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**DEVELOPMENT AND EVOLUTION OF HIGHER BRAIN CENTERS IN
INSECTS**

Xiangyi Zhao

**Thesis submitted to the College of Arts and Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Master of Science
in
Biology**

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**Morgantown, West Virginia
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Abstract

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Xiangyi Zhao

Brain structures within an individual can change during development through modification of individual neuron morphology together with alteration of neuron numbers. Differences in the same or analogous brain structures from different species can reflect behavioral pattern variation among these species fixed by natural selection. This study used insects from two different taxonomic groups to address such brain structure changes at these two time scales: developmental and evolutionary. Mushroom bodies, as an integrative sensory center involved in learning and memory in insects, were the main structure surveyed to illustrate changes in higher brain centers in both projects. In the developmental study, the changes of higher brain centers in red flour beetle, *Tribolium castaneum*, were described from late larva through adulthood. The mechanisms detected for change of brain included axon reorganization, compartment enlargement and adult neurogenesis. In a second study to comparing brain structure differences across species, with an effort to address an evolutionary question, a significant difference in mushroom body volumes from different species of eusocial termites was found. This suggests that it is possible to independently test the social brain hypothesis, which was developed for very different animal lineages including primates, within this group of successful social insects.

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Part I: Introduction

Brains can change in response to the behavioral demands of an individual. These changes can also be favored in a species over evolutionary time in response to selective pressures. Thus, if the same type of change repeatedly occurs in different species, which are facing the same behavioral challenges, it may be possible to correlate this type of change in brain with that behavioral factor.

Changes in brain can happen in a single neuron with the modification of its morphology and gene expression. This modification usually occurs in synapses, dendrites and axons of neurons via branching, pruning, or retracting (Scelfo and Buffelli, 2009; Jan and Jan, 2010). Remodeling of higher brain centers in an individual can also occur by adding or reducing the number of neurons, which are called neurogenesis and apoptosis respectively. Neurogenesis is usually considered uneconomical (Lindsey and Tropepe, 2006) as it involves wiring reconstruction to integrate new neurons into pre-existing neural circuitry. At the same time, neurogenesis requires keeping neuronal stem cells, which have the full potential to develop into a specific type of neuron as needed. All these changes in neurons and neuron numbers can be combined and reflected by the alteration, such as change in volume, of structures in higher brain centers. Thus, tracing an individual's brain structure development may help us understand mechanisms underlying brain change. And if such effort continues to an evolutionary level across many different species, it may even tell us what type of neural circuit modification is associated with a certain behavior.

Insects, with the largest number of species in the animal kingdom, are well-suited for comparative studies. Insects are an extremely successful and diverse taxon that are found in nearly every terrestrial and freshwater environment on earth (Grimaldi and Engel, 2005). At the same time, as a well studied group, knowledge of insect brain anatomy has already reached the point that makes it possible to perform comparative studies (Farris and Sinakevitch, 2003; Farris, 2005; Strausfeld et al., 2009). More importantly, insects offer a good opportunity to find connection between behavior and brain structure. In holometabolous insect species dramatic changes, including a whole body reconstruction which involves remodeling the nervous system, happen during one individual's life span (Truman and Riddiford, 2002). For instance, in the *Drosophila* mushroom body, a well-studied higher brain center in insects, remodeling includes production of a new type of neuron and axon retraction of the preexisting neurons during metamorphosis (Lee et al., 1999). Although different species may use different mechanisms for remodeling processes in higher brain centers, by comparative study of a group of closely related species from different environmental niches, we may be able to associate structural changes in the insect brain with the acquisition of adaptive behavior. With the abundant number of insect species

and great variation of behavior in insects, if such a pattern occurs in different insect groups, it could be evidence for natural selection for structural alterations in the brain.

At the evolutionary level, enlargement of the size of the total brain or a specific brain structure, regardless of what mechanisms are used, appears to have occurred in many animal groups. This phenomenon especially arouses interest as primates, particularly human beings, have exceptionally large brains according to their body size (Lande, 1979). Several hypotheses have been proposed to explain why such large brains exist, typically grouped into one of four categories: epiphenomenal, developmental, ecological, and social hypotheses (Dunbar, 1998). The brain is an energetically expensive organ. Thus, it is generally agreed that a large brain must result from natural selection related to the acquisition of complex behaviors or the needs of ecological impacts (Shultz and Dunbar, 2006). However, in order to realize the final result of large brains, it may be necessary to overcome developmental constraints.

Between the competing hypotheses about large brains, social factors drew particular attention as sociality is a special feature of primates. One hypothesis first proposed during the late 1980s is the Machiavellian Intelligence hypothesis (Byrne and Whiten, 1988). It attributed the large brains in primates to the computational demands of complex social systems. It came to be called the “Social brain hypothesis” to emphasize the connection between sophisticated and enlarged brain structures in species with complex social structures (Dunbar, 1998). As research about this hypothesis progressed, it was tested against other ecological factors (Dunbar, 1998; Byrne and Bates, 2007) and turned out to be favored even in non-primate animals (Connor et al., 1998; Molina and O'Donnell, 2007; O'Donnell et al., 2007; Perez-Barberia et al., 2007).

In insects, social behaviors have evolved independently several times (Wilson, 1971). For instance, among bees it is likely sociality evolved independently at least eight times, although the actual number is unclear (Michener, 2000; Danforth, 2002). At the same time, the presence of particularly large mushroom bodies in both eusocial Hymenoptera (ants, bees and wasps) and in termites (order Dictyoptera, suborder Isoptera) suggests that the acquisition of social behaviors may require the evolution of large brain regions in these species. But the social brain hypothesis has never been systematically tested in insects, partially due to the lack of well-understood phylogenies trees.

Among all the brain regions studied so far in insects, mushroom bodies are the most extensively studied higher brain center. Mushroom bodies, lobed neuropils symmetrically distributed on both brain hemispheres (Fig 1A-B), were first described in honeybees and were associated with ‘intelligent’ behavior from the very beginning (Dujardin, 1850). In an

entomology text book, *The Insects: structure and function*, the large volume of the wasp mushroom bodies relative to other brain structures placed wasps at the top of the “smartest” insect as, mushroom bodies generally dominated the brains of social Hymenoptera (Chapman, 1998). An important reason mushroom bodies are useful for comparative studies in insects was that while they are homologous across the insects, the morphology of mushroom bodies is diverse and in some cases strongly associated with behavioral ecology (Farris and Sinakevitch, 2003; Farris, 2005; Fahrbach, 2006).

This thesis is divided into two parts with both focused on the anatomy of mushroom bodies. The first part is a developmental study in red flour beetles. It introduces the necessary techniques and also constructs a profile of how higher brain centers can be altered as one species faces varied behavioral demands. The second part attempts to test the social brain hypothesis in insects. It is an evolutionary study in termites benefiting from a newly available phylogenetic tree (Inward et al., 2007). Together, these will help us understand how insect neural networks are constructed to fulfill their behavior needs.

Part II: Metamorphosis and adult development of the mushroom bodies of the red flour beetle, *Tribolium castaneum*.^{*1}

1. Introduction

A relationship between insect mushroom bodies and higher cognitive functions was first posited by Dujardin (1850), who noted that the largest mushroom bodies were to be found in the brains of the social Hymenoptera. In the intervening decades a wealth of studies revealed the importance of the mushroom bodies in complex behaviors such as sensory integration (Schildberger, 1984; Li and Strausfeld, 1997, 1999), place memory and motor control (Mizunami et al., 1993), higher-level olfactory processing (Laurent and Naraghi, 1994; Perez-Orive et al., 2002; Cassenaer and Laurent, 2007), and associative and context-dependent learning and memory (Liu et al., 1999; for reviews see Waddell and Quinn, 2001; Heisenberg, 2003). Using the powerful tools for the manipulation of gene expression that are available in the fruit fly *Drosophila melanogaster*, it has also been possible to elucidate the molecular pathways for memory formation and their localization within subpopulations of mushroom body neurons (Zars et al., 2000; Pascual and Prat, 2001; McGuire et al., 2003; Krashes et al., 2007; Schwaerzel et al., 2007; Thum et al., 2007).

Mushroom body intrinsic neurons, called Kenyon cells, undergo a dramatic reorganization during metamorphosis in *Drosophila*, and display adult plasticity of axonal and dendritic outgrowth that is associated with behavioral experience in both *Drosophila* and the honey bee *Apis mellifera* (Technau and Heisenberg, 1982; Technau, 1984; Armstrong et al., 1998; Lee et al., 1999; Farris et al., 2001). Another form of neural plasticity, adult neurogenesis, is of great interest in the vertebrate literature due to the possibility that newborn neurons mediate higher cognitive processes such as learning, and the potential for neural stem cells to be utilized in a therapeutic context (Van Praag et al., 2002; Van Praag et al., 2005; Aimone et al., 2006; Bruel-Jungerman et al., 2006; Sohur et al., 2006; Okano and Sawamoto, 2008). In this area, however, *Drosophila* has not been a useful model as the available evidence suggests that the mushroom body neuroblasts do not persist beyond adult eclosion (Technau and Heisenberg, 1982; Technau, 1984). Adult neurogenesis is present in the mushroom bodies of crickets (*Acheta domesticus*), and neuron production has been linked to environmental enrichment, olfactory stimulation and

¹ Copyright (2008) Wiley. Used with permission from (Zhao X, Coptis V and Farris SM. "Metamorphosis and adult development of the mushroom bodies of the red flour beetle, *Tribolium castaneum*." Developmental Neurobiology), Wiley Periodicals, Inc.

performance on olfactory learning tasks in both associative and operant contexts (Scotto-Lomassese et al., 2000; Scotto-Lomassese et al., 2002; Scotto-Lomassese et al., 2003; Cayre et al., 2007). Tools for the manipulation of gene expression are not available for the cricket, however, so although neurogenesis in this insect has been shown to be under the control of both external and internal factors (Cayre et al., 1997; Cayre et al., 1997; Cayre et al., 2001; Cayre et al., 2005), it has not been possible to gain more detailed insight into the regulatory pathways that mediate adult neurogenesis. The red flour beetle *Tribolium castaneum* is a stored products pest that has recently proven valuable as a point of comparison with *Drosophila* in studies of the genetic control of body plan organization and the specification of appendage identity (deCamillis et al., 2003; Schröder, 2003; Wheeler et al., 2003; Copf et al., 2004; Tomoyasu et al., 2005; van der Zee et al., 2005; Choe et al., 2006; Choe and Brown, 2007). Like *Drosophila*, *Tribolium* is amenable to genetic manipulation through germ line transformation, transgene expression vectors and RNAi. The genome has also been sequenced and assembled into linkage maps that are available online via Beetlebase (Browne and Scholtz, 1999; Lorenzen et al., 2002; Lorenzen et al., 2003; Lorenzen et al., 2005; Wang et al., 2007). Unlike the fruit fly and similar to the cricket, the mushroom body neuroblasts of beetles persist into adulthood and undergo active neurogenesis as indicated by BrdU incorporation (Cayre et al., 1996). Little else is known about the mushroom bodies of *Tribolium*, however, and mushroom body morphology has been described in detail for only one family of beetles (the Scarabaeidae; Larsson et al., 2004; Farris and Roberts, 2005) despite the fact that the beetles (Coleoptera) are the most speciose taxon on Earth.

