lip comparing dendritic spines in freshly emerged bees reared at **A)** lower (31°C) and **B)** higher (37°C) temperatures than naturally occur within the hive (32 – 36°C). Scale bar: 20µm.

Dendritic spines were clearly visible along the length of KC dendrites in the calyx lip. No conspicuous differences in dendritic spine morphology in the calyx lip of all temperature treated bees were found, not even at extreme rearing temperatures (Fig. 31A and B). Temperature-mediated effects on the branching and length of KC dendrites in the olfactory lip are possible, but need to be analyzed in future studies according to Farris et al. (2001).

6.3 Temperature-dependent developmental plasticity of synaptic complexes in the calyx of seven-day-old workers

To find out whether changes in MG numbers persist, I performed similar measurements in the MB calyx of 7-day-old worker bees. The brains were taken from bees that had been tested in a previous study (Tautz et al., 2003).

The estimated numbers of MG profiles yielded similar values to one-day-old bees (34.5°C vs. 32°C, n=4, $p<0.02$; 34.5°C vs. 36°C, n=4, $p<0.03$). Absolute numbers and densities of MG were not different from those in freshly emerged bees (Fig. 32). In the collar, similar as in one-day-old bees, changes in MG were not detectable. The results indicated that temperature-mediated effects on MG numbers are not compensated during the first week of adult life.

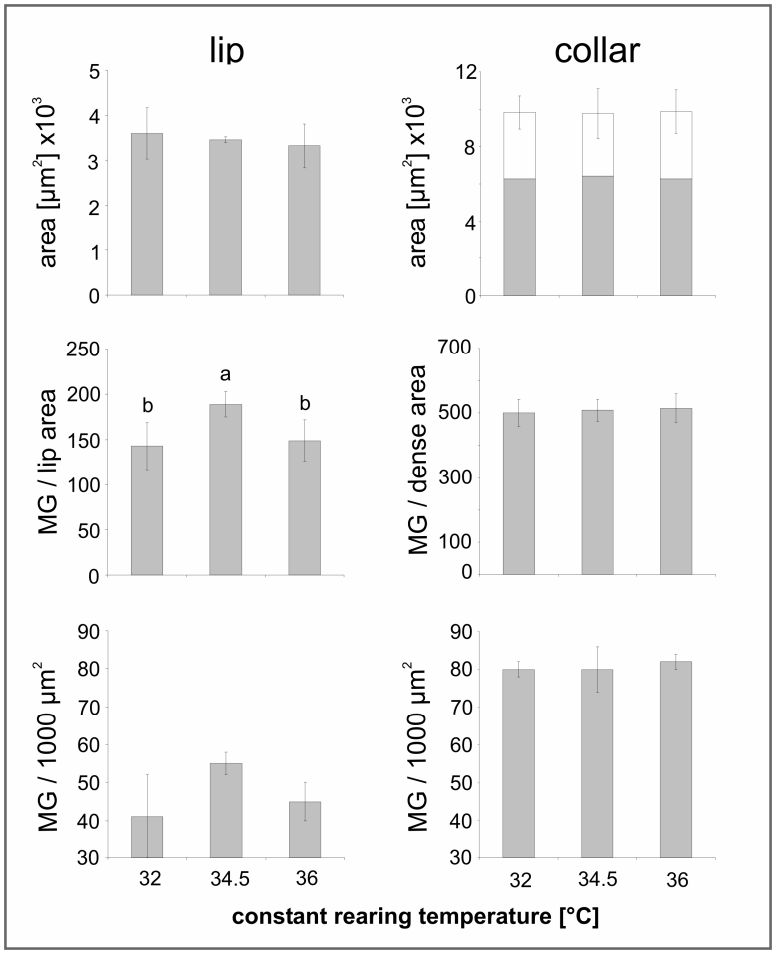

Fig. 32: Changes in MG with different rearing temperatures (7-day-old bees). Cross-sectional areas in the lip (top, left) and collar (top, right; white bars: loose region, grey bars: dense region). Number of MG profiles in the lip (middle, left; a and b indicate significance) and the dense region of the collar (middle, right). Estimated density of MG in the lip (bottom, left) and the dense region of the collar (bottom, right; normalized to 1,000µm^2).

6.4 Effects of two-step temperature treatments during post-capping period on calycal synaptic complexes of freshly emerged workers

Developing honeybee workers are very sensitive to temperature changes before prepupal ecdysis and during the first half of the subsequent pupal metamorphosis (Gontarski, 1957; Weiss, 1962). To gain insight into the effect of the incubation temperature during the first days of post-capping period on the synaptic organization in the adult brain, freshly capped brood cells were reared at different two-step temperature regimes. Developing workers were temperature treated (within the range of naturally occurring temperatures) during the first six days of post-capping period and then reared at 34.5°C until emergence.

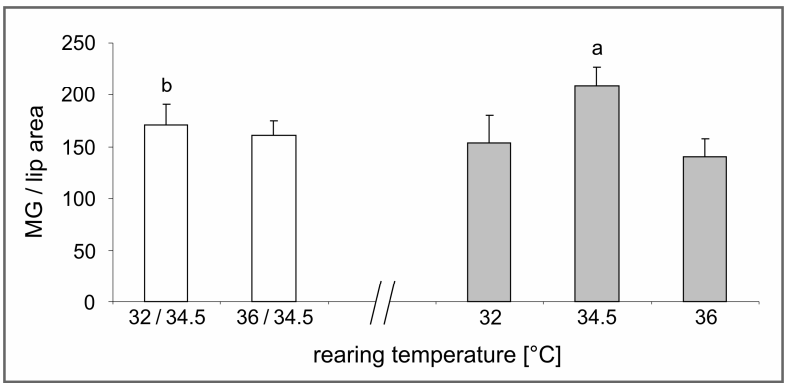

Fig. 33: Effect of two step temperature regimes during post-capping period on MG numbers in the olfactory lip region of one-day-old workers. Changes in MG are significant even within the range of naturally occurring temperatures (white bars: two-step temperature treatment, grey bars: constant rearing).

In workers that had been incubated at different constant temperatures during the entire post-capping period, the numbers of calycal MG were different in the olfactory lip. As shown in Fig. 30, the number of MG was significantly highest in bees raised at 34.5°C and decreased above and below (see also Fig. 33, grey bars). In addition, even within the temperature range maintained in the hive, changes in MG numbers were detectable in bees that were temperature-treated only during the first six days of post-capping period (Fig. 33, white bars). Temperature treatment within this thermosensitive period of metamorphosis significantly affected the number of calycal MG in the olfactory lip region of freshly emerged workers (32/34.5°C vs. 34.5°C, n=4, $p<0.025$). In comparison with constant rearing conditions, the two-step temperature treatments revealed intermediate MG numbers in the olfactory lip region. General effects of temperature manipulations that vary randomly within naturally occurring temperatures on MG numbers need to be examined more closely in further studies.

7 Temperature- and age-dependent plasticity of synaptic complexes in the adult mushroom body calyx of queens

Inside the honeybee society, the queen and the worker caste differ substantially in anatomy, physiology, life-span and behavior. As the higher order neuropils in the brain contribute to differences in behavior, I analyzed environment- and age-dependent effects on the synaptic organization within the MBs of adult queens.

7.1 Characterization of microglomeruli in the calyx of young queens

In order to compare the anatomy of synaptic neuropils within the MBs in brains of freshly emerged queens with those of workers reared under normal conditions (34.5°C) (Fig. 34), queen brains were double-labeled with fluophore-conjugated phalloidin and an antibody to synapsin.

In general, phalloidin labeling in the brain of freshly emerged queens was comparable to those conditions found in workers (compare Fig. 34A and Fig. 17A). I found intense phalloidin labeling in all major brain neuropils such as the ALs, the MBs, the central body and the protocerebral lobes (Fig. 34A). Scaled drawings of a frontal view of the dorsal brain of a queen and a worker illustrate differences in the size and shape of the MBs and the central body (Fig. 34B). All subcompartments of the MBs (calyces, peduncle and lobes) were noticeably smaller in queens compared to workers (Figs. 34B – D). The upper and lower subdivisions (fan-shaped and ellipsoid body) of the central body were slightly smaller in queens, and phalloidin labeling was more intense in the ellipsoid body than in the fan-shaped body (Fig. 34A). Nuclear staining with propidium iodide revealed that in queens the area occupied by KC bodies within the calyx cup appeared more flattened in the lateral and medial calyx of both hemispheres compared to workers (Figs. 34C, D). As already shown in crickets and honeybee workers (*Gryllus bimaculatus*: Frambach et al., 2004; *Apis mellifera*: Groh et al., 2004), double-labeling with phalloidin and anti-synapsin-IR visualized pre- and postsynaptic elements of individual MG at high magnification in the MB calyx (Figs. 34E – H). Each MG comprises a synapsin-IR central core formed by axonal terminals of PNs surrounded by a phalloidin-labeled ring-like structure resulting from F-actin aggregation in KC spines surrounding the central boutons. Although this pattern of phalloidin and anti-synapsin labeled MG in the MB calyx of honeybee queens was comparable to that of workers (Groh et al., 2004), the distribution of MG in the MB

calyx appeared more sparse in queens (Fig. 34E, F). Furthermore, the outer diameter of the phalloidinergic profile of MG was slightly larger in queens (3.29 ± 0.3 µm) compared to workers (3.09 ± 0.2 µm) (60 randomly selected MG from brains of 3 queens and 3 workers).

