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Neuroecology of social organization in the Australasian weaver ant, Oecophylla smaragdi

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NEUROECOLOGY OF SOCIAL ORGANIZATION IN THE AUSTRALASIAN WEAVER ANT, *OECOPHYLLA SMARAGDINA*

by

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ABSTRACT

The social brain hypothesis predicts that larger group size and greater social complexity select for increased brain size. In ants, social complexity is associated with large colony size, emergent collective action, and division of labor among workers. The great diversity of social organization in ants offers numerous systems to test social brain theory and examine the neurobiology of social behavior. My studies focused on the Australasian weaver ant, *Oecophylla smaragdina*, a polymorphic species, as a model of advanced social organization. I critically analyzed how biogenic amines modulate social behavior in ants and examined their role in worker subcaste-related territorial aggression. Major workers that naturally engage in territorial defense showed higher levels of brain octopamine in comparison to more docile, smaller minor workers, whose social role is nursing. Through pharmacological manipulations of octopaminergic action in both subcastes, octopamine was found to be both necessary and sufficient for aggression, suggesting subcaste-related task specialization results from neuromodulation. Additionally, I tested social brain theory by contrasting the neurobiological correlates of social organization in a phylogenetically closely related ant species, *Formica subsericea*, which is more basic in social structure. Specifically, I compared brain neuroanatomy and neurometabolism in respect to the neuroecology and degree of social complexity of *O. smaragdina* major and minor workers and *F. subsericea* monomorphic workers. Increased brain production costs were found in both *O. smaragdina* subcastes, and the collective action of *O. smaragdina* majors appeared to compensate for these elevated costs through decreased ATP usage, measured from cytochrome oxidase activity, an endogenous marker of neurometabolism. Macroscopic and cellular neuroanatomical analyses of brain development showed that higher-order sensory processing regions in workers of *O. smaragdina*, but not *F. subsericea*, had age-related synaptic reorganization and increased volume. Supporting the social brain hypothesis, ecological and social challenges associated with large colony size were found to contribute to increased brain size. I conclude that division of labor and collective action, among other components of social complexity, may drive the evolution of brain structure and function in compensatory ways by generating anatomically and metabolically plastic mosaic brains that adaptively reflect cognitive demands of worker task specialization and colony-level social organization.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-HT	Serotonin
AL	Antennal lobe
CC	Central complex
CIN	Serotonergic mushroom body calyx input neuron
COX	Cytochrome oxidase
DA	Dopamine
DMF	Dimethylformamide
EPN	Epinastine
HPLC-ED	High performance liquid chromatography with electrochemical detection
HW	Head width
OA	Octopamine
OL	Optic lobe
MB	Mushroom body
MB-mc	Mushroom body medial calyx
MB-lc	Mushroom body lateral calyx
MB-ped	Mushroom body peduncle
MG	Microglomeruli
ROCB	Rest of central brain
SEZ	Subesophageal zone

CHAPTER ONE: INTRODUCTION

Can the social brain hypothesis be applied to eusocial insects?

Social complexity and its accompanying cognitive challenges are considered to have greatly influenced brain evolution. Large group size and collateral increases in the quantity of encounters and individualization of information transfer among group members are associated with an increase in brain size in vertebrates (Burish et al., 2004, Dunbar and Shultz, 2007a, b). In eusocial ("truly social") insects such as ants, however, our understanding of the effect of group living and social complexity on brain size, structure, and neurochemistry is very limited. Ants, in contrast to vertebrates, are exemplars of cohesive and cooperative colony-level behaviors that benefit the colony as a whole. Workers, which are typically sterile, may partition tasks among workers that are morphologically similar and/or form morphologically distinct worker subcastes. Workers may change task sets or build repertoires among as they age, yielding specialized behaviors. The reproductive success of the colony, rather than the individual, is therefore targeted by selection (Bourke and Franks, 1995), and social selection should impact worker brain evolution.

Brain miniaturization must also be considered in applying social brain theory to eusocial insects. One paradigmatic eusocial insect, the honey bee, has a brain that contains roughly 960,000 neurons (Menzel and Giurfa, 2001). Ant brains are even more minute at about one tenth the size of a bee brain or less (Muscedere et al., 2014) and have approximately 100,000 neurons. In striking contrast, a human brain has approximately 86 billion neurons (Herculano-Houzel, 2012). Because brain tissue is metabolically expensive (Aiello and Wheeler, 1995), brain size is predicted to be optimized to reduce metabolic costs while maintaining adaptive behavioral performance. Even in cases of extreme brain miniaturization, behavior does not appear to be compromised (Polilov, 2012, 2015). Ant brain size, scaled to body size, follows Haller's rule (Seid et al., 2011), which states that as body size decreases relative brain size increases. However, in minute ants, brain size is smaller than would be predicted from the allometric relationship of brain to body size in larger species (Seid et al., 2011). These results indicate that the costs associated with higher investment in neural tissue at very small worker body size in ants may outweigh the fitness benefits of greater cognitive capability or, alternatively, brain metabolic costs could be significantly reduced or more efficient in miniaturized brains. Moreover, small-bodied workers could have reduced brain tissue by expanding sensory systems in the peripheral nervous system.

Relatively large brain size does not appear to have provided as pronounced of an individual fitness benefit in ants as it has in group-living vertebrates. The increase in brain size corresponding to increased group-size in vertebrates appears to facilitate adaptive responses to social information processing involved in mdsore complex and individualized social interactions (Dunbar and Shultz, 2007b). The benefit of increased cognitive capability, which may enhance mate choice, parental care, and other social behaviors (Dunbar and Shultz, 2007a), may thus outweigh increased brain metabolic costs. In ants, social complexity is associated with large colony size and worker division of labor (Thomas and Elgar, 2003, Holbrook et al., 2011, Ferguson-Gow et al., 2014) and emergent collective action (Sumpter, 2006, Couzin, 2009). As colony size increases, division of labor becomes more prevalent, resulting in workers that partition colony tasks and are behaviorally specialized according to task performance. Cognitive processing is thus distributed throughout the colony according to worker phenotypes. Additionally, collective actions may emerge, leading to more efficient and accurate decision-making occurring at the level of the group rather than individual worker. Brain architecture, including macroscopic anatomy and synaptic organization, is predicted to be tuned to ecological and social challenges associated with worker task performance, and investment in neural processing is predicted to decrease with increased behavioral specialization (Gronenberg and Riveros, 2009). Because worker division of labor is predicted to be associated with canalized behavior, this social organization may be correlated with experience-expectant brain development. Reduced cognitive capability may also arise from collective intelligence (Feinerman and Traniello, 2015): theories of self-organization propose that collective actions emerge from interactions of simple components (Anderson and McShea, 2001, Jeanson et al., 2012). Patterns of brain investment in socially complex species could reduce metabolic costs and provide a fitness benefit. An alternative, but not mutually exclusive explanation, the ecological challenges hypothesis, predicts that brain and brain compartment size as well as the extent of experience-dependent neuroplasticity corresponds to the cognitive demands of worker behavioral repertoire independent of degree of social complexity. Accordingly, workers within the same colony could have different brain sizes reflecting the degree of behavioral specialization and cognitive requirements of task performance.

Neuroanatomical studies provide evidence of adaptive brain organization corresponding to worker division of labor. Ant brains, like vertebrate brains, are mosaics of functionally distinct regions (Gronenberg, 2008) that are not constrained in compartment size by total brain size (Muscedere et al., 2014). Macroscopic and cellular neuroanatomy vary intra- and interspecifically and reflect cognitive demands associated with task performance (Seid et al., 2005, Stieb et al., 2010, Muscedere and Traniello, 2012, Stieb et al., 2012, Giraldo et al., 2013). Volumes of brain compartments increase in size with age, corresponding to increases in behavioral repertoire size and/or behavioral competence, and vary according to the specialized tasks performed by morphologically differentiated subcastes (Gronenberg et al., 1996, Muscedere and Traniello, 2012). Synaptic organization in higher-order sensory integration regions can be modulated by task-related sensory experience, suggesting that experience-dependent neuroplasticity can occur as workers transition from inside-nest tasks such as nursing to foraging outside of the nest (Stieb et al., 2010, Stieb et al., 2012).

Altering brain region volume modulates the number of synaptic connections (Chittka and Niven, 2009), and as a result may affect neurochemical production and receptor expression, which are integral for social behaviors in ants. Division of labor is associated with neuromodulatory regulation by biogenic amines such as dopamine, serotonin, and octopamine (Cuvillier-Hot and Lenoir, 2006, Muscedere et al., 2012, Kamhi and Traniello, 2013, Smith et al., 2013). Neurotransmitter levels vary with worker age (Seid and Traniello, 2005), corresponding to the transition from within-nest tasks such as brood care to foraging and other extranidal activities. Serotonin, specifically, modulates sensitivity to trail pheromone, the chemical signal used to coordinate social foraging (Muscedere et al., 2012). Biogenic amines also correlate with body size and task specificity (Smith et al., 2013), suggesting that these neurotransmitters play an integral role in modulating task-related behaviors in workers. Indeed, octopamine is necessary and sufficient for morphological subcaste-related territorial defense (Kamhi et al., 2015).

While neuroanatomical organization correlates with behavioral repertoire size and biogenic amines regulate task performance associated with division of labor, it is unknown how these neurobiological features result from selection associated with social complexity. Recent studies exploring how social insect brains adaptively reflect variation in components of social complexity such as division of labor and colony size have yielded mixed results (Riveros et al., 2012, Amador-Vargas et al., 2015, O'Donnell et al., 2015). My dissertation research aimed to test theories of social brain evolution (Dunbar and Shultz, 2007a, Gronenberg and Riveros, 2009) by investigating whether morphologically and neuroanatomically distinct workers in a socially complex species also have specialized neurochemical mechanisms underlying worker division of labor, a key component of advanced social organization, and exploring how encephalization, brain compartment metabolic investment, synaptic organization, and neuroplasticity correspond to the level of social complexity in ants.

Dissertation overview

To investigate the effect of social organization on brain architecture, I explored the neuroecology of the Australasian weaver ant, *Oecophylla smaragdina*, which is considered a pinnacle of ant social evolution due to its highly coordinated social actions and division of labor by physical subcastes (Crozier et al., 2010). Task specialization based on worker size appears to be an ancient social trait in weaver ant biology (Wilson and Taylor, 1964) and is critically important to colony organization. Small minor workers specialize in brood care within nests, and larger major workers leave nests after maturity to participate in all other colony tasks. Major workers are highly territorial, patrolling and effectively defending their arboreal nests and foraging areas (Hölldobler and Wilson, 1977b, Hölldobler, 1983, Crozier et al., 2010, Newey et al., 2010a, b). As their name implies, workers weave leaf nests by first forming living chains to draw leaves together, shortening the chain and anchoring leaves in position, then shuttling silk-secreting larvae to hold them together (Crozier et al., 2010). Colonies of *O. smaragdina* are ecologically dominant in the Australian rainforest canopy: as many as ~500,000 workers inhabit a single colony, whose territory may span 1500 m^2 (Hölldobler, 1983).

To comparatively analyze the evolutionary relationship between neuroanatomy and degree of social complexity, I performed parallel neuroanatomical studies on a phylogenetically closely related species pair with less social organization. In contrast to *O. smaragdina*, the sister clade *Formica subsericea* (Moreau et al., 2006) is characterized by monomorphic workers that are similar in size to an *O. smaragdina* major. Workers scavenge for arthropod prey and tend homopterans for carbohydrates, sometimes recruiting nestmates to foraging sites. Group size does not appear to affect worker performance (Klotz, 1986), and collective decision-making in this species appears limited. *F. subsericea* reside in relatively small colonies (several 100 to ~8,000 workers) in Nearctic woodlands. *F. subsericea* and *O. smaragdina* thus provide appropriate contrasts in social phenotypes, enabling an evolutionary analysis of the impact of various aspects of social complexity on brain organization.

Chapter 2 (Kamhi and Traniello, 2013) synthesizes and critically analyzes the literature on the role of the biogenic amines, serotonin, dopamine, and octopamine in ant social behavior. Biogenic amine levels have been shown to correlate with nestmate recognition, division of labor, social food flow, and predatory aggression – all integral to colony organization. The role of biogenic amines in modulating collective behavior is for the first time considered. Future research directions that would expand our understanding of the genetic regulation of biogenic amines and determine whether biogenic amines reflect social complexity and modulate social behaviors, behavioral phenotypes, and division of labor are presented.

Given the role of biogenic amines in age-related task performance in ants (Muscedere et al., 2012) and the correlation of amine titers with task specialization, in Chapter 3 (Kamhi et al., 2015) I investigated whether neuromodulators regulate subcaste-related task performance, specifically territorial aggression, in *O. smaragdina*. Major workers, which engage in territorial defense, had higher titers of octopamine than minor workers, which are typically more docile and remain within the nest. Behavioral assays and biogenic amine titer quantifications revealed a strong correlation between octopamine and territorial defense. Pharmacological manipulations of brain octopamine action in both worker subcastes demonstrated that this amine is necessary and sufficient for aggression in *O. smaragdina* workers, suggesting that octopamine modulates subcaste-related task performance. These findings are the first to demonstrate a causal role of a neuromodulator in subcaste-related division of labor, and thus illustrate how aminergic control systems are coupled to social complexity.

Chapter 4 (Kamhi et al., to be submitted) explores the hypothesis that brain production and operations costs decrease with increasing social complexity. I performed neuroanatomical studies in *O. smaragdina* and *F. subsericea* workers to compare total brain mass, brain compartment volumes, and cell rind volume, density, and neuron size as a measure of brain investment (production costs). To infer operations costs, I quantified cytochrome oxidase activity, an endogenous proxy for ATP usage. Results indicated that brain production costs were higher in the socially complex *O. smaragdina* workers, consistent with the vertebrate social brain hypothesis. *O. smaragdina* major workers, however, showed decreased metabolic operations costs compared to minor and *F. subsericea* workers. These findings are the first to demonstrate a compensatory relationship between neurometabolism and neuropil investment patterns in the context of social evolution. Results suggest that increased colony size and its associated cognitive demands may increase brain size while the collective action of *O. smaragdina* major workers, among other aspects of social phenotype of weaver ants, may decrease brain metabolic requirements.

Chapter 5 (Kamhi et al., in preparation) examines the relation of neuroplasticity to worker subcaste evolution as a metric of social complexity. Quantifying brain compartment volumes in newly eclosed and mature *O. smaragdina* and *F. subsericea* workers, I explored intra- and interspecific patterns of neural plasticity at the macroscopic level. To further investigate whether sociality is associated with experience-expectant or experience-dependent brain development, I experimentally reared ants in control and light-deprivation conditions and quantified synaptic complexes in higher-order sensory input regions. Results indicate *O. smaragdina* workers of both subcastes showed age-related volume increases in higher-order sensory processing regions when scaled for total brain size and changes in synaptic complexes in sensory input regions. I found no macroscopic or cellular changes in *F. subsericea* worker brains with development. Neither species showed experience-dependent synaptic plasticity within the visual system. These findings suggest that division of labor may be correlated with increased neural reorganization during development.

Chapter 6 summarizes the results of my dissertation research and suggests modifications to the social brain hypothesis as it pertains to ants and other eusocial insects. Future research directions are discussed, specifically focusing on how investigation of the role of neuromodulators in social behavior will provide a more thorough understanding of the control of phenotypic differences that underlie division of labor and emergent action. I discuss how the patterns I found in *O. smaragdina* and *F. subsericea* workers may be used to model macroevolutionary neuroanatomical and neurochemical phylogenetic comparisons and enhance our understanding of social brain evolution at multiple levels of analysis.

CHAPTER TWO: BIOGENIC AMINES AND COLLECTIVE ORGANIZATION IN A SUPERORGANISM: NEUROMODULATION OF SOCIAL BEHAVIOR IN ANTS

Abstract

The ecological dominance of ants has to a great extent been achieved through their collective action and complex social organization. Ants provide diverse model systems to examine neural underpinnings of individual behavior and group action that contribute to their evolutionary success. Core elements of ant colony structure such as reproductive and ergonomic division of labor, task specialization, and social integration are beginning to be understood in terms of cellular neuroanatomy and neurochemistry. In this review we discuss the neuroethology of colony organization by focusing on the role of biogenic amines in the control of social behavior in ants. We examine the role of neuromodulation in significant sociobiological characteristics of ants, including reproductive hierarchies, colony foundation, social food flow, nestmate recognition, territoriality, and size- and age-related sensory perception and task performance, as well as the involvement of monoamines in collective intelligence, the ultimate key to the global dominance of these remarkable superorganisms. We conclude by suggesting future directions for the analysis of the aminergic regulation of behavior and social complexity in ants.

Introduction

Invertebrates have long served as important models in neuroethology. The comparative simplicity of roundworm, mollusk, and arthropod neural architecture, wide range of behaviors, relative ease of culture, and experimental utility have revealed molecular and cellular underpinnings of sensory and motor processes that improve the survival and fitness of individual animals. Ants, as social invertebrates, provide novel opportunities to neuroethologically examine the genesis of complex social behavior from a miniscule brain composed of a small number of neurons and relatively simple circuitry (Gronenberg, 1996). Ants are ubiquitous models of biological complexity renowned for their highly organized colonies and ability to exceed individual behavioral capacities by integrating activities of single workers into group behaviors, thus acting as a superorganism. For example, leaf-cutter ants, one paradigm of social complexity in this clade, maintain an agricultural system with a mutualistic fungus and sustain colonies of more than a million individuals. Individual ants make local contributions to colony labor: workers cut or transport leaves, implant fungal hyphae, fertilize the fungal comb with fresh mulch, maintain sanitation to control infection, and provide for colony security (Wilson, 1980). The summed actions of colony members result in an extraordinarily well-organized farming community without centralized supervision.

Ants can provide insight into the neurobiological mechanisms of such sophisticated cooperative and collective action and are emerging as powerful models to explore the neuroanatomy and neurochemistry of development, plasticity, evolution, and ecology of behavioral differentiation and sociality. Given their remarkable species richness (>12,500 identified species) and dominance in abundance and biomass, ants present diverse opportunities to elucidate neural underpinnings of adaptive individual- and group-level actions in an animal long reputed for its relatively simple neural architecture but extraordinary behavioral capability. Ant brains can be smaller than 1/100th of the volume of a honey bee brain, which itself is minute (0.64±0.12 mm^3; Mares et al., 2005). The genesis of adaptive behavior from such a miniscule amount of nervous tissue has long captivated researchers. Darwin (1871) considered the ant brain to be "one of the most marvelous atoms of matter in the world, perhaps more so than the brain of a man." Ant brains reflect evolutionary responses to social selection, and they possess "social brains" in the same sense that nervous systems in all group-living animals reflect cognitive adaptations to challenges of social life (Dunbar and Shultz, 2007a). Methods enabling analyses of the cellular and macroscopic neuroanatomy and neurochemistry of ant brains are well-developed (Groh and Rössler, 2011, Muscedere et al., 2012, Muscedere and Traniello, 2012, Muscedere et al., 2013). There is currently unprecedented interest in brain miniaturization and its relation to sociality (Wehner et al., 2007, Chittka and Niven, 2009, Seid et al., 2011, Eberhard and Wcislo, 2012, Niven and Farris, 2012). There are also compelling studies on the influence of monoamines on social behavior; for example, swarm formation has been causally linked to the release of neurochemicals in desert locusts (Anstey et al., 2009). Monoamines may play a significant role in processing stimuli to enable monitoring the social environment and perhaps serve important functions in the control of behavior and colony organization in social insects such as ants, bees, and wasps (Order Hymenoptera) and termites (Order Isoptera) (Giurfa, 2006, Neckameyer and Leal, 2009, Tedjakumala and Giurfa, 2013).

The orchestration hypothesis (Hoyle, 1985) notes that neurochemical release stimulates specific neural circuits underlying behavior and makes testable predictions about the coordination of social behavior by monoamines. Research on invertebrate neurochemical circuitry (Kravitz and Huber, 2003, Libersat and Pflueger, 2004, Neckameyer and Leal, 2009, Riffell et al., 2013) includes studies of the regulation of flight (Orchard et al., 1993), grooming (Weisel-Eichler et al., 1999), hygienic behavior (Spivak et al., 2003), mate attraction (Christensen et al., 1992), aggression (Huber et al., 1997, Baier et al., 2002, Huber, 2005), and learning (Tedjakumala and Giurfa, 2013) in crickets, honey bees, locusts, roaches, and crustaceans. In honey bees, workers show chronological and physiological age-related variation in brain structure and monoamine levels, which adjust colony labor profiles in relation to colony needs and behavioral development (Schulz and Robinson, 2001, Schulz et al., 2002, Schulz et al., 2003, Barron and Robinson, 2005). Comparable research has only recently begun in ants; however, there is evidence supporting the hypothesis that social organization is based on neurally differentiated worker phenotypes (Wnuk et al., 2010, Muscedere and Traniello, 2012) whose patterns of task performance are dependent on aminergically controlled sensitivity to social signals (Vander Meer et al., 2008, Muscedere et al., 2012).

The confluence of interests in insect social brain evolution, brain miniaturization and mechanistic research in the neuromodulation of behavior offers the possibility of novel explorations of worker task capability, colony-level performance, neuroarchitecture, and neurochemistry in ants to advance our understanding of the underpinnings of complex social behavior. This is the emphasis of our review. We develop an integrative sociobiological and neuroethological framework to examine the role of the biogenic amines serotonin (5-HT), dopamine (DA), and octopamine (OA) in the modulation of social behavior, colony-level division of labor in ants, and collective intelligence. We conclude with a prospectus for future studies on the aminergic regulation of ant behavior.

The organization of ant colonies: sensory environments of worker behavior

All ants, representing a single hymenopteran family, the Formicidae, are *eusocial*. Colony social phenotype is universally defined by three traits: reproductive specialization (the coexistence of a fertile queen and sterile worker offspring; Figure 2.1), allomaternal care (shared nursing of immature offspring by adult workers), and overlapping generations (queen longevity sufficient for coexistence with her offspring). Mating behaviors are thus absent from worker repertoires, and queens do not contribute to colony labor. In a typical colony life cycle, winged gynes (virgin queens) disperse from their parent colony and outbreed with winged males, which die soon after mating. Newly inseminated queens shed their wings, construct a nest, and rear their first worker brood using energy gained from metabolizing their wing muscles (Hölldobler and Wilson, 1990). After the maturation of the first brood, queens become wholly specialized on reproduction and workers perform nursing, foraging, nest maintenance, and defense tasks. Workers recognize nestmates and aggressively discriminate against individuals from other colonies encountered while patrolling their territory or intruding in the nest. The biology of reproductive and sterile castes and the themes of cooperation, altruism, intense social interaction, and elaborate social networks are core elements of ant sociobiology.

Division of labor among workers is the basis of ergonomic organization in ant colonies. Through worker task specializations, division of labor enhances efficiency and thus the productivity and reproductive success of the colony (Hölldobler and Wilson, 1990, 2009). Worker social roles can be correlated with morphological variability, at times leading to the evolution of size-differentiated *subcastes*. For example, relatively large-bodied workers, "soldiers," or majors, are typically aggressive and involved in nest defense and territory security, whereas smaller workers (minors, e.g.) nurse brood, maintain the nest, manage infectious waste, and forage (Seid and Traniello, 2006; Figure 2.2). Task performance may also be coupled to age (reviewed in Seid and Traniello, 2006) and genetic architecture (Snyder, 1992, Libbrecht et al., 2011, Schluns et al., 2011). Worker behavior is not fixed according to size, age, or task load, and can be modified to allow flexible responses to colony needs (Seid and Traniello, 2006, Muscedere et al., 2009, Gordon, 2010). Worker attendance to a given task or task set may depend upon thresholds of responsiveness to cues in the environment and interactions with nestmates (Robinson, 1992, Beshers and Fewell, 2001, Gordon, 2010). Patterns of task attendance may be narrow, as in the case of specialist workers in a polymorphic species that may only process or store food, or broad, as is true for species characterized by monomorphic caste distributions and generalist workers (Hölldobler and Wilson, 1990).

Division of labor reflects energetic needs first associated with somatic growth and then the reproductive phases of the colony life cycle. Eggs produced by the queen develop into nutritionally dependent larvae that increase in size from worker provisioning, molt through a number of instars, and subsequently pupate. Each developmental stage likely has a chemical signature identifying colony identity and perhaps instar and nutritional state (Brian, 1975, den Boer and Duchateau, 2006, Cotoneschi et al., 2007). Immatures must be maintained in temperature and humidity regimes favorable to the requirements for development of each stage and eggs, larvae, and pupae are transported accordingly among nest chambers (Anderson and Munger, 2003). The information content of larval or pupal chemical cues and signals induces behavioral responses dependent on worker developmental stage, social role, experience, and colony need (Hölldobler and Wilson, 1990, Gordon, 2010). Beginning with adult eclosion, workers respond to their physical and social world by processing information encoded in olfactory, tactile, seismic, and visual signals and sensing chemical, thermal, and hygrometric clines (Billen and Morgan, 1998, Roces and Kleineidam, 2000, Kleineidam et al., 2007). Processing sensory input is thus critical to social behavior.

The first environment of task performance of a newly eclosed adult worker ant is the dark, labyrinthal interior of a nest chamber typically in a log or soil nest, perhaps deep beneath the surface. At this early stage of adulthood, it is likely that most workers interact through chemical communication with their nestmates in only a few basic ways and have the physical ability to attend to only the least demanding of tasks. Newly eclosed workers, or callows, are typically light in color due to incomplete cuticular sclerotization. The unhardened integument renders them vulnerable to attack, and muscle groups supporting the movement of the mandibles - the tools needed to work productively - are poorly developed (Muscedere et al., 2011). Callows have a relatively low level of movement and task activity. With increasing sclerotization of the exoskeleton and neural and myological maturation, workers develop efficiency in their task performance (Muscedere et al., 2009). As age increases, the sensory environment changes and workers are capable of more extensive locomotion. Behavioral development is typically marked by the onset of positive phototaxis, departure from the nest, and participation in food collection and territorial defense. These extranidal tasks involve the genesis of search patterns, pedometry, navigation, pheromone trail following, food recognition, food retrieval, predatory aggression, enemy identification, and interpretation of additional social signals. Worker size- and age-related patterns of task performance and specialization are presumably underscored by neural characteristics that enable sensory processing and integration (Libersat and Pflueger, 2004, Seid and Traniello, 2005, Wnuk et al., 2010, Giraldo et al., 2013). Ants are renowned for their cooperation and advanced sociality, but the neurobiological processes that give rise to their individual actions and colony organization are poorly understood.

The ant nervous system

Brain anatomy

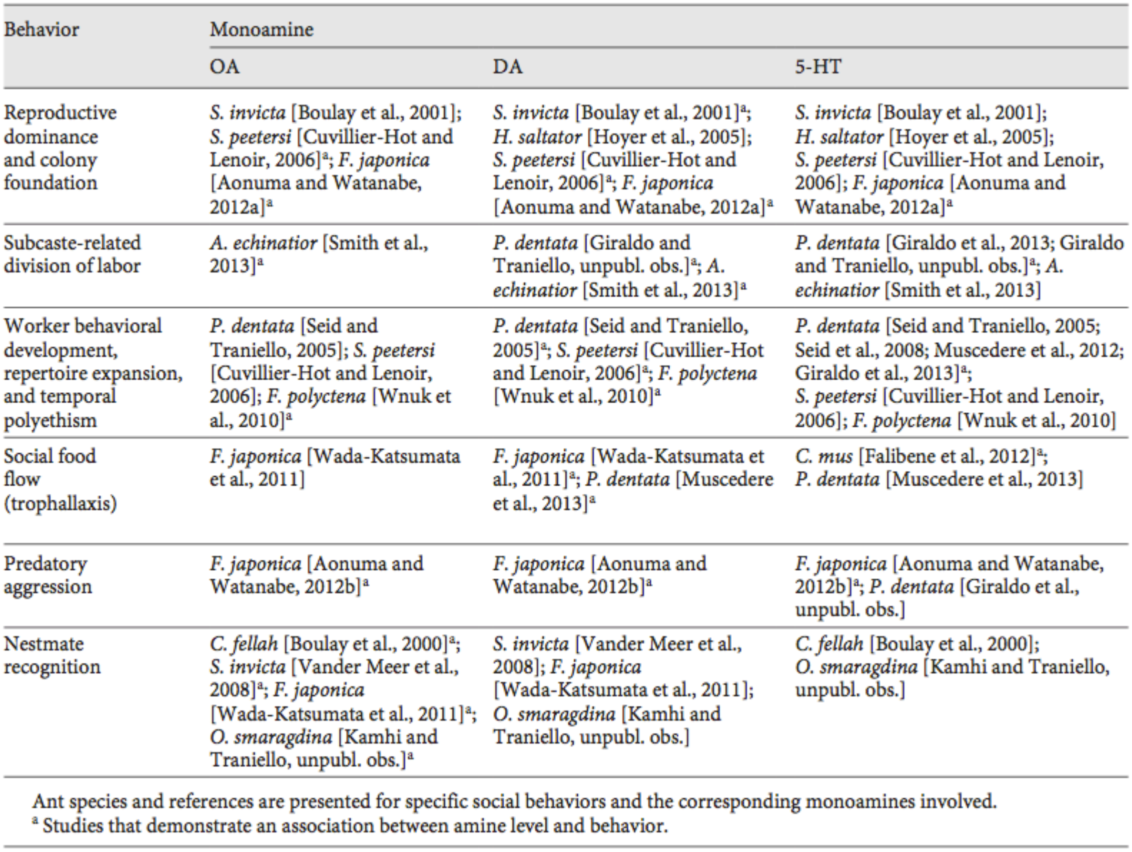
Hymenopteran brains exhibit a mosaic of functionally distinct compartments (Gronenberg, 2008; Figure 2.3A,B). Sensory regions are the optic lobes, comprised of the lamina (which attaches to ommatidia), the medulla and lobula, and the antennal lobes, made up of glomeruli that have distinct spatial patterns of activation to encode specific odors (Zube et al., 2008, Brandstaetter and Kleineidam, 2011). Mushroom body neuropil is dedicated to sensory integration, learning, and memory; typically, the calyx receives sensory input and the peduncle contains axons that project to various other brain regions (Fahrbach, 2006). Chemical and visual inputs are segregated in the mushroom body calyx: the lip receives olfactory input from the antennal lobes, axons from the optic lobes innervate the collar, and the basal ring receives information from both primary sensory regions (Gronenberg, 2001). The central body and protocerebral lobes complete the supraesophageal brain. The subesophageal ganglion is fused ventrally, and the ventral nerve cord, which extends throughout the body and controls movement, is comprised of three thoracic and a varying number of abdominal ganglia (Niven et al., 2008).