The present account of *Tribolium* mushroom body structure and development is intended to put this species at the forefront of comparative studies that will lay the groundwork for investigation of the genetic regulation and experimental manipulation of adult neurogenesis. Immunohistochemistry and BrdU labeling reveals a mushroom body organization much like that of *Drosophila* but with key differences in cellular composition and developmental events. The mushroom bodies undergo significant remodeling of dendritic structures but not of axons during metamorphosis, and continuous proliferation of mushroom body neuroblasts is observed until nearly three months post-eclosion. An unexpected result of this study is that, unlike in *Drosophila* (Ito and Hotta, 1992; Ito et al., 1997), neuroblast number is variable across individuals and appears to impact mushroom body size. Should the variation be heritable, the *Tribolium* model system will be uniquely suited to future studies of the regulation of neuroblast number and cell division dynamics, the effects of different sized populations of intrinsic neurons on afferent and efferent connections, and the relationship between mushroom body structure and behavioral output.

2. Methods

Insects

Tribolium castaneum beetles were purchased from Carolina Biological Supply Company (Burlington, North Carolina) and kept in an incubator at 28 °C, with a 12:12 light:dark cycle. Prepared *Tribolium* medium was purchased from the same company and was composed of 4 parts white flour, 4 parts whole wheat flour, and 1 part brewer's yeast. Last instar larvae were identified by their relatively large body size, and selected for immunostaining. Pre-pupae were selected from the media based on their large size and restricted movement and kept separately in Petri dishes until pupation. Pre-pupae were monitored daily so that upon metamorphosis, the new pupae were moved to a separate dish and marked as Day 0. Different ages of pupae were determined primarily by the time from pupation, but also by the degree of eye pigment darkening.

Tissue dissection and fixation

All dissections were done in insect physiological saline (O'Shea and Adams, 1981) after chilling the insects on ice. The head capsules of beetles were removed from the body and the brains were partially dissected. This made the tiny brains more visible throughout the immunostaining procedure, and reduced the number of brains that were lost. Fixation took place in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.2) for 1 hour at room temperature. The tissue was then washed in PBS and stored at 4 °C in the dark until further processing.

Immunostaining and fluorescent labeling of F-actin (phalloidin staining)

Before applying any antibodies, whole fixed brains were washed in 1% PBST (1% Triton X-100 in PBS) and then permeabilized in collagenase-dispase (Sigma-Aldrich, St. Louis, MO) for 20 minutes. The concentration of enzyme that was used differed according to age, as brains of younger beetles were damaged by the higher concentrations needed to permeabilize older tissue. For brains from late larval to new born adult beetles, the collagenase concentration used was 0.125 mg/ml. For adults up to two weeks old the concentration used was 0.25 mg/ml, and brains from all older beetles were treated with 0.5 mg/ml collagenase. After the enzyme treatment, all brains were washed several times in 1% PBST and then incubated in a blocking solution of 10% normal goat serum (NGS) in 1% PBST for at least 1 hour.

Anti-DC0 primary antibody, a polyclonal antibody against the catalytic subunit of *Drosophila melanogaster* protein kinase A, was a gift provided by Dr. Daniel Kalderon

(Columbia University). This antibody has been shown to have a high affinity for mushroom body intrinsic neurons in a range of insect species (Skoulakis et al., 1993; Farris, 2005). In the present study anti-DC0 was diluted 1:1 in glycerol for storage, and a 1:500 concentration of this stock was used in 1% NGS in 1% PBST for staining. The following day, sections were washed with 1% PBST three times for 10 min each followed by incubation in a 1:200 concentration of goat anti-rabbit secondary antibody tagged with Texas Red (Invitrogen (Molecular Probes), Eugene, OR), also in 1% NGS solution in PBST. Alexa 488-conjugated phalloidin (Molecular Probes), which labels filamentous actin that is enriched in outgrowing Kenyon cell processes (Kurusu et al., 2002; Farris and Sinakevitch, 2003; Farris, 2004; Farris et al., 2005), was applied at a 1:500 concentration at the same time as the secondary antibody. The brains were incubated in this solution overnight at room temperature. The next day sections were washed in PBST and cleared in 60% glycerol for half an hour, followed by incubation 80% glycerol for one half hour. After mounting in 80% glycerol, brains were dissected free of the remnants of the head capsule using fine forceps, and viewed using an Olympus Fluoview 1000 confocal microscope within 48 hours of coverslipping.

BrdU Treatments and Staining

Adult beetles were chilled on ice and a drop of insect saline containing 25 mg/ml 5'-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO), a thymidine analog and marker of cellular mitotic activity, was placed on the mouthparts. As the beetles recovered from chilling, movements of their mouthparts caused them to ingest the solution. After BrdU treatment, beetles were placed in Petri dishes with food for 4h, 8h or overnight prior to dissection. In place of the enzyme treatment used prior to immunostaining, BrdU treated and dissected brains were immersed in 2N HCl for twenty minutes. This served both to permeabilize the tissue and to denature the DNA to allow access to the anti-BrdU antibody. Monoclonal anti-BrdU was purchased from Becton-Dickinson (San Jose, CA) and used at a 1:200 concentration in 1% NGS in PBST. Anti-DC0 was also applied at a 1:500 concentration as a counterstain. Anti-BrdU was detected using a 1:200 solution of Texas Red conjugated goat anti-mouse secondary antibody in 1% NGS in PBST, and the anti-DC0 was detected using an Alexa 488 conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR). Following incubation in these secondary antibodies, processing and visualization of labeled tissue proceeded as described above.

Confocal Microscopy and Image processing

All stained preparations were viewed on an Olympus Fluoview 1000 confocal microscope. Single 1.5 μ m deep optical sections, or projections made from a small number of optical

sections, were selected from stacks of each stained brain. These images were then processed as needed for brightness and contrast using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

3. Results

Structure and development of the insect mushroom bodies with reference to *Drosophila melanogaster*: a review

Insect mushroom bodies are composed of intrinsic neurons, Kenyon cells, whose soma lay in the dorso-posterior region of the brain. Kenyon cell dendrites form a cup- or bulb-shaped structure called the calyx, which receives sensory afferents (primarily olfactory projection neurons from the antennal lobe; Ito et al., 1998; Gronenberg, 2001; Strausfeld et al., 2003). The axons of Kenyon cells travel anteroventrally through the pedunculus and bifurcate into the medial and vertical lobes, which are commonly thought of as an output region although they also receive input from multimodal protocerebral afferents (Ito et al., 1998; Li and Strausfeld, 1999; Strausfeld, 2002).

During development, four neuroblasts per hemisphere each produce at least three types of Kenyon cells, the γ , α'/β' and α/β cells (Ito et al., 1997; Crittenden et al., 1998; Lee et al., 1999). More recent studies suggest that there are several more Kenyon cell subpopulations in *Drosophila* based on dendritic morphology and afferent input (Strausfeld et al., 2003; Zhu et al., 2003; Lin et al., 2007). The first-born Kenyon cell subpopulation is produced from stage 15 of embryonic development until the middle of the third larval instar and forms the medially projecting γ lobe of the adult mushroom bodies (Tettamanti et al., 1997; Armstrong et al., 1998; Lee et al., 1999; Noveen et al., 2000). Noveen et al. (2000) report that the mushroom body neuroblasts are present in the embryonic brain and actively dividing prior to stage 15, however, the identity of these earliest progeny has yet to be determined. The second-born subpopulation of Kenyon cells form the α'/β' lobes with their bifurcated axons and are born in the short interval between the mid-third instar and the onset of puparium formation (Lee et al., 1999). The last-born subpopulation of Kenyon cells forms the α/β lobes with their axons and is produced after puparium formation; neurogenesis ends shortly before eclosion and the neuroblasts do not persist in the adult brain (Ito and Hotta, 1992; Lee et al., 1999). The last-born Kenyon cells within the α/β subpopulation, called the α/β core neurons after the projection pattern of their axons in the core of the α/β lobes, may also be differentiated morphologically and by distinct gene expression patterns (Kurusu et al., 2002; Lin et al., 2007).

During metamorphosis in holometabolous insects, much of the larval nervous system is remodeled through a combination of neuronal birth and death, and process

degeneration, and regrowth (Truman, 1990; Consoulas et al., 2000). In the brain, the antennal lobe is the primary olfactory processing center and is composed of densely packed glomeruli containing the synapses of olfactory receptor neurons from the antennae, inhibitory local interneurons and projection neurons that synapse onto Kenyon cell dendrites in the calyx (Stocker et al., 1990; Stocker, 1994). The antennal lobe undergoes a dramatic reorganization during metamorphosis during which the larval glomeruli are dissolved and reformed into the adult configuration and the connections of projection neurons in the calyx are similarly reshaped (Jefferis et al., 2002; Marin et al., 2005). Kenyon cell dendrites and axons are also pruned and re-established (Zhu et al., 2003; Marin et al., 2005), and the axons forming the larval γ lobe, which is composed of vertical and medial projecting components formed by the bifurcating axons of γ Kenyon cells, are engulfed by glia and regrow in the medial direction only (Armstrong et al., 1998; Lee et al., 1999). The α'/β' Kenyon cells that are born shortly before puparium formation serve as guidance cues for the re-extending γ axons (Zhu et al., 2003). Large-scale reorganization of the mushroom body lobes like that in *Drosophila* has also been observed in the wasp *Polistes apachiensis*, but in the honey bee *Apis mellifera* the γ Kenyon cell axons only undergo a slight pruning during metamorphosis (Farris et al., 2004).