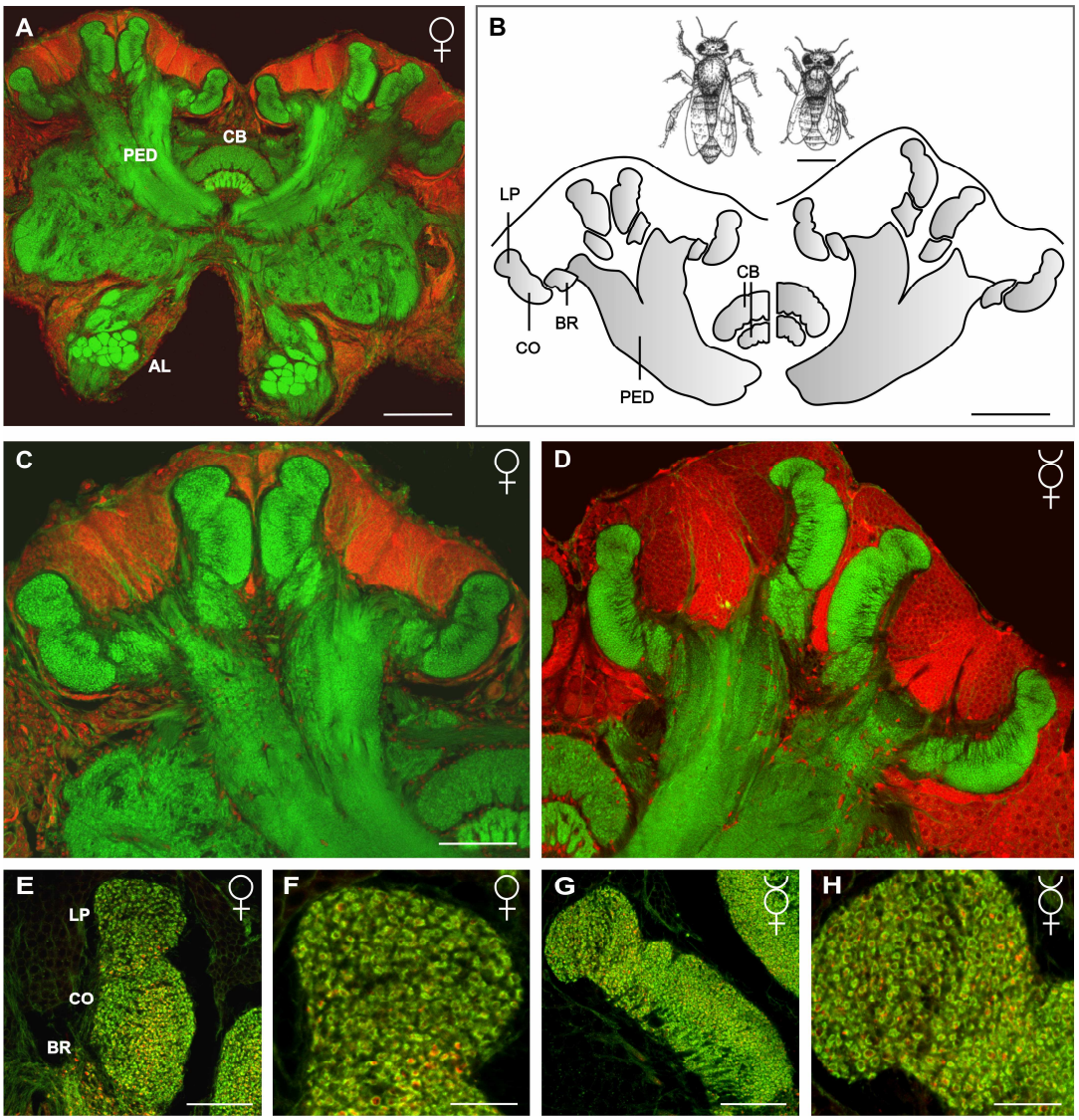

Fig. 34: Morphology of the MB calyx comparing one-day-old queens and workers reared at 34.5°C (single optical sections). **A)** Frontal view of the central neuropils of a queen brain visualized with phalloidin (green) and propidium iodide (red). **B)** Lower: Comparison of the lateral and medial calyces, peduncles and central body shown in the queen (left) and worker (right) brain drawn at the same scale. Upper: Difference in the outer morphology drawn by M. Obermayer. **C**, **D)** Comparison of the MB calyces and peduncles double-labeled with phalloidin (green) and propidium iodide (red). **E – H)** Comparison of calycal microglomeruli (MG) double-labeled with phalloidin (green) and anti-synapsin IR (red). **E**, **F)** Example showing the calyx subdivisions in queens and the spatial distribution of MG. **G**, **H)** Example showing the distribution of MG in the calyx of a worker. AL: antennal lobe, BR: basal ring, CB: central body, CO: collar, LP: lip, PED, peduncle. Scale bars: A and B: 200 µm (animals in B: 5 mm), C and D: 100 µm, E and G: 50 µm, F and H: 20 µm.

7.2 Temperature-dependent developmental plasticity of microglomeruli in the calyx of young queens

Only slight differences in the pupal rearing temperature were sufficient to affect the number of MG in the MB-calyx lip of freshly emerged queens (Fig. 35, left). Temperature-mediated differences in the number of MG profiles were significant among tested groups (Kruskal-Wallis, χ^2=31.526, df=7, p=0.001). The number of MG was highest in queens reared at 33.5°C (Mann-Whitney, U=8.500, n=16, p=0.016). Interestingly, the highest number of MG occurred at a lower temperature compared to workers (34.5°C in workers) as was shown by Groh et al. (2004).

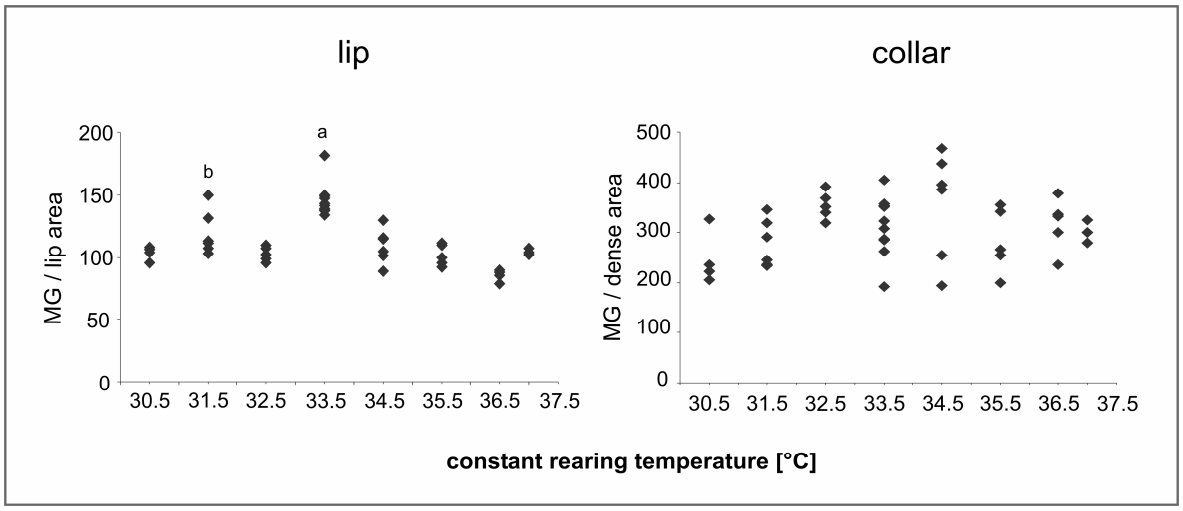

Fig. 35: Effect of pupal incubation temperature on the number of MG in the MB calyx of freshly emerged queens. The numbers of MG profiles in the calyces of each brain (1 – 4 in Fig. 17A) were averaged and are represented by a single symbol for each brain (a and b indicate significance between datasets). Numbers of MG were counted in the lip (left) and the dense area of the collar (right) (see area marked in Fig. 17B).

In the collar, MG were arranged in two distinct subdivisions (Fig. 17B). MG numbers were estimated for three circular areas (20µm in diameter) in the dense portion of the collar and extrapolated to the total dense area (see outlines in Fig. 17B). Numbers of MG in the visual collar did not significantly differ within the tested temperature range (Fig. 35, right). Compared to workers, queens had a lower number of MG in both observed areas and at all tested temperatures.

7.3 Characterization of microglomeruli in the calyx of reproductive queens

Honeybee queens remain reproductively capable and stay inside the hive for up to three years (Seeley, 1978). I was interested in the question whether this period is accompanied by long-term plastic changes in the organization of calycal MG.