Three features distinguish ant brains from those of other hymenopterans: smaller optic lobes (with prominent exceptions in the highly visual ants *Myrmecia, Gigantiops*, and *Cataglyphis*), larger and more complex antennal lobes, and large mushroom bodies (Gronenberg, 1996). These neural characteristics reflect the chemical basis of social life in ants, particularly in phylogenetically derived species. Most ant species rely heavily on pheromones to communicate in multiple contexts, identify nestmates and interact socially, mark territory, and orient.

Neuromodulation

The modulation of behavior by the biogenic amines 5-HT, DA, and OA depends on the nature of G-protein-coupled receptors to which they bind. Each monoamine may have several associated receptor types acting through a different molecular mechanism. Little is understood about monoamine receptors in ants; however, immunolabeling monoamines has revealed distinct expression patterns in some species. In the ponerine ant *Harpegnathos saltator*, DA fibers innervate all regions of the mushroom bodies and densely innervate the central body, but not the optic or antennal lobes (Hoyer et al., 2005; Figure 2.4). In *Pheidole dentata*, newly eclosed and mature minor workers have similar dopaminergic innervation, suggesting these circuits are conserved during development and across ant species [S. Ibarraran and E. Abouheif personal communication]. DA therefore may have a limited role in behavioral development and related changes in sensory processing.

5-HT immunoreactive cell bodies have been identified in major and minor workers in the protocerebrum, subesophageal ganglion, and optic lobes, with diffuse innervation of all primary neuropil (Seid et al., 2008). The number of serotonergic cell bodies increases significantly with age in the optic lobes of workers in both subcastes, implicating 5-HT in the development of the visual system (Seid et al., 2008). Similarly, the extrinsic serotonergic mushroom body calyx input neuron (CIN) has more elaborate axonal arborization in mature majors than newly eclosed majors although there is no variation in age-related arborization in either subcaste when brain volume differences were controlled (Giraldo et al., 2013). Subcaste distinctions also occur in the CIN: more elaborate axonal arborization was found in majors than minors. These findings, along with those of neuropharmacological studies (Muscedere et al., 2012), suggest that 5-HT is associated with age- and subcaste-related task specializations, possibly by regulating olfactory sensitivity. *H. saltator* worker brains exhibit similar 5-HT expression (Hoyer et al., 2005): serotonergic neurons innervate most of the prominent neuropil, with dense labeling of the central body and mushroom body penduncle and calyx lip. These studies relate age, subcaste, and 5-HT expression patterns, suggesting that worker behavior is under aminergic regulation (Table 2.1).



Behavior	Monoamine		
	OA	DA	5-HT
Reproductive dominance and colony foundation	*S. invicta* [Boulay et al., 2001]; *S. peetersi* [Cuvillier-Hot and Lenoir, 2006][a]; *F. japonica* [Aonuma and Watanabe, 2012a][a]	*S. invicta* [Boulay et al., 2001][a]; *H. saltator* [Hoyer et al., 2005]; *S. peetersi* [Cuvillier-Hot and Lenoir, 2006][a]; *F. japonica* [Aonuma and Watanabe, 2012a][a]	*S. invicta* [Boulay et al., 2001]; *H. saltator* [Hoyer et al., 2005]; *S. peetersi* [Cuvillier-Hot and Lenoir, 2006]; *F. japonica* [Aonuma and Watanabe, 2012a][a]
Subcaste-related division of labor	*A. echinatior* [Smith et al., 2013][a]	*P. dentata* [Giraldo and Traniello, unpubl. obs.][a]; *A. echinatior* [Smith et al., 2013][a]	*P. dentata* [Giraldo et al., 2013; Giraldo and Traniello, unpubl. obs.][a]; *A. echinatior* [Smith et al., 2013]
Worker behavioral development, repertoire expansion, and temporal polyethism	*P. dentata* [Seid and Traniello, 2005]; *S. peetersi* [Cuvillier-Hot and Lenoir, 2006]; *F. polyctena* [Wnuk et al., 2010][a]	*P. dentata* [Seid and Traniello, 2005][a]; *S. peetersi* [Cuvillier-Hot and Lenoir, 2006][a]; *F. polyctena* [Wnuk et al., 2010][a]	*P. dentata* [Seid and Traniello, 2005; Seid et al., 2008; Muscedere et al., 2012; Giraldo et al., 2013][a]; *S. peetersi* [Cuvillier-Hot and Lenoir, 2006]; *F. polyctena* [Wnuk et al., 2010]
Social food flow (trophallaxis)	*F. japonica* [Wada-Katsumata et al., 2011]	*F. japonica* [Wada-Katsumata et al., 2011][a]; *P. dentata* [Muscedere et al., 2013][a]	*C. mus* [Falibene et al., 2012][a]; *P. dentata* [Muscedere et al., 2013]
Predatory aggression	*F. japonica* [Aonuma and Watanabe, 2012b][a]	*F. japonica* [Aonuma and Watanabe, 2012b][a]	*F. japonica* [Aonuma and Watanabe, 2012b][a]; *P. dentata* [Giraldo et al., unpubl. obs.]
Nestmate recognition	*C. fellah* [Boulay et al., 2000][a]; *S. invicta* [Vander Meer et al., 2008][a]; *F. japonica* [Wada-Katsumata et al., 2011][a]; *O. smaragdina* [Kamhi and Traniello, unpubl. obs.][a]	*S. invicta* [Vander Meer et al., 2008]; *F. japonica* [Wada-Katsumata et al., 2011]; *O. smaragdina* [Kamhi and Traniello, unpubl. obs.]	*C. fellah* [Boulay et al., 2000]; *O. smaragdina* [Kamhi and Traniello, unpubl. obs.]

Ant species and references are presented for specific social behaviors and the corresponding monoamines involved.
[a] Studies that demonstrate an association between amine level and behavior.

Table 2.1: Monoamines and social behavior in ants

Neuromodulation of ant social behavior

Reproductive dominance and colony foundation

The distinction between the non-reproductive (worker) caste and the fertile (queen) caste is a defining feature of eusociality. Queenless ant species – those in which reproductive and worker forms are not morphologically well-differentiated – are particularly insightful to study the neuromodulation of behaviors related to fertility and reproductive status because all females develop as reproductively capable workers. Dominance hierarchies among colony members in queenless ants, and thus reproductive or worker roles, are created by outcomes of conflict. Through aggressive confrontations, the most dominant female becomes the sole reproductive, or queen, in a colony. A variety of studies have explored the involvement of monoamines in reproductive dominance in ants. In *Harpegnathus saltator*, expression of dopaminergic and serotonergic fibers were found to be independent of reproductive state, suggesting neural architecture is unaffected by fertility and social hierarchy in this species (Hoyer et al., 2005). However, in *Streblognathus peteersi* dominant females have higher OA titers indicating OA involvement in ovarian activity (Cuvillier-Hot and Lenoir, 2006). DA can also be positively correlated with reproductive behavior in insects (Neckameyer, 1998, Boulay et al., 2001, Sasaki et al., 2007). In gynes of the fire ant *Solenopsis invicta* high levels of DA are associated with asexual reproduction (Boulay et al., 2001). Higher levels of DA are correlated with increased dealation, the shedding of wings that occurs during colony foundation, and growth of oocytes in gynes separated from a queen. Production of oocytes and haploid egg-laying, but not dealation, can be decreased by treatment with a tyrosine hydroxylase inhibitor, which leads to lowered DA titers. In honey bees, queen mandibular pheromone reduces DA levels of young workers inside the nest; decreased DA reduces locomotion, which may increase worker brood care (Beggs et al., 2007). Thus, the actions of OA and DA may both be involved in reproductive division of labor, and more broadly, in worker task attendance.

Queen insemination is followed by significant physiological, anatomical, and behavioral changes, including reduction of brain regions (Julian and Gronenberg, 2002), induction of cooperation or competitive aggression (Aron et al., 2009), maternal care

(Mas and Kölliker, 2008), and fertility signaling (Peeters and Liebig, 2009). In *Formica japonica* queens, levels of DA, 5-HT, and OA in the brain decrease after mating, while tyramine increases (Aonuma and Watanabe, 2012a). Tyramine, the precursor to OA often considered a signaling molecule in its own right (Roeder, 2005), may be involved in reproduction (as in honey bees; Salomon et al., 2012), but it is unclear whether the decrease of other biogenic amines is due to the act of mating itself or other behavioral changes such as the completion of flight activity and dealation or isolation from nestmates, which are both temporally coincident with mating. The observed decrease in DA could also be due to starvation stress (Wada-Katsumata et al., 2011), as claustrally founding queens establish colonies without access to food, relying only on their own energy reserves to provision the first brood. OA may decrease throughout a queen's life, corresponding with either reproductive activities or social environment (Wada-Katsumata et al., 2011). In addition to a decrease after mating as in *F. japonica* queens (Aonuma and Watanabe, 2012a), established *Messor pergandei* queens have less OA than foundresses [M. Muscedere, S. Cahan, and J. Traniello, in prep]. Biogenic amines in gynes increase during 10 days of isolation (Aonuma and Watanabe, 2012a), suggesting that biogenic amines likely play a role in the transition from virgin status to a mated, colony-founding queen, but it is unknown how mating and accompanying physiological changes are controlled or affect biogenic amine titers.

Worker polymorphism and task specialization

The majority of ant species have a monomorphic worker caste in which worker size variation is low. In a relatively small number of ant species, however, multiple worker subcastes differentiated by morphology and body size perform different tasks. Studies of the organization of division of labor in ants feature *Pheidole* (Traniello, 2010), renowned for its hyperdiversity, prevalence, and striking subcaste specializations, which are hypothesized to have been key to the adaptive radiation of this genus (Wilson, 2003). The majority of *Pheidole* species exhibit complete dimorphism in the worker caste (Figure 2A); major workers are relatively large in body size and have disproportionately large heads, adaptations that improve fighting or food processing ability but compromise behavioral plasticity (Mertl and Traniello, 2009). Minor workers, in comparison, are smaller and more morphologically uniform task generalists. Biogenic amine titers, scaled for brain size variation, may differ among subcastes, suggesting a role in modulating division of labor in relation to caste polyphenism. 5-HT and DA titers, for example, are significantly higher in *P. dentata* major worker brains than minor worker brains [Y. Giraldo and J. Traniello, unpublished observations] and may influence major worker defensive behaviors.

The coupling of worker body size, task specialization, brain structure, and monoamines is illustrated by leaf-cutter ants, which have a coevolutionary relationship with symbiotic fungi and bacteria (Schultz and Brady, 2008). *Atta* form colonies of potentially millions of strongly polymorphic workers (0.8 mm to 3.5 mm in head width; Figure 2.2D) that divide tasks associated with leaf collection and the culturing of a mutualistic fungus according to body size (Wilson, 1980). Tiny *minim* workers implant fungal hyphae and care for brood (Figure 2.2B), slightly larger *media* workers dispose of colony waste, larger workers gather leaves that are reduced to mulch to cultivate fungus (Figure 2.2C), and the largest *major* workers engage solely in defense (Wilson, 1980, Camargo et al., 2007, Waddington and Hughes, 2010). Following Haller's rule, which notes that smaller individuals have brains that are large relative to their body than individuals of larger body size, smaller *Atta columbica* workers have relatively larger brains than larger workers despite miniaturization of body size (Seid et al., 2011). Brain scaling in minim workers, however, does not follow the same slope as that of larger ants; minims therefore have relatively smaller brains, perhaps due to energetic costs or morphological constraints. Similar brain-to-body scaling trends are found in *Atta cephalotes* (Figure 2.5A). Analysis of brain DA and 5-HT content show a single allometric relationship between biogenic amine titers and brain mass, indicating biogenic amines do not scale diphasically as does body size. Instead, these data suggest a 1:1 relationship between monoamine level and the absolute amount of neural tissue (Figure 2.5B). An energetic cost constraint on brain size (Seid et al., 2011, van der Woude et al., 2013) could result in smaller workers having relatively smaller brains than would be predicted. Brain size could, in turn, limit the amount of biogenic amine production in *A. cephalotes* workers due to limited availability of precursors and energy costs of neural signaling. The energetic cost of signaling increases at a greater rate than performance in fly photoreceptors (Niven et al., 2007); the costs of increased aminergic signaling could similarly outweigh the benefits of greater processing capability. Decreased energy availability associated with smaller brain size could limit sustained neural firing capacity (Niven and Farris, 2012), thus rendering the smallest workers physiologically incapable of having relatively higher levels of monoamine signalling per brain mass than larger workers.

Little is known about how amines scale with brain size and how variation in task performance is correlated with variation in monoamine titers. In the leaf-cutter *Acromyrmex echinatior*, a crucial function of division of labor is the reduction of fungal comb contamination by pathogens or toxic substances in waste (Waddington and Hughes, 2010). Biogenic amine levels positively correlate with body size in *A. echinatior*, but DA and OA titers are significantly higher in foragers than midden workers of the same size range suggesting that amine levels correlate with task specificity (Smith et al., in press). 5-HT is similar between middens workers and foragers in this species. However, 5-HT appears to increase sensitivity to odors of decomposition in midden workers in the closely related *Acromyrmex octospinosus* [M. Muscedere and J. Traniello, unpublished observations]. All three biogenic amines may play an integral role in waste management role differentiation.

Behavioral development, task repertoire expansion, and temporal polyethism

Temporal polyethism is division of labor related to worker age. The typical progression of task attendance is initiated after adult eclosion with the performance of inside-nest tasks. As adults mature they transition to tasks outside of the nest that appear more cognitively demanding such as searching for and collecting food and defending territory. These extranidal tasks require greater biomechanical and locomotory skills as well as perception and processing of multimodal stimuli. In *P. dentata*, minor workers begin to forage at roughly three weeks of age while retaining their role as nurses and improving their ability to care for brood (Seid and Traniello, 2006, Muscedere et al., 2009, 2013). Mature minor workers respond to signals and cues associated with both inner-nest tasks such as nursing and stimuli associated with predation and scavenging outside the nest, as opposed to responding sequentially to discrete sets of task-related stimuli. Age-related repertoire expansion appears to have a neuronal basis: the mushroom bodies and antennal lobes of *P. dentata* minor workers increase in size (Muscedere and Traniello, 2012) and synaptic boutons decrease in number but enlarge and increase in volume and vesicle number (Seid et al., 2005). Studies of microglomeruli – synaptic clusters formed between afferent sensory projection neurons and efferent mushroom body Kenyon cells in the mushroom body calyces – show that plasticity in neural architecture may be accompanied by changes in signaling molecules. As honey bee workers age and gain sensory experience, the density of microglomeruli decreases as their size increases indicating growth in dendritic processes and strengthening of select synapses (Groh et al., 2012). Associated increases in ribbon synapses in the postsynaptic portion of the microglomeruli suggest increased rates of neurotransmitter release. Neuroplastic changes associated with the development of behavioral capabilities may be reliant on experience-dependent (Stieb et al., 2012) and experience-expectant processes (Fahrbach et al., 1998) and may be accompanied by changes in neurotransmitter synthesis and release.

Although OA titers increase in the worker brain as honey bees age and begin to forage and are causally linked to polyethism (Schulz et al., 2002), few studies correlate age-related division of labor to brain levels of monoamines in ants or causally link neurochemistry and behavior. In *P. dentata*, 5-HT and DA levels increase significantly with minor worker age; OA levels remain constant (Seid and Traniello, 2005). Increasing brain monoamine levels in 20-day old individuals coincide with the transition from inner- to outer-nest tasks. Moreover, 5-HT appears to control olfactory sensitivity to trail pheromone: *P. dentata* minor workers with pharmacologically decreased 5-HT initiated following behavior on an artificial trail fewer times and followed the trail for shorter distances than control minors (Muscedere et al., 2012). These findings indicate that 5-HT is an important neuromodulator of age-related behavior. In *Streblognathus peetersi*, DA may be involved in foraging: comparisons of biogenic amine levels among fertile dominant ants, young gynes, and subordinate workers that become foragers show that subordinate workers have significantly higher levels of brain DA (Cuvillier-Hot and Lenoir, 2006). Because these workers are older, their brain DA may increase with age and regulate their behavior. Alternatively, the DA increase could be due to their subordinate status within the colony.

Mature workers can be induced to perform tasks generally performed by younger workers (Seid and Traniello, 2006), raising questions concerning the role of monoamines in the regulation of social plasticity. In *Formica polyctena*, analyses of biogenic amine levels showed significantly higher titers of OA and a non-significant trend toward lower DA in nurses than in foragers and foragers that engage in brood care following exposure to brood in the absence of young workers (termed "reverted nurses"; Wnuk et al., 2010). However, it is unclear whether individuals considered to be reverted nurses were actually altering their behavior. Perhaps nursing simply remains in the repertoire of mature worker behavior and therefore the trend toward changes in OA and DA levels recorded in foragers and reverted nurses could be more parsimoniously explained by a general age-related change in amine levels, as occurs in *P. dentata* in association with the expansion of task attendance (Seid and Traniello, 2005).

Social food flow

The nutritional status of individual workers and the colony as a whole is mediated in part by trophallaxis, the exchange and distribution of liquid food and nutrients among workers, between workers and the queen, and between workers and larvae. 5-HT and DA have been implicated in food intake and nutrition-related trophallaxis. *Camponotus mus* workers fed 5-HT consumed less sucrose solution than controls in contexts of individual and group feeding (Falibene et al., 2012). These authors also show the presence of 5-HT immunoreactivity in the crop and proventriculus, alimentary organs that store liquid food and control bidirectional pumping through valves to take in and regurgitate liquids, indicating that 5-HT regulates trophallaxis.

DA may play a role as well: *Formica japonica* workers show lower DA levels and decreased trophallaxis after seven days of starvation (Wada-Katsumata et al., 2011). After consuming a solution of sucrose, trophallaxis was reinstated and DA increased. DA may be associated with satiation, which occurs following the distribution of food:

lower titers of DA are found in brood-deprived *P. dentata* minor workers, possibly due to not having brood to provide nutrition (Kloppenburg and Hilderbrand, 1995, Muscedere et al., 2013). However, in *F. japonica*, DA returned to control levels only after trophallaxis suggesting its level may be modulated by both social interaction and nutritional state (Wada-Katsumata et al., 2011). It is unclear whether DA levels would return to control levels in the absence of trophallaxis if workers were allowed more time for digestion. On this point, Wada-Katsumata et al. (2011) showed that three and five minutes after imbibing a sucrose solution, levels of DA were stable suggesting that DA may be implicated in food intake and trophallaxis. However, DA titers decreased, rather than increased as in the prior observation, from titers measured in the absence of sucrose feeding indicating the precise role of DA requires resolution in this system.

Predatory aggression

One context in which aggression can occur is foraging: ants are important predators of arthropods, including other social insects, and sometimes, small vertebrates. Few studies identify a role for biogenic amines in predatory aggression. In *P. dentata*, serotonin does not modulate aggression toward fruit flies used as experimental prey [Y. Giraldo, A. Rusakov, and J. Traniello, unpublished observations]. Aonuma and Watanabe (2012b) suggest that OA is involved in prey capture by *Formica japonica* foragers due to correlational differences between aggression and the ratio of OA to OA catabolite N-acetyloctopamine in queens and workers. However, workers also have higher titers of 5-HT and DA and lower levels of tyramine than queens, which do not typically forage. It is therefore unclear whether these signaling molecules play a role in predatory aggression or are associated with other physiological differences between workers and queens.

Nestmate recognition

The ability to distinguish nestmates from non-nestmate conspecific and heterospecific ants is crucial to the social cohesiveness of a colony and its ability to defend the nest and territory. Ecological interactions among ants may involve aggressive confrontations among colonies resulting in unoccupied buffer zones (“no ants land”) between adjacent territories. Territorial disputes are mediated by nestmate recognition systems that may have species- and/or colony-specificity (Newey et al., 2010b). Nestmate recognition systems have traditionally been analyzed by recording the frequency and level of aggressive responses to non-nestmates (Blomquist and Bagnères, 2010). Cuticular hydrocarbons found on the exoskeleton surface serve as recognition signals (Blomquist and Bagnères, 2010, Sturgis and Gordon, 2012) and can be transferred by allogrooming and trophallaxis. Cuticular hydrocarbons providing cues for nestmate recognition are detected by olfactory processing in antennal receptors and the antennal lobes (Stroeymeyt et al., 2010, Brandstaetter et al., 2011).

OA, which improves nestmate recognition in honey bees (Robinson et al., 1999), appears to serve a similar neuromodulatory role in the fire ant *Solenopsis invicta* (Vander Meer et al., 2008). OA levels are correlated with levels of aggression directed toward conspecifics that change with the presence of a queen. Of three treatment groups (queenless workers, queenless workers fed OA, and queenright workers), OA levels were highest in workers administered OA, which were similar to those of workers in queenright groups. OA levels were significantly lower in workers in the untreated queenless group, suggesting that OA is maintained at high levels in the presence of the queen possibly due to pheromonal cues (Beggs et al., 2007). Queens do not appear to directly provide cues needed for nestmate recognition because they produce significantly lower amounts of hydrocarbons than workers and hydrocarbon transfer is primarily from worker to queen (Howard and Blomquist, 2005). Queenright workers showed significantly more aggression toward non-nestmates than queenless groups, and OA-treated queenless workers, while initially exhibiting similar aggressive levels as queenless controls, increased their aggression to levels similar to those of queenright workers over a 10-week period. Therefore, decreased aggression (and hence nestmate discrimination) due to losing a queen may be caused by a decrease in OA.

OA may enhance aggressive responses in the Australian weaver ant, *Oecophylla smaragdina*, as well. Nestmate recognition, as noted, is a mechanism associated with territorial defense. In *O. smaragdina*, workers are in effect dimorphic: minor workers remain within leaf nests and care for brood whereas major workers patrol territory, attack intruders, and harvest food. Major workers show higher levels of territorial aggression than minor workers and have significantly higher levels of OA [unpublished observations]. These results suggest OA induces aggressive responses to alien ants or increases discrimination of non-nestmates, corroborating studies implicating OA in aggression, in *Drosophila* (Zhou et al., 2008) and crickets (Stevenson and Schildberger, 2013).

In *Camponotus fellah,* experimentally isolated workers appear to lose their colony-specific chemical signatures, but re-assimilation into their parent colony may be facilitated by trophallactic exchanges. OA may be involved in the regulation of this isolation-induced trophallaxis (Boulay et al., 2000). Ants isolated for five days and administered a high dose of OA exhibited trophallaxis for short durations, similar to those of non-isolated controls, whereas isolated control workers engaged in significantly longer durations of trophallaxis. Brain titers of monoamines were not quantified in these experiments to confirm the effect of pharmacological manipulations on OA levels. However, administration of phentolamine, an OA antagonist, returned durations of trophallactic exchanges to isolation control levels when given in combination with OA, suggesting that decreasing OA titers does increase isolation-induced trophallaxis. In *Formica japonica*, however, OA has the opposite effect and increased the rate of trophallaxis after workers were isolated for two days (Wada-Katsumata et al., 2011). In this species, OA could be reduced to control levels after contact with nestmates. These apparently opposing actions of OA in closely related species accentuate the need for further investigations.

Collective intelligence: neuromodulation of individual behavior and the organization of group decision making

Cooperative behaviors allow a colony to respond to environmental challenges that individual workers cannot effectively meet by acting alone. The collective intelligence of a colony – its capacity for accurate and precise group decision making – is hypothesized to emerge from interactions which integrate activities of simple individuals following basic behavioral algorithms (Camazine et al., 2001). Relying only on local information shared through communication signals, colonies behave as cohesive units, reducing individual errors and rendering rapid decisions despite the limited knowledge and experience of individual workers comprising the group. Workers lack an understanding of the global, fitness-enhancing objective, but nevertheless assemble collective actions through self-organization. The relationship of social interactions, colony-level behavior, and the neuromodulation of individual worker behavior is poorly understood.

Cooperative foraging in ants, one commonly studied system of collective intelligence, requires the behavioral integration of potentially thousands of workers to make adaptive decisions that maximize colony nutritional intake by harvesting the most profitable food resources or minimize energetic expenditures by selecting shorter foraging routes (Garnier et al., 2009). The role of monoamines in such collective behavior can be appreciated from an analysis of recruitment communication, which is initiated following the discovery of a new food source (reviewed in Hölldobler and Wilson, 1990). Workers unaware of the location of the food source are first recruited from the nest to assist in retrieving food; the number of workers arriving at the food source increases as more workers are recruited, and the food is eventually depleted or the colony becomes sated. Foraging then ceases.

The social organization of food recruitment and foraging varies among ant species, and includes basal and derived signaling systems. Tandem running is a simple form of recruitment communication, where a successful scout returns to the nest and leads a single nestmate to a new food source (Möglich et al., 1974). Physical contact and surface pheromones on the gaster of the leading ant maintain tandem movement by the pair. In group recruitment, an activating motor display delivered directly to nestmates upon contact in the nest and a trail pheromone that provides only an orientation cue are social signals required in combination to recruit ants. Mass recruitment, considered to be the most highly evolved mode of chemical recruitment communication in ants, involves the deposition of a pheromone trail between the food source and the nest; the chemical properties of the trail encode information necessary to both excite workers to depart from the nest and orient them to the food (Hölldobler and Wilson, 1990). As trail pheromone evaporates, traffic on the trail decreases, and the recruitment response of the colony and food collection are terminated. In this evolutionary model, nestmate stimulation and orientation are necessary to organize foraging. Either behavioral displays of successful scouts or perception of excitatory components of chemical trails could initiate orientation within the active space of the trail pheromone.

Tactile excitation (Anstey et al., 2009) and/or pheromonal stimulation may cause neurotransmitter release or workers may show age-related changes in neuromodulators to prime ants to initiate foraging (Seid and Traniello, 2005, Seid et al., 2008) or respond to trail pheromones (Muscedere et al., 2012). DA release, for example, is implicated in arousal in *Drosophila* (Kume et al., 2005) and leads to increased locomotion, and monoamines are known to modulate olfactory sensitivity (Kloppenburg and Hilderbrand, 1995). The level of biogenic amines in the brains of individual workers is thus critical to their ability to participate in foraging and is therefore significant in the organization of collective behavior. Aminergic control of task-related sensory responsiveness illustrates one neuroethological mechanism of collective decision making. The role of the metaphorical "colony brain" in emergent group behavior is therefore at least in part constructed from the neurochemistry of the brains of individual workers.

Couzin (2009) suggested ant workers are "mobile neural networks" and described parallels in neuron-like and neural network properties of workers that can be modeled from the characteristics of neurons and neural circuits to understand social network structure. Coordination of actions among ants could be modulated by exposure to different stimuli, stimulus intensities, and interactions among workers coupled physiologically by brain monoamine levels. The induction of gregariousness in desert locusts offers a simple model: tactile and visual stimuli from conspecifics increase brain levels of 5-HT, causing the transition from solitary to social life-history phases (Anstey et al., 2009). Locusts thus form a social swarm from solitary individuals due to spatial proximity. Boi et al. (1999) showed that spontaneously active ants can excite inactive ants, leading to cyclical periods of group activity in a nest that are not retained in workers outside the nest. Interactions among ants can lead to initiation of specialized labor within the colony (Duarte et al., 2011). Such synchronization of worker activity may be comparable to neuronal synchronization (Couzin, 2009): spontaneously firing neurons form an oscillating neuronal network creating cyclical periods of increased sensory processing (Buzsáki and Draguhn, 2004). We hypothesize that interactions among workers may cause neuron-like synchronization of behaviors that in turn synchronize monoamine titers in workers and contribute to the coordination of collective behavior. This neuromodulatory sychronization could lead to the alignment of worker behavior to format group activity. Some ants could act as "pacemaker neurons," setting periods of cyclical activity (Boi et al., 1999) and synchronizing biogenic amine titers. For example, when a successful forager returns to the nest, DA could be activated to synchronize trail pheromone responsiveness among workers having variable thresholds and thus coordinate the foraging effort. Synchronization of neurotransmitter release occurs in vertebrate brains (Pace-Schott and Hobson, 2002) and can be internally driven from the oscillating system or externally driven from a neural center (Buzsáki, 2006). Neural synchronization could parallel collective action within an ant colony. Driven by external sensory stimuli or internal "pacemaker" ants, monoamines could become synchronized within a group of ants and lead to coordinated collective action. There is sufficient interindividual variability in worker behavior to support this hypothesis (Robson and Traniello, 1999, 2002).

Research opportunities

The analysis of social behavior in ants can clearly benefit from studies of neurochemical regulation that integrate physiology, development, ecology, and evolution and thus provide models for the study of the neuromodulation of behavior in animal societies in general. Recent research indicates biogenic amines regulate several important social behaviors and serve significant functions in the genesis of individual behavior and complex social organization. This work provides a basis for rigorous experimental studies to advance our understanding of the role of monoamines in social organization and the emergence of collective action. Apparent contradictions in some results need to be resolved and key questions remain unanswered. In addition to the social behaviors discussed here that require continued and more detailed investigation, we present six additional areas of research that we believe will be highly insightful and productive. We note, however, that we are constrained by a lack of data that would allow the details of the phenomena we suggest be explored to be more readily identified and discussed in detail.