Structure of the mushroom bodies of *Tribolium castaneum*

As in other insects, the mushroom bodies of *Tribolium castaneum*, as observed in this study, were composed of densely packed intrinsic neurons, the Kenyon cells. The Kenyon cell somata resided around a single calyx in each hemisphere. Fig 2-1 summarizes and illustrates the general structures of mushroom bodies in *Tribolium*. Specifically, Fig. 2-1A illustrates the structure of the calyx (Cx) in one hemisphere of the brain, and the location of the two large neuroblasts (Nb). The ovoid shape of the calyx is similar to that of *Drosophila* and is similarly composed of the dendrites of Kenyon cells. In contrast to *Drosophila*, the neuroblasts were clearly visible at all stages of development, from the late larva through adulthood. Phalloidin labeling, which has been demonstrated to reveal the ingrowing axons of newborn Kenyon cells in several insect species (Kurusu et al., 2002; Farris and Sinakevitch, 2003; Farris, 2004; Farris et al., 2005), showed two axon tracts entering the *Tribolium* calyx, one associated with each of the neuroblasts (Fig. 2-1A, arrows). Ventral to the calyx, Kenyon cell axons projected into the pedunculus (Pe, Fig 2-1A). The vertical and medial lobes were formed by the vertical and medial branches of Kenyon cell axons (Fig. 2-1B-E). Staining with the anti-DC0 antibody allowed the identification of five Kenyon cell types based on axon morphology and antibody affinity. Figures 2-1B-E show four optical sections from anterior to posterior through the mushroom body lobes of a single individual, and differently colored arrows indicate the five subpopulations of Kenyon cells. A summary of the Kenyon cell subpopulations observed is

represented in the schematic in Fig.2-1F, with different colors representing the arrows in Figs. 2-1B-E. The red " δ " cells, whose far anterior location suggests that they are among the first-born Kenyon cells (Farris and Sinakevitch, 2003), likely correspond to the Class III Kenyon cells that have been described in many insects, but not in *Drosophila* (Farris, 2005). Orange, green, blue and purple arrows indicate cell populations that, based on axon morphology and birth order (see below), are likely homologs of the γ , α'/β' , α/β and α/β core Kenyon cells of *Drosophila* (Crittenden et al., 1998; Lee et al., 1999; Kurusu et al., 2002; Arendt et al., 2007). Some differences in morphology between *Drosophila* and *Tribolium* were observed. Most notably, the γ neurons provided axon branches to both the medial and vertical lobes in the adult *Tribolium* mushroom bodies in contrast to those of *Drosophila* that branch only medial lobe (Armstrong et al., 1998; Lee et al., 1999). The following result sections will illustrate in further detail the brain structure changes in *Tribolium* during development.

Development and reorganization of the *Tribolium* mushroom bodies during metamorphosis

The present study began with phalloidin labeling and anti-DC0 immunohistochemistry of the brain of the last instar feeding larva, which was identified as the largest larval instar that was observed moving freely through the flour medium. In these last instar larvae just two groups of Kenyon cells, γ and δ , were observed in the lobes (Fig.2-2A). Phalloidin labeling revealed a tract of newborn Kenyon cell axons emerging from a small group of cell bodies immediately surrounding each of the two neuroblasts (Fig. 2-2B, arrows). The two tracts passed through the calyx and fused at the origin of the pedunculus to form an ingrowth core that continued into the vertical and medial branches of one lobe pair (arrows, Fig. 2-2 A-C). This lobe pair was termed γ , after their resemblance to the larval γ Kenyon cells of *Drosophila*. Kenyon cells making up a smaller, more anterior lobe pair that lacked a core of ingrowing axons were termed δ . As mushroom body neuroblasts produce Kenyon cell subpopulations sequentially (Lee et al., 1999) and no ingrowing axons were observed in the δ lobe, these Kenyon cells are likely born prior to the γ cells.

Clear glomeruli were also present in the antennal lobe of the last instar larvae (arrows, Fig. 2-2D), as were microglomeruli in the mushroom body calyx (arrowheads, Fig.2-2B). Glomeruli and microglomeruli are structures associated with synaptic associations in both the antennal lobe and the mushroom bodies.

Dramatic changes were observed in the structure of the antennal lobes and mushroom body calyces of non-feeding prepupae, which were identified as last instar

larvae that were immobile save for rotational movements of the abdomen. The ingrowth core was now observed to contain axons of two Kenyon cell subpopulations in the vertical lobes (arrows, Fig 2-2E), suggesting that the neuroblasts had begun producing a new subpopulation of Kenyon cells (termed α'/β' after those born in the late third instar *Drosophila* larva). The persistence of phalloidin labeling in the γ lobes, however, suggested that the outgrowth of γ Kenyon cell axons was not completed before neuroblast production of α'/β' neurons had begun. Interestingly, the medial lobe had a phalloidin labeled core only in the medial γ lobe, with no apparent medial α'/β' ingrowth at this stage (Fig 2-2 G). This suggested that α'/β' axon outgrowth into the vertical lobes preceded branching into the medial lobe, the opposite of what has been observed in *Drosophila* (Tettamanti et al., 1997; Noveen et al., 2000; Kurusu et al., 2002).

In the prepupal mushroom body calyces and antennal lobes, all traces of glomerular structure had dissolved (Fig 2-2H, F). This is suggestive of synaptic reorganization of these two sequential components of the olfactory processing pathway, also reminiscent of *Drosophila* (Marin et al., 2005). In contrast to *Drosophila*, however, there was no apparent axon degeneration or pruning in the mushroom body lobes during the prepupal or pupal stages.

The appearance of the pupal mushroom bodies was similar to those of the prepupa for the first two days of the eight-day pupal stage (Fig. 2-3A inset). At day three, phalloidin labeling of extending γ Kenyon cell axons disappeared from the vertical lobe so that only α'/β' axons were labeled (arrows, Fig 2-3A). In the medial lobe, axons of newborn α'/β' cells were now labeled along with those of γ Kenyon cells (arrows, Fig 2-3B). Again, this result suggests a delay in Kenyon cell branching into the medial lobe relative to outgrowth into the vertical lobe, as γ axon extension into the vertical lobe was completed prior to extension into the medial lobe. By the end of pupal day 4, phalloidin labeling of γ Kenyon cell axons in the medial lobe had also disappeared and only labeling of α'/β' axons was observed (Fig. 2-3C).

The ingrowth of α'/β' Kenyon cells into the lobes continued through the remainder of the pupal stage (Fig 2-4). It started in day three and had lasted till day eight. Glomeruli in the mushroom body calyces and antennal lobes gradually coalesced until these structures were indistinguishable from those of adults by pupal day 8, which was the last day of the pupal stage under our rearing conditions at 28 °C. Comparing the calyces of last instar larvae with those of late pupae, it appeared that the late pupal microglomeruli were smaller and finer in structure than were those of the larva, perhaps suggesting a larger number of synaptic connections in the mushroom body calyces of the developing adult.

Adult neurogenesis in the *Tribolium* mushroom bodies

Newly eclosed adults could be differentiated from older adults by their lightly tanned cuticle (Fig 2-5A, newly eclosed adult at top and two week old adult below). Both phalloidin staining and BrdU labeling, as it incorporated into DNA of newly born cells, revealed the two neuroblasts and their newborn Kenyon cell progeny in the adult mushroom bodies (Fig. 2-5B1, 2-5B2). These neuroblasts were actively proliferating for the first two months of adult life. Beyond this age, however, BrdU incorporation and phalloidin labeling of newborn neurons became minimal or was totally lost (Fig. 2-5C). At 88 days of age, only a small number of adults maintained active neuroblasts in the mushroom bodies (data not shown). At adult eclosion, the neuroblasts began production of the last group of Kenyon cells, termed α/β (Fig 2-5C, blue arrow). The axons of newborn Kenyon cells in the adult mushroom bodies formed an ingrowth core at the center of the α/β lobes (Fig. 2-5C, purple arrow). The α/β lobes steadily increased in size throughout adulthood as the axons of these new neurons entered via the ingrowth core (compare Figs. 2-5C-E). The mushroom bodies as a whole appeared to increase in size as adult life progressed, perhaps reflecting the ramification of afferent and efferent processes within the mushroom body neuropil. Interestingly, individual variation in mushroom body neuroblast number was observed in approximately 10% of the fifty larval, pupal and adult brains viewed for this study (Fig. 2-6). A small number of these variants had only one rather than two mushroom body neuroblasts in one of the brain hemispheres (compare Fig. 2-6A with Fig. 2-6B). When only one neuroblast was present, the mushroom body neuropil in that hemisphere appeared to be diminished in size. Most of the variants had three mushroom body neuroblasts in one hemisphere (Fig. 2-6C), with two of the neuroblasts more closely associated with one another, and with their progeny entering the mushroom body calyx via the same phalloidin-labeled tract. In these latter variants, the size of the mushroom bodies appeared to be more similar to that of wild type individuals.

4. Discussion

Comparing mushroom body structure and development in *Tribolium castaneum* with that of *Drosophila melanogaster*.

Mushroom bodies are protocerebral neuropils that are composed of one or more populations of intrinsic neurons, the Kenyon cells (Flögel, 1878; Strausfeld et al., 1995; Strausfeld et al., 1998). The mushroom body calyx is made up of Kenyon cell dendrites that in most insects receive a preponderance of input from the antennal lobes. The lobes are

composed of the axon-like processes of Kenyon cells and represent the primary outputs of information from the mushroom bodies. Kenyon cell subpopulations are generated sequentially during embryonic, larval, pupal and even adult development by the mushroom body neuroblasts (Ito and Hotta, 1992; Farris et al., 1999; Lee et al., 1999; Malaterre et al., 2002). This is the basic organizational groundplan upon which taxon-specific modifications in calyx and lobe morphology, and in the number and type of Kenyon cell subpopulations, have been evolutionarily acquired (Farris, 2005).

The present study identifies five types of Kenyon cells in the mushroom bodies of the red flour beetle *Tribolium castaneum* that are sequentially generated during larval, pupal and adult life. These Kenyon cell populations were identified primarily by the projection patterns of their axons in the lobes, as visualized using anti-DC0 immunohistochemistry and fluorescent phalloidin labeling. In *Tribolium*, Kenyon cells were typically produced by two neuroblasts in each brain hemisphere; mushroom body neurogenesis was continuous from the late larval stage (the earliest stage investigated in this study) until up to 88 days after adult eclosion. Neurogenesis was detected by fluorescent phalloidin labeling of the extending axons of newborn neurons, and by BrdU incorporation by the neuroblasts and their progeny. Several aspects of the structure and development of the *Tribolium* mushroom bodies were very similar to that observed in the fruit fly *Drosophila melanogaster*. In both insects the mushroom bodies are composed of a single calyx, and medial and vertical lobes that are formed by the bifurcated axons of Kenyon cells that are subdivided into distinct lobe systems according to the Kenyon cell subpopulation supplying them. In *Drosophila* the γ Kenyon cells, which supply the vertical and medial γ lobes with their axons, are produced throughout larval life until a few hours before pupariation, at which time the neuroblasts transition completely to the generation of α'/β' Kenyon cells that form the α'/β' lobes with their bifurcated axons (Lee et al., 1999). The production of α'/β' neurons ceases shortly after pupariation and gives way to the production of α/β Kenyon cells that form the α/β lobes, which is completed prior to adult eclosion in *Drosophila*. The very last born Kenyon cells form a core in the α/β lobes of the *Drosophila* mushroom bodies and may be identified by the affinity of their axons to phalloidin labeling and by other molecular and morphological characteristics (Kurusu et al., 2002; Strausfeld et al., 2003; Lin et al., 2007). In *Tribolium castaneum* a very similar sequential pattern of Kenyon cell production is observed, albeit with an expansion of the α'/β' neuron production period to encompass the duration of the pupal stage, thus delaying the onset of generation of α/β neurons until adult eclosion. Unlike *Drosophila*, mushroom body neurogenesis in *Tribolium* persisted post- adult eclosion for as long as 88 days, with α/β Kenyon cells being generated and their axons incorporated into the α/β lobes via an ingrowth or α/β core the entire time. Setting aside this developmental heterochrony when

compared with *Drosophila*, the γ , α'/β' , α/β and α/β core Kenyon cells of *Tribolium* were observed to be very similar to those of *Drosophila* both in morphology and in birth order, and are likely to represent homologous cell populations.