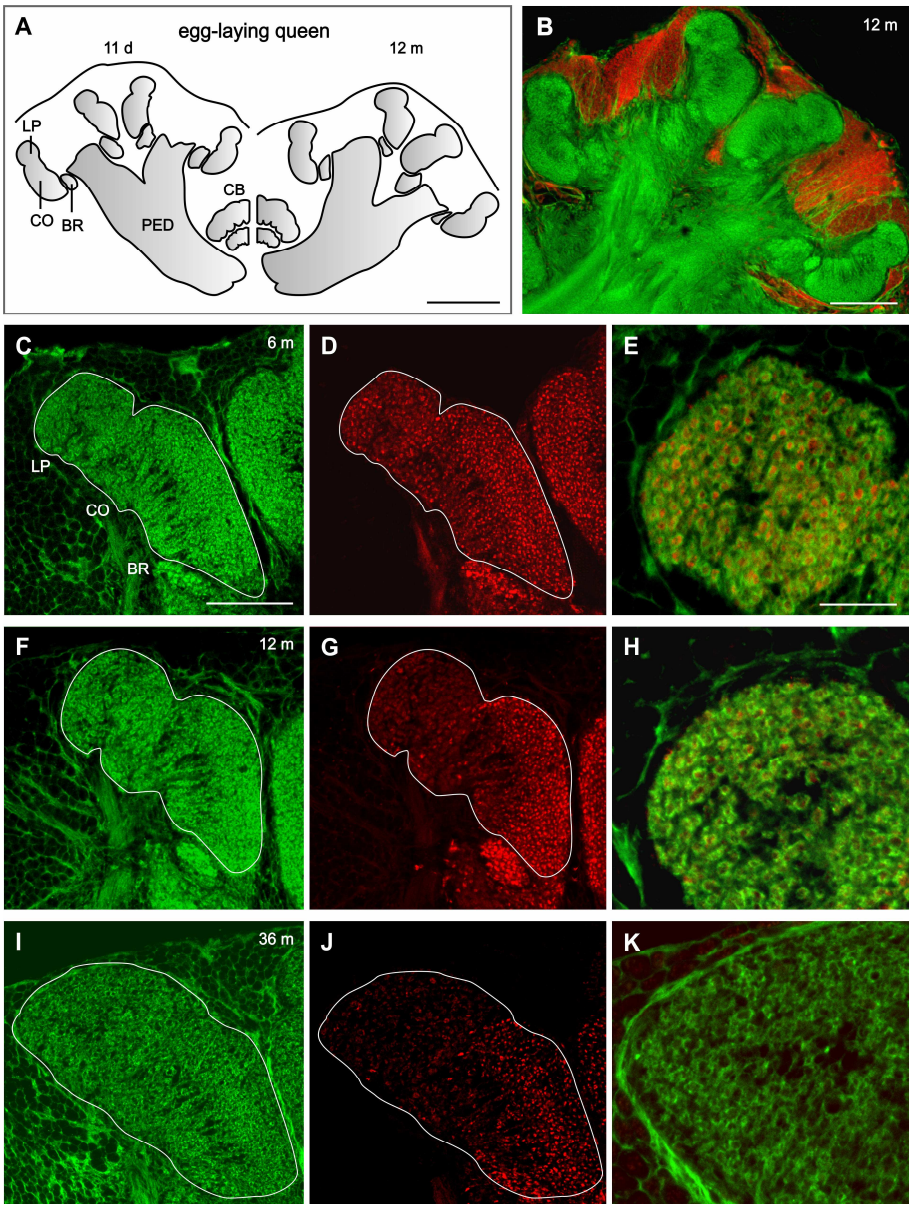

Fig. 36: Age-related structural changes in the MB calyx of reproductive queens. **A)** Scaled drawings of a frontal view of the lateral and medial calyx, peduncle and central body in 11-day (left) and 12-month-old egg-laying queens (right). **B – K)** Single optical sections of the MB calyx. **B)** Overview of the MBs of a 12-month-old queen labeled with phalloidin (green) and propidium iodide (red). **C – K)** Distribution of MG within the calyx in 6-month (C – E), 12-month (F – H) and 36-month-old queens (I – K). Phalloidin labeled F-actin distribution in C, F, I, synapsin-IR in D, G, J and merged images in E, H, K showing the MG distribution in the calyx lip at high magnification. BR: basal ring, CB: central body, CO: collar, d: days, LP: lip, m: months, PED: peduncle. Scale bars: A: 200 µm, B: 100 µm, C, D, F, G, I, J: 50 µm, E, H, K: 20 µm.

Comparisons of brains from naturally mated queens at different ages revealed striking long-term changes in the arrangement of MG in the MB calyx (Fig. 36), although the general organization of the brain showed only slight differences. As an example, the scaled drawing in Fig. 36A gives an impression of the differences among a young (11-day) and an old (12-month) queen. In 12-month-old queens, the MBs appeared slightly more compact than in young queens.

At all ages investigated, the MBs were brightly labeled after phalloidin treatment (Figs. 36B, C, F, I). At high magnification, F-actin labeling revealed a substantial change in the shape of the MB calyx (Figs. 36C, F, I) in older queens. There was a considerable age-dependent increase in the cross sectional area of the MB-calyx lip and a decrease in the collar. Although phalloidin labeling remained more or less similar, synapsin-IR showed a marked decrease with age, especially in the lip region (Figs. 36D, G, J). In contrast to young queens, synapsin-IR associated with synaptic boutons was still bright within the dense area of the collar in old queens, but very faint in the lip region (Figs. 36D, G, J and superimposed images in Figs. 36E, H, K). In queens older than 12 months, I was no longer able to distinguish individual MG because the phalloidin labeled profiles of MG became disintegrated (Figs. 36I and K). In addition to these changes in the microstructures of MG, the characteristic borders between the MB-calyx lip and the collar were no longer distinguishable (Figs. 36I and J).

7.4 Age-dependent plasticity of microglomeruli in the calyx of reproductive queens

I further quantified the changes in MG in 11-day, 6-month, and 12-month-old reproductive queens that had been reared under natural conditions inside the society (Fig. 37). Unfortunately, I was not able to quantify MG in a 36-month-old queen because the borders between both subdivisions were not distinguishable and phalloidinergic MG profiles had disintegrated (Figs. 36I – K).

The numbers of MG in the olfactory lip (left) and the dense part of the visual collar (right) were differentially affected with increasing age (Fig. 37). In the lip, I found a significant increase in the number of MG profiles with age (Spearman-Rho=0.940, n=25, $p<0.001$) (Fig. 37, left, top). Similarly, an increase in the cross-sectional area of the lip significantly correlated with age (Spearman-Rho=0.786, n=25, $p<0.001$) (Fig. 37, left, middle). The estimated density of MG profiles in the lip remained more or less constant (Fig. 37, left, bottom). In the dense region of the collar, I found an opposite age-dependent effect on the

number of MG. The estimated number of MG profiles was highest in 11-day-old queens (Fig. 37, right, top), and the decrease in the MG number correlated significantly with age (Spearman-Rho=-0.709, n=25, p<0.001). In contrast to the lip region, the cross-sectional area of the dense region of the collar did not show a significant increase (Fig. 37, right, middle), but there was a significant correlation between a decrease in the density of MG profiles and age (Spearman-Rho=-0.925, n=25, p<0.001).

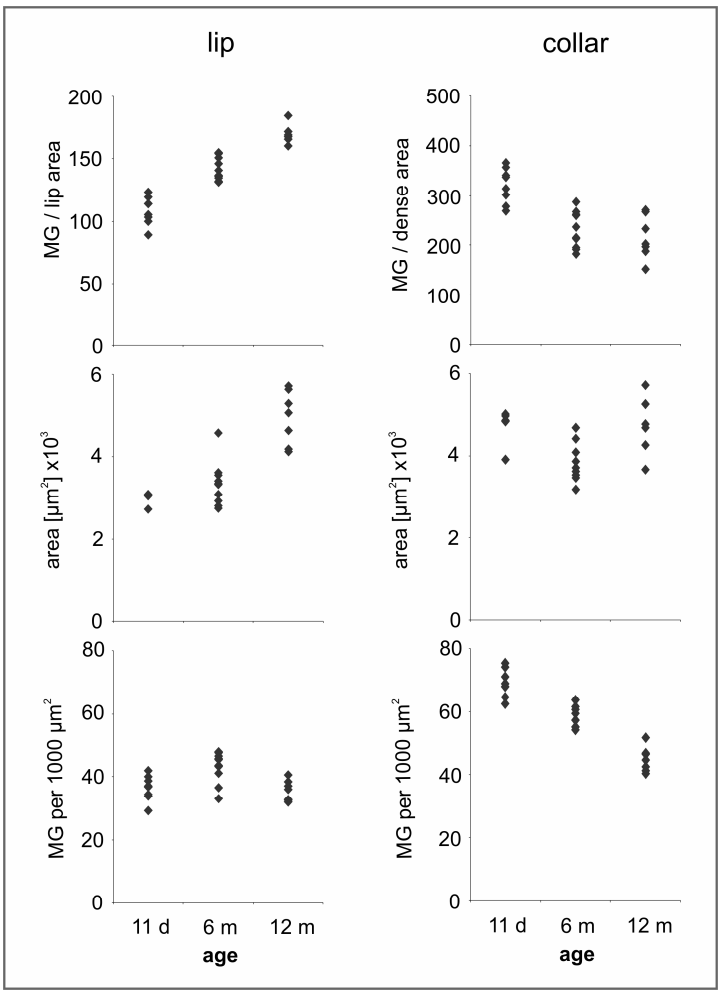

Fig. 37: Age-dependent effects on MG numbers in the olfactory lip (left) and the dense region of the visual collar (right) in reproductive queens: comparison of 11-day (n=8), 6-month (n=10) and 12-month-old queens (n=7). (Top) Numbers of MG profiles counted in the four calyces (1 – 4 in Fig.17A), averaged for each brain and represented by one single symbol. (Middle) Cross-sectional areas of the lip and dense region of the collar (as indicated in Fig. 17B). (Bottom) Estimated density of MG (normalized to 1,000 μm^2).

DISCUSSION

Caste specific, environmentally induced and aging/experience-dependent differences in the brains of honeybees represent prime examples of ontogenetic and adult neuronal plasticity. In the present study, I investigated differences between the queen and the worker caste, thermoregulatory influences during the post-capping period as well as the influence of aging/experience and their consequences for synaptic maturation within the central olfactory pathway (Fig. 38).

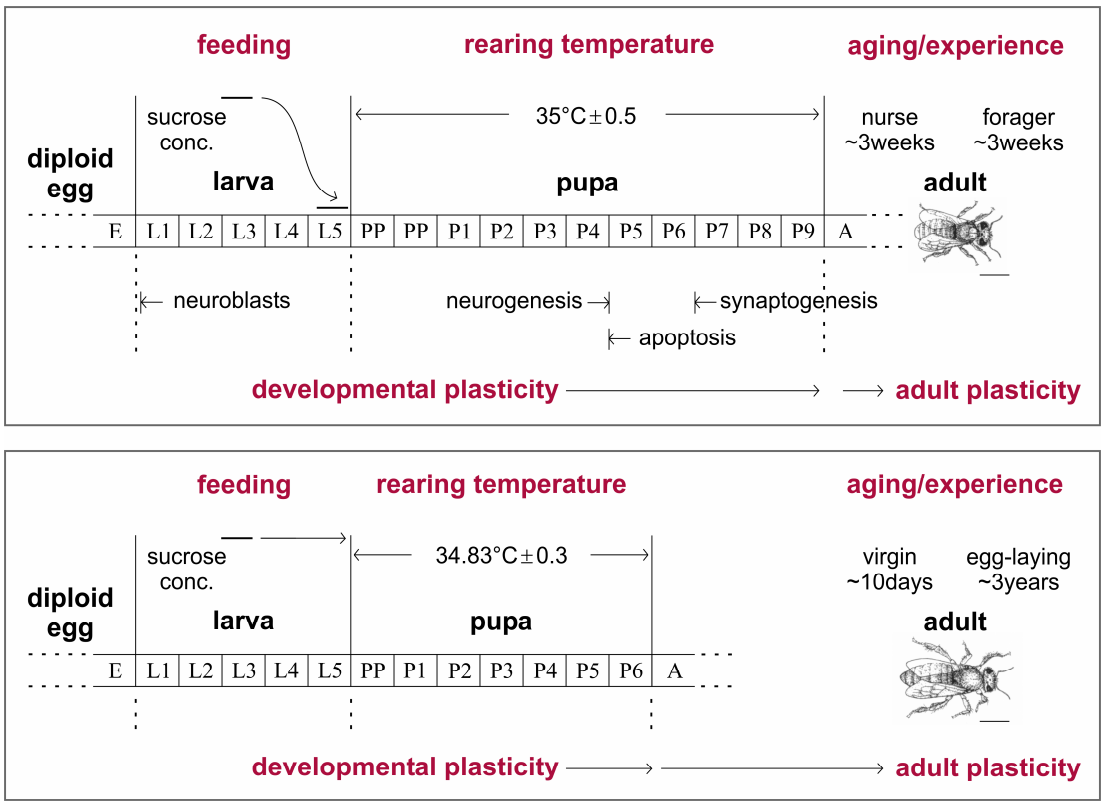

Fig. 38: Workers (upper) and queens (lower) differ substantially in pupal development time, anatomy, aging and behavior. This remarkable phenotypic plasticity is initiated via larval feeding. The underlying developmental and adult neuronal plasticity are of special interest in this thesis. The parameters I focused on are indicated by red letters. A: adult, conc.: concentration, L: larva, P: pupa, PP: prepupa. Scale bars: 5mm.