Gene expression and neuromodulation of task performance

Genes that modulate neurotransmitter action have been implicated in the regulation of social behavior in a variety of species (Caspi et al., 2002, Bortolato et al., 2011, Gurel et al., 2012). Although the upregulation of specific genes in ants has been related to task plasticity (Lucas and Sokolowski, 2009), *in situ* immunolabeling of monoamine receptors that function in the neuromodulation of task performance are limited to studies on honey bees (Hauser et al., 2006). Research linking gene expression and neuromodulation in ants will very likely yield results different from those of honey bees because ants are apterous, potentially strongly polymorphic in the worker caste, and diverse in species number and thus the manner in which brain design reflects adaptation. To date, the genomes for seven ants have been sequenced (Bonasio et al., 2010, Nygaard et al., 2011, Smith et al., 2011a, Smith et al., 2011b, Suen et al., 2011, Wurm et al., 2011), enabling evolutionary analyses of the genetics of social behaviors characteristic of species such as seed-harvesting *Pogonomyrmex* [Smith et al., 2011b] or activities related to the cultivation of symbiotic fungus in *Atta cephalotes* (Suen et al., 2011) and *Acromyrmex echinatior* (Nygaard et al., 2011). By sequencing the genome of *Pheidole dentata*, a species well-studied for its subcaste- and age-related division of labor, we can further our understanding of how monoamines modulate task specialization and explore its genetic basis. Characterization of genes involved in the regulation and function of ant biogenic amines will allow the development of pharmacological reagents to facilitate the development of experimental drug treatments and improve their reliability.

Genetic diversity, neuromodulation, and division of labor

Sociogenomic approaches to the study of the regulation of ant behavior can address a broad range of questions. Genetic diversity in colonies of different ant species, for example, varies with degree of polyandry (multiple mating by the queen) and polygyny (the presence of multiple queens in a colony) and is considered beneficial to the efficiency of a colony's ergonomic operations (Waibel et al., 2006). Behavioral specialization of groups – either castes or worker subcastes and age cohorts – within a colony may reflect genetic variation associated with worker patrilines (Evison and Hughes, 2011, Schluns et al., 2011, Huang et al., 2013, Smith et al., in press) that could code for differences in monoamine synthesis or receptors and thus the neuromodulation of behavior (Donaldson and Young, 2008, Krugel et al., 2009). Variation in monoamine action could be associated with patrilineal or matrilineal differences in worker response thresholds for task attendance, influencing sensitivity to cues or task-related pheromones and other social signals correlated with division of labor. Exploring intracolony genetic diversity could thus provide insight into the origin of neural and physiological mechanisms of social behavior.

Aminergic regulation of size- and age-related division of labor and the cost of brain metabolism

As we have discussed, the evolution of caste- and age-related behavioral specialization can dramatically reduce worker repertoire size and/or flexibility. This characteristic of ant division of labor is striking when one considers the behavioral multipotency of any solitary insect that searches for a mate, reproduces, forages, and rears offspring independently, and the plasticity of more generalist workers in a colony. Caste-specific behavioral adaptations, therefore, could lower metabolic costs associated with maintaining expensive neural tissue by reducing brain size and processing regions needed to support behavioral repertoires (Mares et al., 2005). Reduction in brain tissue could in turn lead to more simplified and specialized aminergic circuitry that either promotes or limits behavioral flexibility. For example, brain monoamine levels could cause increased sensitivity to task-related stimuli such as foraging and, conversely, a decreased ability to respond to stimuli that guide nursing or waste disposal. Our understanding of the relationship of task specialization, aminergic circuitry, and brain metabolism is currently limited.

Aminergic regulation of behavioral syndromes

Although division of labor is often linked to age and subcaste, consistent individual variation in behavior within a subcaste can also lead to task specialization (Jandt et al., 2013). Given that the vast majority of ants have a monomorphic worker caste, it is not surprising that behavioral syndromes may be present within colonies and that ants show personality differences (some workers behave more aggressively than others, for example). Colonies, as well as individual workers, can also exhibit behavioral syndromes, leading to varying abilities in territorial competition and thus access to resources (Jandt et al., 2013). While maturity of both individual workers and colonies and genetic variation within and among colonies may be implicated, the mechanisms underlying behavioral syndromes are largely unknown. Exploring the role of biogenic amines in behavioral syndromes will elucidate if and how amines modulate colony-level behavioral variation and worker personalities that are not solely age- and subcaste-related.

Phylogenetic analysis of the modulation of social behavior

Carlson (2012) recently argued that research in behavioral neuroscience – which has predominantly focused on studies of a limited number of traditional model systems –

could be made more insightful by evolutionary analysis. The great species richness of ants, which are a single monophyletic and ecologically dominant family (Moreau et al., 2006, Moreau and Bell, 2013), is sparsely represented in the current literature on ant neuromodulation. All of the species studied fall into only three subfamilies (Myrmicinae, Formicinae, Ponerinae) of a total of 25, leaving most groups unrepresented. Macroevolutionary trends in aminergic control and microevolutionary patterns therefore are currently not possible to identify due to a lack of relevant data. Broadening species sampling, comparing sister clades with contrasting degrees of social complexity, and adding phylogenetic analyses of ant social behavior and its control will allow independent contrasts and offer multiple systems to aid in distinguishing the relationship of aminergic regulation, evolutionary relatedness, ecology, social organization, and functional neuroplasticity. Research in this area has been beneficial in the study of biogenic amines and social decision making in vertebrate lineages (O'Connell and Hofmann, 2012). In the eusocial Hymenoptera, neuroanatomy has been contrasted in solitary and social bees (Mares et al., 2005), but few studies have examined the evolution of aminergic control of social behavior (Hauser et al., 2006). It is unknown how ancestral character states relate to derived functions of monoamines in ants, and how they have differentiated in respect to sociality. *Cataglyphis fortis*, for example, has served as a neuroethological model for detailed behavioral and neuroanatomical studies of solitary action (Stieb et al., 2010, Stieb et al., 2011), but neurochemistry has yet to be explored. Sister clade comparisons between the socially complex weaver ants, *Oecophylla*, and various species of *Formica*, which have strikingly lower degrees of complexity, could provide instructive models for comparative studies. By combining our understanding of the molecular phylogeny of ants (Moreau and Bell, 2013) with sociobiological and neuromolecular data, comparisons can be made across ant genera to reveal neuroethological trends in the evolution of caste division of labor and emergent colony intelligence. It is also possible to more deeply explore the origin of the aminergic control of ant social behavior through comparative studies of solitary wasps that show extensive parental care or other hymenopteran lineages, including semisocial and quasisocial forms, in which females may exhibit diverse and complex repertoires.

Monoamines and parasite manipulation of social host behavior

Diverse parasites use ants as hosts and manipulate worker behavior for their fitness advantage. The fungus *Ophiocordyceps unilateralis*, for example, infects *Camponotus leonardi,* causing workers to become "zombies" and descend from their arboreal nests, become disorientated, and latch onto a leaf in a "death grip" to promote fungal growth and dispersal (Hughes et al., 2011). The mechanisms by which parasites alter host behavior may involve quantitative and/or qualitative changes in brain monoamine levels (Skallova et al., 2006, Shaw et al., 2009). Ant behavior could change due to infection-related monoamine action caused by immune response to infection or substances of parasite origin that acts on the ant brain to affect neuromodulation. The neurogenetic architectures underscoring these parasite-induced alterations of brain chemistry could also be examined. Exploring parasite host systems and the neuromodulatory mechanisms underlying behavioral manipulation by parasites may also generally elucidate the role of monoamines in ant behavior.

Conclusions

Ants, as a diverse clade, provide numerous model systems in socioneuroethology. Workers possess relatively simple brain structures that nonetheless give rise to elaborate and adaptive individual and collective behaviors, enabling ants to establish themselves ecologically as "the little creatures that run the world." Understanding the role of monoamines in individual and collective actions performed by diverse species of ants can provide insight into the mechanisms by which biogenic amines can adaptively modulate social behavior. Examining neuromodulation in the ant brain will broadly inform our knowledge of mechanisms underlying behavior and neural circuitry.

Acknowledgements

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Figure 2.1: Reproductive caste and worker subcastes of the Australian weaver ant, *Oecophylla smaragdina*. Clockwise from the top: queen, winged male, mature minor worker, newly eclosed major worker. Not illustrated: winged gyne (virgin female) [Photo courtesy of J. Evans].

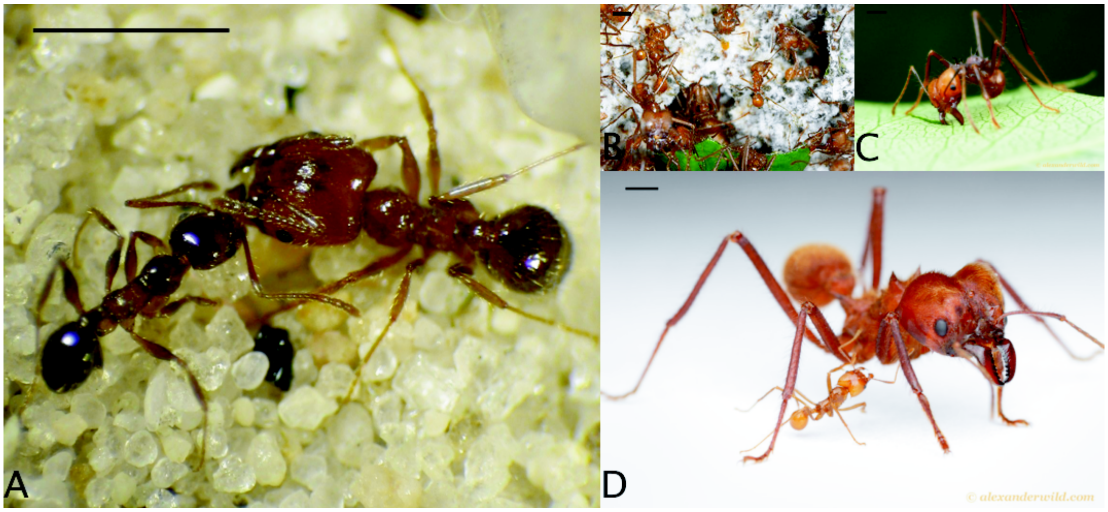

Figure 2.2: Polymorphism in worker castes. (A) *Pheidole dentata* minor and major worker (40x) and *Atta cephalotes* workers: (B) minim workers on fungal comb, (C) a media cutting a leaf, and (D) a minim and a soldier [1mm scale bars; *A. cephalotes* photos used by permission ©Alex Wild].

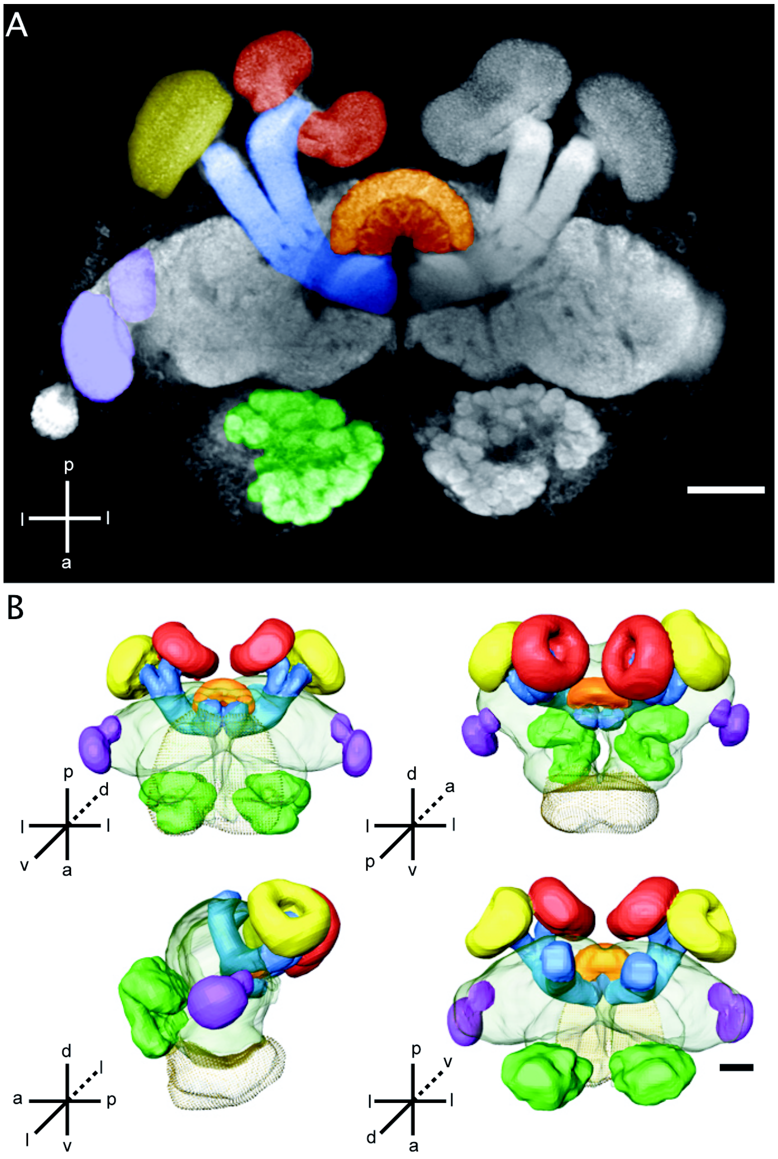

Figure 2.3: Ant brain structure. (A) Confocal image and (B) 3D reconstructions of the brain of a *Pheidole dentata* mature major worker stained with anti-synapsin. Brain regions are false colored. Optic lobe (purple), antennal lobe (green), mushroom body mid calyx (red), lateral calyx (yellow), and peduncle (blue), central body (orange). Subesophageal ganglion is not pictured (50 μm scale bar; image reprinted with permission from Muscedere and Traniello, 2012).

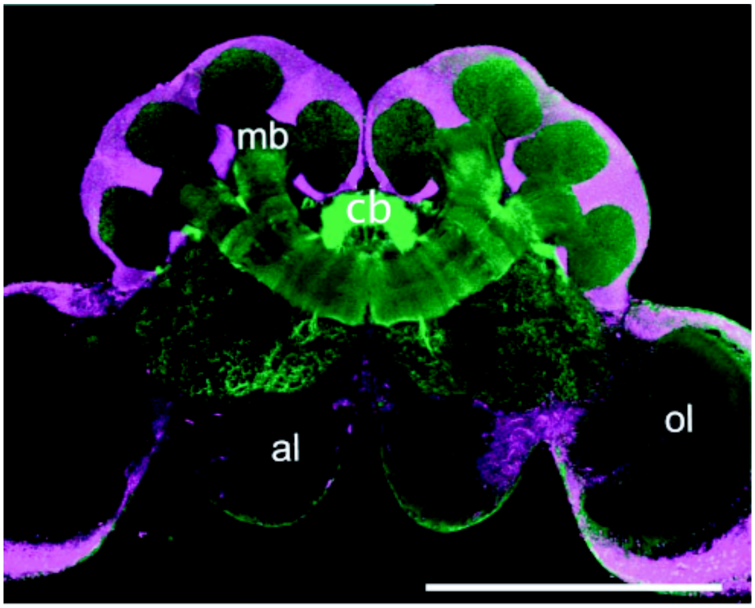

Figure 2.4: *Harpegnathos saltator* worker brain illustrating dopaminergic immunoreactivity (green) and prodium iodide labeled nuclei (red). Dopaminergic fibers project throughout the mushroom bodies (mb), heavily label the central body (cb), but do not innervate the optic (ol) and antennal lobes (al). 500μm scale bar (image reprinted with permission from Hoyer et al., 2005).

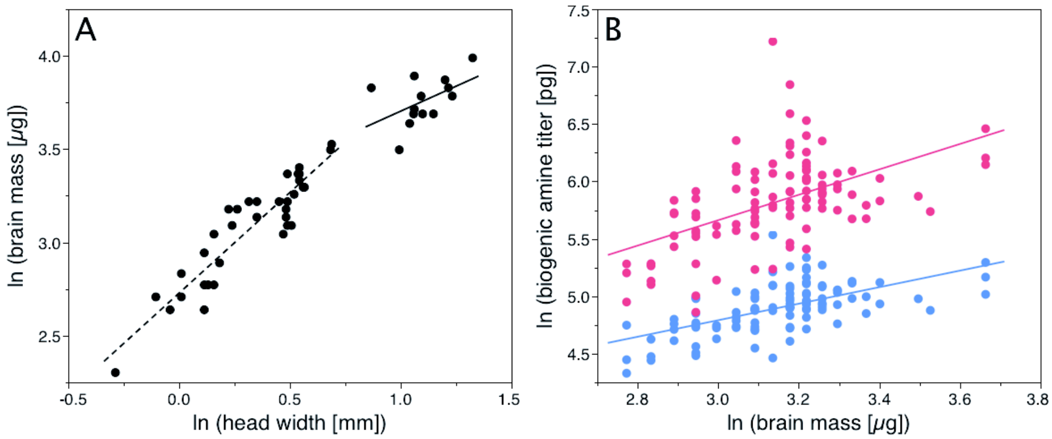

Figure 2.5: Brain scaling relationships in the leaf cutter ant *Atta cephalotes*. (A) Diphasic relationship between head width and brain mass in workers (larger: y= 0.53x+3.17 (R^2=0.25, solid line); smaller: y=1.08x+2.73 (R^2=0.85, dashed line). (B) DA (red) and 5-HT (blue) titers correlate with brain mass throughout the polymorphic range of workers (DA: y=1.11x+9.99 (R^2=0.27); 5-HT: y=0.72x+7.60 (R^2=0.37)).

CHAPTER THREE: POLYMORPHISM AND DIVISION OF LABOR IN A SOCIALLY COMPLEX ANT: NEUROMODULATION OF AGGRESSION IN THE AUSTRALIAN WEAVER ANT, *OECOPHYLLA SMARAGDINA*

Abstract

Complex social structure in eusocial insects can involve worker morphological and behavioral differentiation. Neuroanatomical variation may underscore worker division of labor, but the regulatory mechanisms of size-based task specialization in polymorphic species are unknown. The Australian weaver ant, *Oecophylla smaragdina*, exhibits worker polyphenism: larger major workers aggressively defend arboreal territories, whereas smaller minors nurse brood. Here we demonstrate that octopamine modulates worker size-related aggression in *O. smaragdina*. We found that the brains of majors had significantly higher titers of octopamine than those of minors and that octopamine was positively and specifically correlated with the frequency of aggressive responses to non-nestmates, a key component of territorial defense. Pharmacological manipulations that effectively switched octopamine action in major and minor worker brains reversed levels of aggression characteristic of each worker size class. Results suggest that altering octopamine action is sufficient to produce differences in aggression characteristic of size-related social roles. Neuromodulators therefore may generate variation in responsiveness to task-related stimuli associated with worker size differentiation and collateral behavioral specializations, a significant component of division of labor in complex social systems.

Introduction

Colony organization in complex insect societies is based on division of labor. Differentiation of sterile workers into polymorphic subcastes specialized on tasks according to morphology is widespread and has evolved independently in diverse eusocial insect taxa (Aoki, 1987, Hölldobler and Wilson, 1990, Crespi, 1992, Eggleton, 2011, Grüter et al., 2012). Some ant species have evolved morphological variation based on growth allometries in association with discretized and potentially size-constrained worker behavioral repertoires (Wilson, 1980, Mertl and Traniello, 2009). Although the number of polymorphic ant species is relatively small, striking physical caste structures characterize the most socially advanced and evolutionarily successful species including the fungus-growing ants, army ants, and members of the hyperdiverse genus *Pheidole* (Hölldobler and Wilson, 1990). Concomitant with increased behavioral specialization, size-variable workers may respond differently to social signals and cues (Wilson, 1985, Mertl and Traniello, 2009); this variation has a significant function in the collective organization and ecology of social insect colonies (Robinson, 1992). Worker polyphenism has been demonstrated to be under hormonal (Nijhout and Wheeler, 1982, Robinson et al., 1992) and genetic (Hughes et al., 2003, Schluns et al., 2011) control. Neuroanatomical differentiation accompanies polymorphism (Gronenberg et al., 1996, Muscedere and Traniello, 2012), and neuropeptides have been suggested to regulate division of labor (Castillo and Pietrantonio, 2013). The role of neuromodulators in worker size-based behavioral differentiation, however, remains poorly understood.

Biogenic amines such as dopamine (DA), serotonin (5-HT), and octopamine (OA) are neurotransmitters that affect sensorimotor systems and behavior in insects (Schulz et al., 2002, Libersat and Pflueger, 2004, Barron and Robinson, 2008, Anstey et al., 2009, Kamhi and Traniello, 2013). Recent investigations in ants suggest linkages between neurotransmitters and colony organization by correlating monoamine brain titers and social behavior (Cuvillier-Hot and Lenoir, 2006, Smith et al., 2013) or causally linking amines to behavior through pharmacological manipulations of brain neuromodulator levels (Falibene et al., 2012, Muscedere et al., 2012). Monoamine titers in the brains of worker ants are associated with age-related task performance (Seid and Traniello, 2005) and possibly genetic variation (Smith et al., 2013). Studies indicate that biogenic amines can be correlated with worker body size (Giraldo et al., 2013, Kamhi and Traniello, 2013, Smith et al., 2013), but their causal role in the genesis of behavioral diversity among polymorphic workers has not been demonstrated.

The morphological differentiation of worker subcastes in ants often involves variation in worker aggression in the context of defense of nest and territory (Powell and Clark, 2004, Mertl and Traniello, 2009). Worker phenotypes specialized on colony security are larger in body size, physically adapted for fighting (Wilson, 1980, Mertl et al., 2010), and quick to respond aggressively to threats from intruders. The Australian weaver ant, *Oecophylla smaragdina*, considered to be one of the most socially complex invertebrates (Crozier et al., 2010), can serve as a model to advance our understanding of the neuromodulation of worker size-related division of labor. *O. smaragdina*, which cooperatively construct arboreal nests from leaves “woven” together by silk produced by larvae, dominate rainforest canopy through their large colony size, social organization, and territoriality (Crozier et al., 2010). Weaver ant task specialization is based on worker size: small minor workers generally care for brood within nests whereas larger major workers participate in nest construction, foraging, and aggressively maintain exclusive territories by preventing intrusion from neighboring conspecific (Hölldobler, 1983, Crozier et al., 2010, Newey et al., 2010b, a) and interspecific (Majer, 1976) competitors. As many as ~500,000 workers inhabit a single colony, and territories may span 1500 m^2 (Hölldobler, 1983). The iconic aggressive threat posture of majors during territorial defense is marked by flaring the mandibles while raising the gaster (Figure 3.1). We tested the hypothesis that division of labor by morphologically differentiated workers in complex insect societies is regulated by biogenic amines. We examine variation in levels of monoamines between *O. smaragdina* minor and major workers and determined their role in controlling subcaste-specific differences in aggressive behaviors associated with territorial defense.

Methods

Colony collection and maintenance

O. smaragdina colonies were collected from ~1km^2 of savannah woodlands on the James Cook University campus in Townsville, Queensland, Australia. Depending on colony size multiple 19 x 10 x 13.5cm Fluon®-lined plastic boxes connected by plastic tubes or a single 52 x 30 x 40cm box were used to house colonies. Colonies were fed 1:3

honey water and flies or crickets every other day ad libitum and were kept on a 12:12 light dark cycle at 55% humidity and 25.5°C.

Aggression assays

Fully pigmented, mature major (average head width 1.57mm, range 1.31-1.71mm) and minor (average head width 1.02mm, range 0.93-1.16mm) *O. smaragdina* workers from two colonies were allowed to habituate in a 5 cm diameter closed petri dish for 1 minute. A non-nestmate conspecific mature major worker collected from a colony not adjacent to the colony of the focal worker was used as an intruder stimulus and was gently placed in the dish after removing a small segment of one pretarsus to distinguish focal ants. Behavioral categories were defined to characterize the full range of *O. smaragdina* aggressive actions (Carlin and Hölldobler, 1986, Newey et al., 2010b, van der Burg et al., 2014). Behavioral responses directed toward non-nestmates were quantified for 5 minutes. The number of times a focal worker performed a particular action was tallied according to the following categorization of aggression using a behavioral scale of 1-6, with 6 being the highest level of agonistic response:

1: non-aggressive behavior such as reversing direction to avoid a non-nestmate or not altering behavior when located within an antenna length from a non-nestmate

2: olfactory assessment, marked by antennae waving in the direction of a non-nestmate (Hölldobler and Wilson, 1990)

3: flaring the mandibles in the direction of a non-nestmate

4: adopting a threat posture directed toward a non-nestmate, characterized by an immediate stop, flaring the mandibles, and raising the gaster

5: lunging toward a non-nestmate a single time or several times in a rapid burst

6: prolonged biting of a non-nestmate

To compare response frequencies, we divided behaviors into three categories: non-aggressive behaviors, olfactory assessment, and aggressive actions (e.g. flared mandibles, startle posture, lunging, and biting), as described in our categorization. Within these categories, frequencies of behaviors performed were recorded and analyzed. To account for variation in activity levels and time spent interacting with non-nestmates, each worker was also given an aggression score calculated as the product of the number of times a worker performed an action in a given category and the level of aggression, divided by the total number of responses recorded during the assay (Newey et al., 2010b).

The degree of aggression between *O. smaragdina* and conspecifics may vary depending on colony proximity (Newey et al., 2010b), genetic relatedness (Newey et al., 2010a), and the intensity of ecological interaction between *O. smaragdina* and interspecific competitors (Majer, 1976). Therefore, we performed additional aggression assays with behaviorally mature major and minor workers from three weaver ant colonies and workers from queenright colonies of *Atta cephalotes*, an ant species that does not overlap geographically but nevertheless elicits a strong aggressive response. Using *A. cephalotes* workers, which are polymorphic, as a model stimulus also allowed us to match intruder body size to that of *O. smaragdina* major workers. A single *A. cephalotes* media worker was grasped with forceps (Dumont No. 5, Inox, standard) at the petiole and inserted through a 2mm hole in the side of a petri dish (5 cm diameter). A weaver ant worker was then placed in the closed dish. Ants acclimated for 5 minutes and were then observed for 10 minutes. Behaviors were quantified using the scale described above. All behavioral assays were filmed using a Canon FS400 camcorder. Petri dishes were cleaned with 70% ethanol between trials. Each focal ant was tested once and stimulus ants were not used more than 3 times.

Biogenic amine analysis

Immediately after interspecific assays were completed, worker head width was measured and brains of majors and minors were prepared to quantify monoamine titers using High Performance Liquid Chromatography with electrochemical detection (HPLC-ED). Workers from two colonies were used in these analyses. Brains were dissected from the head capsule in cold Insect Ringer's solution following established monoamine quantification methods (2012). Cuticle, which contains DA, was meticulously removed to ensure samples were not contaminated and brain concentrations of DA were thus accurately measured. Brains were then homogenized in a microcentrifuge tube containing 55μL of mobile phase solution (50mM citrate/acetate buffer, 1.5mM sodium dodecyl sulfonate, 0.01% triethylamine, and 22% acetronitrile in MilliQ water; modified from (Muscedere et al.) to optimize OA quantification) and kept on ice to prevent amine degradation. Solutions were centrifuged at 1500rpm for 10 minutes at 0°C and injected into the HPLC-ED system (model 584 pump, model 5020 guard cell, model MD-150 reversed-phase analytical column, model 5011A dual-channel coulometric analytical cell, and a Coulochem III electrochemical detector [ESA Inc., Chelmsford, MA]). Electrode potentials were 375mV for the first channel, which measured 5-HT and DA, and 600mV for the second channel, which measured OA. External standards of 5-HT, DA, and OA were run daily.

Workers from two colonies of *O. smaragdina* encompassing the full body size spectrum were analyzed separately to determine the relationship between head width and brain mass. Brains were dried for 48 hours and weighed on a microbalance (Sartorius CP 2P). The data displayed a linear trend (brain mass = [31.3 x head width] +13.45; $F_{1,39}=12.64$, $R^2=0.24$; $p<0.01$; $n=41$; Figure 3.S1). Environmental humidity and the limit of accuracy and resolution of the microbalance likely account in part for variability in brain measurements. Because brain sizes varied between and within subcastes, titers of biogenic amines were scaled to both head width and estimated brain mass.

Pharmacological manipulations of OA

Epinastine (EPN), a specific OA receptor antagonist (Roeder et al., 1998), was used to decrease OA action in mature majors. OA-specific synthesis inhibitors have not been developed and thus could not be employed to lower OA titer. To orally administer EPN, 30-50 workers were placed in a Fluon®-lined box and provided 10 mM EPN in a 3:1 honey water solution or 3:1 honey water as a control. Both solutions were dyed with red food coloring to confirm that ants had ingested drug-treated and control solutions. Only ants with red-colored fluid visible in the crop were tested. Dosage was determined in a series of trials that began with a concentration of 20mM EPN in 1:4 honey water

(Rillich and Stevenson, 2011). However, this solution produced a strongly aversive response and the dose was lowered to increase its palatability to majors. Intraspecific aggression assays were conducted using workers from two colonies 1-3 hours after solution ingestion as described above, except observation time was increased to 10 minutes for more robust behavioral quantification. Intraspecific assays were used because of their ecological relevance; they also produced similar results to those of interspecific assays. Additionally, worker activity was assayed to determine whether gross locomotion was systemically affected by treatments. Workers were placed in a closed petri dish (8.5 cm diameter) with lines dividing the bottom into four equal quadrants; ants were allowed 1 minute to habituate. Worker movement among quadrants made during a 5-minute period was recorded to quantify activity.