A fifth Kenyon cell subpopulation, named δ , made up a pair of anterior lobes that appeared fully developed in the late *Tribolium* larva, indicating that δ Kenyon cells are born prior to the γ Kenyon cells whose phalloidin-labeled axons were entering the γ lobes at that stage. While no Kenyon cell population with a birthdate prior to that of the γ Kenyon cells has been definitively identified in *Drosophila* (but see Noveen et al., 2000), insects such as cockroaches, crickets and moths have an early-born cell population called Class III Kenyon cells (Malaterre et al., 2002; Farris and Strausfeld, 2003; Sjöholm et al., 2005). This subpopulation of Kenyon cells is the first born in development, prior to Class II Kenyon cells (the γ Kenyon cells of *Drosophila*), and appear to have a specialized function in processing gustatory input to the mushroom bodies (Weiss, 1981; Frambach and Schurmann, 2004; Farris, submitted). To further build the case for the homology of *Tribolium* Kenyon cell subpopulations with those of other insects, future studies must characterize their dendritic morphologies and the types and sources of their afferent inputs.

Neural circuits in the ventral nerve cord of holometabolous insects are extensively reorganized during metamorphosis, as the larval body plan is reshaped into that of the adult (Truman, 1990; Consoulas et al., 2000). In the brain, some neuropils are formed *de novo* during metamorphosis for processing input from adult-specific sensory structures such as the eyes (Monsma and Booker, 1996), while larval brain centers such as the antennal lobes are reorganized and new neurons are incorporated to form circuitry suited to the processing needs of adults (Jefferis et al., 2002; Marin et al., 2005). The *Drosophila* mushroom bodies are also reorganized during metamorphosis, a process in which the axons and dendrites of γ Kenyon cells that were produced in the larva are pruned by engulfing glia and reshaped into adult configurations (Armstrong et al., 1998; Lee et al., 1999; Watts et al., 2003; Awasaki and Ito, 2004; Watts et al., 2004). Notably, the bifurcated axons of larval γ neurons are pruned back to the pedunculus and then regrow unbranched in the medial direction, so that the adult has only a medial γ lobe. The *Tribolium* mushroom bodies are also reshaped during metamorphosis, as microglomeruli formed by larval Kenyon cell dendrites in the calyx are dissolved during the prepupal stage and reformed gradually in the late pupal stage. One synapse before the calyx in the olfactory processing pathway, the antennal lobe glomeruli are reshaped following the same timeline. In contrast to *Drosophila*, however, no pruning could be observed in the *Tribolium* lobes using anti-DC0 immunohistochemistry and phalloidin labeling, and the γ Kenyon cells retain their bifurcated axons into adulthood. The degree of mushroom body metamorphosis thus seems

to be variable according to species, as also evidenced by studies in the Hymenoptera in which the mushroom bodies of the honey bee *Apis mellifera* undergo just moderate pruning at the tips of γ axons, while the wasp *Polistes apachiensis* displays massive pruning and regrowth of the entire lobe system similar to that observed in *Drosophila* (Farris et al., 2004).

Most holometabolous insect larvae lack compound eyes, meaning that these structures and the optic lobe neuropils that process visual information in the adult must be generated almost in their entirety during metamorphosis (Monsma and Booker, 1996; Friedrich, 2006). Hundreds of thousands of new neurons that will form the adult lamina, medulla and lobula are produced at this time by optic lobe neuroblasts in the optic anlagen adjoining the lateral protocerebrum. In the mushroom bodies of both *Drosophila* and *Tribolium* one Kenyon cell subpopulation, the α/β neurons, are produced during or after metamorphosis, and so like the optic lobe neurons these Kenyon cells must be serving adult-specific functions. In contrast to optic lobe neurons, however, it is not as clear what the adult specific functions of these α/β Kenyon cells may be. It has been hypothesized that Kenyon cells produced after adult eclosion in the moth *Ephestia kuniella* may be involved in processing information about sex pheromones, since in males the ability to respond to pheromone matures at the same time as the production and incorporation of adult Kenyon cells (Dufour and Gadenne, 2006). While this appears to be a logical function for adult-specific neurons in an olfactory processing center, assigning definitive role to these Kenyon cells awaits more detailed anatomical studies of their connections, in conjunction with physiological recordings of their outputs.

Adult neurogenesis and plasticity of *Tribolium* mushroom bodies

Neurogenesis in adult insect mushroom bodies appears to be relatively uncommon (Cayre et al., 1996), and thus cannot be a basis for neural plasticity in most species. Like vertebrates, neural plasticity in the insect mushroom bodies in response to environmental stimuli often takes the form of neuronal process outgrowth, branching, and increased synaptogenesis (Diamond et al., 1972; Greenough et al., 1985; Black et al., 1990; Greenough and Black, 1992). For example, sensory stimuli and behavioral experience shape the adult *Drosophila* mushroom bodies in part through changes in fiber number in the pedunculus, which may reflect delayed axon outgrowth of Kenyon cells born before adult eclosion (Technau, 1984). In honey bees, which also lack neurogenesis in the adult mushroom bodies (Fahrbach et al., 1995), volume increases associated with age and behavioral experience are due in part to outgrowth and branching of Kenyon cell dendrites in the calyx (Farris et al., 2001).

Although adult neurogenesis occurs to widely varying extents in the brains of both invertebrate and vertebrate animals, when present, it indeed appears to play an important role in mediating brain plasticity (Van Praag et al., 2002; Van Praag et al., 2005; Aimone et al., 2006; Bruel-Jungerman et al., 2006; Sohur et al., 2006; Okano and Sawamoto, 2008). In insects, adult neurogenesis is more often observed in the more basal ametabolous and hemimetabolous species such as the primitively wingless firebrats (Farris, 2005), cockroaches (Gu et al., 1999) and crickets (Cayre et al., 1994). As described above, honey bees and the fruit fly *Drosophila*, both holometabolous, lack adult neurogenesis. Adult neurogenesis has been observed in various species of the holometabolous beetles (Cayre et al., 1996), but given the enormous diversity of this taxon the loss of this capacity by some species is likely (as is the case in the Scarabaeidae; Miller and Farris, unpublished data).

What is the function of newborn neurons in adult insect mushroom bodies? In the cricket, several lines of evidence indicate that these newborn cells mediate plasticity in response to olfactory input and environmental enrichment, and in the formation of olfactory associative memories (Scotto Lomassese et al., 2000; Scotto-Lomassese et al., 2002; Scotto-Lomassese et al., 2003; Cayre et al., 2005). It is therefore likely that adult α/β Kenyon cells in the *Tribolium* mushroom bodies perform some of the same functions. Some important and thus far unanswered questions that apply to the brains of both vertebrates and invertebrates is, why does neurogenesis persist in some species, in some brain regions, and not in others? Does adult neurogenesis represent an ancestral holdover (since in both vertebrates and invertebrates, more basal lineages are more likely to have adult neurogenesis in the brain, and to a greater extent)? Is plasticity mediated through process outgrowth an improvement over plasticity mediated through neurogenesis, in terms of functionality, energetics, or other factors? Are there some brain regions and some functions for which neurogenesis is better suited to mediate plasticity? Answering these questions, via both model systems and comparative studies, will be important for broadening our understanding of the mechanisms of neural plasticity, from an evolutionary perspective down to the cellular and molecular levels.

Individual variation in neuroblast number in the *Tribolium* mushroom bodies

While most *Tribolium* brains observed in this study displayed two mushroom body neuroblasts per hemisphere, a small number (10%) had either one or three neuroblasts (Fig. 2-7). This variation typically occurred in just one hemisphere of the brain of each individual affected. In contrast, years of developmental studies in *Drosophila* have failed to turn up any variation from the four neuroblasts per hemisphere that is typical for this species (Ito and Hotta, 1992; Ito et al., 1997; J.S. deBelle, personal communication). If this variation in mushroom body neuroblast number in *Tribolium* is heritable, selective

breeding may be utilized to directly test the impact of neuroblast number on mushroom body structure, afferent and efferent connections, and even behavioral output.

Part III: Higher brain centers in termites with different social structures

1. Introduction

Brains are composed of higher centers processing sensory cues from outside and sending out commands for a corresponding behavior. As with other aspects of an organism, brains are shaped by the environment over evolutionary time. The reshaping mechanisms can include morphological changes in individual neurons and alterations in neuron numbers.

The neuroanatomical changes in brains can be reflected in volume changes of higher brain centers. Several hypotheses have been proposed to explain why certain species have larger brains (Dunbar, 1998). Epiphenomenal hypotheses attributed larger brains to larger body size. Developmental hypotheses emphasized the energetic constraints needed to be overcome during the development of brains. Both are true to certain level, but do not explain why the expansion of such an expensive organ is necessary. It is unlikely that brain centers just get bigger whenever there is a chance. It is much more plausible that there is a need for such bigger brain structures. A third category of hypotheses correlated larger brains with ecological factors such as diet, mental maps and foraging.

No matter how, enlargement of higher brain centers accompanied by acquisition of new and complex behaviors appears to be a common adaptation in many lineages (Shultz and Dunbar, 2006). One explanation for this pattern is the Social Brain Hypothesis. Originally named the “Machiavellian Intelligence hypothesis” during the late 1980s, it seeks to explain why large brains existed in certain species but not others (Byrne and Whiten, 1988). The computational demands from complex social systems were proposed as the main driving force for large brains in primates. Subsequently, studies have shown that this is a better explanation than diet, mental maps and extractive foraging (Dunbar, 1998; Byrne and Bates, 2007). Explicitly, the social brain hypothesis explained why certain species within a subgroup of primates, which faced the same ecological jobs, had larger brain structures than others. Gradually, the social brain hypothesis has also been expanded to include non-primate animals such as whales (Connor et al., 1998), paper wasps (Molina and O'Donnell, 2007; O'Donnell et al., 2007) and, in a recent study, three orders of non-primate mammals (Perez-Barberia et al., 2007).