Considerable morphological and behavioral differences exist among queens and workers. This study gives the first account of the underlying developmental as well as adult neuronal and synaptic structural plasticity. First of all, I revealed clear differences between the two female castes in both the timetable of developing olfactory centers during post-capping period (formation of olfactory glomeruli in the AL and microglomeruli [MG] in the MB calyx) and the resulting number and density of MG in the MB calyces upon adult

emergence. Secondly, I showed that the rearing temperature experienced during the post-capping period significantly affects the synaptic organization within higher order neuropils of adult workers and queens. Quantitative analysis of neuroanatomical data focused on a major sensory input-region of the MBs, the calyces. The number of MG in the olfactory lip was affected by temperature changes as small as 1°C, and comparison with the neighboring visual collar showed that these effects are regionally and modality specific. In queens, the highest number of MG in the olfactory lip developed at a lower temperature compared to workers, and pupal developmental time in queens was less sensitive to changes in the rearing temperature. Thirdly, adding to this developmental neuronal plasticity, I found a striking long-term adult plasticity of MG throughout the extended life span of queens. Whereas the number of MG in the olfactory lip significantly increased over a period of one year, MG in the visual collar continuously decreased with age. Considering these facts, this study revealed a profound developmental and adult neuronal plasticity in the synaptic organization within major sensory input regions of the MB calyx in the female honeybee brain.

1 Methodical strategy

To unravel possible neuronal correlates for environmentally mediated and caste dependent influences on olfactory behavior, I comparatively analyzed neuroanatomical characters of olfactory synaptic neuropils using honeybee queens and workers as a model system. Honeybees are a fascinating and important model in neurobiology. This is due to the detailed description of the gross and fine anatomy of the honeybee brain (e.g., Witthöft, 1967; Mobbs, 1985) and their highly evolved and elaborate behavior that includes division of labor, landmark orientation and navigation, communication, and learning and memory (e.g., von Frisch, 1967; Wilson, 1971; Menzel and Erber, 1978; Seeley, 1985; Menzel 1993). Attempts to discover the underlying neuronal basis for such complex behavioral capabilities have evoked correlations between the volume or size of prominent brain neuropils and the behavioral elaboration of different castes inside the honeybee society (e.g., Sigg et al., 1997; Farris et. al., 2001; Brown et al., 2002; Ehmer and Gronenberg, 2002). However, the role of synapses in behavioral maturation has rarely been investigated (Farris et al., 2001). In this study, quantitative analysis and detailed comparisons of neuroanatomical data on synaptic characters were performed, thereby providing the opportunity for correlating changes in the neuronal structure with brain function.

I utilized a double-labeling technique using fluophore-conjugated phalloidin in combination with anti-synapsin immunocytochemistry to investigate the structural organization of particular proteins involved in neuronal and synaptic plasticity in the brain. The toxin phalloidin, a small bicyclic peptide isolated from the mushroom *Amanita phalloides*, binds specifically to the multiple isoforms of F-actin (Wieland, 1987; Haugland, 1996). Since the synthesis of fluorescent analogs that retain actin binding (Wulf et al., 1979), fluophore-conjugated phalloidin has provided a useful tool to localize the distribution of F-actin in permeabilised cells. In neurons, the distribution of neuronal F-actin depends on neuron type as well as developmental and functional states (Harris, 1999; reviewed in Matus, 2000; Luo, 2002). F-actin is associated with the synaptic network in vertebrates as well as invertebrates and is highly concentrated in presynaptic terminals, dendritic spines and growth cones (Fifkova and Delay, 1982; Lee and Cleveland, 1996; Matus, 1999; reviewed in Matus et al., 2000; de Camilli et al., 2001; Rössler et al., 2002; Frambach et al., 2004). The characterization of presynaptic elements was feasible with a monoclonal antibody specific for *Drosophila* synapsin I (SYNORF1; kindly provided by Dr. E. Buchner, University of Würzburg, Germany) (Klagges et al., 1996). Synapsin I, the most abundant protein of a small family of synaptic vesicle-associated phosphoproteins, is involved in the maturation of synaptic contacts during synaptogenesis and in short-term regulation of neurotransmitter release from adult nerve terminals (e.g., reviewed in Bähler et al., 1990; Chin et al., 1995; Valtorta et al., 1995). As regulation of transmitter release is important for synaptic plasticity, synapsin was shown to be involved in olfactory associative learning in *Drosophila* larvae (Michels et al., 2005). Using phalloidin and anti-synapsin as synaptic markers in developing as well as mature honeybee brains, I was able to detect the distribution of F-actin and synapsin at the cellular level via high-resolution laser scanning confocal microscopy.

At present, the immunofluorescence staining with phalloidin can only be applied to thick vibratome sections (100 – 200µm) because the mushroom toxin is washed out by methylsalycilyate necessary for clearing the tissue. In contrast, much progress has been made in staining the presynaptic central boutons with anti-synapsin in whole mount preparations (Müller, 2005).

2 Brood incubation differentially affects metamorphic growth in female castes

This study, for the first time, revealed general effects of brood temperature control during the post-capping period on the development time and emergence rate in queens (Fig. 39A and B, circles). In workers, previous results were confirmed (Eichmüller, 1994) and extended. Brood care, by providing energy through elevated and constant warming, resulted in short developmental times as well as low mortality rates. Benefits of brood incubation on worker metamorphosis were previously documented. It has been described that active brood incubation influences the duration of the post-capping period (reviewed in Jay, 1963) as well as the rate of mortality, malformation (e.g., Himmer, 1927a) and chalkbrood disease caused by the fungus *Ascophaera apis* (Maurizio, 1934). Taken together, this illustrates an extreme temperature sensitivity of honeybee worker brood which has adapted to a stable microclimate.

The stable microclimate in the central brood area which is achieved via endothermic heat production (Esch, 1960; Heinrich, 1981) or wing fanning (Hazelhoff, 1954; Southwick and Moritz, 1987) is maintained at 35±0.5°C (e.g., Hess, 1926; Himmer, 1927a; Heinrich, 1993). A recent study shows that brood nest temperature in genetically uniform honeybee colonies exhibits more fluctuations than in genetically diverse colonies (Jones et al., 2004). Jones et al. (2004) hypothesize that genetically determined diversity in workers may broaden the range of temperature response thresholds of individual worker honeybees for the initiation of thermoregulatory behavior. Thermoregulatory capabilities via endothermic heat production are not confined to honeybees, as active brood incubation is known in bumble bees (Vogt, 1986) and vespine wasps (Ishay and Ruttner, 1971). Thermoregulatory control of brood rearing is also performed in other social insects. In many ant species this includes the selection of a suitable nest site by the founding queen (Brian, 1952) and the nest architecture (Steiner, 1929; Scherba, 1962). Ant nurses are not able to actively incubate the brood, but they do transport immobile pupae to selected thermal values within the nest (e.g., Roces and Núñez, 1989), indicating that during larval-adult metamorphosis the organism is most sensitive to temperature deviations.

In queens, brood-temperature control had different effects on the duration of the post-capping period. Up to now, studies on effects of brood rearing in honeybee queens have been limited. The breeding technique introduced at our apiary was very reliable and produced a large number of synchronized queen brood. The emerging adults were

homogeneous in size, weight and ovary development. Compared to workers, I found that the post-capping period is significantly shorter in queens and less sensitive to changes in temperature, especially at lower temperatures (Fig. 39A, triangles). This might be adaptive because under natural conditions queen-destined larvae are reared only after the loss of a queen or in anticipation of colony splitting (Allen, 1965; Laidlaw, 1992). Fast development is crucial under these circumstances because the queen that emerges first will attempt to kill all successors in order to become the next single reproductive queen (Seeley, 1985; Pflugfelder and Koeniger, 2003). Therefore, queens might have evolved greater flexibility from fluctuations in rearing temperature than workers.