To increase brain levels of OA, 0.5μL of 10.5mM OA in dimethylformamide (DMF) was topically applied to the thoraces of mature minors from two colonies following methods established in honey bees (Barron et al., 2007). The amount of solution applied was reduced due to the smaller body size of minor workers. OA application was used rather than a receptor agonist to allow quantification of brain OA titers, ensuring titers were elevated after treatment. Controls received topical applications of 0.5μL DMF. The timing of behavioral assays and dissections were based on sampling times described in (Barron et al., 2007). Minors were tested 15-30 minutes after treatment. Intraspecific aggression assays and activity assays were conducted as described in the EPN experiment. Immediately after testing, ants were placed on ice. Treated workers were repeatedly washed in MilliQ water to rinse any OA potentially remaining on the cuticle and minimize contamination. Brains were then dissected and prepared for HPLC-ED analysis 45-60 minutes after treatment. Biogenic amines were measured as described above, except the electrode potential for the first channel was 425 mV and 600 mV for the second channel. Changes in sensitivity associated with routine equipment maintenance prevented the accurate comparison of absolute amine titers in interspecific and OA manipulation assays.

Statistical analysis

Aggression scores and the frequency of behaviors in response categories (non-aggressive behaviors, olfactory assessment, aggressive actions) of subcaste or treatment groups were compared using non-parametric Mann-Whitney U tests. Simple linear regression analyses were used to determine the relationship between amine titer and the natural log of the frequency of behaviors in each category. We added one to all frequencies to correct for frequencies of zero within behavioral categories (Box and Cox, 1964). We used Bonferroni correction (Rice, 1989) for multiple comparisons of behavior frequencies and simple linear regression analyses: $p<0.017$ and $p<0.0056$, respectively, were considered statistically significant. Biogenic amine titers and movement assays were compared with Student's t-tests. Summary statistics are presented as mean±95% confidence intervals and all statistical analyses were performed using JMP Pro 11 statistical software.

Results

Worker subcaste-related aggression in response to intraspecific and interspecific ants

When presented with non-nestmate conspecifics, majors had significantly higher aggression scores than minors (major: 2.57±0.44, minor: 2.18±0.3; Mann-Whitney U, $W_{20,20}$=315.5, $p<0.05$; Figure 3.2a). Majors showed a significantly higher frequency of aggressive behaviors (major: 3.10±1.5, minors: 1.25±1.06; $W_{20,20}$=302.5, $p<0.01$; Figure 3.2b); there were no subcaste differences in the frequency of non-aggressive behaviors (major: 2.7±2.93, minor: 1.05±0.77; $W_{20,20}$=392, $p=0.56$) or olfactory assessment (major: 7.2±2.72, minor: 6.8±3.67; $W_{20,20}$=366.5, $p=0.24$).

In interspecific assays major worker aggression scores were significantly greater than those of minors (major: 3.50±0.61, minor: 1.76±0.53; Mann-Whitney U test, $W_{17,23}$=323, $p<0.001$; Figure 3.2c). Majors exhibited aggressive displays more frequently than minors (major: 8.76±2.66, minor: 2.71±1.27; $W_{17,23}$=361, $p<0.001$; Figure 2d). There were no subcaste differences in the frequency of non-aggressive behaviors (major: 5.24±3.18, minor: 9.0±4.16; $W_{17,23}$=324.5, $p=0.39$) or olfactory assessment (major: 4.24±2.12, minor: 3.46±1.45; $W_{17,23}$=484.5, $p=0.60$).

Brain biogenic amine titers and behavioral responses to interspecific intruders

Absolute titers of all biogenic amines were significantly higher in majors than minors (DA: major: 676.72±59.15 pg, minor: 507.55±40.42 pg; 5-HT: major: 398.25±16.92 pg, minor: 304.60±12.56 pg; OA: major: 296.37±14.79 pg, minor: 181.71±14.79 pg; t-test, all $p<0.001$; Figure 3.S2). OA level, controlled for brain size, was significantly different between subcastes (major: 4.73±0.28 pg/μg, minor: 3.89±0.18 pg/μg; t-test, t_{28}=5.39, $p<0.001$; Figure 3.3); on average, majors had 19.5% higher OA levels than minors. DA and 5-HT titers of majors and minors were not significantly different when scaled to brain mass (DA: major: 10.79±0.87 pg/μg, minor: 10.87±0.88 pg/μg; t_{28}=0.13, p=0.89; 5-HT: major: 6.35±0.22 pg/μg, minor: 6.52±0.26 pg/μg; t_{28}=1.03, p=0.31). There were no significant relationships between behavior and either DA or 5-HT (all $p>0.02$). There were no relationships between the frequency of non-aggressive behaviors and olfactory assessment recorded and OA titer (Simple linear regression, $F_{1,28}$=0.04, p=0.84 and $F_{1,28}$=1.53, p=0.23, respectively). Only the frequency of aggressive behaviors showed a significant positive correlation with OA level ($F_{1,28}$=27.33, R^2=0.49, $p<0.001$; Figure 3.4). When scaled to head width, OA was higher in majors than minors (major: 188.55±11.21, minor: 170.87±7.95; t_{28}=2.76, $p<0.05$; Figure 3.S2). DA level was similar between subcastes (major: 430.37±34.82, minor: 477.82±39.25; t_{28}=1.94, p=0.063), whereas 5-HT was significantly higher in minors than in majors (major: 253.54±9.39, minor: 286.66±11.95; t_{28}=4.68, $p<0.001$). Only the positive correlation of OA titer scaled for head width and frequency of aggressive behavior was significant ($F_{1,28}$=11.49, R^2=0.29, $p<0.005$; all other $p>0.01$).

Effects of EPN treatment on behavioral responses of major workers

EPN-treated majors had significantly decreased aggression scores compared to those of control majors (control: 3.08±0.34, EPN: 2.64±0.38; Mann-Whitney U test, $W_{17,20}$=258.5, $p<0.05$; Figure 3.5a). With respect to the frequency of behaviors, both groups showed similar levels of non-aggressive acts (control: 0.70±0.57, EPN: 0.82±0.78; $W_{17,20}$=376, p=0.89; Figure 3.5b), olfactory assessment (control: 6.30±1.33, EPN: 7.24±2.34; $W_{17,20}$=373, p=0.83), and aggressive behaviors (control: 12.70±8.95, EPN: 6.35±4.2; $W_{17,20}$=274, p=0.13). Workers in both groups showed similar activity levels (control: 16.10±9.44, EPN: 23.18±7.96; t-test, t_{35}=1.18, p=0.25).

Effects of OA treatment on biogenic amine titers and behavioral responses of minor workers

OA-treated minors had significantly increased titers of brain OA compared to control minors when correcting for brain mass (control: 5.11±1.14 pg/μg, OA-treated: 8.00±1.76 pg/μg; t-test, t_{31}=2.96, p<0.01); the average increase in brain OA level was 44% percent. DA and 5-HT levels were similar between groups (DA: control: 10.47±0.87 pg/μg, OA-treated: 10.22±0.82 pg/μg, t_{37}=0.45, p=0.65; 5-HT: control: 6.06±1.07 pg/μg, OA-treated: 5.56±0.47 pg/μg, t_{37}=0.92, p=0.36). Similar relationships were found between absolute biogenic amine titers and titers scaled to head width (Table 3.1; Figure 3.S3). OA-treated minors were significantly more aggressive than controls, with higher aggression scores (control: 2.49±0.28, OA-treated: 2.74±0.19; Mann-Whitney U test, $W_{20,20}$=335, p<0.05; Figure 3.5a) and an increased frequency of aggressive acts (control: 10.45±5.02, OA-treated: 16.70±4.59; $W_{20,20}$=318, p<0.05; Figure 3.5c). Frequencies of non-aggressive behaviors (control: 2.70±2.05, OA-treated: 1.65±0.89; $W_{20,20}$=396.5, p=0.70) and olfactory assessment (control: 14.65±3.79, OA-treated: 13.35±3.5; $W_{20,20}$=396.5, p=0.71) were similar between groups. Workers in both groups showed similar activity levels (control: 15.59±8.83, OA-treated: 8.95±4.88; t-test, t_{36}=1.44, p=0.16).

Discussion

Morphological subcastes and behavioral differentiation have coevolved in social insects (Wilson, 1953). To understand the control mechanisms associated with division of labor by physical caste we assayed major and minor worker aggression in the context of intruder recognition and territorial defense, quantified monoamine neurotransmitter levels in individual worker brains, and pharmacologically altered monoamine action. Using a socially complex ant species as a model, we determined that OA can act as a modulator of task specializations characteristic of worker physical castes. Major workers of *O. smaragdina*, renowned for their stereotypical territorial aggression, had significantly higher titers of OA than minors, whose labor appears to be primarily focused on within-nest tasks such as nursing. 5-HT was significantly higher in minors when scaled to head width. However, only OA titer scaled to estimated brain mass was positively and specifically correlated with frequencies of aggressive responses triggered by nestmate recognition stimuli that allow territorial intruders to be detected. DA and 5-HT did not appear to regulate aggression, olfactory assessment, or non-aggressive responses toward intruders. These results could be explained by a direct effect of OA on aggression, an effect of OA on nestmate recognition (Vander Meer and Morel, 1998), and/or a positive effect of aggression on OA expression (Adamo and Baker, 2011, Rillich and Stevenson, 2011), all supporting a function of OA in the task specializations of polymorphic weaver ant workers. Effectively switching OA action in major and minor worker brains by pharmacological treatments reversed the aggression level characteristic of each worker subcaste. Although we cannot rule out the possibility that aggressive encounters caused an increase in OA titers (Rillich and Stevenson, 2011), we nonetheless provide pharmacological evidence that OA contributes to the behavioral differentiation of major and minor workers with respect to aggressive behavior. Our study is the first to demonstrate that neuromodulators can underscore size-related variation in worker responsiveness to task-related stimuli – a core element of division of labor for socially complex polymorphic insects.

The results of our pharmacological manipulations of OA in both major and minor workers suggest that OA specifically impacts aggressive behavior without affecting general activity. We are confident that using interspecific assays for correlative analyses and intraspecific assays involving drug manipulations did not bias our results or interpretation because significant subcaste differences were seen in both assays. The use of an OA antagonist to block monoamine action and OA topical application to increase monoamine titer produced results consistent with those of studies in other insects: our treatments therefore effectively altered OA action in the manner intended (Roeder et al., 1998, Barron et al., 2007). Majors treated with EPN had lower aggression scores than controls, suggesting that inhibiting OA action decreased aggressive behavior. There was no difference in the frequency of aggressive actions between treatment groups, which may be due to a change in the ratio of aggressive to non-aggressive acts rather than a change in the absolute frequency of aggressive behaviors. Inter-individual variation in both groups may also lead the discrepancy. Variation in the experimental group could have been a result of majors ingesting different amounts of EPN, which we could not quantify. Minors with increased OA titers exhibited higher aggression scores than controls and had a greater frequency of aggressive acts. Non-aggressive actions, olfactory assessment, and rates of movement were similar across treatment groups indicating that increasing OA specifically increased aggressive actions and did not affect overall activity or behavioral responsiveness. Contrasting with our results, OA has been shown to affect general activity in other insects (Stevenson et al., 2005, Fussnecker et al., 2006). However, worker activity during the aggression assay, which was not measured, may be affected by OA.

The causal relationship between OA and aggression in *O. smaragdina* could be due to an enhanced ability to recognize non-nestmate cuticular hydrocarbon profiles, an effect on motor outputs generating aggression (Stevenson et al., 2005), or both. These behaviors are closely linked in ants: nestmate recognition enables workers to distinguish colony-specific chemical signatures and is therefore crucial for defending nest and territory from intruders (Vander Meer and Morel, 1998). OA has previously been implicated in both nestmate recognition and aggression in insects and other invertebrates (Stevenson et al., 2005, Hoyer et al., 2008, Vander Meer et al., 2008, Zhou et al., 2008, Jones et al., 2011, Kamhi and Traniello, 2013, Simpson and Stevenson, 2015). The causal coupling of OA and aggression toward non-nestmates in *O. smaragdina* worker subcastes is consistent with the conserved neuromodulatory function of OA. However, the role of biogenic amines in modulating aggression is complex and varies among invertebrates (Kravitz, 2000, Alekseyenko et al., 2010, Okada et al., 2015). For example, the role of 5-HT in decreasing aggression in some species (Kravitz, 2000, Alekseyenko et al., 2010) is supported by our data, which show significantly higher 5-HT titers in minors than majors when scaled to head width. 5-HT may be modulating behaviors associated with decreased aggression such as retreat or reduced impulsivity (Simpson and Stevenson, 2015). Allometric relationships of brain mass and head width may account for the observed inconsistencies in amine titer comparisons between subcastes. The role of monoamines in the control of social behavior in ants and other eusocial insects is unclear, largely due to the sampling of only ~10 of the more than 14,000 described species of ants and the lack of broader phylogenetic analysis of the evolution of aminergic systems in the Hymenoptera.

Aminergic systems affected by gene expression (Tsalik et al., 2003) could regulate the behavioral differentiation that accompanied the evolution of physical castes and task specialization in eusocial insects (Young et al., 1999, Smith et al., 2013). The mechanistic relationships between genes, neuromodulators, and behavioral specializations associated with worker morphology, however, are poorly understood. Genetic techniques developed in *Drosophila* have targeted specific aminergic circuitry, enabling detailed analyses that may provide insight for ants (Alekseyenko et al., 2010, Andrews et al., 2014). Direct relationships between genes and phenotypic differentiation are nevertheless not well defined in ants. Gene expression has been suggested to control subcaste differences in task performance and plasticity (Hughes et al., 2003, Corona et al., 2013, Libbrecht et al., 2013). In the completely dimorphic ant *Pheidole pallidula* changes in foraging or defense in major workers are correlated with the expression of the *ppfor* protein in a small number of cells; this does not occur in minors (Lucas and Sokolowski, 2009). Downstream neurobiological effects contributing to division of labor between subcastes and the higher defensive aggression shown by major workers have not been determined in *P. pallidula* or other ants; however, studies on honey bees suggest the nature of the mechanisms involved (Alaux et al., 2009).

Morphology, neuroanatomy, and behavior are interrelated in brain evolution (Farris and Roberts, 2005, Striedter, 2005, Ilies et al., 2015). Subcaste differences in macroscopic and cellular neuroanatomy may interact with neuromodulators to generate specialized behavior in social insects. In *O. smaragdina* the relative sizes of brain compartments that function in the primary processing of olfactory signals and cues and visual information are larger in majors, reflecting their subcaste-specific sensory ecologies [Kamhi and Traniello, unpubl. data]. OA receptor expression is likely widespread throughout the neuropil (Sinakevitch et al., 2011) and receptor expression density, which varies by brain region, could correlate with social role (Reim and Scheiner, 2014). Differences in relative investment in brain regions may therefore affect OA receptor profiles and consequently OA-regulated tasks. The association of serotonergic circuitry with subcaste behavioral differentiation in the dimorphic ant *Pheidole dentata* supports this model. In comparison to minor workers, defensive majors have more 5-HT-immunoreactive cells in primary visual regions, more extensive serotonergic varicosities in sensory and integrative brain regions (Seid et al., 2008), and more elaborate axonal arborization in a serotonergic neuron integral to sensory integration (Giraldo et al., 2013). Although relationships between neuromodulators and circuit development have been described in other taxa (Shakiryanova et al., 2011, Neckameyer and Bhatt, 2012), the influence of neuroanatomical differences in 5-HT circuitry on 5-HT signaling and behavior remains to be studied in ants. In *O. smaragdina,* major and minor worker differences in endogenous OA titers may alter circuitry to generate subcaste-related aggression or workers may retain behavioral flexibility through similar distributions of OA receptors (Young et al., 1999) that enable responses to changing titers of OA acting on neural circuits underscoring aggression despite differences in endogenous OA titers and brain compartment sizes. Behavioral differentiation of polymorphic workers may thus be based on variation in neuromodulator levels to parsimoniously produce worker size-related task responsiveness and thus distributed intelligence at the colony level.

Acknowledgements

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Figure 3.1: *Oecophylla smaragdina* major worker. Mandibles are flared and the gaster is raised in a threat posture (Photo courtesy of P. Smallhorn-West).

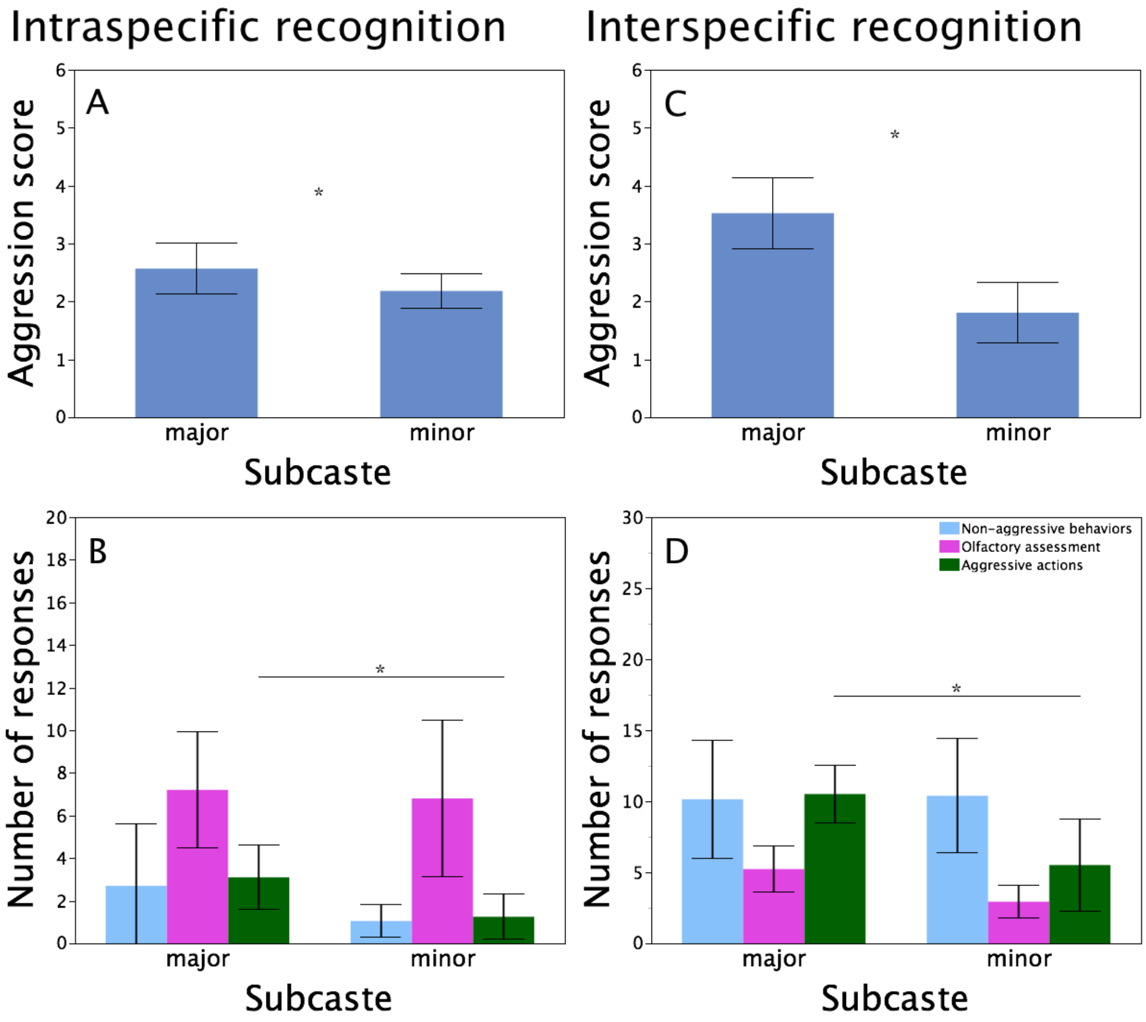

Figure 3.2: Average aggression scores (A, C) and frequency of responses in three behavioral categories (B, D) are presented for intraspecific assays (A, B) and interspecific assays (C, D) for major and minor workers. Error bars indicate 95% confidence intervals and asterisks denote statistical significance.

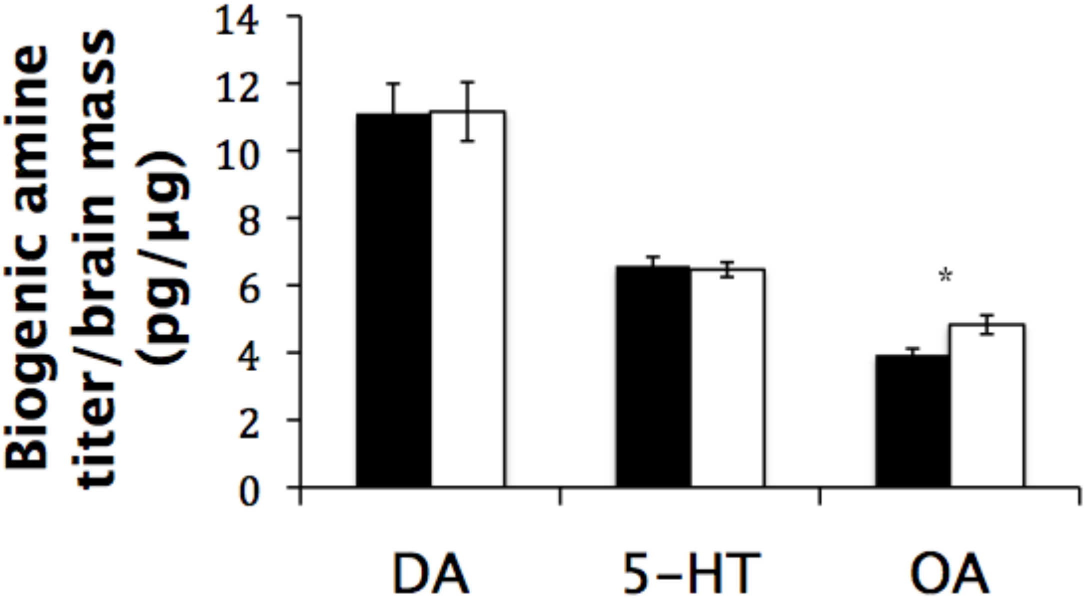

Figure 3.3: Mean ± 95% confidence intervals of dopamine (DA), serotonin (5-HT), and octopamine (OA) brain titers corrected for estimated brain mass are presented for minors (black) and majors (white). Asterisk denotes statistical significance.

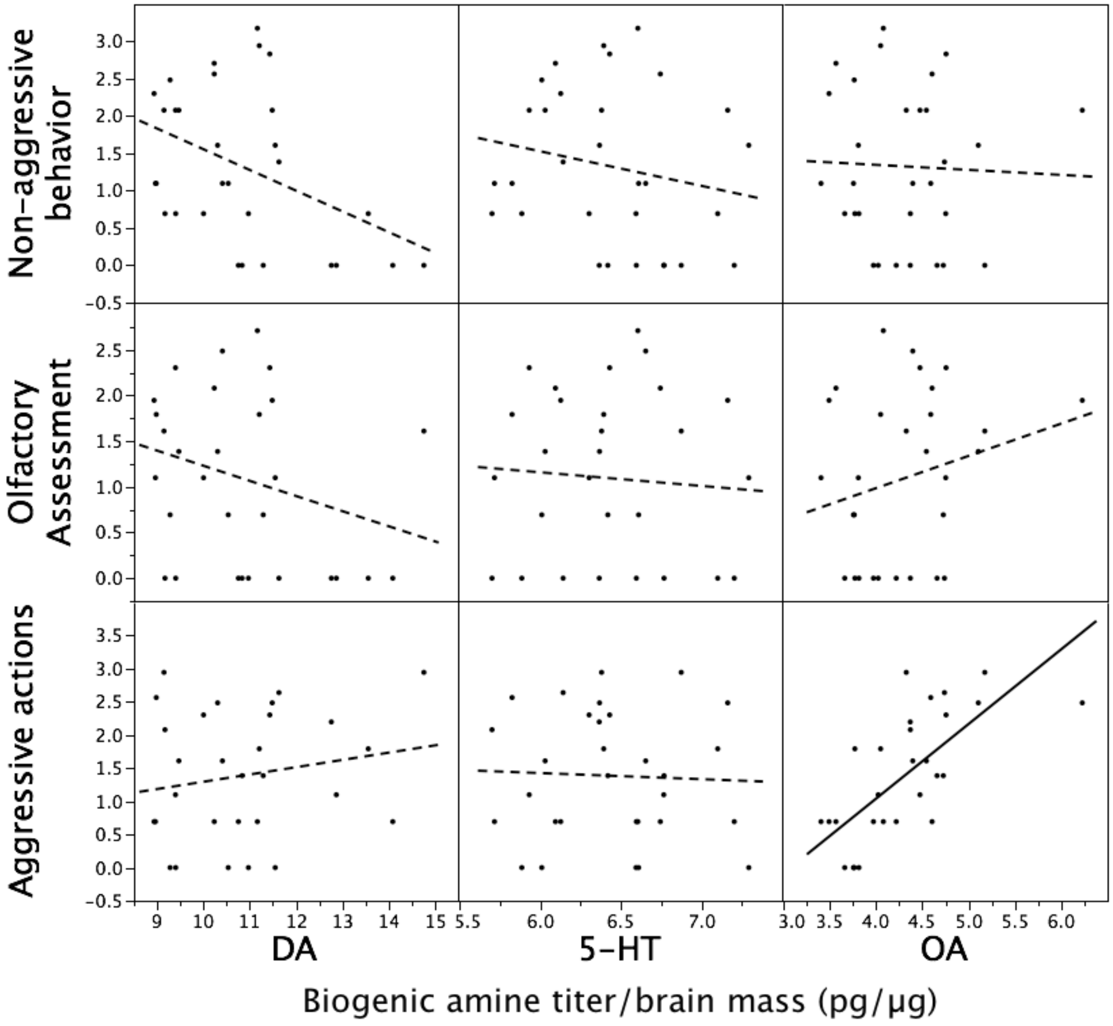

Figure 3.4: Best-fit regression lines of dopamine (DA), serotonin (5-HT), and octopamine (OA) titers in both majors and minors and the number of times a worker performed non-aggressive behaviors, olfactory assessment, and aggressive action (ln). Solid line indicates regression of statistical significance.

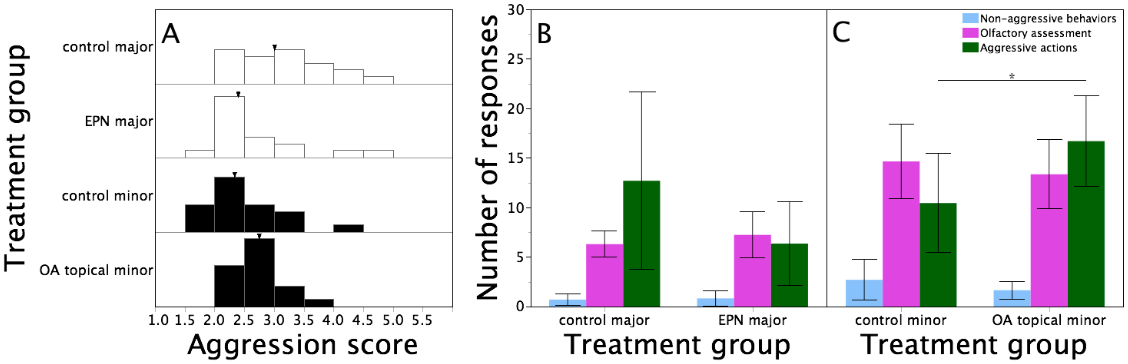

Figure 3.5: Distribution of aggression scores for control and pharmacologically treated majors (white) and minors (black) are presented with arrowheads indicating mean (A). The mean frequencies of behaviors are shown in control and epinastine (EPN)-treated majors (B) and control and OA topically treated minors (C). Error bars indicate 95% confidence intervals and asterisks denote statistical significance.

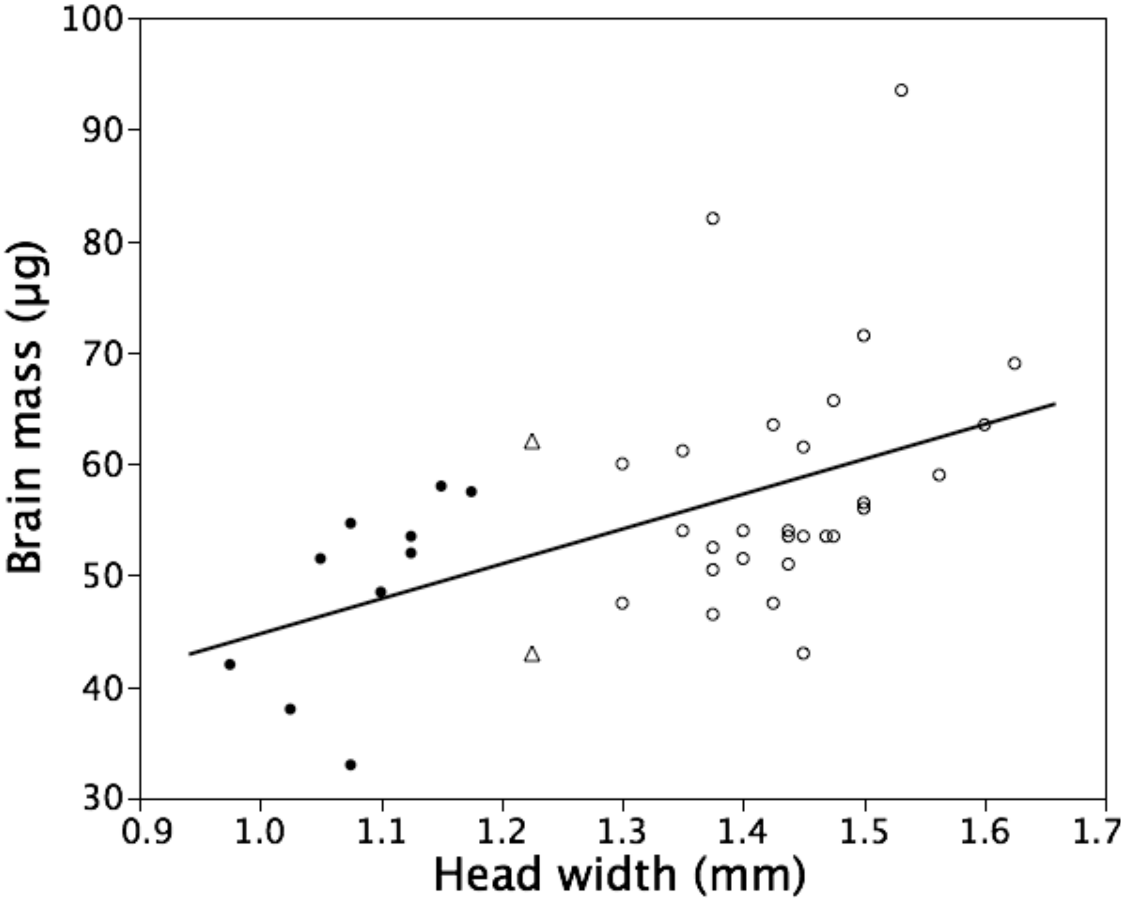

Figure 3.S1: Best-fit regression line of head width and brain mass of *O. smaragdina* minor (black) and major (open circles) workers. Workers of intermediate head width are labeled with open triangles.