Social behaviors evolved independently several times within insects (Wilson, 1971). Just among bees, sociality is proposed to have evolved at least eight times

independently (Michener, 2000; Danforth, 2002). This would seem to make insects attractive subjects for studying the acquisition of such behaviors. However, the social brain hypothesis has never been systematically tested in insects. This is partially due to the lack of well-constructed phylogenetic trees and behavioral data. For example, wasps and bees include species whose behaviors range from solitary to eusocial. But we do not know their phylogeny very well. It is also the reason the actual number of independent acquisitions of sociality in bees is under debate (Michener, 2000; Danforth, 2002). Also, we do not know how different volumes of higher brain centers are distributed among all these bee species except for a few such as bumblebees and honey bees (Mares et al., 2005).

No direct relationship between social behavior and higher brain centers in insect has been demonstrated so far. But the presence of particularly large mushroom bodies in both eusocial Hymenoptera (ants, bees and wasps) and in termites (order Dictyoptera, suborder Isoptera) implies that the acquisition of social behaviors may require the evolution of large brain regions in these species. Thus, insects could be a good model system to test whether higher brain enlargement is specially needed for social behavior.

Termites, as a group of eusocial insects with a well-constructed phylogeny (Inward et al., 2007) offer a good opportunity to test the social brain hypothesis in insects. According to recent phylogenetic studies, termites arose from within the cockroaches (Dictyoptera), and the phylogenetic relationships within the termites have been determined with some confidence (Grimaldi, 2005; Inward et al., 2007). They are generally divided into 7 families according to both morphological and molecular characters: Mastotermitidae (the most basal group), Kalotermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae, Serritermitidae and the family containing the most termite species, the Termitidae (Fig3-1). The family Termitidae can be further divided into 6 subfamilies (Donovan et al., 2000).

Social life in termites has a really long history, longer than ants, and evolved independently. They are the oldest social insects with fossil evidence dated to the Cretaceous (130 Ma, Million years ago) suggesting social structures similar to modern basal species (Thorne et al., 2000). At the same time, termites' complex and variable social structures, which reflect evolutionary changes in behavior patterns, are likely to have shaped brain morphology including size during evolution.

Division of labor is a feature of eusocial insects including termites (Wilson, 1971). The social insect colony requires extensive communication between workers, as well as across castes of workers, soldiers and reproductives. As in other social insects, such as ants, termite workers may fall into multiple discrete phenotypes that are determined by responses to environmental cues, a state called polyphenism (Noirot and Pasteels, 1988).

Among different termite species, the degree of labor division varies according to colony structure. In basal termites that live within a single piece of decomposing wood, there is no complex tunnel or chamber construction performed by workers. Also, there is not much foraging, since the wood piece offers both food and protection (Korb, 2008). In these species, the nymphs care for themselves from a very young age due to the ready availability of food (Korb and Schmidinger, 2004), so less parental care is needed. Additionally, the workers of these wood-dwelling species retain the developmental potential for becoming reproductives (Korb, 2005). Differentiation into reproductives is then determined mainly by the availability of food resources within the piece of wood that contains the nest (Korb, 2004).

In more derived termite taxa, the social organization and nest have become more complex. There are two other types of nests: intermediate and separate, in termites. Separate nests are completely distinct from the food source, requiring workers to forage for food outside of the colony. The intermediate nests are still within pieces of wood but there can be multiple pieces, which distinguish them from the one piece nesters. In more advanced termites such as separate nest termite species, nest construction requires highly coordinated group work. There are functional differences between constructed chambers in the nests of these termites, with some serving as chambers for the reproductives, some for food storage and some for rearing nymphs (Abe and Bignell, 2000). Parental care of nymphs by workers is more extensive in these species compared with the wood dwelling species. Independent foraging has not been described in any advanced termite species in which colonies nest away from their sources of food (Korb, 2008). In other words, they always forage as a group of workers with diverse group sizes according to species.

Further complexity of nest construction by higher termites is reflected by the presence of tunnels that connect all of the different chambers, and that also fulfill heat and gas exchange needs (Gould, 2007). The general construction or the shape of any two termite mounds, even when they are formed by the same species, may be only vaguely similar, perhaps reflecting flexibility in construction according to the local environment and needs of the colony (Noirot et al., 1986; Korb and Linsenmair, 1998). This distinguishes termite colonies from those of social insects such as honeybees whose colonies look relatively similar to one another. The randomness of colony design is the result of multiple local independent decisions by worker termites (Gould, 2007). Thus, the termite worker in the non-dwelling species must be able to assess many different sensory cues from the environment, and to translate this into the cooperative construction of the appropriate nest architecture. In addition, since the detection of colony status directly by assessing

conditions within a single piece of wood is no longer possible, it seems essential that workers of non wood-dwelling species be able to communicate efficiently.

While all termites are eusocial, they vary in other aspects of their social organization, such as nest size (group size) and construction, parental care, and foraging range. This variability offers an opportunity to test the social brain hypothesis in insects against other ecologically-related hypotheses that could possibly have driven higher brain center evolution. In addition, the evolution of sociality in termites was accompanied by the transition from workers that retain the potential to develop into reproductives to species with fully infertile true workers (Thompson et al., 2004). A recent phylogeny (Figure 3-1) suggests three independent emergences of a true worker caste, in Mastotermitidae, Hodotermitidae, and at least once in the Rhinotermitidae + Termitidae (Noirot and Pasteels, 1988; Thompson et al., 2004; Inward et al., 2007). All these species are intermediate nesting (Mastotermitidae and Rhinotermitidae) or separate-piece nesting (Hodotermitidae and Termitidae), which suggests that the need for foraging outside of the nest is associated with the evolution of true workers.

In order to test the social brain hypothesis in termites, this study focused on two higher brain centers, the central complex (Strausfeld, 1999; Urbach and Technau, 2003) and mushroom bodies (Farris, 2005; Strausfeld et al., 2009). The central complex is a midline neuropil of the protocerebrum (Fig 1-1A-B). As the name suggests, it is actually made up of a collection of neuropils: the protocerebral bridge, the ellipsoid body, the fan-shaped body, and the paired noduli (Fig 3-2) (Loesel et al., 2002; Strauss, 2002). The central complex has been shown to play a role in visual associative learning in *Drosophila* (Liu et al., 2006; Wang et al., 2008), in regulating locomotion in grasshoppers (Wolf, 1992), cockroaches (Ridgel et al., 2007) and *Drosophila* (Strauss, 2002), and in responses to polarized light during flight in a grasshopper (Heinze and Homberg, 2007).

The insect mushroom bodies have been the subject of extensive study in many species (for reviews see Strausfeld et al., 1998, Farris 2005, Fahrbach 2006). Generally mushroom bodies are paired neuropils composed of intrinsic neurons called Kenyon cells (Pearson, 1971; Schürmann, 1973; Strausfeld, 1976; Mobbs, 1982), whose cell bodies are located in the dorsoposterior protocerebrum. The number of Kenyon cells is species-specific and varies from 2500 in *Drosophila* to 170,000 per hemisphere in *Apis mellifera* (honey bee) (Urbach and Technau, 2003; Farris, 2005). Dendrites of Kenyon cells form a structure called the calyx that is organized into microglomeruli-like substructures that contain the synaptic contacts between the dendrites and their inputs, similar to the glomeruli of the antennal lobe. The shape and number of calyces in each mushroom body varies in different insect lineages (Farris, 2005). Kenyon cell axons exit posteriorly from

the calyx in a structure called the pedunculus. The axons usually bifurcate at the distal pedunculus and enter medial and vertical lobes that project towards the central complex and the anterior edge of brain, respectively (Fig 1-1A-B; Fig 3-3A-B).

The mushroom bodies are best known for their role in olfactory learning and memory (Davis et al., 1995; Heisenberg, 1998; Heisenberg, 2003). Mushroom bodies are also very important in odor discrimination (Cassenaer and Laurent, 2007; Turner et al., 2008) and receive inputs from modalities besides olfaction, for instance acoustic, visual motion, and tactile stimuli (Li and Strausfeld, 1997; Li and Strausfeld, 1999). While homologous across the insects, the morphology of mushroom bodies is extremely diverse and in some cases strongly correlated with behavioral ecology (Fahrbach, 2006; Farris, 2005; Farris and Sinakevitch, 2003). Functional studies have also implicated the mushroom bodies in processes ranging from motor control to spatial learning to context-dependent learning (Mizunami et al., 1993; Liu et al., 1999). Physiological data support a role for the mushroom bodies as an integrative brain center for different sensory inputs (Li and Strausfeld, 1997; Li and Strausfeld, 1999). However, the primary functional role for the mushroom bodies appears to be olfactory processing and olfactory-related learning and memory (Davis, 1993; Gerber et al., 2004).

As trail pheromone is used as a signal for foraging in all of termite species, olfactory centers such as the mushroom bodies are likely to have been modified during the evolution of nest complexity. Furthermore, in separate-piece nesting, spaces for food storage and brood care are usually in different parts of the nest. Thus more brood care is required in these species in comparison with one piece and intermediate nesters, whose nymphs can obtain their own food. Among separate-pieces species, the nest size and architecture also varies extensively. Workers forage through tunnels they construct within and around the colony. For certain extreme cases, with very large population and complex nest, in Termitidae, they form a large closed system that extends a great distance outside of the colony. Maintenance of such a colony will require efficient communication and coordination among many individual termites (Abe and Bignell, 2000).

By closely analyzing differences in behavior of workers representing these different species, it may be possible to detect if complex sociality, represented by the existence of true workers and nesting types, is linked to evolutionary modifications in brain structure. More specifically, if the social brain hypothesis holds in termites, given the role of the mushroom bodies in sensory integration and different types of learning and memory, the largest mushroom bodies are expected to occur in termite taxa with true workers and separate-nesting type. The mushroom bodies in non-true worker species and single piece nesting should be the smallest. Similar correlations would not be predicted in volumes of

the central complex. Thus, this study uses termites to test the social brain hypothesis explicitly, by measuring both mushroom body and central complex volume, and Kenyon cell number, of workers from phylogenetically key termite species.

2. Methods

Insects

Six termite species, *Mastotermes darwiniensis*, *Coptotermes kalshoveni*, *Amalotermes phaeocephalus*, *Cubitermes heghi*, *Kalotermes sinaiicus*, *Macrotermes renouxi*, representing species with different complexity of sociality were used. Among all the analyzed termites, *Mastotermes darwiniensis* is the most basal species and represents the sister group of all other extant termites (Abe and Bignell, 2000). All termite specimens were provided by Dr. Paul Eggleton at the London Natural History Museum, except *Reticulitermes flavipes*, which was collected in Morgantown, WV.

Immunostaining and fluorescent labeling of F-actin (phalloidin staining)

Brains of a local termite species, *Reticulitermes flavipes* (family: Rhinotermitidae) were dissected in insect physiological saline (O'Shea and Adams, 1981) and immediately fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.2) for 1 hour at room temperature. Then, the brains were washed in 1% PBST (1% Triton X-100 in PBS) and then permeabilized in collagenase-dispase (Sigma-Aldrich, St. Louis, MO) for 20 minutes with a concentration at 0.25 mg/ml. After the enzyme treatment, all brains were washed three times in 1% PBST and then incubated in a blocking solution of 10% normal goat serum (NGS) in 1% PBST for 1 hour.