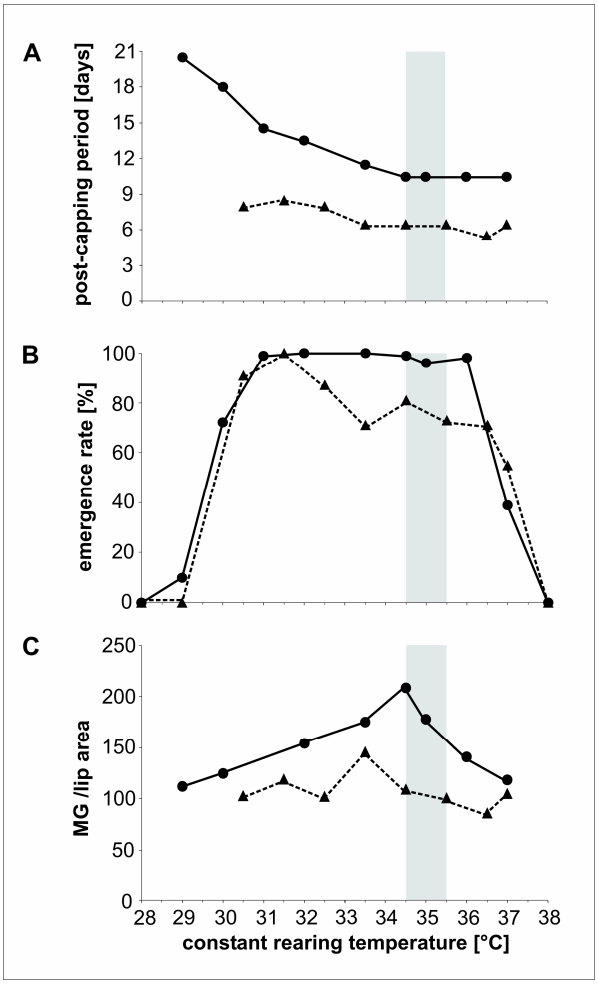

Fig. 39: Influence of the incubation temperature on the duration of post-capping period, emergence rates and number of microglomeruli in the mushroom body calyx lip of freshly emerged queens (triangles) in comparison with data from workers (circles). The shaded area indicates the temperature range maintained in the central brood area. **A)** The prepupal and pupal development of queens is significantly shorter and less dependent on temperature compared to workers, especially at lower temperatures. **B)** In both castes emergence rates are high over a broad temperature range. **C)** Temperature dependent influences on the number of MG in the calyx lip differ among workers and queens. In queens, the overall number of MG is lower and the maximum is shifted toward a lower temperature. MG: microglomeruli.

3 Developmental plasticity of the olfactory pathway

3.1 F-actin as a marker for neuronal growth in both female castes

The goal of this experiment was to comparatively analyze the postembryonic differentiation of primary and secondary olfactory neuropils in the CNS of worker and queen prepupae and pupae using synaptic markers. Neuroanatomical analyses revealed clear differences between both female castes in the formation of the AL synaptic neuropil and olfactory input regions of the MB calyces.

In honeybees, as in other holometabolous insects, larval neuronal circuits that for example underlie feeding and simple locomotor activity are rebuilt during pupal metamorphosis to provide structural and functional modifications at all levels of adult behavioral control (e.g., Levine et al., 1995). Previous studies focused on either the compartmentalization of primary (Gascuel and Masson, 1991; Hähnlein and Bicker, 1997; Schröter and Malun, 2000) or secondary olfactory neuropils (Menzel et al., 1994; Farris et al., 1999; Schröter and Malun, 2000) in the CNS of workers. In this thesis, labeling of F-actin and synapsin in the brain of developing workers revealed a precise timetable of the formation of AL synaptic neuropil and calycal sensory input regions. The homogenously textured AL neuropil that is already invaded by ORN axons (Hähnlein and Bicker, 1997) started to coalesce into preglomeruli from late P2/early P3 onward at peripheral regions of the AL. As in *Manduca sexta* (reviewed in Tolbert et al., 2004), glomeruli are not formed synchronously in the honeybee. In *Apis*, their development was initiated within that AL region where T1 ORN axons provide olfactory input and in *Manduca* within an area near the entrance of the antennal nerve (e.g., Malun et al., 1994; Rössler et al., 1998 and 1999). Similar in vertebrates, ORN axons are shown to aggregate into protoglomeruli in the future glomerular layer of the olfactory bulb (e.g., Valverde et al., 1992; Malun and Brunjes, 1996). At pupal stage 6, glomeruli had formed and, as in most insect species, were arranged in the periphery around a central fibrous core (*Apis*: Arnold et al., 1985; *Drosophila*: reviewed in Stocker et al., 1990; *Manduca*: reviewed in Tolbert et al., 2004). Double-labeling with anti-synapsin that is restricted to presynaptic elements (Klagges et al., 1996) revealed that from P7 onward the distribution of synapsin-IR resembled the distribution of phalloidin. This coincides with the aggregation of ORN fiber terminals in the cortical layer of the mature glomeruli (Gascuel and Masson, 1991). It also indicates that in the AL F-actin is mostly aggregated in axonal terminals of ORNs as was found in adults (Rössler et al., 2002). At this time, electroantennogram measurements and

intracellular recordings of AL neurons detected electrical activity of ORNs and PNs (Masson and Mustaparta, 1990).

Phalloidin turned out as a very useful marker to visualize the compartmentalization of the MB calyx. I found that the so-called calyx-developing-zone (Menzel et al., 1994) divided into lip and collar directly after prepupal ecdysis, whereas the basal ring emerged at P5 (according to earlier observations summarized in Schröter and Malun, 2000). Within this time, different KC populations are born in a specific sequence (Malun, 1998; Farris et al., 1999) and their neurogenesis lasts until P5 (Ganeshina et al., 2000). Thus, the KC dendrites may be in the correct place for laying down a template for ingrowing MB extrinsic neuron terminals. At the subsequent pupal stage, simultaneous labeling with anti-synapsin enabled the visualization, for the first time, of the formation of distinct synaptic complexes (microglomeruli [MG], Yusuyama et al., 2002) in the developing calyx. Although olfactory and visual PNs innervate strictly separated regions within the developing calyx from the first pupal stage on (Schröter and Malun, 2000), their large synaptic boutons (visualized by synapsin-IR) were not encircled by phalloidinergic profiles until P6. At P6, MG became clearly visible within the subdivisions of the calyx (lip, collar, basal ring). They exhibited intense phalloidin-labeled F-actin around the central bouton that appeared to be devoid of phalloidin. In adult insects, F-actin clearly seemed to be accumulated in the MG postsynaptic elements, representing KC dendritic spines synapsing on PN boutons (Frambach et al., 2004; Groh et al., 2004). A striking resemblance between the insect calycal MG and those MG in the vertebrate cerebellum has been noted (Schürmann, 1974; Yusuyama et al., 2002; Frambach et al., 2004). Vertebrate cerebellar MG appear as patches with presynaptic boutons of mossy fibers surrounded by dendritic spines (Dino and Mugnaini, 2000; Capani et al., 2001) and, as documented via electron microscopy of actin filaments, F-actin is most abundant in dendritic spines.

Dendritic spines have been suggested to be primary sites of structural and synaptic plasticity in the brain (Fischer et al., 1998). Since it is assumed that actin filaments constitute the structural basis of spine plasticity (Matus, 1999; Halpain, 2000), labeling of the synaptic contact zones with phalloidin enables the visualization of potential sites for structural plasticity of the MB calyx. Quantitative analyses of the calycal organization of MG in workers reared at 34.5°C are possible from P6 onward. Pupal rearing temperatures below the naturally occurring temperatures affected the timetable of the compartmentalization of the calyx neuropil and delayed the formation of MG.

The results indicate that the rearing temperature may affect the formation of both pre- and postsynaptic elements of MG. This could be caused via changes in neuronal processes, but also via effects on neurogenesis or apoptosis. Temperature may act either directly on neurons and glial cells or indirectly via effects on neurosecretory cells and the hormonal system (Rössler and Bickmeyer, 1993; Fahrbach and Weeks, 2002), which needs be explored in further experiments.

This study gives the first account on formation of AL glomeruli and the MB calyx in metamorphosing queens. The established timetable of the developing worker brain with the focus on the olfactory pathway served as reference to describe the neuronal differentiation in queens. Comparative analysis of the AL development showed that the formation of the first olfactory glomeruli appeared earlier in queens than in workers. Upon adult emergence, the quantitative analysis showed the total number of AL glomeruli, the volume of individual glomeruli (innervated via T1 and T3) and the total AL volume is smaller in queens compared to workers (Müller, 2005). A faster timetable in neuropil formation was also true for the MB calyx in queens. All three calycal subdivisions as well as the formation of distinct MG became visible one day (lip and collar) and two days (basal ring and distinct MG) earlier. The resulting overall number of MG in the olfactory lip and visual collar was significantly lower in one day old queens than in one day old workers (Groh et al., 2004; Groh et al., 2006, in press).

Many studies have focused on the role of ecdysteroids and juvenile hormone, as general morphogenetic insect hormones, during caste determination in honeybees (e.g., Rembold et al., 1992; Rachinsky and Hartfelder, 1995; Hartfelder and Engels, 1998). These hormones are likely to affect caste specific neuronal development. Analyses of the dynamics of cell proliferation in worker-destined larvae provided insights into the hormonal regulation of neurogenesis during female caste differentiation (reviewed in Page and Peng, 2001). Using BrdU (5-bromo-2-deoxy-uridine) to investigate the proliferation activity of neuroblasts and their progeny in the ALs and MBs, it was revealed that the pattern of neurogenesis in the larval antennal centers differs from that observed in the MBs during the first larval instar and prepupal stage (Vitt and Hartfelder, 1998). Vitt and Hartfelder (1998) also showed that the mitotic activity in the brain during last larval instar coincides with a low JH titer (Rachinsky et al., 1990) and high titers of ecdysteroids (Remboldt, 1987). How these hormones influence caste-specific neuronal differentiation in female honeybees is still under debate.

3.2 Serotonin-immunoreactivity in developing worker brains

There is abundant evidence that the biogenic amine serotonin acts as neurotransmitter and neuromodulator in vertebrates and invertebrates. With respect to social insects, serotonin may be involved in the regulation of reproductive states (e.g., Bloch et al., 2000), aggressive behavior (e.g., Kostowski and Tarchalska, 1972), and learning and memory (e.g., Erber et al., 1993).