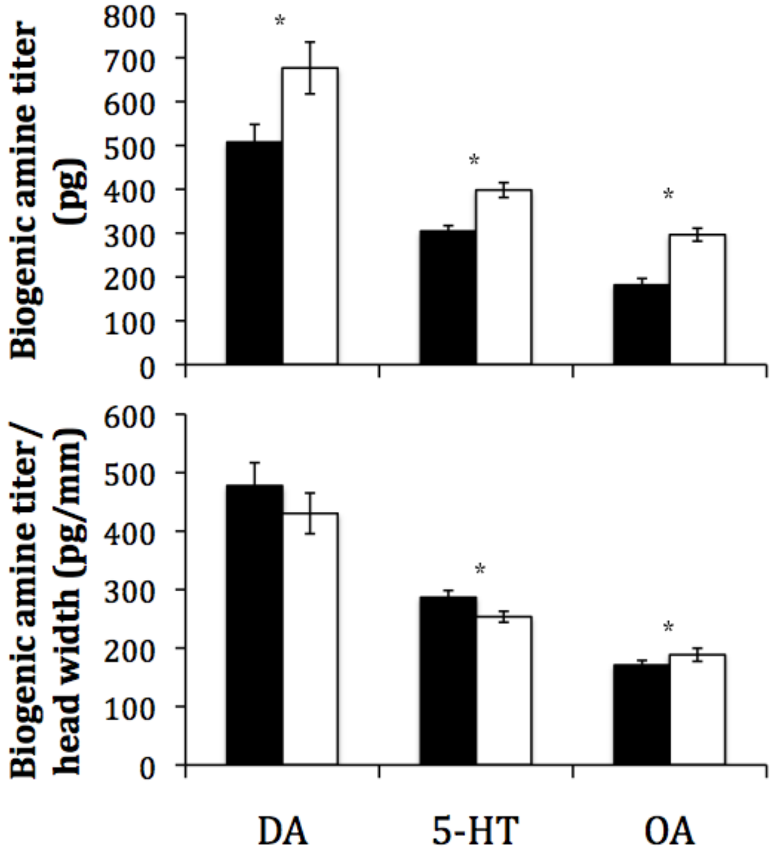

Figure 3.S2: Mean ± 95% confidence intervals of dopamine (DA), serotonin (5-HT), and octopamine titers (top) and titers corrected for head width (bottom) for minor (black) and major (white) workers. Asterisks denote statistical significance.

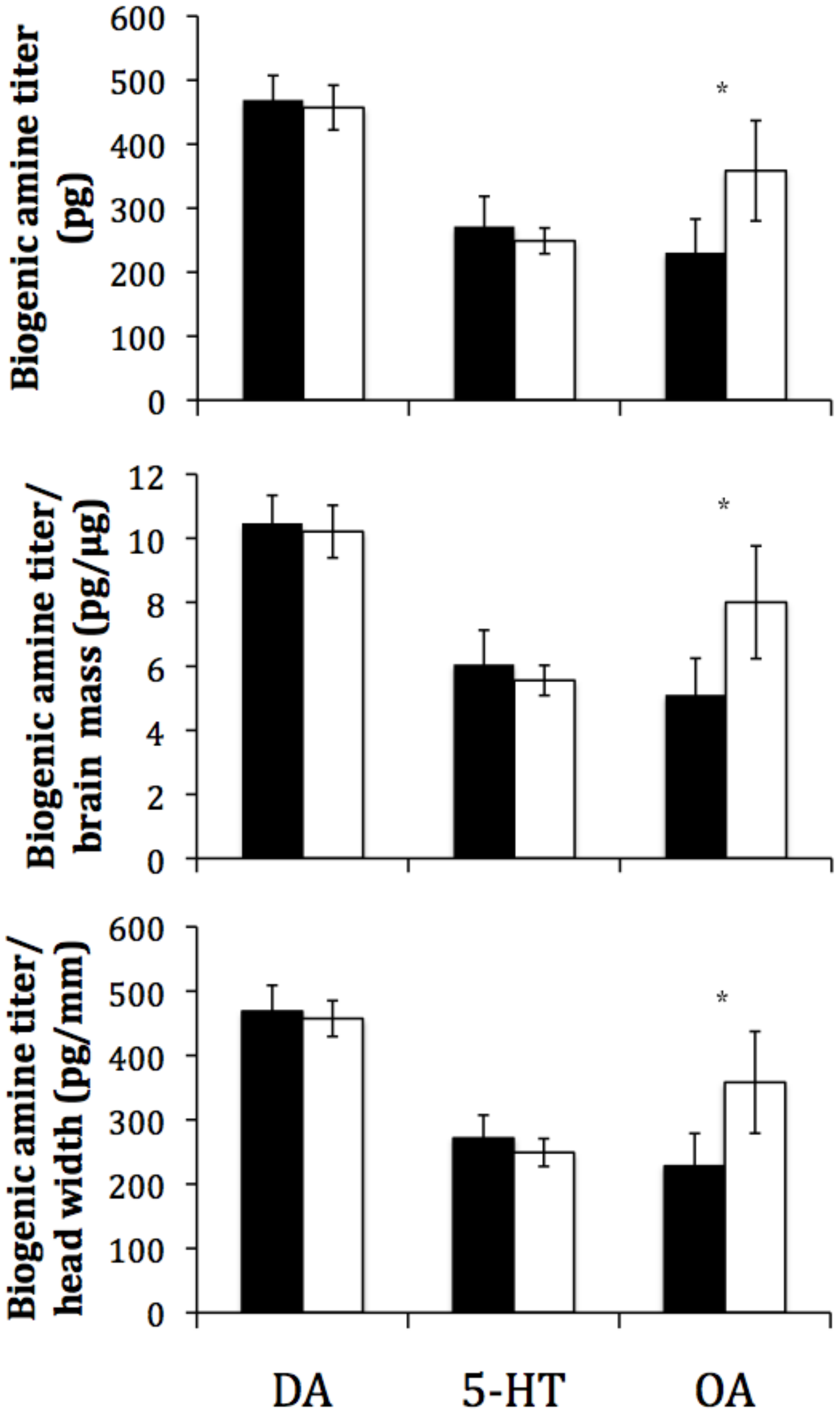
Biogenic amine titer (pg)
600
500
400
300
200
100
0
*
Biogenic amine titer/ brain mass (pg/µg)
12
10
8
6
4
2
0
*
Biogenic amine titer/ head width (pg/mm)
600
500
400
300
200
100
0
*
DA
5-HT
OA

Figure 3.S3: Mean ± 95% confidence intervals of dopamine (DA), serotonin (5-HT), and octopamine (OA) titers (top), titers corrected for estimated brain mass (Cheng et al.), and corrected for head width (bottom) for control (black) and OA topically treated (white) minors. Asterisks denote statistical significance.

	Biogenic amine	**Control**	**OA-treated**	**t-statistic**	**p-value**
Absolute titer (pg)	**DA**	469.14±38.11	457.03±34.92	0.49	0.63
	5-HT	271.45±47.10	248.93±20.08	0.94	0.35
	OA	230.56±52.43	358.49±78.39	2.92	**<0.01**
Titer/brain mass (pg/µg)	**DA**	10.47±0.87	10.22±0.82	0.45	0.65
	5-HT	6.06±1.07	5.56±0.47	0.92	0.36
	OA	5.11±1.14	8.00±1.76	2.96	**<0.01**
Titer/head width (pg/mm)	**DA**	468.73±40.3	457.37±37.87	0.43	0.67
	5-HT	271.42±35.78	249.10±21.59	0.91	0.37
	OA	228.06±50.72	358.17±79.21	2.98	**<0.01**

Table 3.S1: For control and OA-treated minors, the mean ± 95% confidence intervals of dopamine (DA), serotonin (5-HT), and octopamine (OA) titers, titer corrected by brain mass, and titer scaled by head width are presented. T-statistics and p-values are also shown. Bolded p-values indicate statistical significance.

CHAPTER FOUR: SOCIAL COMPLEXITY INFLUENCES BRAIN PRODUCTION AND OPERATION COSTS IN ANTS

Abstract

The metabolic expense of producing and operating neuropil required for adaptive behavior is considered to be a significant selective force in brain evolution. However, the relationship between brain size and social complexity in eusocial insects such as ants is poorly understood. Social complexity in ants is characterized by large colony size, collective action, and division of labor, which could lower cognitive demands and reduce metabolic costs by enabling workers to invest in neuropils specific to the requirements of their particular task performance. To investigate the extent to which brain size (production) and metabolic (operation) costs correspond to social complexity and associated task specializations related to division of labor we analyzed brain compartments involved in sensory, integrative, and motor processing in brains of workers of the Australasian weaver ant *Oecophylla smaragdina*, a paradigm of advanced social life in invertebrates, and the socially basic *Formica subsericea*. *O. smaragdina* form large, polymorphic colonies and partition labor between large-bodied, behaviorally diverse, and collectively intelligent major workers and smaller minor workers that specialize in brood care. *F. subsericea*, in contrast, live in small, monomorphic colonies, and workers appear to engage in little collective action. We compared brain mass, neuron size, number, and density, cell rind and neuropil volumes, and cytochrome oxidase (COX) activity, an endogenous marker of ATP production and thus neurometabolism. Contrary to our hypothesis, *O. smaragdina* major and minor workers

had significantly larger brains, including larger higher-order processing compartments, than *F. subsericea* workers when scaled to body size. *O. smaragdina* majors, however, had reduced COX activity in the whole brain and in higher-order processing regions compared to the other two groups. As predicted, the volumes of primary sensory and motor neuropils and their patterns of ATP usage reflected cognitive demands of worker task repertoires in both species. Our study suggests that larger colony size may be associated with increased worker brain size and that collective action may be associated with reduced brain operations costs.

Introduction

The costs of producing and operating neural tissue needed for socially and ecologically adaptive behavior are critically important to brain evolution. Although brains are metabolically expensive (Aiello and Wheeler, 1995, Laughlin et al., 1998), brain size has been found to increase with group size in vertebrates due to the greater cognitive challenges associated with more complex social life (Dunbar and Shultz, 2007a). Eusocial insects such as bees, wasps, ants, and termites also form large, complex societies, but workers are typically sterile and their labor benefits the fitness of the colony as a whole. Reproductive competition, mate selection, and pair bonding, which are considered to be among the primary drivers of brain evolution in vertebrates (Dunbar and Shultz, 2007a), are thus absent or reduced in most eusocial insects. Although behavioral challenges do not appear to be similar to those that characterize large vertebrate societies, social interactions among workers in large colonies may nevertheless involve social selection that may impact brain evolution. For example, groups of workers can rapidly render decisions that are more precise and accurate than those of individuals (Seeley, 2010, Sasaki and Pratt, 2011, Jeanson et al., 2012, Sasaki and Pratt, 2012, Sasaki et al., 2013), suggesting that fitness benefits in insect societies can accrue from collective intelligence. Our understanding of the impact of social complexity, decentralized organization, collective and/or distributed intelligence, and other colony-level processes contributing to social complexity on brain size and structure, neurochemistry, and metabolism is very limited (Muscedere and Traniello, 2012, Kamhi and Traniello, 2013, Ilies et al., 2015, O'Donnell et al., 2015).

Large colony size and division of labor (Thomas and Elgar, 2003, Holbrook et al., 2011, Ferguson-Gow et al., 2014), sophisticated communication systems (Hölldobler and Wilson, 1978, Billen and Morgan, 1998, Hölldobler, 1999), emergent collective action (Sumpter, 2006, Couzin, 2009), and ecological dominance (O'Donnell, 1998, Kronauer, 2009, Crozier et al., 2010, Hölldobler and Wilson, 2011) are characteristics of social complexity in ants. Division of labor is correlated with the evolution of morphologically differentiated workers (subcastes) and age-related schedules of behavioral development, both of which may result in task specialization and therefore reduced repertoire size (reviewed in Hölldobler and Wilson, 1990, Mertl and Traniello, 2009). Worker morphological phenotypes, or subcastes, and worker chronological age are underscored by mosaics of neuropil volume variation and brain compartment covariance (Muscedere and Traniello, 2012, Ilies et al., 2015), which appear to reduce brain metabolic costs through adaptive investment in functionally specialized brain regions according to worker social roles (Gronenberg and Riveros, 2009, Muscedere et al., 2013, Ilies et al., 2015, O'Donnell et al., 2015). In addition, eusociality itself may result in reduced investment in the mushroom bodies (MB), a region of sensory integration, learning, and memory (Fahrbach, 2006, O'Donnell et al., 2015). Behavioral specialization of worker subcastes may require less investment in the MBs due to decreased task plasticity and increased investment in primary sensory input regions that underscore requirements specific to worker task environments. Furthermore, self-organization theory suggests that simple components are sufficient for group decision-making (Anderson and McShea, 2001, Jeanson et al., 2012); collective intelligence may thus collaterally reduce brain investment in individuals due to reduced cognitive demands (Feinerman and Traniello, 2015).

To examine the relationship between social complexity and brain evolution, we quantified patterns of neural investment by measuring brain mass and the volumes of functionally distinct neuropil regions as a proxy for brain production (development) costs. These brain production costs include investment in both neurons and glia, which are indistinguishable in mass and neuropil measurements. In addition, we measured the volume of the cell rind, the cell density, and neuron soma size of select brain compartments to complete our assessment of brain production costs. We defined operational metabolic costs as the energy usage needed for neural maintenance and functioning (Bassett et al., 2009), including but not limited to costs of sustaining membrane resting potential and synthesizing macromolecules necessary for signaling and recovery ("house-keeping," Du et al. [2008]) as well as activating neural circuitry. We measured the activity of cytochrome oxidase (COX), a catalyst for ATP synthesis and thus an endogenous marker of neurometabolism (Wong-Riley, 1989, Li et al., 2006, Hall et al., 2012, Wong-Riley, 2012), in brain compartments as a proxy for operation costs. We contrasted neuropil investment patterns and metabolic activity in the brains of workers of the socially complex ant *Oecophylla smaragdina* and the socially basic sister clade *Formica subsericea*, two species that have significantly diverged in formicine ant evolutionary trajectories (Moreau et al., 2006). *O. smaragdina* is considered a pinnacle of social complexity in ants (Crozier et al., 2010): colonies may be composed of up to ~500,000 workers that show size-based specialization in task performance. Small minor workers (average head width [HW] 1.02 mm, range 0.93-1.16 mm) specialize in caring for brood within the nest, whereas larger major workers (average HW 1.57 mm, range 1.31-1.71 mm) are task generalists. Major workers forage throughout large (<1500m^2), collectively defend arboreal territories and cooperatively build leaf nests by bridging their bodies between leaves to form a living ratchet to draw leaves together, then binding them in place with larval-secreted silk. *F. subsericea* workers, in sharp contrast, are monomorphic (average HW 1.46 mm, range 1.2-1.7 mm), show generalized behavioral repertoires that encompass nursing, foraging, and nest construction, and live in relatively small colonies (several 100 to ~8,000 workers). Moreover, cooperation in groups of more than two workers does not enhance overall work effort (Klotz, 1986), suggesting that collective capabilities are limited.

We predicted that *O. smaragdina* majors have small brain mass and less investment in integrative processing due to their division of labor and emergent collective actions. Majors are also predicted to invest substantially in sensory input regions involved in visual navigation in a three-dimensional canopy environment as well as the pheromonal coordination of territorial defense and foraging. Similar to majors, weaver ant minor workers were predicted to show decreased investment in overall brain size and higher order sensory integration regions due to their nursing specialization; investment in sensory system brain compartments was expected to be lower than that of majors because of a presumed decrease in reliance on visual and chemical cues for performing brood-care and other tasks within the nest. Alternatively, according to the cognitive ecology of *O. smaragdina* majors, these workers may invest significantly in total brain size and the MBs, specifically, because they perform their tasks in rich sensory environments and collective action and worker polyphenism may not substantially reduce cognitive demands. In contrast, *F. subsericea* workers are predicted to require large brains and investment in sensory, motor, and integrative processing regions because of concurrent cognitive demands for both brood care and other tasks performed within the nest, as well as foraging and related tasks performed outside the nest. The striking contrasts in individual worker and colony phenotypes of these species provide an excellent opportunity to comparatively analyze neurobiological correlates of social complexity and test the hypothesis that increased division of labor through subcaste evolution is associated with more specialized investment in sensory processing as well as decreased production and operation costs in the total brain and higher order processing regions.

Methods

Colony collection and operation

O. smaragdina nests were collected from savannah woodlands on the James Cook University campus in Townsville, Queensland, Australia. Nests were considered separate colonies based on worker nestmate recognition tests. Workers collected for brain volume and body mass analyses were obtained directly from the field. For other analyses, colonies of several hundred *O. smaragdina* were transported to Boston University. Samples from workers in queenless nests were processed shortly after collection. *F. subsericea* queenright colonies of several dozen workers were collected in temperate mixed-hardwood forests in Hammond Woods and the Middlesex Fells Reservation in Massachusetts, USA. All colonies were housed in Fluon®-lined plastic boxes and fed carbohydrates (1:3 honey water or 1M sugar water) and insect prey ad libitum every other day. Colonies were maintained on a 12:12 light dark cycle at 55% humidity and 25.5°C.

Brain and body mass scaling

To determine brain scaling, we measured brain mass and body mass of fully pigmented, mature workers of both species. *O. smaragdina* brain mass data from Kamhi et al. (2015), using workers from two colonies (n=29, 12 from each colony), were used in scaling studies. Brains were dissected in Insect Ringer (Nation, 2002) after head width measurements were taken at the widest point of the head capsule, not including the eyes. Brains were placed in a tin capsule of known weight and dried at 55°C and weighed at 48 hours. Following the same protocol, *F. subsericea* worker brains from three colonies (n=10, 8, 9 from each colony) were measured. Brain mass and HW displayed a significant linear correlation (*O. smaragdina*: brain mass = [30.43 x HW] + 14.83; $F_{1,39}$ = 12.22, R^2=0.24; p<0.01 [Kamhi et al., 2015]; *F. subsericea:* brain mass = [24.72 x HW] + 7.62; $F_{1,26}$ = 6.43, R^2=0.71; p<0.001). Body masses were obtained by placing whole, intact bodies in tin capsules of known weight. The same drying and weighing protocol was used as noted above. Three *O. smaragdina* colonies (n=97, 100, 99 from each colony) and four *F. subsericea* colonies (n=17, 18, 5, 12) were used. Brain weight measurements were acquired during prior neural studies; brain and body mass therefore were quantified in separate individuals. Body mass and HW also showed a highly significant linear correlation (*O. smaragdina*: body mass = [4.18 x HW] – 3.68; $F_{1,294}$ = 2010.88, R^2=0.87; p<0.001; *F. subsericea:* body mass = [9.65 x HW] – 8.15; $F_{1,50}$ = 110.12, R^2=0.69; p<0.001). Using the equations describing the correlation between body mass and HW, we estimated body mass for workers of known brain mass for each species. Standardized major axis regression was used to compare scaling of species and subcaste measurements with the program (S)MATR v. 2.0 (Warton et al., 2006). Between-group effects were tested using ANCOVA as in Muscedere et al. (2012). All analyses were performed using JMP Pro 11 statistical software unless otherwise noted.

Histochemical and statistical analysis of neuropil production costs

Fully pigmented, mature major and minor *O. smaragdina* brains from 4 colonies were dissected in HEPES buffer (150mM NaCl, 5mM KCl, 5mM CaCl2, 25mM sucrose, 10mM HEPES [Ott, 2008]) and tissue was fixed for either 3 hours at room temperature or overnight at 4°C in Zinc 4% paraformaldehyde (0.25g $ZnCl_2$, 0.788g

NaCl, 1.20g sucrose, 25mL 16% paraformaldehyde for 100mL solution in dH_20). Whole brains were post-fixed in Dent's fixative (4:1 methanol:dimethyl sulfoxide) for 1 hour at room temperature. Brains were stored in 100% methanol at -20°C until further processing. After rehydration with a graded methanol series (95%, 75%, 50%, 30% methanol in 1M phosphate buffer solution [PBS] for 5 min each), brains were washed (6 x 10 min) with 0.5% Triton X in PBS (PBST), incubated in a blocking solution (10% Normal Goat Serum [Sigma]) in PBST for 1 hour at room temperature, and incubated with the primary antibody anti-synapsin (SYNORF1, DSHB, 1:50) in PBST for at least 48 hours on a shaker. After incubation, brains were washed (6 x 10 min) in PBST, incubated in secondary antibody (Alexa Fluor 488, Invitrogen, 1:200 in 10% Normal Goat Serum [NGS] in PBST) for at least 48 hours at room temperature on a shaker, washed with PBST, PBS, and then dehydrated in the same methanol series as noted above. About 50μL of methyl salicilate was added to the methanol for approximately 5 minutes before brains were transferred to custom-made double-sided stainless steel well slides, immersed in methyl salicilate, and allowed to clear. Brains were imaged using a confocal microscope (Zeiss LSM 710) with a 10x objective and optically sectioned at 12.34 μm intervals. Brains of fully pigmented, mature *F. subsericea* workers from 4 colonies collected while foraging in the arena of a nest box were similarly processed, but brains were rinsed with 0.1M Tris buffer (pH=7.4) instead of being rehydrated in methanol. These brains were dehydrated after staining in an ethanol series and optically sectioned at 3.1 μm intervals using an Olympus FluoView 1 confocal microscope with a 10x objective.

Brain images on confocal scans were traced blind using Amira (v3.1) to measure the volume of the following functionally distinct neuropil regions (Figure 4.1a,b): the lobula and medulla of the optic lobes (OL: primary visual processing), antennal lobes (AL: olfactory input [Hansson and Anton, 2000]), subesophageal zone (SEZ: mandible and mouthpart function [Chapman, 2013]), mushroom body (MB: higher order regions of sensory input, learning, and memory, partitioned into the lateral (MB-lc) and medial calyces (MB-mc) and the peduncle (MB-ped) [Fahrbach, 2006]), the central complex (CC: visual and motor aspects of navigation, sensory integration, and learning [Pfeiffer and Homberg, 2014]), and the remainder of the undifferentiated central brain (ROCB). The MB-lc and MB-mc were measured separately due to possible functional differences (Riveros and Gronenberg, 2010). For bilateral structures, one hemisphere selected at random was measured; for regions located along the brain midline (SEZ and CC) the whole structure was measured.

Whole brain and brain compartment comparisons were performed using repeated measures ANOVA with mixed models, with the eight brain regions as the within-subjects factor and species and subcaste as between-subjects factors. Student's t-tests were performed for post-hoc comparisons between groups (species and subcastes) and brain regions. These analyses were also used to compare relative brain compartment volume, which was calculated by dividing the volume of the region of interest by the total brain volume. The ROCB was used as a baseline measurement for relative brain compartment comparisons because of its multifunctionality. Analyses were performed using JMP Pro 11 statistical software.

Neuron number, density, and size

Brains from mature workers from both species were dissected in HEPES buffer solution (130 mM NaCl, 5 mM KCl, 4 mM MgCl2, 5mMCaCl2, 15mM Hepes, 25mMglucose,160mM sucrose; pH 7.2 [Groh and Rössler, 2011]) and incubated in 4% zinc formaldehyde overnight at 4°C. Brains were rinsed (3 x 20 min) in 1M phosphate buffer solution (PBS), embedded in 5% (weight/volume) low melting point agarose, and sectioned at 100μm in the horizontal plane with a vibratome (Series 1000, Technical Products International). Sections were then permeabilized in 2% PBST for 20 min, washed in 0.2% PBST for 20 min, blocked in 2% NGS in 0.2% Triton X in PBS for 1 hour, and incubated in 3C11 (anti-SYNORF1, DHSB; 1:50) in blocking solution for at least 3 nights at 4°C. After incubation, sections were washed in PBS (5 x 20 min) and incubated in Alexa Fluor 568-conjugated goat anti-mouse secondary antibody (Life Technologies; 1:250) in 1% NGS in PBS overnight at room temperature. Sections were then washed in PBS (4 x 20 min), incubated in DAPI (NucBlue®) for 30 min, and incubated in 60% glycerol in PBS overnight at room temperature. After incubation in 80% glycerol in PBS for 30 min, sections were mounted on slides in 80% glycerol, and imaged with a FV10i confocal microscope using a 60x objective. Several overlapping confocal stacks of 2μm optical sections were imaged to ensure that the entire MB and AL cell rind was represented. These two regions were chosen due to their importance to social interactions in ants (Ilies et al., 2015) and because cell bodies can be unambiguously identified with their target neuropils.

The volume of the cell rind, the cell “density” (numbers of cell bodies per unit volume), and the cell size were quantified blind to species and subcaste. The cell rind was traced using Amira (v6.0) to measure volume. Density was determined by an unbiased stereology protocol using ImageJ 1.48s. A 30μm^2 grid was overlaid on confocal images and 3-4 boxes per region were chosen randomly for counting for each tissue section. Because cells were more than 2μm in depth, we used 4μm as our “section” depth. Every other 4μm section was counted, with each counting section having a “look-up” section. Cells found in both the counting and “look-up” section were not counted, those found only in the counting section were counted. Cells that were within the counting square or touched the top and right edges were counted while those that touched the bottom or left edges were not. Total cell number estimates were calculated by multiplying the volume of the cell rind of that region by the density of cells. Cell size was determined by measuring the longest length of the cells that were counted for density measurements in one counting box per tissue section. JMP Pro 11 statistical software was used to perform ANOVAs to analyze variation among subcaste and species.

Histochemical and statistical analysis of brain operation

Fully pigmented mature workers from each species and subcaste were analyzed for COX activity. Two colonies of *O. smaragdina* and three colonies of *F. subsericea* were used. COX reflects functional characteristics of neurons because it catalyses ATP synthesis in oxidative phosphorylation (Wong-Riley, 1989, Hevner and Wong-Riley, 1993, Li et al., 2006) and its transcription is regulated by neuronal activity (Wong-Riley, 2012). Oxidative phosphorylation occurs primarily in neurons, therefore our measurement of brain operation costs excludes the integral glial contributions to brain metabolic function (Volkenhoff et al., 2015). COX activity has been used to analyze metabolism in visual processing regions (Wong-Riley, 1979, Mimura, 1988, Wong-Riley et al., 1993, Edwards et al., 1995) and may be associated with behavioral phenotype (Sakata et al., 2005), circadian rhythms (da Silva et al., 2000), and olfactory learning (Armengaud et al., 2000, Deglise et al., 2003). To limit effects of circadian rhythm and activity on COX measurements, *O. smaragdina* majors and *F. subsericea* were collected while actively foraging in laboratory nest arenas and *O. smaragdina* minors were collected from nest compartments. All workers were sampled between dawn and dusk, a period of sustained, but not maximal, foraging activity (Lokkers, 1990, North, 1993). Brains were dissected in HEPES buffer and fixed in 4% paraformaldehyde in 1M PBS for 40 minutes. The fixative was removed with a series of four 15-minute washes in PBS. Brains were incubated in 30% sucrose in PBS (g/mL) overnight at 4°C and sectioned at 30μm on a cryostat (Leica CM 1520). Equal amounts of cytochrome c solution (4mg cytochrome c in 4mL HEPES buffer with 0.3 mL 1%$CoCl_2$) and diaminobenzidine solution (5mg diaminobenzidine in 4mL HEPES buffer) were placed on dried slides. Sections were incubated in the solutions for 15-30 minutes in the dark and monitored for exposure time. Sections were rinsed three times in distilled water and incubated for at least 10 minutes in distilled water before drying and coverslipping with Permount. Sections were imaged at with a 10x objective using a Canon Rebel t3i with an adaptor for an Olympus BX40 light microscope.

Images were quantified using ImageJ 1.48s. Whole brain sections were first outlined to determine minimum and maximum intensity due to variability in overall staining among sections. COX activity is concentrated in neuropil (Wong-Riley, 1989); neuropils measured for volume analyses were also measured for COX activity. Individual brain regions of each section were delimited and average staining intensity was recorded. Similar to volume analyses, whole measurements of the SEZ and CC were made while all other brain regions were measured in one hemisphere selected at random. Each brain region in a section was scaled to the range of staining intensity in that section. Regions were measured in 1 – 6 sections, as available. COX activity per unit area for each brain region was calculated by averaging the scaled intensities of each compartment across sections. Because species and subcastes varied in brain compartment size, we measured absolute metabolic investment as well as relative investment compared to total brain volume. Mean brain region volumes were used because not every section of the brains stained for COX activity was present. These estimates of brain operation costs take into account differential regional investment patterns between species and subcastes. We use the term *metabolic cost* to describe results of scaling COX activity to brain compartment volume.

For COX comparisons we performed repeated measures ANOVA with mixed models using the eight brain regions as within-subjects factors and species and subcaste as the between-subjects factors, similar to the analysis of volume data. No differences were found between brains of workers from queenright and queenless *O. smaragdina* colonies; therefore colonies were combined for analyses. Student's t-tests were performed for post-hoc comparisons between groups (species and subcastes) and brain regions. JMP Pro 11 statistical software was used for all statistical analyses.