Anti-DC0 primary antibody, a polyclonal antibody against the catalytic subunit of *Drosophila melanogaster* protein kinase A, was a gift provided by Dr. Daniel Kalderon (Columbia University). This antibody has been shown to have a high affinity for mushroom body intrinsic neurons in a range of insect species (Skoulakis et al., 1993; Farris, 2005). In the present study anti-DC0 was diluted 1:1 in glycerol for storage, and a 1:500 concentration of this stock was used in 1% NGS in 1% PBST for staining. The following day, sections were washed with 1% PBST three times for 10 min each followed by incubation in a 1:200 concentration of goat anti-rabbit secondary antibody tagged with Texas Red (Invitrogen (Molecular Probes), Eugene, OR), also in 1% NGS solution in

PBST. Alexa 488-conjugated phalloidin (Molecular Probes), which labels filamentous actin(F-actin), is enriched in outgrowing Kenyon cell processes (Kurusu et al., 2002; Farris and Sinakevitch, 2003; Farris, 2004; Farris et al., 2005), was applied at a 1:500 concentration at the same time as the secondary antibody. The brains were incubated in this solution overnight at room temperature. The next day sections were washed in PBST and cleared in 60% glycerol for half an hour, followed by incubation in 80% glycerol for one half hour. After mounting in 80% glycerol, brains were dissected free of the remnants of the head capsule using fine forceps, and viewed using an Olympus Fluoview 1000 confocal microscope within 48 hours of coverslipping. This allowed precise definition of the selected brain regions for volume estimations in the ethanol-fixed materials.

Paraffin sectioning and staining

All termites, except *Reticulitermes flavipes*, were collected from all over the world by Dr. Paul Eggleton and preserved for varying lengths of time in 70% ethanol. Whole brains were dissected in 70% -80% fresh ethanol and fixed for one hour in Carnoy's fixative. Fixed tissues were embedded in paraffin, frontally sectioned and then stained using a Cason's stain (Kiernan, 1990). Depending on the brain size of each species, the larger species and smaller species were sectioned at 8 and 6 μm thickness respectively.

Data Analysis

In order to determine volumes of higher brain centers, brain sections stained by Cason's stain were traced using the software AxioCam and Neurolucida 7. After tracing the perimeters of each structure, its area was estimated using the Cavalieri method (Gundersen et al., 1988; Michel and Cruz-Orive, 1988; Withers et al., 1995; Farris and Roberts, 2005). Every section was measured for all termite specimens. These areas were multiplied by the thickness of the section to give the volume per section, and then the section volumes were added together to provide an estimate of the total volume for each structure. The volume of mushroom bodies in each individual was estimated for one randomly selected hemisphere. One way ANOVA was used to determine the significance of differences between four species in absolute volumes of mushroom body and central complex.

Before embedding and sectioning, the head width was measured for each termite worker. The volumes of brain regions were then normalized to head capsule maximum width (distance between outer margins of left and right eye/degenerated eye dots, measured in each individual with a stereomicroscope ocular micrometer). This standardization corrects for the effect of both species body size and individual variance on brain region volume. Due to the loss of samples during embedding, sectioning and staining, the sample sizes for head width estimation were usually larger than those for brain volume estimates.

Total numbers of Kenyon cells within six species were also estimated. By measuring the diameter of Kenyon cells in each preparation, an average volume for a Kenyon cell was estimated for each species. Volumes of total Kenyon cell bodies (2 species where only one brain available) or its mean (the four species with three brains available) within each species were also estimated. The number of Kenyon cells within one hemisphere was estimated by dividing the volume of total Kenyon cell bodies by the average volume of a Kenyon cell in each species.

3. Results

General Mushroom bodies and central complex structures represented in termites

Immunostaining and fluorescent labeling of F-actin (phalloidin staining) of fresh *Reticulitermes flavipes* brains provided high quality information on the neural structures involved in this study. General brain structures including the central complex and mushroom bodies are illustrated in Fig 3-4. The medial lobes of mushroom bodies bifurcate where this higher brain center meets the central complex (Fig 3-4 A,B). The calyx of mushroom bodies is doubled in a fused pattern in each hemisphere (Fig 3-4 C). Sub-compartments are present in the medial lobes (Fig 3-4 A,B,D). The growing axons of Kenyon cells, indicated by high affinity to F-actin, are present at the edge of the medial lobes (Fig 3-4 D, white arrows). These sub-compartments may indicate different Kenyon cell sub populations (Farris, 2005). This high F-actin affinity indicated the Kenyon cell subpopulation undergoing axon extension or rearrangement right at the time of fixation. But further study may be needed to clarify whether actual representatives of Kenyon subpopulations within media lobes correspond to these sub-compartments.

General brain structures of the six preserved termite species were shown by paraffin sectioning and Cason's staining (Fig 3-5 A-D, Fig3-6 A,C). Kenyon cell bodies, calyx and medial lobe structures were very obvious (Fig 3-5 A, B). Central complex (Fig 3-5 C and D) had the columned sub-compartment structure of fan-shaped body. *Hodotermes mossambicus*, one of the few termite species with compound eyes, had clear optical nerves connected to brain (Fig 3-5 E). All brain sections processed by Cason's staining showed a great shrinkage or fragility of tissues (Fig 3-5 A-D, 3-6 A,C). Great variation existed in body size of workers in different termite species while their heads and brains were less variable (Fig 3-6 B, Table 3-1).

Mushroom bodies and central complex volumes in termite

Macrotermes renouxi, a species with true workers and separate-pieces nesting, had the largest mushroom body together with central complex (Table 3-1). It is also one of termite species with the most complex nest structures and largest colony size. The second largest mushroom body was observed in *Mastotermes darwiniensis* with true workers and intermediate-piece nesting. However, two other true worker and separate-piece nesting species *Amalotermes phaeocephalus* and *Cubitermes heghi* had a far smaller mushroom body and compared to a wood-dwelling species *Kaloterms sinaicus* as single piece nest and without true worker. This ranking did not change whether brain volumes were standardized or not.

However, using one way ANOVA analysis, a significant variation was detected in mushroom body volumes of the four species with sample size of three (Table 3-1, Fig 3-7 A-B). In contrast, there were no significant differences of central complex volumes ($P=0.060$, Table 3-1, Fig 3-7 C). For pair-wise differences in means of higher brain centers in the same four species, *Mastotermes darwiniensis* stood out for its significantly enlarged mushroom body ($P<0.001$) to all the other species tested so far. A significant difference of central complex size was also detected in this species against *Coptotermes kalshoveni* and *Cubitermes heghi* ($P<0.05$) but not *Amalotermes phaeocephalus* ($P=0.394$).

The brain volumes of *Coptotermes kalshoveni* and *Cubitermes heghi* were less variable than those of *Mastotermes darwiniensis* and *Amalotermes phaeocephalus* (Table 3-1). However, the individual head capsule width was also far less variable.

Kenyon cell numbers in one hemisphere ranged from about 4,500 to 116,000 in all six termite species surveyed. This is far fewer than the average 170,000 estimated in honey bee (Farris et al., 1999). The estimated Kenyon cell numbers showed a great variation among different species while there was little variation of individual cell volumes within each species. The estimated Kenyon cell numbers showed a different trend compared with volumes changes in mushroom bodies and central complex (Table 3-3). *Macrotermes renouxi* had the smallest Kenyon cell number despite its largest mushroom body size. The second largest mushroom body, in *Mastotermes darwiniensis*, had the largest population of Kenyon cells. This may suggest that mushroom body enlargement might not only involve neuron number increasing but also morphological changes in individual neurons in termites. Finally, separating the mushroom bodies into calyx and lobes, the input and output regions respectively, the volumes of calyx was less than the lobes in all the species analyzed (Table 3-4).

4. Discussion

As shown above, the prediction based on social brain hypothesis for the six species distributed along the updated phylogenetic trees in termite did not generally hold. The conflict came with one non-true worker species, which should have the smallest mushroom body among the six if the social hypothesis were correct. This species actually had a larger mushroom body compared with two other true worker and separate piece nesting species, whether the brain volumes were standardized by individual head width or not. However, the data collected do not allow us to draw a strong conclusion on the social brain hypothesis in termites with the species surveyed so far. This is because it is unclear if the reverse of mushroom body enlargement in this non-true worker and wood-dwelling species is a specialized case or if it represents a general pattern. If the evolution of mushroom body had been driven by other factors than sociality, it could actually over-written the original small volumes which should be observed in non-true worker and single-piece species with far more less complexity of sociality. Further collections of brain volumes in species that also lost true workers from the most common ancestor could fortify the test of the social hypothesis in termite. If the mushroom body volume within such species actually shows a decrease in future investigation, it will be a piece of evidence to support the social brain hypothesis in termite.

Despite the conflict with the social brain hypothesis predictions, there were significant differences between species in volumes of mushroom bodies detected. And the number of Kenyon cells in different termite species showed a wide range. *Macrotermes renouxi* (Termitidae: Macrotermitinae), with the largest group size and complex nest construction and parental care among the species analyzed, has the largest mushroom bodies together with central complex. But the mushroom bodies of *Mastotermes darwiniensis* were also relatively large even though this is the most basal species in the phylogenetic tree of termites. It could be explained by the fact that true workers exist in this basal species, a signature of collaboration among individuals. However, this trend did not persist in the rest of species analyzed as the sophisticated true workers in *Amalotermes phaeocephalus* (Termitidae: Apicotermitinae) and *Cubitermes heghi* (Termitidae: Termitinae) had far smaller mushroom bodies and central complex compared to *Kalotermes sinaiicus* (Kalotermitidae), which was considered as less social, with more primitive features among termite species and without the existence of true workers. So far, as the one individual surveyed in *Macrotermes renouxi* had the smallest kenyon cell number and the largest mushroom body size, suggesting Kenyon cell number might not be associated with mushroom bodies size (Table 3-3).

With these morphology differences, it is reasonable to speculate on possible factors that could cause such changes. Some behaviors, such as feeding strategies and foraging from a central nest have been found to be correlated with alterations in higher brain centers. In scarab beetles, species with generalist feeding ecologies have greatly enlarged mushroom bodies, perhaps as an adaptation to the increased demand on sensory integration and learning and memory capabilities in species that must select from many food options (Farris and Roberts, 2005). At the individual level, both social honey bees and solitary orchard bees show an enlargement of the mushroom bodies over their lifetimes as they acquire foraging experience (Withers et al., 1995; Withers et al., 2008). Whether these factors also contributed to the evolution of higher brain centers in termites, is worth further investigation. Ideally, with brain volume data from more termite species, these speculations can be further tested against the social brain hypothesis in a finer scale. For instance, a better index of sociality may help differentiate other non-social related behavior pattern changes in termites that could also affect higher brain volumes. In this study social complexity was represented by the existence of true workers and nesting types in each species. In order to separate other possible computationally-demanding tasks such as nest construction and foraging behavior of workers, it would be ideal if there were termite species that could be surveyed with variation in these behaviors. In other words, detailed behavioral study in different termite species will greatly improve the quality of the social brain hypothesis testing in this unique group of insect.