The influence of serotonin on the regulation of neuronal outgrowth has been suggested in a variety of species (e.g., Haydon et al., 1987; Lipton and Kater, 1989; Lauder, 1993; Mercer et al., 1996). As serotonin-immunoreactive (serotonin-IR) neurons are already present in the last larval instar in honeybee workers (Boleli et al., 1995; Seidel and Bicker, 1996), I investigated the development and distribution of serotonin-IR fibers in metamorphosing workers with regard to the olfactory pathway. As revealed by serotonin-immunocytochemistry, serotonin-IR processes were detected within the AL before prepupal ecdysis and persisted throughout pupal development into the adult stage. The developmental pattern of serotonin-IR fibers during glomerulus formation is strikingly similar to the extensively studied one in the brain of *Manduca sexta* (e.g., Oland et al., 1995). First of all, serotonin-IR processes that arise from one prominent serotonergic neuron (*Apis*: Rehder et al., 1987; *Manduca*: Kent et al., 1987) arborize quite homogenously throughout the Al neuropil at early stages of metamorphic development but do not yet innervate preglomeruli (in *Manduca* so-called protoglomeruli). Secondly, serotonin-IR fibers innervate the developing glomeruli at least one stage later than when olfactory PNs have reached their target area within the AL (for details in *Apis* see Schröter and Malun, 2000; for details in *Manduca* see Malun et al., 1994). Thirdly, once glomeruli have formed, the dendritic branches of the serotonin-IR neuron invade, apparently, all glomeruli with varicose and large presynaptic terminals (e.g., Sun et al., 1993). However, the role of serotonin in glomerulus development is still unknown in the honeybee. In *Manduca sexta*, by means of experimentally manipulated ALs, glomeruli form properly during pupal development in the absence of serotonin-processes (Oland et al., 1995). These findings strongly support the current view that the serotonin-IR neuron might promote the fine arborizations of developing AL neurons since serotonin has been shown to enhance the outgrowth of cultured AL neurons (Mercer et al., 1996), and ORN axons might mediate communication to serotonin-immunoreactive neurons via nitric oxide signaling (Gibson et al., 2001).

Within the developing MBs, weak serotonin-immunoreactivity was restricted to the MB output region, the peduncles, from P2 onward. At pupal ecdysis, serotonin-immunoreactivity in the peduncles corresponded to different layers formed by tiny serotonin-IR fiber bundles from different KC types (Schürmann and Klemm, 1984; Seidel and Bicker, 1996). Focusing on the sensory input regions of the MBs, the calyces, no serotonergic processes were detected during larva-adult metamorphosis which stands in contrast to findings in ants. In the brain of ant species from different subfamilies (*Harpegnathos saltator*, *Atta sexdens, Myrmecia gulosa*), the MB calyces displayed a striking innervation pattern concerning the olfactory lip region (Hoyer et al., 2005). This indicates that, in contrast to the honeybee, serotonin in ants might affect olfactory processing at a further level.

4 Temperature-dependent plasticity of synaptic complexes in the mushroom body of workers

I showed that brood-temperature control can influence synaptic organization in the brain of adult honeybee workers. The most important finding is that temperature-mediated effects are both position and/or modality specific, even in adjacent areas of the MB calyx. Temperature-mediated changes occurred within the range of natural variations in brood temperature and persisted after the first week of adult life. The temperature-based plasticity in the synaptic circuitry might affect behavioral performance and the start and/or rate of behavioral transitions.

In the lip of the MB calyx, the numbers of MG were different in freshly emerged workers that were raised in incubators at different temperatures. Changes in MG occurred at temperature differences ≤1°C. MG, therefore, represent a potential neuronal substrate for temperature-mediated effects observed in adult behavior (Tautz et al., 2003). In the lip, MG numbers were highest between 33.5 and 35°C (Fig. 39C, circles), which overlaps with the narrow temperature range maintained in central brood cells (e.g., Hess, 1926; Himmer, 1927a). Pupae were kept at constant temperatures, whereas in the natural situation temperatures can fluctuate in the range of ~3°C, and may be higher in peripheral brood cells (Kleinhenz et al., 2003). Therefore, effects in a natural population may be more subtle than in experimental animals. In the MB collar, MG numbers were less affected by

temperature. This indicates that thermoregulation has a differential influence on different brain regions and/or sensory modalities.

MG numbers were maintained after one week of sensory experience within the hive, indicating that bees were equipped with different numbers of synaptic units at the time they performed tasks inside the hive. This developmental induced polyethism is new in addition to age polyethism. The division of labor in honeybee colonies is based on age polyethism and represents a most important feature of social life (e.g., Wilson, 1971). Polyethism, however, was shown to be sensitive to environmental cues (Visscher and Seeley, 1982; Seeley, 1985). Different workers spend different amounts of time on nest activities compared with foraging activity, whereas others may never forage at all (Seeley, 1995). I propose that brood incubation may contribute to flexibility of division of labor, as the observed difference in the number of MG may affect subsequent maturation of the synaptic circuitry. Pupal rearing temperature, therefore, may have important consequences concerning the ability of the adult MBs to express plastic changes in synaptic structures and neuronal processes. The fact that temperature-mediated changes in the MBs are position and modality-specific and do occur in a physiological temperature range (32 – 36°C) suggests a potential role in mediating behavioral plasticity. Behavior studies suggest that especially lower rearing temperatures within this range cause deficits in waggle-dance performance and olfactory learning in adult workers (Tautz et al., 2003). Interestingly, similar effects of rearing temperature (at larval stage) on adult learning capabilities were observed in the parasitic wasp *Anaphes victus* (Hymenoptera). Low temperature exposure results in a decrease in their capacities for learning external landmarks while localizing the host habitat (van Barren et al., 2005).

The number of MG in the adult MBs changed with the temperature experienced during pupal period, which could be caused by presynaptic (PN boutons) and/or postsynaptic (KC dendrites) modifications (Fig. 40). Previous studies have shown that neurogenesis is active until P4, decreases drastically after onset of apoptosis and is absent in the adult brain (Fahrbach et al., 1995b; Ganeshina et al., 2000). As shown in fruit flies, ants, and honeybees, the adult MBs exhibit age- and experience-related volume changes (Withers et al., 1993; Durst et al., 1994; Fahrbach et al., 1995a; Heisenberg et al., 1995; Gronenberg et al., 1996). In the fruit fly, the MB volume was affected by visual experience, and in ants and honeybees a volume increase was associated with age and behavioral maturation. In the bee and in *Drosophila*, volume measurements in the ALs also revealed age- and experience- related changes in distinct olfactory glomeruli (Sigg et al., 1997; Devaud et al.,

2001). In the honeybee, volume changes in the MBs were suggested to be caused mainly by dendritic growth of KCs (Farris et al., 2001). Changes in neuronal processes during experience-dependent maturation of the nervous system are common to many sensory systems. In the vertebrate visual system and olfactory bulb, excess dendritic branches are pruned during maturation (Shatz, 1990; Malun and Brunjes, 1996). My results indicate that temperature-induced changes in the number of MG are the underling cause of volume changes in the calyx and might include both pre-and postsynaptic elements, most likely caused by changes in the branching pattern. This could be achieved via effects on neurogenesis or apoptosis, which should be explored by future studies on the ultrastructural level.

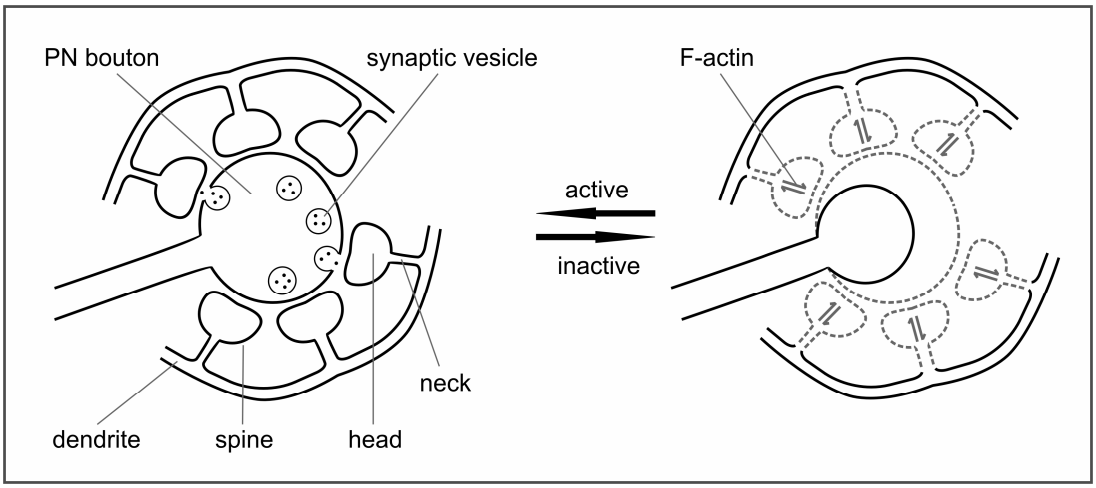

Fig. 40: Hypothetical model of structural plasticity within a microglomerulus in the mushroom body calyx. For simplicity, inhibitory and potential neuromodulatory neurons are not included. Synaptic consolidation and destabilization most likely involves pre- and postsynaptic elements. PN: projection neuron.

Temperature mediated effects on the developing neuronal network rarely have been investigated in insects. Recently, a study by Zhong and Wu (2004) in *Drosophila* demonstrated that terminal arborizations of motor neurons at larval neuromuscular junctions exhibit a temperature-dependent developmental plasticity of axonal outgrowth. In this case, structural changes in neuronal processes were mediated via changes in neuronal activity linked to the cAMP pathway (Zhong and Wu, 2004). Future studies which block neuronal activity may show whether activity dependent mechanisms are involved in the honeybee. However, other temperature-dependent pathways could interfere as well with neuronal outgrowth and synaptic development. In developing pupae of *Manduca sexta*, temperature was shown to affect axon-glia interactions and proper neuropil formation

within the antennal lobes (Rössler et al., 1999 and 2000). Some of these cell-cell interactions are mediated by nitric oxide (Gibson et al., 2001). Temperature effects may be mediated via changes in neurosecretory systems. Electrophysiological recordings in the locust *pars intercerebralis* neurosecretory cells showed that the firing probability of these cells was affected by temperature which could affect the release of neuropeptides in the neurohemal organs (Rössler and Bickmeyer, 1993). The fact that temperature-mediated changes differ in the visual and olfactory input regions indicates that the mechanisms and/or threshold in different types of neurons or brain regions may vary. The different developmental timetables and/or sensitive periods of the olfactory and visual pathways could also underlie this discrepancy in the sensitivity to temperature during pupal metamorphosis.

5 Temperature- and age-dependent plasticity of synaptic complexes in the mushroom body of queens

Reproductive division of labor plays a crucial role for eusociality. This behavioral polymorphism in female honeybees offers an excellent model system to address fundamental questions about the underlying developmental and adult neuronal plasticity. This study showed a profound developmental and adult plasticity in the synaptic organization within major sensory input regions of the MB calyx in the honeybee brain.