Results

Relationships between brain mass and body mass

Brain mass to HW scaling showed that both species have a common slope not statistically different from 1 (Mean slope 1.13, range 0.95 – 1.37; $\chi^2 = 1.98$, $p=0.16$). There was a significant grade shift ($W^2 = 104.27$, $p<0.001$) and x-axis shift ($W^2 = 5.38$, $p<0.05$) indicating that *O. smaragdina* workers of both subcastes have significantly larger brains than *F. subsericea* workers when scaled for body size ($F_1=78.22$, $p<0.001$). There was insufficient statistical evidence to support a common slope between species groups for body mass to HW scaling ($P<0.05$); further analyses comparing groups therefore could not be performed.

A statistically significant linear relationship of brain mass to estimated body mass was found for both species (*O. smaragdina*: brain mass = [7.28 x body mass] + 41.63; $F_{1,39} = 12.22$, $R^2=0.24$; $p<0.01$; *F. subsericea:* brain mass = [2.56 x body mass] + 28.50; $F_{1,25} = 60.43$, $R^2=0.71$; $p<0.001$). Species and subcaste groups shared a common slope that was significantly different from 1 (Mean slope 0.38, range 0.32 – 0.46; $\chi^2 = 78.01$, $p<0.001$; Figure 4.2). There was a significant grade shift between species ($W^2 = 187.74$, $p<0.001$) and a significant shift along the x-axis ($W^2 = 5.55$, $p<0.05$). Between-group analyses indicate that both *O. smaragdina* subcastes had significantly greater brain mass for their body size than *F. subsericea* workers (F_1=93.19, p<0.001).

Neuropil investment patterns between species and subcastes

There was a significant main effect of group in brain compartment volume (Repeated measures ANOVA: F_2=9.59 p<0.001): *O. smaragdina* major workers (n=16) had larger total brain neuropil volume than minors (n=11) and *F. subsericea* workers (n=11; post-hoc t-test: major, *F. subsericea*: t_{35}=4.06, p<0.001; major, minor: t_{35}=3.14, p<0.01; minor, *F. subsericea*: t_{35}=0.84, p=0.40;). There was a significant interaction of group (subcaste and species) by brain region (F_{14}=7.61, p<0.001; Table 4.1). The MB calyces were significantly larger in both subcastes of *O. smaragdina* than in *F. subsericea* workers whereas *O. smaragdina* majors had larger MB-lc than minors, but there were no significant differences between subcastes (MB-lc: major, *F. subsericea*: $t_{128.21}$=4.76, p<0.001; major, minor: $t_{128.21}$=1.95, p=0.053; minor, *F. subsericea*: $t_{128.21}$=2.58, p<0.05; MB-mc: major, *F. subsericea*: $t_{128.21}$=4.80, p<0.001; major, minor: $t_{128.21}$=1.51, p=0.13; minor, *F. subsericea*: $t_{128.21}$=3.03, p<0.01). The MB-lobes were larger in both subcastes of *O. smaragdina* than in *F. subsericea* workers, although there was no significant difference between *O. smaragdina* minors and *F. subsericea* workers (major, *F. subsericea*: $t_{128.21}$=3.30, p<0.01; major, minor: $t_{128.21}$=1.17, p=0.24; minor, *F. subsericea*: $t_{128.21}$=1.95, p=0.053). The OL was significantly larger in *O. smaragdina* majors and *F. subsericea* workers than *O. smaragdina* minors; *F. subsericea* workers did not have statistically different OL volume compared to *O. smaragdina* majors (major, *F. subsericea*: $t_{128.21}=1.26$, $p=0.21$; major, minor: $t_{128.21}=3.42$, $p<0.001$; minor, *F. subsericea*: $t_{128.21}=1.98$, $p<0.05$). *O. smaragdina* majors had significantly larger ALs than minor workers and both subcastes had similar AL volume compared to *F. subsericea* workers, although *O. smaragdina* majors tended to have larger AL than *F. subsericea* workers (major, *F. subsericea*: $t_{128.21}=1.67$, $p=0.098$; major, minor: $t_{128.21}=2.26$, $p<0.05$; minor, *F. subsericea*: $t_{128.21}=0.54$, $p=0.59$). There were no significant differences between groups in either the SEZ or the CC (all $p>0.1$). The ROCB was significantly larger in *O. smaragdina* majors than either comparison groups and there was no significant difference between *O. smaragdina* minors and *F. subsericea* workers (major, *F. subsericea*: $t_{128.21}=7.04$, $p<0.001$; major, minor: $t_{128.21}=5.87$, $p<0.001$; minor, *F. subsericea*: $t_{128.21}=1.07$, $p=0.29$).

There was no significant main effect of group when relative brain region volumes were compared ($F_2=2.98$, $p=0.064$). *O. smaragdina* majors showed significantly greater relative investment in brain compartments than *F. subsericea* workers, but there was no significant difference between *O. smaragdina* minors and either comparison group (major, *F. subsericea*: $t_{35}=2.44$, $p<0.05$; major, minor: $t_{35}=1.11$, $p=0.27$; minor, *F. subsericea*: $t_{35}=1.22$, $p=0.23$). There was a significant interaction of subcaste and species by brain region ($F_{12}=23.24$, $p<0.001$; Table 4.1). Workers of both *O. smaragdina* subcastes had significantly greater relative investment in the MB calyces than *F. subsericea* workers: no differences between subcastes were found (MB-lc: major, *F. subsericea*: $t_{240.22}=6.92$, $p<0.001$; major, minor: $t_{240.22}=0.091$, $p=0.93$; minor, *F. subsericea*: $t_{240.22}=6.44$, $p<0.001$; MB-mc: major, *F. subsericea*: $t_{240.22}=7.60$, $p<0.001$;

major, minor: $t_{240.22}=1.34$, $p=0.18$; minor, *F. subsericea*: $t_{240.22}=8.21$, $p<0.001$). The MB-lobes were also significantly larger in *O. smaragdina* than *F. subsericea* relative to their respective total brain volume; weaver ant minor workers had significantly larger relative MB-lobe volumes than major workers (major, *F. subsericea*: $t_{240.22}=2.57$, $p<0.05$; major, minor: $t_{240.22}=2.37$, $p<0.05$; minor, *F. subsericea*: $t_{240.22}=4.54$, $p<0.001$). *F. subsericea* workers had significantly greater relative investment in the OL than *O. smaragdina* majors, which had greater relative investment than minor workers (major, *F. subsericea*: $t_{246.92}=2.92$, $p<0.01$; major, minor: $t_{246.92}=5.25$, $p<0.001$; minor, *F. subsericea*: $t_{246.92}=7.42$, $p<0.001$). *O. smaragdina* minor workers had significantly smaller relative AL volume than *F. subsericea* workers and minors had smaller relative AL volume compared to *O. smaragdina* majors, although the difference was not significant. There was no difference in relative AL volume between *O. smaragdina* majors and *F. subsericea* workers (major, *F. subsericea*: $t_{240.22}=0.76$, $p=0.45$; major, minor: $t_{240.22}=1.83$, $p=0.068$; minor, *F. subsericea*: $t_{240.22}=2.38$, $p<0.05$). The SEZ was relatively larger in *F. subsericea* than in the two *O. smaragdina* subcastes (major, *F. subsericea*: $t_{240.22}=7.85$, $p<0.001$; major, minor: $t_{240.22}=0.90$, $p=0.37$; minor, *F. subsericea*: $t_{240.22}=6.38$, $p<0.001$). There were no significant differences between subcaste and species groups in relative CC volume (all $p>0.90$).

Species and subcaste patterns of neuron size, number, and density

There was no significant difference in cell rind neuron density between species or subcaste ($n=3$ for all groups) in the AL ($F_2=2.37$, $p=0.17$) or the MB ($F_2=4.00$, $p=0.08$).

AL and MB cell rind volume were not statistically different between species and subcaste (AL: F_2 =0.46, p=0.65; MB: F_2 =0.46, p=0.65). Neuron number in these two regions was estimated to be similar across species and subcaste (AL: F_2 =0.67, p=0.55; MB: F_2 =0.30, p=0.75). Neurons in the AL of both subcastes of *O. smaragdina* were larger than those of *F. subsericea* workers, although the difference between *F. subsericea* workers and majors was not significant (F_2 =4.00, p=0.08; post-hoc Student's t-test: major, *F. subsericea*: t_6=2.44, p=0.05; major, minor: t_6=0.03, p=0.98; minor, *F. subsericea*: t_6=2.46, p<0.05; mean diameter [±sd] for majors: 5.17±0.82μm, minors: 5.19±1.27μm, *F. subsericea*: 3.26±0.49). *O. smaragdina* major workers had significantly larger MB neurons than either other comparison group; *O. smaragdina* minors had larger neurons than *F. subsericea* workers but this difference was not significant (F_2 =37.90, p<0.001; post-hoc Student's t-test: major, *F. subsericea*: t_6=8.37, p<0.001; major, minor: t_6=6.25, p<0.001; minor, *F. subsericea*: t_6=2.12, p=0.08; mean diameter [±sd] for majors: 5.37±0.25μm, minors: 4.24±0.16μm, *F. subsericea*: 3.86±0.24μm).

Species and subcaste patterns of COX activity

Comparisons of COX activity between *O. smaragdina* major and minor workers and *F. subsericea* workers (n=10 for all groups) did not reveal a statistically significant main effect (Repeated Measures ANOVA mixed model: F_2=2.77, p=0.081; Figure 4.1c,d). *O. smaragdina* major workers, however, had significantly lower total brain COX activity per unit volume than *F. subsericea* workers (t_{27}=2.20, p<0.05). Total COX activity in minors showed a trend of being greater than majors, although the difference was non-significant (t_{27}=1.82, p=0.080). Total COX activity was similar in *O. smaragdina* minors and *F. subsericea* workers (t_{27}=0.38, p=0.70).

A significant interaction effect of species, subcaste, and regional COX activity was found (F_{14}=2.27, p<0.01; Table 4.2, Figure 4.3A). Post-hoc Student's t-tests showed that *O. smaragdina* minors had significantly greater COX activity in the AL than majors ($t_{100.02}$=3.29, p<0.01). AL COX activity was lower in *O. smaragdina* majors than *F. subsericea*; the difference showed borderline significance ($t_{100.02}$=1.96, p=0.052). There was no significant difference between minors and *F. subsericea* ($t_{100.02}$=1.33, p=0.19). *O. smaragdina* major workers had significantly lower COX activity than *F. subsericea* workers in both the MB-lc ($t_{100.02}$=3.68, p<0.001) and MB-mc ($t_{100.02}$=3.26, p<0.01). *O. smaragdina* minor workers had significantly greater COX activity in the MB-lc than majors ($t_{100.02}$=2.38, p<0.05), and there was a similar trend in the MB-mc, but the result was not significant ($t_{100.02}$=1.77, p=0.079). There were no significant differences in COX activity in the MB calyces between weaver ant minors and *F. subsericea* workers (MB-lc: $t_{100.02}$=1.31, p=0.19; MB-mc: $t_{100.02}$=1.48, p=0.14)). All other brain regions had similar COX activity levels across species and subcastes (all p>0.1).

Total metabolic costs

There was a non-significant main effect of species and subcaste when COX activity was scaled by absolute brain region volume (F_2=3.00, p=0.067). *O. smaragdina* major workers showed greater total metabolic cost than *F. subsericea* workers (t_{27}=2.32, p<0.05) and a trend of greater metabolic cost compared to minor workers (t_{27}=1.85,

$p=0.076$). There was no significant difference between *O. smaragdina* minors and *F. subsericea* workers ($t_{27}=0.47$, $p=0.64$). The interaction of subcaste and species and brain region volume was significant ($F_{14}=5.61$, $p<0.001$; Figure 4.3B). Both *O. smaragdina* majors and *F. subsericea* workers had greater metabolic cost than weaver ant minors in the OL (major: $t_{143.32}=3.36$, $p<0.01$; *F. subsericea*: $t_{143.32}=2.56$, $p<0.05$). There was no significant difference in OL total metabolic cost between major and *F. subsericea* workers ($t_{143.32}=0.80$, $p=0.42$). *O. smaragdina* minors had significantly lower metabolic cost in the ROCB than *O. smaragdina* majors ($t_{143.32}=4.83$, $p<0.001$) and *F. subsericea* workers ($t_{143.32}=2.07$, $p<0.05$). Majors had significantly greater metabolic cost in this region than *F. subsericea* workers ($t_{143.32}=6.90$, $p<0.001$). Metabolic cost was similar across species and subcaste in other brain regions (all $p>0.05$).

There was no significant main effect of species and subcaste when COX activity was scaled to relative brain compartment volume ($F_2=2.39$, $p=0.11$), but there was an interaction effect of species and subcaste by brain region ($F_{14}=2.68$, $p<0.01$; Figure 4.3C). Post-hoc tests showed that *F. subsericea* workers had significantly higher relative OL metabolic cost than *O. smaragdina* minors ($t_{143.32}=3.49$, $p<0.001$). Majors did not have significantly different metabolic cost in the OL than minors ($t_{143.32}=1.57$, $p=0.12$), and showed a trend toward less metabolic cost compared to *F. subsericea* ($t_{143.32}=1.93$, $p=0.056$). *F. subsericea* workers had significantly higher relative energy consumption in the SEZ than both weaver ant subcastes; there was no significant subcaste difference in the SEZ (major, *F. subsericea*: $t_{143.32}=2.95$, $p<0.01$; majors, minor: $t_{143.32}=0.17$, $p=0.86$; minor, *F. subsericea*: $t_{145.30}=2.78$, $p<0.01$). *O. smaragdina* minors had significantly higher relative energy consumption than majors in the MB-lc ($t_{143.32}$=2.03, $p<0.05$) and a trend in the same direction in the MB-mc ($t_{143.32}$=1.81, p=0.073). Neither *O. smaragdina* subcaste had significantly different investment in either the MB-lc (major: $t_{143.32}$=0.90, p=0.37; minor: $t_{143.32}$=1.13, p=0.26) or MB-mc (major: $t_{143.32}$=0.25, p=0.80; minor: $t_{143.32}$=1.56, p=0.12) compared to *F. subsericea*. *O. smaragdina* majors had significantly lower relative metabolic cost in the ROCB than both minors ($t_{143.32}$=2.06, $p<0.05$) and *F. subsericea* workers ($t_{143.32}$=2.14, $p<0.05$). There was no significant difference between minors and *F. subsericea* in this region ($t_{143.32}$=0.076, p=0.94). There were no significant differences among subcaste and species in all other brain regions (all $p>0.1$).

Discussion

Large colony size, division of labor by worker morphological subcastes, and collective action characterize higher levels of social complexity in insects; associated cognitive challenges are considered to be significant forces that shape worker brain evolution. To examine how complex colony phenotypes influence brain evolution, we estimated brain production and operations costs by scaling total brain mass to body mass, quantifying volumes of functionally distinct neuropil compartments, determining neuron number, density, and soma size, and measuring COX activity in brain regions in closely related but socially and ecologically differentiated ants. Our comparisons suggest brain production and operation costs are associated with complexity in colony-level social organization as well as the cognitive demands that correlate with worker ecology and task variation. Contrary to the hypothesis that greater social complexity correlates with smaller brains and lower investment in higher-order processing (Gronenberg and Riveros, 2009, Riveros et al., 2012, O'Donnell et al., 2015), our results show that workers of both *O. smaragdina* subcastes invested significantly more in total brain size and MB production than *F. subsericea* workers, although they are nearly identical in body size. Cell rind volume and density were similar across subcaste and species, while *O. smaragdina* workers had significantly larger AL neurons compared to *F. subsericea*, and *O. smaragdina* majors had larger MB neurons than either comparison group. Total brain, MB, and AL operation costs in *O. smaragdina* majors were significantly lower than those of minors and *F. subsericea* workers. These findings suggest that collective action may provide a fitness benefit for *O. smaragdina* majors by lowering operation costs to compensate for higher neural production costs that may be associated with large colony size.

Social brain theory and ant brain evolution

Social brain theory predicts a positive correlation between group size and brain size (Dunbar, 1998). Support for this hypothesis in ants is mixed. Worker brain size in fungus-growing ants decreased in larger colonies of monomorphic species, but brain size and its covariation with some brain regions, colony size, and task specialization appears to be complex (Riveros et al., 2012). Similar to our findings, a significant positive relationship between colony size and individual worker brain mass was found in the ant genus *Cataglyphis* (Wehner et al., 2007); brain size does not appear to correspond to navigational capability or other challenges potentially associated with interspecific variation in foraging range size. Wehner (2007) suggested that larger colonies may have more resources available to support larger worker brains and considered increased social interactions to be the driving force of brain evolution in this genus.

In vertebrates, large group size positively correlates with brain size due to the cognitive demands associated with increased individualized social interactions (Dunbar, 1998) and challenges involved in mate choice (Dunbar and Shultz, 2007a) and parental care (Shultz and Dunbar, 2010). The social brain hypothesis, therefore, cannot be directly applied to ants and other eusocial insects because the cognitive demands of mate choice and parental care are absent in workers, which are typically sterile. Moreover, workers do not appear to be able to recognize individuals (Hölldobler and Wilson, 1990, Vander Meer and Morel, 1998), and social selection of this type is likely to be absent. However, workers engage in alloparental care, which likely has similar cognitive requirements as parenting. Cuticular hydrocarbons function in nestmate discrimination (Hölldobler and Wilson, 1977a, Blomquist and Bagnères, 2010, Newey et al., 2010b) as well as discriminating groups of workers in different social roles (Greene and Gordon, 2003). This recognition mechanism may be more highly developed in socially complex species because advanced division of labor can generate more morphologically or behaviorally variable worker groups and greater discriminatory capabilities to support colony-level functions. The neural mechanisms underlying these social processes, however, remain unclear, and it is therefore difficult to determine their precise role in brain evolution.

Emergent colony-level behavior and brain evolution

Social complexity is thought to emerge from individual simplicity (Delgado and Sole, 1997, Robinson et al., 2011). Theories of group decision-making and collective action, however, do not make direct predictions concerning brain size and structure in relation to emergent properties of social insect colonies. We predicted that worker brain size in socially complex ants would be relatively small due to lower cognitive demands on workers, which function as individually simplistic components of an emergent complex system. Moreover, neurometabolic costs that might typically limit investment in total brain size, as well as in its cognitive processing centers, could also be lowered if behaviorally "simple" workers are required to assemble a collectively functioning colony. Behaviorally complex individuals, nevertheless, can perform collective actions (Jeanson et al., 2012) and may contribute to complex colony cooperation (Czaczkes et al., 2015a). Behavioral algorithms coordinating collective action may be simple, but the underlying neural mechanisms of individual action could be sophisticated. For example, weaver ant nest construction is governed by a straightforward positive-feedback system (Bochynek and Robson, 2014); there is a strong visual component to worker chain formation, and tactile and pheromonal cues also may be involved in this coordinated action (unpub. data). Collective actions of this type may require substantial sensory perception and processing even if decisions are made at the level of the group.

Although this analysis of individual cognition in respect to emergent behavior suggests that collective action may represent an added layer of complexity that may not itself selectively reduce brain production costs, COX analyses showed that *O*.

smaragdina major workers had significantly lower total brain metabolic activity than minors and *F. subsericea* workers. The number of neurons in the MB and AL appear to be similar across subcaste and species; given that neurons utilize a consistent amount of energy regardless of size (Herculano-Houzel, 2011), our results suggest that neural investment does not affect operation costs. The significant variation in brain operation costs between *O. smaragdina* majors and minors suggests that colony size, social interactions, and division of labor do not significantly contribute to overall costs of neural operation. Additionally, differences in brain metabolic activity between *O. smaragdina* majors and *F. subsericea* workers suggest that task performance may not be the sole determinant of brain operation costs due to interspecific overlap in the behavioral organization of task performance and related neurobiological requirements. Although specific neural circuits underscoring emergent, colony-level behavior have yet to be identified, our data nevertheless suggest that the collective action preeminent in the organization of work by *O. smaragdina* majors provides a fitness benefit by decreasing neural operation costs, thus energetically compensating for the higher production costs of their larger brains. Reducing brain metabolic costs can be achieved by decreasing redundancy in neural circuitry, leading to a reduced signal-to-noise ratio (Niven and Laughlin, 2008). In the context of workers engaging in collective action, any resulting decrease in worker processing capability may not have a colony-level cost because of cooperation: groups of workers acting in concert may be able to compensate for potential cognitive deficiencies in individual workers (Couzin, 2009). Recognizing that the relationship between behavioral ability and brain size is not well established (Healy and

Rowe, 2007, Chittka and Niven, 2009, Lihoreau et al., 2012, Healy and Rowe, 2013), we hypothesize that controlling behavioral responses by collective cognition may be less energetically expensive than evolving a brain with the capacity to enable greater individual action.

Social brain structure in ants

1. Sensory processing: ecological demands on individuals versus social challenges

Ecological task demands faced by individual workers may affect brain evolution. *O. smaragdina* majors visually navigate and chemically orient between nests and worksites in their large arboreal and terrestrial territories (Hölldobler, 1983, Jander and Jander, 1998, Crozier et al., 2010). Visual navigation may require tracking landmarks and celestial cues (Jander and Jander, 1998, Wehner, 2003, Graham and Cheng, 2009). Accordingly, tissue volume and COX activity in the OLs were both greater in majors than minors. Minors, whose primary social role appears to be limited to caring for brood within leaf nests, also had smaller relative OL neuropil volumes and significantly less total and relative metabolic cost in this region than *F. subsericea* workers. The significantly higher production costs in the less socially complex *F. subsericea* compared to *O. smaragdina* majors may be due to neural requirements for navigation independent of the specific nature of the environment, or the evolution of specialized circuitry in weaver ants that does not contribute significantly to their total neuropil volume in weaver ant majors. This hypothesis is supported in part by studies of desert ants, which show that worker brain size does not correspond to foraging distance (Wehner et al., 2007).

AL volume variation appears to reflect the role of pheromonal cues in the ecology of *O. smaragdina* majors and *F. subsericea* workers compared to that of minors. Olfactory processing requirements may be greater in *O. smaragdina* major and *F. subsericea* workers than *O. smaragdina* minor workers. *O. smaragdina* workers have minimally four distinct exocrine glands (Hölldobler and Wilson, 1977b) associated with at least five different forms of chemical and tactile recruitment (Hölldobler and Wilson, 1978). Pheromonal recruitment in *O. smaragdina* is considered to be predominately within the behavioral repertoire of major workers and not minors (Hölldobler and Wilson, 1978); majors therefore must be able to distinguish between pheromones as well as pheromone blends to respond adaptively, adjusting individual and group-level behavior to the perception of multiple chemical cues in a variety of contexts (Czaczkes et al., 2015b). While *F. subsericea* workers seem to have little chemical communication and use a combination of trophic cues and tactile signals for recruitment (Wallis, 1964), their engagement in tasks both within and outside of nest may require similar levels of AL production costs as *O. smaragdina* major workers.

The increased metabolic cost of the ALs in *O. smaragdina* minors and *F. subsericea* workers relative to *O. smaragdina* majors suggests that brood care also may require considerable investment in olfactory processing in both species. We at first considered that major worker involvement in multiple tasks distributed throughout a structurally complex arboreal environment would be more cognitively demanding than those performed by *F. subsericea* workers that nurse, forage, and excavate nests, and *O. smaragdina* minors that appear to predominately nurse. This characterization, however, may underestimate the complexities of brood care and hence the demands they place on the brain. Efficient brood care requires accurately assessing the needs of multiple developmental stages of brood, which includes determination of larval nutritional state and maintaining immatures in optimal temperature and humidity regimes within the structural complexities of a nest (Cassill and Tschinkel, 1999, Lopes et al., 2005, Ruchty et al., 2010, Tschinkel, 2015), and assisting metamorphosis. Cognitive requirements of nursing and the performance of other tasks within the dark labyrinths of nest chambers as well as influences of social interactions (Wehner et al., 2007) may be comparable to or exceed those of outside-nest task work. *O. smaragdina* minors and *F. subsericea* workers therefore may require behavioral abilities in addition to the cognitive demands immediately associated with the care of eggs, larvae, and pupae.

2. Higher-order processing

Socially basic ant species exhibit relatively small colony size, reduced and/or less elaborate communication and a lower degree of collective capability. Selection in these species appears to have favored diverse task repertoires (Traniello, 1978, Jaisson et al., 1992), leading to more totipotent and plastic task performance in different sensory environments within and outside of the nest. Workers in these species were therefore predicted to invest more in the MB in response to increased needs for higher-order processing. Indeed, total brain size is positively correlated with behavioral capability in individual workers (Gronenberg and Riveros, 2009), and intra- and interspecific comparisons of the MB, in particular, show that this region is adaptively scaled to brain size and varies according to cognitive requirements associated with task performance (Muscedere and Traniello, 2012). Large colony size and greater task specialization correlate with decreased MB volume in monomorphic *Pseudomyrmex spinicola* workers (Amador-Vargas et al., 2015). Consistent with these findings, we predicted that *F. subsericea* workers would invest more in the MB because they have a broader task repertoire encompassing both inside and outside nest tasks, and thus may require more behavioral capability and flexibility than workers of either *O. smaragdina* subcaste. Results did not support this hypothesis, suggesting that behavioral plasticity may not affect MB circuitry in a way that modifies neuropil volume, or *O. smaragdina* may in fact be more behaviorally flexible and thus require more higher-order processing than *F. subsericea*. *O. smaragdina* MB may undergo experience-dependent MB growth (Fahrbach et al., 1998) that is absent or less demanding in *F. subsericea*. MB organization and development in relation to behavioral plasticity may underlie the sensory integration required for task performance. Like mammals, weaver ants use colony-specific secretions to mark territories and major workers will fight more aggressively when they recognize the scent of their own colony (Hölldobler and Wilson, 1977a). This scaling of aggressive behavior requires perception of a chemical signature likely acquired during development, as well as higher-order processing and neural signaling between brain compartments to control motor patterns of fighting. Further neuroecological studies are needed to determine the extent of behavioral and neural plasticity in relation to MB circuitry.

3. Motor control

We did not make *a priori* predictions concerning social complexity and motor control systems, and variation in all such neural supports was not measured in our study. Locomotion pattern generators, for example, are located in the ventral nerve cord (Heinrich, 2002). CC volume and metabolic activity were similar across subcaste and species, possibly due to functional convergence; workers may have similar demands for motor coordination and visual integrative processes controlled by this compartment. The SEZ, which controls motor outputs to the mandibles (Chapman, 2013) - the principle appendages used to work – had significantly greater volume and estimated energy consumption in *F. subsericea* workers than workers of both *O. smaragdina* subcastes. *O. smaragdina* minors may require less mandibular muscle power for manipulating and caring for brood (Muscedere and Traniello, 2012). Majors, however, appear to use their mandibles for task performance in similar ways as *F. subsericea* workers, in addition to pulling and anchoring leaves and delicately handling larvae during cooperative nest construction (Crozier et al., 2010). The decreased energy consumption found in weaver ant majors compared to *F. subsericea* is therefore surprising and may be a consequence of collective action decreasing metabolic investment in the SEZ. Alternatively, subcaste and species similarities in CC investment and similar subcaste SEZ investment may be a product of developmental constraints on motor processing at the subfamily or species level (Finlay and Darlington, 1995).

Conclusion

Our data are the first to demonstrate a relationship between brain production and neurometabolic operation costs and worker behavior in the context of the evolution of complex social organization in social insects. Previous studies have suggested that larger colony size is associated with greater task specialization and lower brain investment (Riveros et al., 2012), which should reduce brain metabolic cost and enhance fitness. However, our comparative study suggests that components of social complexity, such as division of labor, large colony size, and collective intelligence, may be driving forces with compensatory effects on brain evolution. We found an association between the MB, AL, and total brain investment and compartment-specific operation costs suggesting that these regions, which are integral to social information processing, are important to responding to cognitive challenges associated with living in complex social groups. These brain compartments thus appear to be metabolically plastic with respect to regional investment and requirements for signaling and recovery. In *O. smaragdina*, large colony size as well as social interactions and behavioral repertoires that require integration of multiple sensory modalities at the level of the individual worker are associated with increased investment in these brain regions, consistent with the vertebrate social brain hypothesis. Compartment size also appears to reflect sensory requirements of worker task performance, thus providing a neuroecological basis for patterns of neuropil investment. Developmental constraints on brain compartment size and neurometabolic activity may limit variation associated with task-specific cognitive requirements. Although we do not know if metabolic lability is a general feature of social insect brain evolution, the resulting increase in brain production costs appear to be compensated for by lower operational metabolic costs in brain compartments in major workers that engage in collective action. Collective intelligence, which is associated with large colony size and socially advanced colony organization, may be one mechanism enabling ant colonies to conserve metabolic investment in brain compartments that perceive and process social information. Both collective as well as individual action in the context of the ecology of individual task performance therefore may be driving the evolution of information-processing circuitry, represented in brain mass, compartment size, neuron size, number, and density, and ATP usage.