Several caveats must be added to these findings. First, the detected variations of brain volumes among species were only standardized by individual head width. Although for different termite workers, the head width of individuals was generally used for the determination of caste and nymphal instar (Katoh et al., 2007). The head width measurements showed consistencies in these fully developed workers in each species of the six species used. Only the head width varied while the head thickness and axis length from mouth to the posterior of head were uniform within a species. Thus, together considering head width as a regular measurement for determining developmental stage of termite worker, this study chose to use head width instead of total brain volume as a standardizing factor. Due to the lack of the research on magnitude of genetic or lineage variations in each termite species, it is unclear whether variations detected actually occurs within species naturally. Even it was unknown whether the workers measured for each species were from the same colony.

Second, the variation within and between species could also come from preservation of specimens in ethanol. The empty spaces among different brain structures and individual Kenyon cells occasionally made the estimation ambiguous along the edges. These may be artifacts due to preservation in ethanol for different times before staining

(Fig 3-5 A-D, 3-6 A,C). If all the individuals in a species have a similar level of diversity in brain volumes, as seen in the volumes for *Cubitermes heghi* (Table 3-1), it is possible that the magnitude of variation depends more on the freshness of sample than the natural variation within that species as the preservation varied in these samples.

Third, perhaps mushroom body volume does not reveal brain differences important for termite sociality. It has been proposed that mushroom bodies in insects are analogs to hippocampus in vertebrates (Strausfeld et al., 1998). In primates, when correlating sociality with brain structures, the relationship is strongest with neocortex (Dunbar, 1998). In birds, the correlation is between range area and hippocampus volumes, a brain center involved in space learning and memory (Krebs et al., 1989). Combined with functional and behavioral research in insects, mushroom bodies are the best choice so far. But whether mushroom bodies as a whole are a suitable region for brain volume measurements for the social brain hypothesis? Or maybe lobes only are better, since lobes generally contribute more to the volume changes (Table 3-4). More functional and behavioral research about mushroom bodies, especially in termites, will help us clarify this. Additionally, the impact of sociality can spread into whole brain enlargement in certain species. The largest mushroom bodies, together with central complex, in *Macrotermes renouxi* (Termitidae: Macrotermitinae (Table 3-1, 3-2) came along with its largest group size among all the termite species analyzed. At the same time, it is also a species with the most complex nest construction and parental care. It might be more suitable to actually assign the enlargement of the whole brain with its social complex changes in this species.

Thus, as termites possess different social structures compared to other social species including well-studied social insect as honey bee and human beings, whether the social brain hypothesis is generally true, especially in insects, is still a wide open question.

Part IV: Conclusion and Discussion

The data presented in this thesis describe neuranatomical changes in brains of insects over both developmental and evolutionary timescales.

At the developmental level, Part II characterized mushroom body structure and development in a new genetic model system, the red flour beetle *Tribolium castaneum*. The presence of adult neurogenesis in this species makes it a promising new model system for studies of the cellular and molecular regulation of this process, and of the functions of adult specific neurons. *Tribolium* is now an insect model system possessing adult neurogenesis in the mushroom bodies, quantifiable variation in neural precursor number, and is tractable for experiments utilizing genetic manipulations. Further studies of mushroom body development in this species will provide insight into the mechanisms of behavioral and neural plasticity that span evolutionary to cellular and molecular levels of organization.

The functional role of new-born neurons during adulthood in higher brain centers has been a question drawing attention for a long time. It has reached a peak since the confirmation of adult neurogenesis in human brain in the 1990s (Kempermann, 2006). One hypothesis, that adult neurogenesis is needed for control of a growing body (Jander and Jander, 1994; Harrison et al., 2001) is not suitable for *Tribolium castaneum* as its body size never changes during adult life. Similarly, examples of seasonal change in neuron number (Clayton, 1998; Hansen and Schmidt, 2004), or neuron development responses to environmental stimuli (Ramirez et al., 1997; Penafiel et al., 2001; Scotto Lomassese et al., 2000; Kempermann, 2006) do not seem relevant given that individuals in this experiment were not subject to environmental variation. However, the addition of new neurons in adult cricket mushroom bodies required for olfactory learning (Scotto-Lomassese et al., 2003) might be relevant for *Tribolium*.

Whatever the benefit of adult neurogenesis in *Tribolium*, its occurrence begs the question of why use such an expensive remodeling mechanism rather than subtle morphological changes in existing cells? This may be related to adult longevity (Beltz et al., 2005), and may explain the difference between *Tribolium castaneum*, which can live for years, and *Drosophila*, which can live for months. As pointed out in Beltz's work, lobster, with much longer life span compared with its closely related crustacean species such as crayfish and crab, had more vigorous and longer adult neurogenesis. This actually can be a plausible explanation in insect. Honeybee, another well-studied insect species,

does not use such a mechanism in its worker's adult life as it also lives somehow similar life length compared with *Drosophila*.

At the evolutionary level, a comparative study of termite species (Part III), with a focus on the brain changes associated with social behaviors, was also included in this thesis to examine the driving force for brain structure alterations. This work represents the first detailed comparative investigation of the brain structures in an important eusocial insect order, the Isoptera (termites). The total Kenyon cell numbers estimated in different termite species varied greatly, which may suggest very different computational networks in mushroom bodies of these termite species. It could also be possible that the large mushroom bodies detected were caused by other factors which confounded the brain volumes in this species other than social index and types of nests. A better index of social complexity, as behavioral research in different termite species progressing, will ultimately enable us testing the social brain hypothesis in depth. It will add to our knowledge about what actually drives the brain structures to change in the luxurious form of enlargement.

Part I: Introduction

Fig 1. Three-dimensional reconstruction of central brain neuropils in insect. Insect brain structures are represented by 3D reconstruction in the cockroach *Diploptera punctata*. A. anterior view B. posterior view. Blue indicates the central complex, yellow indicates the mushroom bodies and purple is the antennal lobes (Chiang et al., 2001).

Part II: Metamorphosis and adult development of the mushroom bodies of the red flour beetle, *Tribolium castaneum*

Figure 2-1. Structure and intrinsic neuron composition of the mushroom bodies in *Tribolium castaneum* adults. A. The calyx (Cx) is composed of the dendrites of Kenyon cells that are produced by two neuroblasts (Nb) in each brain hemisphere). The granular appearance of the calyx reflects the presence of microglomeruli, sites of synaptic interaction between afferent boutons and Kenyon cell dendrites (Yasuyama et al., 2002). Kenyon cell axons form the pedunculus (Pe) and lobes (B-E). Alexa 488- conjugated phalloidin (green) strongly labels the ingrowing axons of newborn Kenyon cells (arrows in A). Anti-DC0 staining of mature Kenyon cells and their processes is indicated in purple. The DC0 antibody is directed against the catalytic subunit of protein kinase A (PKA) of *Drosophila melanogaster* (Skoulakis et al., 1993). B-E. Anterior (B) to posterior (E) optical sections of the lobes labeled with anti-DC0 (green). Arrows indicate lobe subdivisions made up of different Kenyon cell populations. Red- δ , orange- γ , green- α'/β' , blue- α/β , purple- core. F. Schematic diagram of Kenyon cell populations in the *Tribolium* mushroom bodies, indicated using the same color scheme as in B-E. Neuroblasts (Nb) are in bright green and are surrounded by the soma of newborn Kenyon cells (small purple circles). Newborn Kenyon cells extend their axons as two tracts into the calyx, which fuse into a single core in the pedunculus and lobes. Scale bars = 20 μ m.

Figure 2-2. Mushroom bodies of the feeding last instar larva (A-C) and prepupa (E-G). A. Anti-DC0 immunostaining (purple) reveals two Kenyon cell populations, δ and γ , in the vertical lobes of the feeding last instar larva. Ingrowing axons form a thin tract in the γ lobe as revealed by phalloidin labeling (green, indicated by arrows). B. Phalloidin labels

synaptic microglomeruli of the calyx (Cx, arrowheads) and extending axons of newborn Kenyon cells (arrows) generated by the mushroom body neuroblasts (Nb). C. Axons of newborn Kenyon cells (arrows) in the medial γ lobes of the feeding larva. D. Glomeruli in the antennal lobe of the feeding larva (AL; arrows). E. Phalloidin labels two tracts of ingrowing Kenyon cell axons in the prepupal vertical lobe: one into γ (white arrows) and one into the newly forming α'/β' lobe (black arrows). F. The prepupal calyx lacks definite microglomeruli and has a uniform consistency. G. In the prepupal medial lobe, ingrowing Kenyon cell axons (arrows) are observed only in the γ lobe; no β' lobe is visible. H. Like the calyx, the prepupal antennal lobe has also lost its glomerular structure. Cc- central complex, Pe- pedunculus. Scale bars = 20 μm .

Figure 2-3. Axon outgrowth into the mushroom body lobes of the early pupa. A inset. As in the prepupa, the pupal stage begins with two phalloidin labeled axon tracts (green, arrows) growing into the vertical γ and α'/β' lobes. Anti-DC0 immunostaining is indicated by purple labeling A. By pupal day 3, axon growth into the vertical γ lobe is completed and phalloidin labels a single tract in the α'/β' lobes of each mushroom body (arrows). B. In the day 3 pupa, phalloidin labels two tracts of ingrowing axons in the medial lobes corresponding to the γ and α'/β' Kenyon cells. C. By pupal day 4, phalloidin labels only a single tract of axons entering the medial lobes, corresponding to the ingrowing axons of newborn α'/β' Kenyon cells. Scale bars = 20 μm .

Figure 2-4. Mushroom bodies of the day 8 pupa. A. Phalloidin labeling (green) reveals that microglomeruli have reformed in the calyx (Cx, arrows) and axogenesis of newborn Kenyon cells (arrowheads) is ongoing. B. Anti-DC0 immunostaining (purple) of the γ and α'/β' lobes, the latter of which are still being constructed by ingrowing axons (arrows). C. Reformed glomeruli (arrows) in the antennal lobe (AL). Pe- pedunculus. Scale bars = 20 μm .