Upon adult emergence, I found that the two female castes differ substantially in the number and density of MG in the MB calyces (Fig. 39C). In addition to these caste specific differences, slight variations in pupal rearing temperature affected the number of MG in the MBs of adult queens. Temperature effects were more pronounced in the olfactory lip region compared to the visual collar area supporting previous observations in workers (Groh et al., 2004). In contrast to workers, the highest number of MG in queens developed at 1°C below the maximum found in workers (33.5 vs. 34.5°C). This is surprising because the rearing temperature in natural queen cells, as determined in this study, is in a similar range as the worker brood cells (Kleinhenz et al., 2003). This difference could be caused, though, by differences in hormonal regulation, probably higher levels in juvenile hormone that were found in queens (Hartfelder, 2000; Bloch et al., 2002b).

In addition to developmental plasticity (influences of brood care conditions), this thesis exhibited a striking long-term adult plasticity of MG throughout the extended life-span of

queens. Whereas the number of MG in the olfactory lip continuously increased with age (~55%), MG in the visual collar significantly decreased over a period of 1 year (~35%). Altogether, the results suggest that the synaptic circuitry in the adult MB calyx is shaped to a remarkable extent by brood-care and continuous remodeling throughout adult life. A previous study by Fahrbach et al. (1995a) showed that the volume of the synaptic neuropil in the MBs of queens increased within the first two weeks of adult life, similar to changes in volume reported earlier for honeybee workers (Withers et al., 1993; Durst et al., 1994). A recent study in eusocial wasps showed that age-related task specializations in workers correlate with an expanded volume in the MB calyces (O'Donnell et al., 2004). The basis of these age-related changes in the MBs and whether plastic changes may proceed through later stages of life remained unclear. In honeybee workers, adult volume changes in the MBs are not a consequence of adult neurogenesis (Fahrbach et al., 1995b). Quantitative analyses by Farris et al. (2001) suggest that an increase in the length and branching pattern of KC dendrites contributes to the volume increase in the MB calyx. I found that the numbers of MG decreased in the visual regions and increased in the olfactory regions of the MB calyx, and double-labeling with phalloidin and anti-synapsin indicated that these changes occurred in both pre- and post-synaptic neuronal structures. My results also show that MG remain plastic throughout the extended life-span of a queen. This suggests that volume changes in the MB calyx are caused by the increase or decrease in the number, density and size of MG. Changes in the dendritic arbors of KCs described by Farris et al. (2001) might reflect the reorganization of the synaptic connections of KCs associated with these changes. The reproductive queen lives in the dark, but her sense of smell is likely to be crucial for monitoring the well-being of her brood. This might explain the stability and increase of MG numbers in the olfactory lip region. The increase in the number of MG indicates that an increase in dendritic arbors as shown by Farris et al. (2001) could be induced by newly forming presynaptic boutons. The different effects in the olfactory and visual subregions of the calyx could result from differences in adult maturation of associated sensory pathways. Altogether, the results indicate that volumetric changes in the MB calyx are related to changes in MG numbers and that the number of MG might be defined by changes in presynaptic boutons and associated changes in dendritic spines of KCs.

Behavior studies indicate that structural plasticity in the MBs could have important functional consequences. In honeybee workers, thermoregulatory control during pupal development was shown to affect odor learning abilities and dance communication in adult

bees (Tautz et al., 2003), and similar changes in rearing temperature significantly influenced the synaptic organization in the MB calyx (Groh et al., 2004). The lower number of MG in the MB-calyx lip of queens compared to workers correlates with the difficulties in performing olfactory conditioning experiments using the proboscis extension reflex in queens despite comparable thresholds to sugar (Pankiw and Page, 1999; Dr. A. Brockmann, personal communication, and own unpublished results). However, in addition to functional changes, reduction of neuronal circuitry could also reflect the necessity to reduce metabolic costs caused by the maintenance of these neuronal structures in order to increase fitness (Laughlin et al., 1998).

The fact that phalloidinergic profiles express developmental and adult changes suggests that F-actin dynamics could play a role in structural plasticity of dendritic spines in KCs. KCs represent the majority of neurons intrinsic to the MBs (Strausfeld, 1976; Mobbs, 1982; Strausfeld et al., 1998; Strausfeld, 2002). F-actin was shown to be highly enriched in KC dendritic spines (Frambach et al., 2004; Groh et al., 2004), similar to the aggregation of F-actin in dendritic spines in the vertebrate hippocampus and cerebellum (Matus, 2000; Capani et al., 2001). The presence of F-actin in KC spines suggests a high degree of structural plasticity comparable to F-actin-related structural plasticity found in the vertebrate brain (Matus, 2000; Halpain, 2000). There are two major types of KCs, class I and II (Strausfeld, 2002). Both classes differ in their arborization patterns, developmental time scale and degree of reorganization during postembryonic development (Farris et al., 2004). In future work it will be particularly interesting to differentiate among class I and II KC via retrograde labeling and double-labeling with phalloidin to find out if both contain similar patterns of F-actin. In honeybee queens, an increase of phalloidinergic profiles in the MB-calyx lip was accompanied by a decrease in the intensity of synapsin-IR. This could indicate that dendritic spines might compete for fewer presynaptic structures in the olfactory input region. Interestingly, the opposite effect was observed in the visual collar where a decrease in the number phalloidinergic profiles was accompanied by unchanged synapsin-IR in presynaptic boutons. This is surprising due to the near complete darkness in the hive and the absence of strong visual stimuli. Dissociation of the typical ring-shaped phalloidinergic profiles in old queens may indicate that F-actin in KC spines becomes gradually reduced or that spines disappear. The changes at the subcellular level and molecular composition of MG need to be explored further at the ultrastructural level.

Developmentally induced and/or age-dependent changes in F-actin associated with KC-dendritic spines may result in a different capacity of the MB calyx responses to dynamic

changes to olfactory and visual inputs. Recent work on the mammalian hippocampus showed that F-actin polymerization in synaptic spines following high frequency stimulation was accompanied by changes in spine morphology (Lin et al., 2005). If this were the case in the MB calyx, the resulting changes in the synaptic circuitry could certainly affect information processing in these sensory integration centers. A study on olfactory processing in the locust demonstrated that the information transfer from olfactory projection neurons to KCs is accompanied by significant sparsening of activity, and KCs preferentially respond to coincident input induced by odor stimuli (Perez-Orive et al., 2002). At this stage of olfactory information processing, the transmission of odor evoked activity patterns is also associated with sparsening of the population code in the honeybee (Szyszka et al., 2005). The microcircuitry within MG may be an important feature supporting this function (Ganeshina and Menzel, 2001; Yusuyama et al., 2002). Therefore, changes in MG may well affect filtering properties for incoming sensory information and, potentially, the ability of the MBs to express long-term changes related to imprinting or long-term behavioral plasticity. This in turn might result in different behavioral responses to olfactory and visual stimuli. In addition, they might cause differences in certain qualitative aspects of integrative functions of the MBs as was indicated in a previous study on workers raised at different temperatures (Tautz et al., 2003). Future functional studies using high resolution optical recording techniques in combination with controlled brood rearing and behavioral experiments may be able to make a clear causal link from long-term structural and molecular changes in MG to functional changes of the MBs.

6 Summary

Structural plasticity correlated with behavioral demands and/or age-dependent changes has been described for prominent neuropils in the brain of vertebrates and social insects (e.g., Kolb and Wishaw, 1998; Farris et al., 2001). The rich behavioral repertoire of social insects has evoked correlations between environmental, age- and status-dependent effects and the volume and/or size of diverse brain neuropils (e.g., Withers et al., 1993; Durst et al., 1994; Gronenberg et al., 1996; Brown et al., 2002). Previous studies in insects documented that experience- and age-related development is accompanied by an outgrowth of KC dendritic trees in the MB calyces (Technau, 1984; Farris et al., 2001), but little is known about the role of synapses in structural plasticity. Recently, intense F-actin staining was found in KC dendritic spine parts within calycal synaptic complexes crickets

(Frambach et al., 2004). This indicates that synaptic complexes within the MB calyx constitute sites where synaptic and structural plasticity might occur. The present thesis gives the first account of environment- and age-dependent plasticity of synaptic complexes in the MBs of female honeybee castes, queens and workers.

I found that phalloidin-labeled neuronal F-actin is expressed in the brain of honeybee workers and queens with strong aggregation in synaptic neuropils, as has also been documented for vertebrate brains (de Camilli et al., 2001). F-actin was highly enriched in calycal synaptic complexes named microglomeruli [MG]. The results showed that within MG F-actin is restricted to postsynaptic elements, dendritic spines of KCs. Double-labeling with anti-synapsin enabled localization of synapsin in large boutons of AL projection neurons, the presynaptic site of MG. This allowed me to comparatively visualize the formation of synaptic complexes in the MB calyces during pupal development in both female castes and to quantify environment- and age-dependent plasticity of synaptic complexes within the major sensory input regions of the MBs. I was able to show that the sequence in the formation of subdivisions (lip, collar, basal ring) differs in developing queens and workers. Upon adult emergence, I found that the number of MG in the worker brain changed significantly with slight variations in natural occurring variations in brood temperatures during pupal development (Groh et al., 2004). Significant differences were also evident among the worker and the queen caste (Groh et al., 2006, in press). In addition to this developmental neuronal plasticity, I found a striking long-term adult plasticity of MG throughout the extended life span of queens. In summary, this study clearly demonstrates that quantification of structural plasticity in the synaptic circuitry is essential toward an understanding of the neuronal processes underlying behavioral plasticity.

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ZUSAMMENFASSUNG

Für die Honigbiene spielt der Geruchssinn eine entscheidende Rolle bei der Kommunikation innerhalb des Sozialstaates. Kastenspezifische, auf umweltbedingten Einflüssen basierende sowie altersbedingte Unterschiede im olfaktorisch gesteuerten Verhalten liefern ein hervorragendes Modellsystem für diese Studie, um die Entwicklung und Funktion neuronaler Plastizität im olfaktorischen System zu untersuchen.