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Brain region	Species	Absolute volume (mm^3)	Relative volume	Cell rind volume (mm^3)	Estimated cell number
OL	*F. subsericea*	$6.11\pm1.24x10^{-3}$	$11.35\pm0.75x10^{-2}$	n/a	n/a
	major	$7.10\pm0.84x10^{-3}$	$9.93\pm1.11x10^{-2}$	n/a	n/a
	minor	$4.42\pm0.99x10^{-3}$	$7.61\pm0.93x10^{-2}$	n/a	n/a
AL	*F. subsericea*	$5.24\pm1.87x10^{-3}$	$9.41\pm1.39\ x10^{-2}$	$1.95\pm0.93x10^{-3}$	20934.80±9498.30
	major	$6.55\pm1.06x10^{-3}$	$9.08\pm0.89\ x10^{-2}$	$2.71\pm0.18x10^{-3}$	17515.40±8195.41
	minor	$4.42\pm0.99x10^{-3}$	$8.29\pm1.01\ x10^{-2}$	$2.15\pm1.48x10^{-3}$	25796.8±8684.78
MB-lc	*F. subsericea*	$4.93\pm1.33x10^{-3}$	$9.03\pm0.71\ x10^{-2}$	n/a	n/a
	major	$8.66\pm1.49x10^{-3}$	$12.02\pm1.33\ x10^{-2}$	n/a	n/a
	minor	$7.13\pm2.30x10^{-3}$	$12.06\pm1.47\ x10^{-2}$	n/a	n/a
MB-mc	*F. subsericea*	$4.59\pm1.43x10^{-3}$	$8.32\pm0.86\ x10^{-2}$	n/a	n/a
	major	$8.36\pm1.44x10^{-3}$	$11.61\pm1.35\ x10^{-2}$	n/a	n/a
	minor	$7.17\pm2.07x10^{-3}$	$12.18\pm1.17\ x10^{-2}$	n/a	n/a
MB-lobes	*F. subsericea*	$5.11\pm0.97x10^{-3}$	$9.58\pm1.24\ x10^{-2}$	n/a	n/a
	major	$7.70\pm1.06x10^{-3}$	$10.69\pm0.70\ x10^{-2}$	n/a	n/a
	minor	$6.78\pm1.46x10^{-3}$	$11.71\pm1.38\ x10^{-2}$	n/a	n/a
Total MB	*F. subsericea*	$1.46\pm0.36x10^{-2}$	$26.94\pm1.76x10^{-2}$	$5.87\pm2.09x10^{-3}$	114823±43624
	major	$2.47\pm0.37x10^{-2}$	$34.32\pm2.72x10^{-2}$	$9.58\pm3.21x10^{-3}$	161257±57390
	minor	$2.11\pm0.56x10^{-2}$	$35.96\pm2.76\ x10^{-2}$	$5.28\pm3.15x10^{-3}$	137679±104497
SEZ	*F. subsericea*	$6.73\pm1.52x10^{-3}$	$12.47\pm1.19\ x10^{-2}$	n/a	n/a
	major	$6.51\pm1.22x10^{-3}$	$9.08\pm1.52\ x10^{-2}$	n/a	n/a
	minor	$5.50\pm1.43x10^{-3}$	$9.47\pm1.78\ x10^{-2}$	n/a	n/a
CC	*F. subsericea*	$2.36\pm0.87x10^{-4}$	$0.42\pm0.08\ x10^{-2}$	n/a	n/a
	major	$3.08\pm0.82x10^{-4}$	$0.42\pm0.09\ x10^{-2}$	n/a	n/a
	minor	$2.70\pm0.85x10^{-4}$	$0.48\pm0.16\ x10^{-2}$	n/a	n/a
ROCB	*F. subsericea*	$2.13\pm0.48x10^{-2}$	$39.42\pm2.18\ x10^{-2}$	n/a	n/a
	major	$2.68\pm0.39x10^{-2}$	$37.17\pm1.94\ x10^{-2}$	n/a	n/a
	minor	$2.22\pm0.50x10^{-2}$	$38.19\pm3.02\ x10^{-2}$	n/a	n/a
Total brain	*F. subsericea*	$5.42\pm1.26x10^{-2}$	n/a	n/a	n/a
	major	$7.20\pm0.87x10^{-2}$	n/a	n/a	n/a
	minor	$5.82\pm1.27x10^{-2}$	n/a	n/a	n/a

Table 4.1: Brain production costs estimates across species and subcastes. Means ± standard deviation area presented for all absolute and relative volume analyses. *O. smaragdina* subcastes are labeled major and minor. n/a = non-applicable.

Brain region	**Species**	**Normalized COX activity**	**Total metabolic cost**	**Relative metabolic cost**
OL	*F. subsericea*	0.68±0.13	4.13±0.77x10^{-3}	7.69±1.43 x10^{-2}
	major	0.63±0.11	4.50±0.79x10^{-3}	6.29±1.10 x10^{-2}
	minor	0.68±0.12	2.99±0.52x10^{-3}	5.15±0.90 x10^{-2}
AL	*F. subsericea*	0.57±0.089	2.99±0.47x10^{-3}	5.37±0.84 x10^{-2}
	major	0.47±0.10	3.09±0.65x10^{-3}	4.28±0.91 x10^{-2}
	minor	0.64±0.11	3.05±0.55x10^{-3}	5.30±0.95 x10^{-2}
MB-lc	*F. subsericea*	0.54±0.15	2.64±0.75x10^{-3}	4.85±1.37 x10^{-2}
	major	0.35±0.12	3.02±1.02x10^{-3}	4.19±1.42 x10^{-2}
	minor	0.47±0.07	3.35±0.49x10^{-3}	5.67±0.84 x10^{-2}
MB-mc	*F. subsericea*	0.53±0.16	2.44±0.75x10^{-3}	4.42±1.35 x10^{-2}
	major	0.36±0.12	3.05±1.03x10^{-3}	4.23±1.44 x10^{-2}
	minor	0.46±0.08	3.26±0.58x10^{-3}	5.55±0.98 x10^{-2}
MB-lobes	*F. subsericea*	0.47±0.12	2.39±0.62x10^{-3}	4.48±1.16 x10^{-2}
	major	0.39±0.09	2.99±0.69x10^{-3}	4.15±0.96 x10^{-2}
	minor	0.42±0.07	2.86±0.48x10^{-3}	4.95±0.84 x10^{-2}
SEZ	*F. subsericea*	0.62±0.13	4.17±0.88x10^{-3}	7.73±1.62 x10^{-2}
	major	0.62±0.13	4.01±0.85x10^{-3}	5.60±1.19 x10^{-2}
	minor	0.60±0.11	3.32±0.60x10^{-3}	5.72±0.10 x10^{-2}
CC	*F. subsericea*	0.48±0.14	0.11±0.03x10^{-3}	0.20±0.06 x10^{-2}
	major	0.45±0.09	0.14±0.03x10^{-3}	0.19±0.04 x10^{-2}
	minor	0.49±0.14	0.13±0.04x10^{-3}	0.24±0.07 x10^{-2}
ROCB	*F. subsericea*	0.59±0.08	1.26±0.18x10^{-2}	23.28±3.31 x10^{-2}
	major	0.58±0.09	1.57±0.25x10^{-2}	21.72±3.49 x10^{-2}
	minor	0.61±0.10	1.35±0.23x10^{-2}	23.22±3.99 x10^{-2}

Table 4.2: Brain operations costs estimates across species and subcastes. Means ± standard deviation area presented for all cytochrome oxidase (COX) and metabolic scaling analyses. *O. smaragdina* subcastes are labeled major and minor.

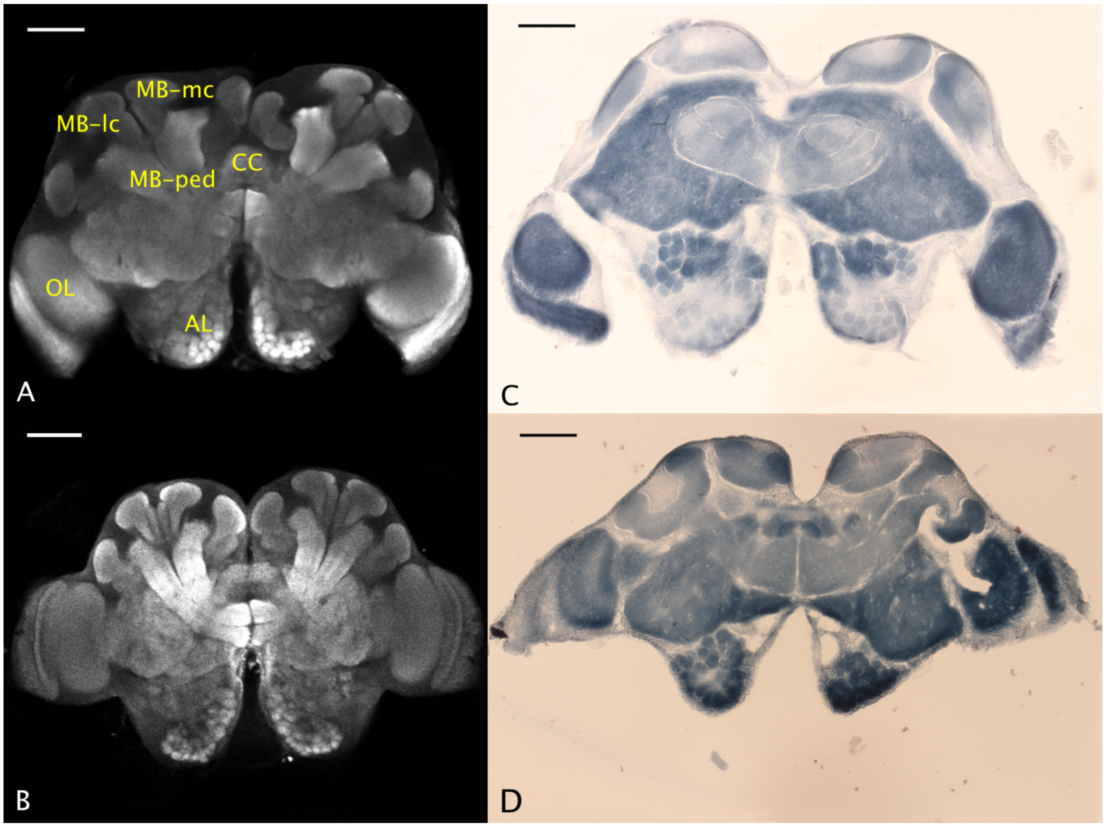

Figure 4.1: Confocal micrographs of synapsin-labelled brains of an *O. smaragdina* major worker (A) and *F. subsericea* worker (B). Labels in one hemisphere of (A) demarcate functionally distinct neuropil regions: optic lobes (OL), antennal lobes (AL), mushroom body (MB) lateral and medial calyces (MB-lc and MB-mc, respectively), MB peduncle (MB-ped), central complex (CC), and the rest of the central brain (unlabeled neuropil). The subesophageal zone (SEZ) is not pictured due to its ventral location with respect to the central brain. Images of (C) *O. smaragdina* major worker and (D) *F. subsericea* worker brain stained for cytochrome oxidase. Darker staining indicates greater COX activity. Scale bars = 100μm.

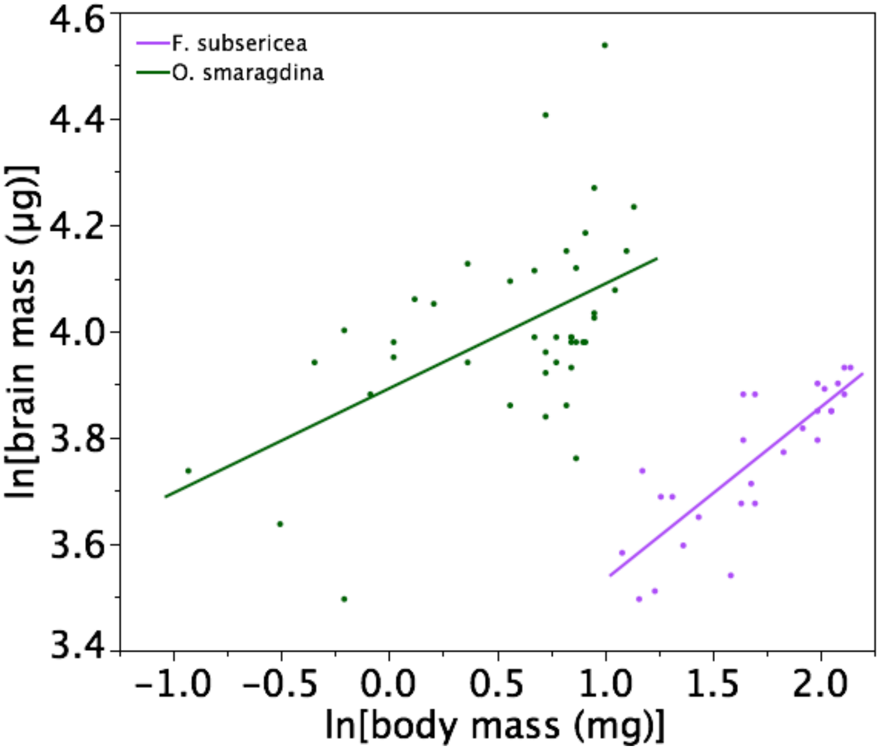

Figure 4.2: Regression of brain mass scaled to estimated body mass for *F. subsericea* and both *O. smaragdina* subcastes.

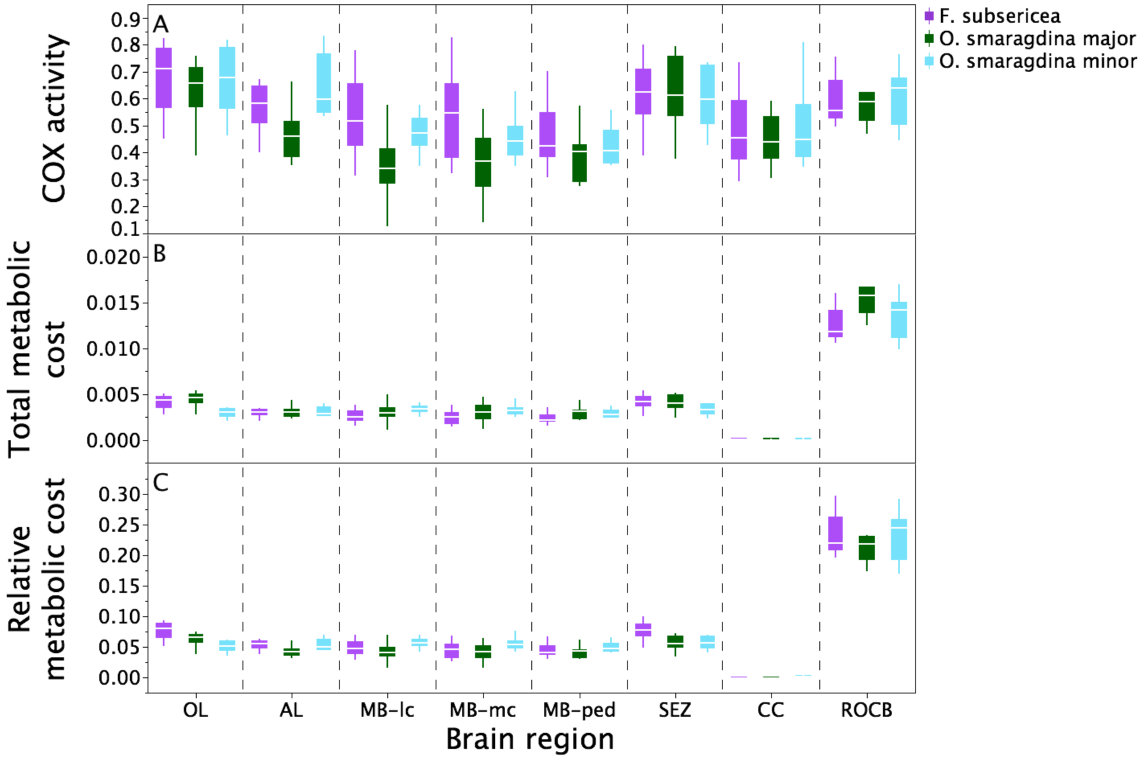

Figure 4.3: Box plots for each functionally distinct brain region for *O. smaragdina* major and minor workers and *F. subsericea* monomorphic workers of (A) cytochrome oxidase (COX) normalized by unit volume, (B) COX activity scaled by total brain region volume to present total metabolic cost (COX activity x mm^3), and (C) COX activity scaled by relative unit of volume, representing relative metabolic cost for each region.

CHAPTER FIVE: SOCIAL COMPLEXITY IN ANTS AFFECTS PATTERNS OF BRAIN DEVELOPMENT

Abstract

In social insects, species-typical patterns of investment in brain- and compartment-size scaling correspond to variation in worker cognitive requirements during behavioral development, which may follow canalized trajectories of neural organization or experience-dependent neuroplasticity. In ants, division of labor reduces the size of task repertoires and the extent of worker neuroplasticity is thus predicted to be negatively associated with higher levels of social complexity that include division of labor by morphologically differentiated workers. Because neural tissue is metabolically expensive, increased behavioral specialization should result in worker brains that are structurally adapted to specific task repertoires; canalized neural development may therefore reduce brain production costs. Sensory inputs and experience, which likely vary with task specialization, have been suggested to contribute to developmental neuroplasticity in ants, but it is unknown how such flexibility relates to division of labor. We compared macroscopic and cellular brain anatomy in two phylogenetically closely related ant species that have very different levels of social complexity: *Oecophylla smaragdina*, whose behaviorally specialized dimorphic workers live in very large colonies and engage in cooperative, collective action, and *Formica subsericea*, whose monomorphic workers show generalized behavioral repertoires and no evidence of collective action among workers, and live in small colonies. We quantified the volumes of functionally distinct brain compartments in newly eclosed and mature workers and quantified density of microglomeruli, synaptic complexes, in the mushroom bodies – regions of higher-order sensory integration – under control and visual deprivation conditions to examine developmental neuroplasticity in these two sister clades. We show that the mushroom bodies increased in volume and had significant age-related synaptic reorganization in both *O. smaragdina* worker subcastes, whereas *F. subsericea* workers showed no such age-related changes. We did not find any changes in synaptic organization that were dependent on visual experience in either species. Our findings suggest that although both ant species exhibited canalized neural development, division of labor was correlated with experience-expectant development in ants.

Introduction

Neuroplasticity enables adaptive responses to changing environments and novel sensory stimuli. Patterns of brain organization underscoring such responsiveness may be canalized, in which case neuroplasticity is experience-expectant (Fahrbach et al., 1998) and associated with environmental predictability. In canalized neural systems, differences in behavioral phenotype arise from intraspecific genetic variability rather than experience-dependent plasticity (Schradin, 2013). Both canalized developmental programs and experience-dependent plasticity may occur in all nervous systems (Greenough et al., 1987, Fahrbach et al., 1998). In group-living vertebrates, cognitive challenges associated with increased individualized interactions and other aspects of advanced sociality may be correlated with increased behavioral flexibility, and thus sensory experience-dependent neuroplasticity (Taborsky and Oliveira, 2012). However, social complexity in insects, as exemplified by some ant species, may not involve the same cognitive challenges that would benefit from behaviorally flexible responses. Ant colonies are composed of workers that are typically sterile and have limited individual recognition capabilities (Hölldobler and Wilson, 1990, Vander Meer and Morel, 1998), and can act collectively to make decisions that are more accurate and precise than those of independent workers (Sasaki and Pratt, 2011, Jeanson et al., 2012, Sasaki and Pratt, 2012, Sasaki et al., 2013). Social selection acting on colonies, therefore, should impact worker behavioral performance and its development.

One of the hallmarks of social complexity in ants is division of labor among morphologically differentiated worker subcastes. The extent to which division of labor influences the plasticity of worker brains during development is unknown. Worker polymorphism is typically associated with size-related, and often limited, behavioral repertoires. Worker brains may reflect the sensory requirements of their specialized task repertoires (Gronenberg et al., 1996, Muscedere and Traniello, 2012). Canalization should be evident in worker brain structures that do not vary with age or undergo neural reorganization with age independent of sensory input during development, and neural processing may differ accordingly. Emergent collective intelligence is predicted to drive brain evolution in the same direction: cooperative colony-level action is considered to be based on simple components (i.e. workers), and therefore may lead to reduced cognitive demands and brain investment (Feinerman and Traniello, 2015). In contrast, workers in socially basic species, which are characterized by small colony size, generalized behavioral repertoires, and more independent actions, may require more experience-dependent neuroplasticity because of their broader task arrays and thus more varied sensory environments.

Brain tissue as well as learning and memory – the principle outcomes of synaptic plasticity – are metabolically expensive (Aiello and Wheeler, 1995, Burns et al., 2011). Investment patterns in functionally specialized brain regions correspond with intra- and interspecific morphological subcaste- and age-related task performance (Gronenberg et al., 1996, Seid and Traniello, 2006, Muscedere and Traniello, 2012, Ilies et al., 2015). Division of labor by polymorphism could provide a fitness benefit by reducing brain metabolic investment in workers with specialized and thus limited cognitive capabilities (Feinerman and Traniello, 2015). The synaptic organization of sensory processing circuitry may change with behavioral repertoire and related sensory experience (Seid et al., 2005, Stieb et al., 2010, Stieb et al., 2012, Falibene et al., 2015), although, task experience does not appear to be necessary for age-related neuroplasticity or efficiency in task performance (Muscedere et al., 2013). Polymorphic workers with more specialized behavioral repertoires may undergo age-related neuropil expansion and/or synaptic reorganization independent of experience because canalized neural development is associated with more predictable environments and behaviors (Schradin, 2013).

We explored the relationship between division of labor and neural development through comparisons of neuropil volumes and synaptic organization in newly eclosed and mature workers of the socially complex Australasian weaver ant, *Oecophylla smaragdina*, and the more socially basic sister clade *Formica subsericea*. The molecular phylogeny of these species (Moreau et al., 2006) indicates close relatedness, but these species show highly significant divergence in social evolution. Considered a "masterpiece" of sociality in ants (Crozier et al., 2010), *O. smaragdina* colonies are large (~500,000 workers), and workers are polymorphic, show size-based task specialization, and are capable of complex collective actions. Large major workers (average head width [HW] 1.57 mm, range 1.31-1.71 mm) are task generalists and forage and defend vast arboreal territories (~1500m^2) and "weave" leaves to build nests by forming a living chain to draw leaves together and bind them with larval silk. Smaller minors (average HW 1.02 mm, range 0.93-1.16 mm) specialize primarily on brood care within nests and are rarely seen outside. *F. subsericea* workers, in contrast, are monomorphic (average HW 1.46 mm, range 1.2-1.7 mm), have generalized behavioral repertoires much like *O. smaragdina* major workers, engage in all colony tasks such as brood care, foraging, and nest construction, and show little collective action (Klotz, 1986). Colonies of *F. subsericea* are very small (100s to ~1,000s of workers) in comparison to *O. smaragdina*.

To determine how neuroanatomy reflects behavioral development in these species, we measured volumes of functionally distinct brain compartments in newly eclosed and mature worker brains. Changes in neuropil volume may indicate reorganization of synaptic processing (Stieb et al., 2010, Muscedere and Traniello, 2012); therefore, we directly assessed synaptic plasticity by quantifying synaptic complexes termed microglomeruli (MG) within both visual and olfactory input regions (collar and lip, respectively; Gronenberg, 1996) of the mushroom bodies (MB), a brain compartment in which higher-order sensory processing and integration occur (Fahrbach, 2006). MG are formed from the post-synaptic dendritic spines of MB Kenyon cells and associated presynaptic axon boutons of afferent sensory neurons (Groh and Rössler, 2011). To distinguish experience-dependent plasticity from canalized developmental plasticity, we raised ants from the pupal stage under control and light-deprivation conditions to determine how species, subcaste, age, and visual experience affect changes in neuropil investment and synaptic organization in the visual system. Ommatidia number and size were quantified to record species and subcaste variation in peripheral receptors. We focused on the visual system because *O. smaragdina* major and minor workers experience very different visual environments. *F. subsericea* workers, however, appear to have similar visual requirements for task performance as those of *O. smaragdina* major workers. This subcaste and species variation provides an ideal opportunity to elucidate the degree of canalized versus experience-dependent neuropil growth and synaptic remodeling.

We hypothesized that both *O. smaragdina* and *F. subsericea* workers would show age-related increases in brain compartment neuropil volumes corresponding to age-related increases in task capabilities (e.g., Gronenberg et al., 1996, Muscedere and Traniello, 2012, Muscedere et al., 2013). *O. smaragdina* workers were predicted to have less neuroplasticity than *F. subsericea* workers due to worker polymorphism and division of labor, and hence greater behavioral specialization, specifically in *O. smaragdina* minor workers. Increases in MG density may indicate the consolidation of long-term memories (Hourcade et al., 2010), while a decrease in MG density suggests synaptic pruning, which may lead to adaptive synaptic processing, tuned for worker sensory experience (Stieb et al., 2010, Scholl et al., 2014). Similarly, increases in MG size suggests either larger or more numerous synapses (Seid et al., 2005, Krofczik et al., 2008). We predicted higher MG density and/or volume in the MB collar of *O. smaragdina* major workers compared to minors, reflecting greater processing capability in the higher-order visual system. We predicted that both *O. smaragdina* major and *F. subsericea* workers would show a decrease in MG density in the MB collar with age in the control condition, corresponding with increased light cues as they leave the nest to forage, and no changes when deprived of light input (Stieb et al., 2010, Scholl et al., 2014). MG in *O. smaragdina* minor workers were predicted to be unaffected by visual experience during development because they should receive minimal light cues within the nest in both conditions.

Methods

Colony collection and maintenance

O. smaragdina nests containing several hundred workers were collected from savannah woodlands on the James Cook University campus in Townsville, Queensland, Australia. Mature workers for brain volume analyses were collected directly from field colonies; newly eclosed workers for brain volume analyses and workers for all other analyses were obtained from freshly collected queenless nests or queenright colonies. *F. subsericea* queenright colonies of several dozen workers were collected in temperate mixed-hardwood forests in Hammond Woods and the Middlesex Fells Reservation in Massachusetts, USA. Colonies were maintained in Fluon®-lined plastic boxes and fed carbohydrates (1:3 honey water or 1M sugar water) and insect prey ad libitum every other day. All colonies and subcolonies (described below) were kept on a 12:12 light dark cycle at 55% humidity and 25.5°C.

Histochemical and statistical analysis of neuropil investment

Mature workers in field-collected colonies were identified by their full pigmentation (from four colonies for each worker group) and newly eclosed (callow) workers were distinguished by their light-yellow color. Callow *F. subsericea* and *O. smaragdina* minor workers were collected from three colonies and callow *O. smaragdina* major workers were collected from two colonies. *O. smaragdina* worker and callow *F. subsericea* worker brains were dissected in HEPES buffer (150mM NaCl, 5mM KCl, 5mM CaCl2, 25mM sucrose, 10mM HEPES [Ott, 2008]). Brain tissue was fixed in Zinc 4% paraformaldehyde (0.25g $ZnCl_2$, 0.788g NaCl, 1.20g sucrose, 25mL 16% paraformaldehyde for 100mL solution in dH_20) for either 3 hours at room temperature or overnight at 4°C. Whole brains were then incubated in Dent's fixative (4:1 methanol:dimethyl sulfoxide) for 1 hour at room temperature and stored in 100% methanol at -20°C until further processing. Brains were rehydrated with a graded methanol series (95%, 75%, 50%, 30% methanol in 1M phosphate buffer solution [PBS] for 5 min each), washed (6 x 10 min) with 0.5% Triton X in PBS (PBST), and incubated in a blocking solution (10% Normal Goat Serum [Sigma]) in PBST for 1 hour at room temperature. They were then incubated with the primary antibody 3C11 (anti-SYNORF1; DSHB, 1:50) in PBST for at least 48 hours on a shaker. After incubation, brains were washed (6 x 10 min) in PBST, incubated in secondary antibody (Alexa

Fluor 488, Invitrogen, 1:200 in 10% Normal Goat Serum in PBST) for at least 48 hours at room temperature, and washed with PBST, PBS, and then dehydrated in the same methanol series as above. Methyl salicylate was added to the methanol at a concentration of approximately 1:10 for approximately 5 minutes, and then brains were transferred to custom-made double-sided stainless steel well slides, immersed in methyl salicylate, and allowed to clear. Brains were imaged using a confocal microscope (Zeiss LSM 710) with a 10x objective and optically sectioned at 12.34μm intervals. Mature *F. subsericea* workers were similarly processed, but post-fixation, they were washed in 0.1M Tris buffer (pH=7.4) prior to washing with PBS. These brains were dehydrated in an ethanol series after staining and imaged using an Olympus FluoView 1 confocal microscope (10x objective) with optical sections of 3.1μm intervals.

To quantify volumes of functionally distinct brain compartments, brain images on confocal scans were traced blind by an observer using Amira (v3.1). The following brain regions were measured: the primary visual (optic lobes [OL]) and olfactory (antennal lobes [AL]) processing regions, the region of mandible and mouthpart control , higher order processing centers of learning and memory (mushroom body calyces, which receive sensory input, and lobes, which are predominately efferent pathways [MB; Fahrbach, 2006]), a region of visual and motor processing involved in navigation, sensory integration, and learning (central complex [CC; Pfeiffer and Homberg, 2014]), and the rest of the multifunctional central brain (ROCB). The MB medial and lateral calyces were combined for measurements because they have similar inputs and functions (Gronenberg, 2001, Fahrbach, 2006). Whole SEZ and CC measurements were made due to the location of these regions along the midline; all other regions were measured in one hemisphere selected at random.

Scaling analyses of each brain region volume to the whole brain volume minus the region of interest (rest of central brain, ROCB), calculated separately for each region, were performed using standardized major axis regression with the program (S)MATR v. 2.0 (Warton et al., 2006). For regions with common slopes, further analyses were performed to test for grade or x-axis shifts and overall relationships between groups. Between-group effects were tested using ANCOVA (Muscedere and Traniello, 2012) with JMP Pro 11 statistical software.

Generating ants of known age and manipulating visual experience

To be able to quantify brain structure in workers of known age, subcolonies were created with marked "caretaker" ants and mature pupae known to be close to eclosion due to their yellow color. Caretaker workers had a small portion of the tarsus of one leg removed to allow identification. *O. smaragdina* subcolonies consisted of 80 majors, 40 minor caretaker workers, 10 larvae, and 10-60 yellow major and minor worker pupae. Six colonies were used for major workers (creating 9 subcolonies) and nine colonies (with 12 subcolonies) for minor workers. Each parent colony was used to create 1 – 4 subcolonies, depending on the size of the nest. Due to smaller parent colony size, *F. subsericea* subcolonies consisted of 15 caretaker workers, 5 larvae, and 20-30 mature pupae to ensure survival of adequate samples; two colonies were used, each contributing to two subcolonies. Adult eclosions were tracked and age of newly-eclosed workers was noted within ±1 day. Larvae were removed and replaced, as available, because they began to develop into pupae. Subcolonies deprived of light were kept in the same environmentally controlled chambers as control subcolonies (described above) but were surrounded by thick light-occluding curtains. Red light was used to view colonies during experiments. Workers were removed for analysis at 1-10 and 30 days post-eclosion. At 30 days, *F. subsericea* workers and *O. smaragdina* majors begin to leave the nest for exploratory trips (personal obs.).