Figure 2-5. Neurogenesis and continued growth of the mushroom body neuropil after adult eclosion. A. Newly emerged adults (top) have lightly sclerotized cuticle. The tanning

process is completed by day 3 of adult life (bottom). B1-3. Progression of neuroblast activity in the adult. B1. In newly eclosed adults, phalloidin (green) labels axons of newborn Kenyon cells associated with two neuroblasts in each hemisphere (arrowheads). Purple- anti-DC0 staining. B2. BrdU incorporation of neuroblasts and their progeny (arrowheads) in the newly eclosed adult. Green-anti-DC0 staining. B3. BrdU incorporation (arrowheads) is less pronounced in the older adult (44 days old in this example). Green-anti-DC0 staining. C. Production of α/β Kenyon cells (blue arrow) begins at adult eclosion as indicated by the location of the phalloidin labeled ingrowth core (purple arrow). D. Increased size of the α/β lobe (blue arrow) relative to α'/β' (green) and γ (orange), due to continued production of α/β Kenyon cells, is evident in the three week (21 day) old adult. E. Overall growth of the mushroom body lobes is apparent in an 88 day old adult, when compared with the lobes of newly eclosed adults (C). Cx- calyx. Scale bars = 20 μm .

Figure 2-6. Phalloidin labeling reveals variation in neuroblast number in the *Tribolium* mushroom bodies. A. An individual in which the mushroom bodies of one hemisphere are generated by just a single neuroblast (arrow). Protocerebral neuroblasts not associated with the mushroom bodies are visible medially (PcNbs). B. The usual configuration of two neuroblasts per hemisphere (arrows), each associated with newborn Kenyon cells supplying the calyx (Cx) with a phalloidin labeled tract of ingrowing axons (arrowheads). C. Mushroom bodies containing three neuroblasts (arrows). Two of the neuroblasts typically appear to be more closely associated with one another in these individuals. Scale bars= 20 μ m.

Part III: higher brain centers Changes in termite with different social structures

Fig 3-1. Phylogeny of the termites using molecular and morphological markers from workers. A. Termites (Isoptera) arose from within the cockroaches, with the wood roaches (Cryptocercidae) as a sister group (Kato et al., 2007; Lo et al., 2000). B. and C. Phylogenetic tree of the termites built from molecular and morphological characters, with the presence of true workers and nesting type (Inward et al., 2007). Blue branched in B are species without true workers, and red ones are with true workers. In C, blue indicates single-piece nesting, green represents intermediate nesting and red shows separate-piece nesting.

Fig 3-2. A schematic picture of the central complex the in cockroach *P. americana*. The central complex is composed of the ellipsoid body, fan-shaped body, superior arch, protocerebral bridge and paired noduli. The protocerebral bridge is next to the lateral neuropils on each side. Ventral bodies and isthmi are connected by axons to the central complex. (modified from Loesel et al., 2002).

A Double and seperated calyces

B Single calyx

Fig 3-3. Schematic drawings of a mushroom body in one hemisphere of insect brain. The general structure of mushroom body illustrated by the ones in honeybee (A) and fruitfly (B). The structures composed of mushroom body are called calyx (divided into basal ring, collar, lip in honeybee), pedunculus, and lobes (medial and vertical). Abbreviations: (A) BR, basal ring; C, collar; L, lip; M, medial lobe; Ped, pedunculus; V, vertical lobe; γ , gamma layer of the vertical lobe. (B) Ped, pedunculus; α , alpha subdivision of the vertical lobe; α' , alpha prime subdivision of the vertical lobe; β , beta subdivision of the medial lobe; β' , beta prime subdivision of the medial lobe; γ , gamma subdivision of the medial lobe (modified from Fahrbach, 2006).

Fig. 3-4. The organization of the central complex and mushroom bodies in workers of termite species *Reticulitermes flavipes*. A-B. Frontal view of the central complex and media lobes of mushroom bodies. Layers within the medial lobes were visible in both A. and B. Inset: B1. Location of the central complex Cx beneath the medial lobes of the mushroom bodies, immediate anterior view of B. B2. High phalloidin binding affinity in mushroom body medial lobes, immediate posterior of B. C. Mushroom body anatomy in one hemisphere of brain, frontal view. D. Ingrowth core (arrows) visible at the edge of lobes. Cx, enlarged in D1, is

anterior to the two labeled medial lobes (arrows) and medial lobes on both hemispheres of brain. Labeling in MBs, Ca: calyx; Cx: central complex; KC: Kenyon cell; M: medial lobe; Pe: pedunculus; V: vertical lobes. In Cx, EB: ellipsoid body; FB: fan-shaped body; PB: protocerebral bridge; No: noduli. Scale bars in 4E and 5A = 500 μm ; all the other scale bars = 20 μm .

Fig. 3-5. Termite brains stained by Cason's staining. A. Calyx of mushroom body together with pedunculus. B. Medial lobe. C. Fan-shaped body with columnar sub-structures. D. Protocerebral bridge. E. Whole mount showing nerve tracts (Black arrow) that connect the brain with outer layers of the optic lobe in *Hodotermes mossambicus*. All sections were cut frontally at 8 μm thickness.

Fig 3-6. Measurements of brain volumes in termites. A. Frontal brain section of *Coptotermes kalshoveni* at 6 μm thickness. B. Workers of the species *Pericapritermes chiasognathus*, *Coptotermes kalshoveni* and *Cubitermes heghi*. C. A frontal brain section of *Mastotermes darwiniensis* at 8 μm thickness showing how brain regions are traced for volume estimations. Ant: antennal lobe; Ca: calyx; Cx: central complex; KC: Kenyon cell; M: medial lobe; Pe: pedunculus; V: vertical lobes. The scale bars in A and C = 50 μm ; B scale bar = 500 μm .

Fig 3-7. Comparison of mushroom body and central complex volumes for 4 termite species. Mean values and standard deviations of mushroom body (A, B) and central complex (C) absolute volumes in 4 termite species were plotted (volumes in unit of μm^3). Consistent with one way ANOVA analysis in table 3-1, mushroom body volumes were significantly different among the four species analyzed with $P < 0.001^{***}$, in contrast, no significant difference was detected in volumes of central complex $P > 0.05$. As for individual species, mushroom body volume of *Mastotermes darwiniensis* was significantly different from all the other species $P < 0.001^{***}$, as its central complex value was also different from two other species *Coptotermes kalshoveni* and *Cubitermes heghi* with $P < 0.05^*$. No significant difference was detected in all other pair-wised comparison.

Species names	Family	Mushroom body volumes (μm^3)	Central Complex (μm^3)	N 1	Head width (μm)	N 2
<i>Mastotermes darwiniensis</i>	Mastotermitidae	3035151 $\pm$ 2048671***	197630 $\pm$ 91381*	3	2218 $\pm$ 137	3
<i>Coptotermes kalshoveni</i>	Rhinotermitidae	317543 $\pm$ 48873	35399 $\pm$ 8683	3	1068 $\pm$ 79	10
<i>Amalotermes phaeocephalus</i>	Termitidae: Apicotermitinae	229818 $\pm$ 115044	156415 $\pm$ 253316	3	810 $\pm$ 33	10
<i>Cubitermes beghi</i>	Termitidae: Termitinae	419663 $\pm$ 43562	50959 $\pm$ 5334	3	1075 $\pm$ 106	7
<i>Kalotermes sinaicus</i>	Kalotermitidae	2077326	180918	1	1268 $\pm$ 120	3
<i>Macrotermes renouxi</i>	Termitidae: Macrotermitinae	6040694	4800324	1	2768 $\pm$ 130	3

Table 3-1. Mushroom body and central complex absolute volumes and head width in 6 termite species. *Macrotermes renouxi* has the largest mushroom body and central complex. The most basal species *Mastotermes darwiniensis* has the second largest mushroom body, why a non-true worker species, *Kalotermes sinaicus* has the third largest mushroom bodies. *** indicated $P < 0.001$, * indicated $P < 0.05$ (except to *Amalotermes phaeocephalus* with $p = 0.394$) for pair wise comparisons of brain structure volumes in the four species with sample size (N1) of 3. Last column (N2) indicated the sample size for head width measurements (volumes in μm^3 , width in μm).

Species names	Family	Standardized MB	Standardized CC	MB:CC
<i>Mastotermes darwiniensis</i>	Mastotermitidae	22164	1443	15.36
<i>Coptotermes kalshoveni</i>	Rhinotermitidae	4008	447	8.97
<i>Amalotermes phaeocephalus</i>	Termitidae: Apicotermitinae	7024	4780	1.46
<i>Cubitermes beghi</i>	Termitidae: Termitinae	3957	480	8.23
<i>Kalotermes sinaicus</i>	Kalotermitidae	17376	1513	11.48
<i>Macrotermes renouxi</i>	Termitidae: Macrotermitinae	46413	36882	1.25

Table 3-2. Mushroom body (MB) and central complex (CC) standardized volumes in 6 termite species. Standardized volumes were the absolute volumes corrected by means of head width in each species. Last column were corresponding ratios between MB and CC.

Species names	Family	Total Volumes of Kenyon Cell (μm^3)	Individual volume of Kenyon cell volumes (μm^3)	N3	Kenyon cell number
<i>Mastotermes darwiniensis</i>	Mastotermitidae	6724354	25.6 $\pm$ 0.17	164	116076 $\pm$ 41496
<i>Coptotermes kalsboveri</i>	Rhinotermitidae	300440	17.4 $\pm$ 0.01	61	16754 $\pm$ 4219
<i>Amalotermes phaeocephalus</i>	Termitidae: Apicotermitinae	117320	13.6 $\pm$ 0.00	211	8677 $\pm$ 20273
<i>Cubitermes hegghii</i>	Termitidae: Termitinae	456384	24.9 $\pm$ 0.00	255	18386 $\pm$ 6039
<i>Kaloterms sinicus</i>	Kalotermitidae	1553904	19.9 $\pm$ 0.02	18	78159
<i>Macrotermes renouxi</i>	Termitidae: Macrotermitinae	222584	48.5 $\pm$ 0.02	404	4588

Table 3-3. Estimation of Kenyon cell numbers in 6 termite species. Termite species are with a wide range of Kenyon cell numbers from several thousands to about 110,000 per hemisphere. N3 All volumes are in μm^3 .

Species names	Family	Volume of pedunculus and Lobes	Volume of Calyx	Ratio of Lobes:Calyx
<i>Mastotermes darwiniensis</i>	Mastotermitidae	2281567	753583	3.02
<i>Coptotermes kalshoveni</i>	Rhinotermitidae	220621	96923	2.28
<i>Amalotermes phaeocephalus</i>	Termitidae: Apicotermitinae	150815	79004	1.91
<i>Cubitermes heghi</i>	Termitidae: Termitinae	297989	121674	2.45
<i>Kalotermes sinaiicus</i>	Kalotermitidae	1552450	524877	2.96
<i>Macrotermes renouxi</i>	Termitidae: Macrotermitinae	4570818	1469877	3.11

Table 3-4. Volumes and ratios of different sub-structures of mushroom body in 6 termite species. Volumes of lobes and pedunculus are estimations for regions where axons of Kenyon cells localized. Estimation of calyx volumes corresponding to dendrites volumes. All volumes are in μm^3 .

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