Diese Studie konzentriert sich auf Unterschiede zwischen Königinnen und Arbeiterinnen, den beiden weiblichen Kasten innerhalb des Bienestaates, sowie auf umweltbedingte Plastizität. Diploide Eier, aus denen sich Königinnen und Arbeiterinnen entwickeln, sind genetisch identisch. Dennoch entwickeln sich Königinnen wesentlich schneller zum Adulttier als Arbeiterinnen, sind als Imago größer, leben wesentlich länger und zeigen andere Verhaltensweisen. Diese Unterschiede werden durch eine differentielle larvale Fütterung initiiert. Im Anschluss an das Larvenstadium und somit nach erfolgter Kastendetermination, entwickeln sich die Bienen über eine Puppenphase (verdeckelte Phase) zum Imago. Adulte Bienen klimatisieren das zentrale Brutareal auf einer mittleren Temperatur von 35°C konstant. Bienen, die bei niedrigeren Temperaturen innerhalb des physiologisch relevanten Bereichs aufwachsen, weisen Defizite im olfaktorischen Lernverhalten und in der Tanzkommunikation auf. Mögliche neuronale Korrelate für altersbedingte, temperatur- und kastenspezifische Unterschiede im olfaktorisch gesteuerten Verhalten sollten in dieser Arbeit betrachtet werden. Die strukturellen Analysen konzentrierten sich dabei auf primäre (Antennalloben) und sekundäre (Pilzkörper-Calyces) olfaktorische Verarbeitungszentren im Gehirn von sich entwickelnden und adulten Tieren beider Kasten.

Synchron verdeckelte Brutzellen beider Kasten wurden unter kontrollierten Bedingungen im Inkubator herangezogen. Neuroanatomische Untersuchungen wurden an fixierten Gewebeschnitten mittels einer Doppelfluoreszenzfärbung mit Fluor-Phalloidin und anti-Synapsin Immuncytochemie durchgeführt. Diese Doppelmarkierung ermöglichte die Visualisierung und Quantifizierung individueller Synapsenkomplexe (Microglomeruli) im Pilzkörper-Calyx. Phalloidin bindet an verschiedene F-Aktin Isoformen und kann zum Nachweis von F-Aktin im Insektennervensystem verwendet werden. F-Aktin wird während der Entwicklung in Wachstumskegeln und in adulten Gehirnen in präsynaptischen Endigungen und dendritischen Dornen exprimiert. Präsynaptische Elemente wurden durch den Einsatz eines spezifischen Antikörpers gegen das *Drosophila*-Vesikeltransportprotein

Synapsin I charakterisiert. Mit Hilfe der konfokalen Laser-Scanning Mikroskopie wurde die exakte räumliche Zuordnung der Fluoreszenzsignale anhand optischer Schnitte durch die Präparate realisiert. Anhand dieser Methodik konnten erstmals über reine Volumenanalysen hinausgehende Messungen zur synaptischen Strukturplastizität im Pilzkörper-Calyx durchgeführt werden.

Die Untersuchungen an Gehirnen in den verschiedenen Puppenstadien zeigten Unterschiede im Entwicklungsverlauf der Gehirne mit dem Fokus auf die Bildung antennaler Glomeruli und calycaler Microglomeruli. Unterschiede in der Gehirnentwicklung verdeutlichten die ontogenetische Plastizität des Gehirns der Honigbiene. Entsprechend der kürzeren Puppenphase der Königinnen bildeten sich sowohl antennale Glomeruli als auch alle Untereinheiten (Lippe, Collar, Basalring) des Calyx etwa drei Tage früher aus. Direkt nach dem Schlupf zeigten quantitative Analysen innerhalb der Pilzkörper-Calyces eine signifikant geringere Anzahl an Microglomeruli bei Königinnen. Diese neuronale Strukturplastizität auf verschiedenen Ebenen der olfaktorischen Informationsverarbeitung korreliert mit der kastenspezifischen Arbeitsteilung.

Die Arbeit liefert Erkenntnisse über den Einfluss eines wichtigen kontrollierten Umweltparameters, der Bruttemperatur, während der Puppenphase auf die synaptische Organisation der adulten Pilzkörper-Calyces. Bereits geringe Unterschiede in der Aufzuchtstemperatur (1°C) beeinflussten signifikant die Anzahl von Microglomeruli in der Lippenregion des Calyx beider weiblicher Kasten. Die maximale Anzahl an MG entwickelte sich bei Arbeiterinnen bei 34.5°C, bei Königinnen aber bei 33.5°C. Neben dieser entwicklungsbedingten neuronalen Plastizität zeigt diese Studie eine starke altersbedingte Strukturplastizität der MG während der relativ langen Lebensdauer von Bienenköniginnen. Hervorzuheben ist, dass die Anzahl an MG in der olfaktorischen Lippenregion mit dem Alter anstieg (~55%), in der angrenzenden visuellen Collarregion jedoch abnahm (~33%). Die in der vorliegenden Arbeite erstmals gezeigte umweltbedingte Entwicklungsplastizität sowie altersbedingte synaptische Strukturplastizität in den sensorischen Eingangsregionen der Pilzkörper-Calyces könnte kasten- und altersspezifischen Anpassungen im Verhalten zugrunde liegen.

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Malu Obermayer gave me an excellent introduction to neurobiological techniques. Besides her expert technical help, she supplied me with chocolate and other non-healthy food to survive the everyday lab-life. She also simply encouraged me with fancy stories when I thought everything seemed to go wrong and with sending a list of my wishes to Santa Claus.

Breeding honeybees, especially queens, is impossible to do without professional help. With the assistance from our beekeeper Dirk Ahrens and Sven Maier I was able to realize my projects. Dirk, in particular, provided me with synchronized sealed worker and queen brood straightforward from early spring to late autumn and with honey all year around.

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The ultimate thanks go to my husband Christian and my oldest friend Kristin Winkelbach for always reminding me that there is a life beside science. Es lebe der Tegernsee!

APPENDIX

CURRICULUM VITAE

PERSONAL DATA born 12th August 1974 in Schweinfurt, Germany

RESEARCH AREA neuroethology

EDUCATION

2002 – 2005	PhD thesis, University of Würzburg, Germany 'environmental influences on the development of the female honeybee brain *Apis mellifera*' advisor: Prof. W. Rössler
2000 – 2001	practical training in aerodynamics, University of Würzburg, Germany BioFuture Research
1999 – 2000	diploma thesis, University of Würzburg, Germany advisor: Prof. J. Tautz
1994 – 2000	study of biology, University of Würzburg, Germany major: behavioral physiology; minors: microbiology, plant physiology
1994	Abitur, Mozart-Gymnasium Würzburg, Germany majors: biology, German

SCHOLARSHIPS

2001 – 2003	PhD scholarship awarded by the DFG, Germany graduate college 'arthropod behavior'
2000	PhD scholarship awarded by the University of Würzburg, Germany

TEACHING EXPERIENCE

2003 – 2005	advanced course in neurobiology
2000 – 2004	introductory animal physiology
2000	coordinator for the graduate college 'arthropod behavior'
1999 – 2005	advanced course in animal physiology

EXTERNAL INTERNSHIPS

02/1998 – 04/1998	internship at the government of Unterfranken, Germany division of horticulture
02/1997 – 04/1997	internship at the government of Unterfranken, Germany division of viniculture

VOLUNTEERING

08/2001	science education for children, TV series 'Tigerentenclub'
1996 – 2000	student representatives of biology, University of Würzburg, Germany elected member
1995 – 1997	tutor for first-year students, University of Würzburg, Germany
1990 – 1995	team leader, congregation of St. Bruno Würzburg, Germany

PUBLICATIONS

FULL PAPERS

Groh C, Ahrens D, Rössler W (2006) Environment- and age-dependent plasticity of synaptic complexes in the mushroom bodies of honeybee queens. Brain Behav Evol (in press).

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*The results presented in this paper were obtained during my diploma thesis.

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Groh C, Brockmann A, Tautz J (2001) Selektive Ausschaltung antennaler Kontaktchemorezeptoren bei der Arbeiterin der Honigbiene. Apidologie 32:498-499.

FURTHER CONFERENCE PRESENTATIONS

Gräbner M, Groh C, Müller B, Rössler W, Schachtner J (2005) Metamorphosis of the honeybee antennal lobe: general development and A-type allatostatin immunoreactivity. DZG, Bayreuth (*poster*).

Groh C, Müller B, Rössler W (2005) Ontogenetic plasticity of primary and secondary olfactory centers in the honeybee brain. Göttingen Neurobiology Conference, Göttingen (*poster*).

Gräbner M, Groh C, Müller B, Rössler W, Schachtner J (2005) The development of allatostatin-A immunoreactivity in the antennal lobe neurons of the honeybee *Apis mellifera*. Göttingen Neurobiology Conference, Göttingen (*poster*).

Groh C, Rössler W (2004) Developmental plasticity of the olfactory pathway in the honeybee brain. International Society for Neuroethology, Nyborg (*poster*).

Groh C, Rössler W (2004) Thermoregulation during pupal development affects synaptic organization in the adult honey-bee brain. DZG, Rostock (*talk*).

Groh C, Rössler W (2004) Comparison of brain development in honeybee workers and queens. DZG, Rostock (*poster*).

Groh C, Rössler W (2003) Brain development of honeybee workers and queens is influenced by temperature manipulations during pupal stages. IUSSI, Regensburg (*talk*).

Groh C, Brockmann A, Fröhlich B, Tautz J (2001) Effect of $ZnSO_4$ on the antennal chemosensory system of the honeybee *Apis mellifera*. IUSSI, Berlin (*poster*).

Groh C, Altwein M, Brockmann A, Tautz J (2001) Chemical ablation in *Apis mellifera*. European Society of Promoting the Science of Bees and Beekeeping, Patras (*poster*).

WORKSHOPS

Groh C, Rössler W (2004) Thermoregulation during pupal development affects synaptic plasticity. Neurobiologischer Doktorandenworkshop, Marburg (*talk*).

Groh C, Rössler W (2003) Influences on the development of the honeybee brain. Neurobiologischer Doktorandenworkshop, Göttingen (*poster*).

ERKLÄRUNG

Hiermit erkläre ich, die vorliegende Arbeit in allen Teilen selbständig und nur mit den angegebenen Hilfsquellen angefertigt zu haben.

Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.

Ich habe bisher außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben, noch zu erwerben versucht.

Würzburg, den 08.12.2005