Quantification of microglomeruli

Brains were processed for MG visualization following a protocol adapted from Groh and Rössler (2011). Brains were dissected in HEPES Buffer Solution (130 mM NaCl, 5 mM KCl, 4 mM MgCl2, 5mMCaCl2,15mM Hepes, 25mM glucose,160mM sucrose; pH 7.2). After dissection, tissue was incubated overnight at 4°C in 4% Paraformaldehyde in 1M PBS. Brains were rinsed (3 x 20 min) in PBS and incubated in 30% sucrose in PBS for at least two hours for cryoprotection. Tissue was then embedded in O.C.T™ Compound (Sakura Tissue-Tek®) and sectioned at 30μm on a cryostat (Leica CM 1850 or 1520). Sections were permeabilized in 2% Triton X-100 in PBS (PBST) for 20 minutes, followed by 20 minutes in 0.2% PBST. Tissue was incubated in 2% normal goat serum in 0.2% PBST for one hour and incubated in Alexa Fluor 488 phalloidin (1:500) and 3C11(anti-SYNORF1; 1:50) in 0.2% PBST with 2% Normal Goat Serum for at least three nights at 4°C. The tissue was washed (5 x 20 min) in PBS and incubated in Alexa Fluor 568-conjugated goat anti-mouse secondary (1:250) in 1% Normal Goat Serum in PBS overnight at room temperature then washed in PBS (5 x 20 min), incubated in 60% glycerol in PBS overnight, incubated in 80% glycerol in PBS for 30 minutes, and visualized using either a Zeiss LSM 710 confocal microscope with a 63x objective or a FV10i confocal microscope using a 60x objective. One arm, a protuberance of the calyx, was selected at random of both the medial and lateral MB calyx was imaged where the lobes intersect the calyx. As reported by Stieb et al. (2010), we found no differences in MG densities in medial and lateral calyces and therefore averaged the values for each calyx. MG density and size were quantified blind using ImageJ 1.48s. We quantified the MG visible within two 400μm^2 circles overlaid in the calyx lip, which receives olfactory input, and one 400μm^2 circle overlaid in the calyx collar, which receives visual input (Gronenberg, 2001, Farris and Sinakevitch, 2003, Groh and Rössler, 2011). The average number of MG in both regions was divided by the confocal micrograph thickness to estimate the density of MG per μm^3.

MG size was determined blind to experimental group, age, and subcaste by overlaying a grid of 10μm^2 boxes on the images in ImageJ 1.48s and measuring the longest length of the presynaptic, synapsin-labeled portion of the MG within 5 randomly selected boxes. The postsynaptic density, labeled with f-actin, was not measured due to its less-defined staining. MG volume was estimated by assuming MG are spherical and calculating the volume based on the length (diameter) measured. By over-estimating the volume of the presynaptic component of the MG through measuring the maximum length, we account for not measuring the postsynaptic portion of the MG.

For *O. smaragdina*, comparisons of MG density, volume, and ratio of MG to calyx lip or collar volume we performed a multifactor ANOVA with subcaste, visual experience, and age as independent variables. A two-way ANOVA was used for *F. subsericea* comparisons with visual experience and age as independent variables. A two-way ANOVA was used for comparisons of MG between control workers of each species/subcaste and age group. Analyses were performed using JMP Pro 11 statistical software.

Ommatidia quantification

Compound eyes of *O. smaragdina* majors and minors and *F. subsericea* workers from three colonies were coated in New-Skin® (liquid bandage) and allowed to dry. The New-Skin® was carefully peeled off and placed on a slide for imaging using a 10x objective and a Canon Rebel t3i camera fitted with an adaptor for an Olympus BX40 light microscope. Because of the curvature of the eyes, several images (4-7) were taken at different focal depths to ensure that all ommatidia could be visualized. Images were combined and quantified in ImageJ 1.48s. Ommatidia number for each eye was determined using CellCounter; ommatidia were randomly selected for size measurements using a measurement tool. Both right and left eyes were quantified for cell number and size. We found no differences between right and left eye measurements and therefore used the average, or measurements were used from a single eye if only one eye was quantifiable. Scaling analyses of ommatidia number and size to head width were performed using standardized major axis regression with the program (S)MATR v.

2.0 (Warton et al., 2006). Between-group effects were tested with ANCOVAs using JMP Pro 11 statistical software.

Results

Age-related changes in neuropil volumes

Common scaling slopes were found in all brain regions except for the AL (Table 5.1; Figure 5.1a, b, c; Figure 5.2). The MB calyx, the AL, and CC had slopes significantly greater than 1. Further analyses could only be performed on brain compartments with common scaling slopes. Significant grade shifts, indicating different y-intercepts for scaling slopes and thus differences in brain region size, were found in all brain compartments with common slopes. These regions also had x-axis shifts, indicating differences in ROCB size along a common scaling slope. Between-group analyses showed that all brain compartment volumes had a significant, positive relationship with ROCB volume (all $p<0.001$; Table 5.2). *O. smaragdina* minors (callow: $n=10$; mature: $n=11$) had significantly smaller relative OL volumes than *O. smaragdina* majors (callow: $n=11$; mature: $n=16$) or *F. subsericea* workers (callow: $n=9$; mature: $n=11$); *O. smaragdina* majors and *F. subsericea* workers had similar relative OL volumes ($F_2=42.53$, $p<0.001$; major, *F. subsericea*: $t_{61}=0.22$, $p=0.83$; minor, *F. subsericea*: $t_{61}=7.01$, $p<0.001$; major, minor: $t_{61}=6.73$, $p<0.001$). There were no significant effects of age ($F_1=2.25$, $p=0.14$) or interaction of subcaste and age ($F_2=0.32$, $p=0.73$). The MB calyx was significantly larger in relative terms in both subcastes of *O. smaragdina* than *F. subsericea* workers ($F_2=15.72$, $p<0.001$; major, *F. subsericea*: $t_{61}=4.70$, $p<0.001$;

minor, *F. subsericea*: t_{61}=5.10, p<0.001; major, minor: t_{61}=0.15, p=0.89) and was significantly larger in mature workers than callows (F_1=11.49, p<0.01). The significant interactions between subcaste and species group and age for the MB calyx (F_2=15.50, p<0.001) indicated that both *O. smaragdina* subcastes showed a significant increase in relative MB calyx volume with age while the MB calyx in *F. subsericea* workers remained constant in relative volume throughout this developmental period (*F. subsericea*: t_{61}=0.87, p=0.39; major: t_{61}=3.87, p<0.001; minor: t_{61}=5.28, p<0.001). There were no significant subcaste or species differences in relative MB calyx volume in callow workers (major, *F. subsericea*: t_{61}=0.92, p=0.36; minor, *F. subsericea*: t_{61}=0.28, p=0.78; major, minor: t_{61}=1.32, p=0.19). The MB lobes were significantly larger in relative volume in both *O. smaragdina* subcastes compared to *F. subsericea* workers (F_2=18.81, p<0.001; major, *F. subsericea*: t_{61}=4.10, p<0.001; minor, *F. subsericea*: t_{61}=6.06, p<0.001; major, minor: t_{61}=1.39, p=0.17) and increased in volume with age (F_1=30.42, p<0.001). There was no significant subcaste x species x age interaction (F_2=0.08, p=0.92). The SEZ was significantly larger in relative terms in *F. subsericea* workers than *O. smaragdina* workers, with no difference between *O. smaragdina* subcastes (F_2=10.15, p<0.001; major, *F. subsericea*: t_{61}=3.85, p<0.001; minor, *F. subsericea*: t_{61}=4.12, p<0.001; major, minor: t_{61}=0.31, p=0.75). There was no age (F_1=0.11, p=0.73) or subcaste and species group x age interaction (F_2=0.97, p=0.38) in this region. *O. smaragdina* minor workers had significantly larger relative CC volume than *F. subsericea* workers, whereas *O. smaragdina* majors showed no differences compared to the other two groups (F_2=5.11, p<0.01; major, *F. subsericea*: t_{61}=1.71, p=0.09; minor, *F.*

subsericea: t_{61}=3.19, p<0.01; major, minor: t_{61}=1.23, p=0.22). There was no significant effect of age (F_1=0.05, p=0.83), although there was a significant age x subcaste x species interaction (F_2=3.57, p<0.05), indicating that *F. subsericea* callows had a significantly smaller relative CC volume than *O. smaragdina* workers of both subcastes, but mature workers of both species and subcastes had similar volumes in this region (callow: major, *F. subsericea*: t_{61}=2.28, p<0.05; minor, *F. subsericea*: t_{61}=3.92, p<0.001; major, minor: t_{61}=1.36, p=0.18; mature worker comparisons: p>0.4).

Visual experience and MG development in O. smaragdina

There was no significant age-related difference in lip MG density (F_1=2.44, p=0.12; callow major control: n=19, dark: n=10; mature major control: n=13, dark: n=13; callow minor control: n=11, dark: n=7; mature minor control: n=8, dark: n=12; Figure 5.1d, e, f; Figure 5.3a). Mature workers had significantly lower MG density in the collar than callows (F_1=27.90, p<0.001). The volume of MB lip MG increased significantly in mature workers of both subcastes (F_1=29.66, p<0.001; Figure 5.3b); MG volume in the collar did not significantly change with age (F_1=0.25, p=0.80). There were no significant effects of subcaste (all p>0.1), light regime (p>0.4), or age x subcaste x light regime interaction (p>0.1) in any MB lip or collar MG measurements.

Visual experience and MG development in F. subsericea

There were no significant differences in MB lip or collar MG density with age (lip: F_1=0.07, p=0.80; collar: F_1=1.24, p=0.26; Figure 5.3a) or light regime (lip: F_1=0.04,

$p=0.84$; collar: $F_1=0.59$, $p=0.45$; callow control: $n=7$, dark: $n=7$; mature control: $n=10$, dark: $n=6$). MB lip and collar MG volume was similar across age (lip: $F_1=2.14$, $p=0.16$; collar: $F_1=0.25$, $p=0.62$; Figure 5.3b) and light regime (lip: $F_1=0.01$, $p=0.94$; collar: $F_1=0.01$, $p=0.96$). There were no significant differences in the interaction of age and group for the MB lip or collar MG density or volume (all $p>0.3$).

Interspecific comparisons of MG

Because there were no differences of experimental treatment in either species, only control age groups were compared. There was a significant effect of species and subcaste on MB lip MG density in control workers of both age groups ($F_2=18.58$, $p<0.001$): *O. smaragdina* workers had significantly higher MG density than *F. subsericea* workers, and *O. smaragdina* majors had significantly higher MG density than minors in this region (major, *F. subsericea*: $t_{62}=6.09$, $p<0.001$; minor, *F. subsericea*: $t_{62}=3.66$, $p<0.001$; major, minor: $t_{62}=2.11$, $p<0.05$). There was no significant age ($F_1=0.02$, $p=0.89$) or species and subcaste x age interaction ($F_2=0.33$, $p=0.72$). MB collar MG density was significantly higher in *O. smaragdina* workers of both subcastes than *F. subsericea* workers; there was no subcaste difference in this region ($F_2=19.11$, $p<0.001$; major, *F. subsericea*: $t_{62}=5.93$, $p<0.001$; minor, *F. subsericea*: $t_{62}=4.91$, $p<0.001$; major, minor: $t_{62}=0.50$, $p=0.62$). MG density decreased with age in the collar region ($F_1=7.21$, $p<0.01$); there was not a significant species and subcaste x age interaction ($F_2=2.16$, $p=0.12$). *F. subsericea* workers had significantly greater estimated MG volumes than both *O. smaragdina* subcastes in both the MB lip ($F_2=107.08$, $p<0.001$; major, *F.*

subsericea: t_{62}=13.65, p<0.001; minor, *F. subsericea*: t_{62}=12.29, p<0.001; major, minor: t_{62}=0.01, p=0.99) and collar (F_2=117.80, p<0.001; major, *F. subsericea*: t_{62}=14.45, p<0.001; minor, *F. subsericea*: t_{62}=12.68, p<0.001; major, minor: t_{62}=0.39, p=0.70). There were no significant differences in age or species and subcaste x age interaction effects in MG volume.

Ommatidia number, size, and body size

All three groups (*O. smaragdina* major: n=10, minor: n=9, *F. subsericea*: n=10) had statistically similar slopes when ommatidia number was scaled to HW (p=0.54; mean 1.41, range 1.06-2.11) and the slopes were significantly different than 1 (χ^2 =5.09, p<0.05). There was no significant grade shift (W_2 = 2.19, p=0.33), but there was a significant x-axis shift (W_2 = 101.35, p<0.001; Figure 5.4). Ommatidia size scaled to HW also showed statistically similar slopes among all groups (p=0.62; mean 0.86, range 0.57 – 1.27), with slopes not statistically different than 1 (χ^2 =0.63, p=0.43). Again there was no significant grade shift (W_2 = 4.59, p=0.10) while there was a significant x-axis shift (W_2 = 56.52, p<0.001). Both analyses indicate that species and subcaste differences in ommatidia measurements varied according to HW.

Discussion

Cognitive challenges associated with group living are considered to have effects on patterns of neural development (Dunbar and Shultz, 2007b, Gronenberg and Riveros, 2009). To explore how social complexity influences neuroplasticity, we compared volumes of functionally distinct neuropil in newly eclosed and mature workers in two closely related formicine ant species with distinct social phenotypes and examined how MG organization within the MB corresponds to age, subcaste, species, and visual sensory experience during development. Our findings suggest that visual circuitry can exhibit conserved development of ommatidia, the peripheral receptors of the visual system, and experience-expectant organization during development. Additionally, results indicate that *O. smaragdina* workers of both subcastes showed significant experience-expectant neuropil growth and cellular reorganization within the MB when scaled for brain size, whereas *F. subsericea* workers showed no such neuroplasticity. Thus, *F. subsericea* worker brains may be more prepared for behavioral performance at eclosion than *O. smaragdina* worker brains. Species differences in MG density and volume suggest different mechanisms of increasing neural connectivity. Contrary to our hypothesis, division of labor appears to be associated with increased experience-expectant neuroplasticity within higher order processing regions.

Developmental reorganization in the MB

All brain regions of both *O. smaragdina* and *F. subsericea* workers increased in size with age as total brain volume increased. However, the MB in *O. smaragdina* workers of both subcastes increased disproportionately with age. The MB has been shown to increase with age in social insects in association with foraging experience (Withers et al., 1995, Farris et al., 2001, Lutz et al., 2012). However, MB volume may increase in the absence of such experience (Withers et al., 1995), suggesting that a combination of experience-expectant and experience-dependent development occurs (Fahrbach et al., 1998). Experience-expectant neuroplasticity could be due to programmed physiological changes during development that anticipate behavioral changes. Juvenile hormone, which is important for behavioral development (Sullivan et al., 2000), does not appear to alter MB size (Fahrbach et al., 1998, Fahrbach et al., 2003), but other hormones or patterns of gene expression may be linked to MB growth (Ingram et al., 2005, Nelson et al., 2007).

MB volume increase in *O. smaragdina* workers may correspond to expansion of neuropil and synaptic architecture. We found increases in MG volume in the MB lip, which may indicate larger synapses with more vesicles and/or more synapses and therefore enhanced connectivity of MB Kenyon cells (Seid et al., 2005). Larger MG in older workers could be associated with increased responsiveness to pheromones regulating recruitment systems (Hölldobler and Wilson, 1978). In contrast, MG density within the MB collar decreases with age, perhaps due to synaptic pruning (Seid et al., 2005), possibly resulting in refined visual processing for foraging (Groh et al., 2012, Stieb et al., 2012). Alternatively, the increase in MB calyx volume could be due to enlarging synapses; in the absence of synaptic pruning or synaptogenesis, this could lead to decreased MG density. In either scenario, if the increase in volume is consistent throughout the MB calyx, the majority of the MB volume increase in the calyx lip would appear to be due to the enlargement of synapses, whereas the growth in the collar region may be due to an increase in non-neuronal circuitry such as glial cells.

O. smaragdina workers of both subcastes had significantly greater densities of both lip and collar MG compared to *F. subsericea* workers. Increased MG density within the lip is associated with increased odor perception (Falibene et al., 2015), perhaps corresponding to the prominence of pheromonal communication among *O. smaragdina* workers (Hölldobler and Wilson, 1977b, 1978). Accordingly, major workers, which appear to engage in the majority of recruitment actions, had a higher lip MG density than minors. However, *F. subsericea* workers had significantly larger MG volumes and volume ratios than either subcaste of *O. smaragdina* workers. Large MG suggests increased number or size of synapses (Seid et al., 2005). Both increased MG density and volume indicates enhanced synaptic communication between afferent sensory neurons and post synaptic Kenyon cells. These findings suggest that differences in MG organization may not necessarily be associated with interspecific behavioral distinctions; *O. smaragdina* and *F. subsericea* may instead have similar levels of sensory integration and connectivity, albeit by different mechanisms.

Visual processing and experience

Our results suggest that components of the visual system, including ommatidia and the OL, CC, and MB calyx collar, in both *O. smaragdina* and *F. subsericea* are organized with a combination of conserved morphology and canalized development reflecting the sensory demands of the worker. Because we did not manipulate olfactory experience, we predicted no experience-dependent effects on MG in the MB lip, the olfactory input region, in either species. Experience-dependent reorganization is known to be important to visual processing (Stieb et al., 2010, Stieb et al., 2012); however, we show that synaptic plasticity occurs independent of light input. This is consistent with Kroczik et al. (2008), who show that deprivation of pheromonal cues during development does not alter MG organization. The physiological mechanisms and the genetic architectures that regulate experience-expectant MG organization are unknown. Vitellogenin and juvenile hormone do not appear to affect MG organization (Scholl et al., 2014); however expression of the *foraging* gene could be involved in age-related behavioral transitions (Ingram et al., 2005).

The MB volume increases occurred over a longer developmental time course than the time period studied to compare MG. Development during the first 30 days of adult life may not typically involve substantial light exposure; synaptic plasticity may occur after 30 days post eclosion (Seid and Wehner, 2009). However, this explanation is not likely able to account for not finding an effect of visual experience on synaptic organization in our study because *O. smaragdina* majors and *F. subsericea* workers are found outside of the nest by 30 days post eclosion (personal obs.), and as few as two days of intermittent light exposure can produce a significant effect of synaptic plasticity within the MB collar (Stieb et al., 2012). Additional developmental studies of controlled light exposure, however, would elucidate whether visual experience alters synaptic organization in *O. smaragdina* and *F. subsericea* workers after 30 days post-eclosion and thus further contributes to MB plasticity.

Functional implications of MB plasticity

It is unclear how the size of the MB precisely affects behavioral output in social insects. Newly eclosed workers engage in brood care within the nest and may increase their behavioral repertoires with age, incorporating tasks at worksites outside of the nest (Seid and Traniello, 2006). Tasks performed by mature workers may require substantial sensory integration. Visual demands of foraging and olfactory requirements of brood care may be shared among *O. smaragdina* and *F. subsericea* workers; however, *O. smaragdina* have at least five forms of recruitment that utilize a combination of chemical and tactile cues (Hölldobler and Wilson, 1978), while no such sophisticated recruitment system has been observed in *F. subsericea* (Wallis, 1964). Additional environmental chemical compounds would be expected to require similar processing demands in both species; however, *O. smaragdina* may require greater olfactory processing requirements for recruitment. No species differences in AL volumes could be identified; the ability to distinguish between pheromones and pheromone blends to respond adaptively to the perception of multiple chemical cues in a variety of contexts (Czaczkes et al., 2015b) may nevertheless require MB input (Hu et al., 2010).

We predicted that *F. subsericea* workers would require greater behavioral flexibility and therefore higher levels of neural plasticity due to their more solitary actions and broader, generalized repertoire encompassing tasks within and outside of the nest. Our results did not support this hypothesis, suggesting that experience-expectant MB development at both macroscopic and cellular levels occur in both species. Alternatively, the modulation of intracellular architecture, receptor expression, and neurotransmitter release that occurs with learning and memory, and thus behavioral plasticity (Menzel and Muller, 1996), may not be detected by our measurements of MB neuropil volume or MG density or volume. Moreover, behavioral repertoires of *O. smaragdina* workers may not be as circumscribed and may require behavioral plasticity and adaptive sensory processing due to cognitive demands associated with behavioral repertoire and/or increased individualized social interactions. The vertebrate social brain hypothesis suggests that larger brains evolved in members of larger social groups due to more complex and individualized interactions. Although it is difficult to directly apply the hypothesis to social insects, similar cognitive demands may be associated with living in a colony. Workers are not able to recognize individuals (Hölldobler and Wilson, 1990, Vander Meer and Morel, 1998), but they can discriminate nestmates (Hölldobler and Wilson, 1977a, Blomquist and Bagnères, 2010, Newey et al., 2010b) and groups of workers in different social roles (Greene and Gordon, 2003). Interactions, therefore, may be more individualized the greater the polymorphic task specialization present in the colony. Thus, division of labor in ants may be associated with a greater requirement for cognitive processing and behavioral plasticity.

Collective intelligence may also be more cognitively demanding than predicted. Although behaviorally simple system components are considered sufficient for collective action (Anderson and McShea, 2001, Jeanson et al., 2012), individuals with more complex neural processing could enhance cooperative decision-making (Czaczkes et al., 2015a). The cooperative and highly coordinated actions of *O. smaragdina* workers may be regulated by a simple algorithms (Bochynek and Robson, 2014), yet integrative sensory processing may be required to support the individual behaviors that contribute to group-level actions. Therefore, collective action may not necessarily drive a decrease in individual cognitive capability, as predicted from self-organization theory.

Our data demonstrate a relationship between colony-level organization, worker behavioral repertoire, neuropil investment, and synaptic structure in two species characterized by strikingly different social phenotypes. Neuroplasticity occurs in association with worker morphological and behavioral differentiation in respect to subcaste variation in neuropil investment, and in patterns of behavioral development in major and minor workers. Both *F. subsericea* and *O. smaragdina* workers show canalized neural development that may reflect conserved formicine developmental patterns and/or basic ecological similarities. *O. smaragdina* major and minor workers, however, have larger MB volumes that correlate with greater experience-expectant developmental plasticity in the MB perhaps due to division of labor and cognitive demands associated with their preeminent role in collective action.

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Brain volume (y)	**Scaling slope (β)**	**β different than 1?**	**Grade shifts?**	**x-axis shifts?**	**Comparisons**
OL	0.93 (0.80,1.10)	No (χ^2=0.78, p=0.38)	Yes (W^2=103.98; $p<0.001$)	Yes (W^2=142.74; $p<0.001$)	*O. smaragdina* minors < majors and *F. subsericea*
AL	Worker groups differ	Yes	n/a	n/a	n/a
MB-lc	1.21 (1.09,1.35)	Yes (χ^2=12.53, $p<0.001$)	Yes (W^2=54.84; $p<0.001$)	Yes (W^2=186.81; $p<0.001$)	*F. subsericea* < mature *O. smaragdina*
MB-mc	1.31 (1.17,1.46)	Yes (χ^2=20.06, $p<0.001$)	Yes (W^2=124.67; $p<0.001$)	Yes (W^2=207.33; $p<0.001$)	
MB-ped	0.99 (0.86,1.14)	No (χ^2=-0.006, p=0.94)	Yes (W^2=32.25; $p<0.001$)	Yes (W^2=195.24; $p<0.001$)	
SEZ	1.10 (0.95,1.30)	No (χ^2=1.81, p=0.18)	Yes (W^2=51.73; $p<0.001$)	Yes (W^2=129.51; $p<0.001$)	mature *F. subsericea* > *O. smaragdina*
CC	1.74 (1.46,2.09)	Yes (χ^2=26.59, $p<0.001$)	Yes (W^2=31.20; $p<0.001$)	Yes (W^2=126.00; $p<0.001$)	No significant differences

Table 5.1: Scaling analyses of brain regions. The volume of each compartment was compared to the volume of the rest of the central brain (ROCB), or in the case of the SEZ, the central brain, calculated separately for each region. Column 3 shows results of tests for groups sharing a common slope (mean slope, 95% confidence intervals in parentheses). Column 4 shows results of tests of whether the slope differed from 1 (χ^2 tests). Columns 5 and 6 show results of Wald tests. Statistical details are presented in parentheses. n/a = not applicable.

Brain region	**Species**	**Newly eclosed**	**Mature**
OL	*F. subsericea*	3.15±1.04x10^{-3}	6.11±1.24x10^{-3}
	major	5.00±1.55x10^{-3}	7.10±0.84x10^{-3}
	minor	2.83±1.08x10^{-3}	4.42±0.99x10^{-3}
AL	*F. subsericea*	2.10±1.02x10^{-3}	5.24±1.87x10^{-3}
	major	3.77±1.96x10^{-3}	6.55±1.06x10^{-3}
	minor	2.53±1.02x10^{-3}	4.78±0.90x10^{-3}
MB-calyx	*F. subsericea*	3.76±1.08 x10^{-3}	9.52±2.72x10^{-3}
	major	7.91±3.36x10^{-3}	17.01±2.79x10^{-3}
	minor	4.90±1.74x10^{-3}	14.30±4.31x10^{-3}
MB-lobes	*F. subsericea*	1.85±0.55x10^{-3}	5.11±0.97 x10^{-3}
	major	3.78±1.56x10^{-3}	7.70±1.06x10^{-3}
	minor	2.83±0.74x10^{-3}	6.78±1.46x10^{-3}
Total MB	*F. subsericea*	5.61±1.56x10^{-3}	2.50±1.83x10^{-2}
	major	11.69±4.85x10^{-3}	2.77±1.87x10^{-2}
	minor	7.73±2.42x10^{-3}	2.71±2.41x10^{-2}
SEZ	*F. subsericea*	2.66±0.74x10^{-3}	6.73±0.15x10^{-3}
	major	4.05±1.73x10^{-3}	6.51±0.12x10^{-3}
	minor	2.96±1.36x10^{-3}	5.50±0.14x10^{-3}
CC	*F. subsericea*	0.72±0.28x10^{-4}	2.36±0.87x10^{-4}
	major	2.32±1.30x10^{-4}	3.08±0.82x10^{-4}
	minor	1.75±0.55x10^{-4}	2.69±0.85x10^{-4}
Total brain	*F. subsericea*	2.26±0.68 x10^{-2}	5.42±1.26 x10^{-2}
	major	4.19±1.68x10^{-2}	7.20±0.87 x10^{-2}
	minor	2.84±0.85x10^{-2}	5.83±1.27 x10^{-2}

Table 5.2: Mean ± sd of brain region and total brain volumes (mm^3) for newly eclosed and mature *F. subsericea* workers and *O. smaragdina* major and minor workers.

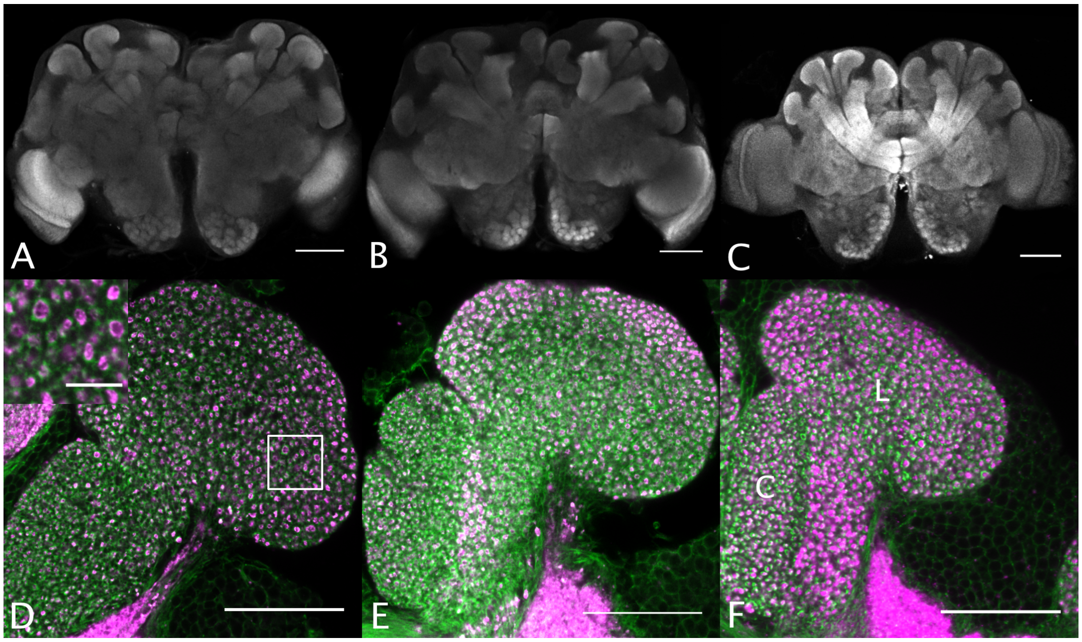

Figure 5.1: Confocal micrographs of synapsin-labeled brains of an *O. smaragdina* minor (A) and major worker (B) and *F. subsericea* worker (C). Scale bar = 100μm. Micrographs of MG of an *O. smaragdina* minor (D) and major (E) worker and *F. subsericea* worker (F). Synapsin in the presynaptic boutons (magenta) and f-actin in the postsynaptic density (green) define MG. Letters in (F) demarcate the calyx lip (L) and collar (C). Scale bar = 50μm. Box in (D) delimits area of inset; inset scale bar = 10μm.

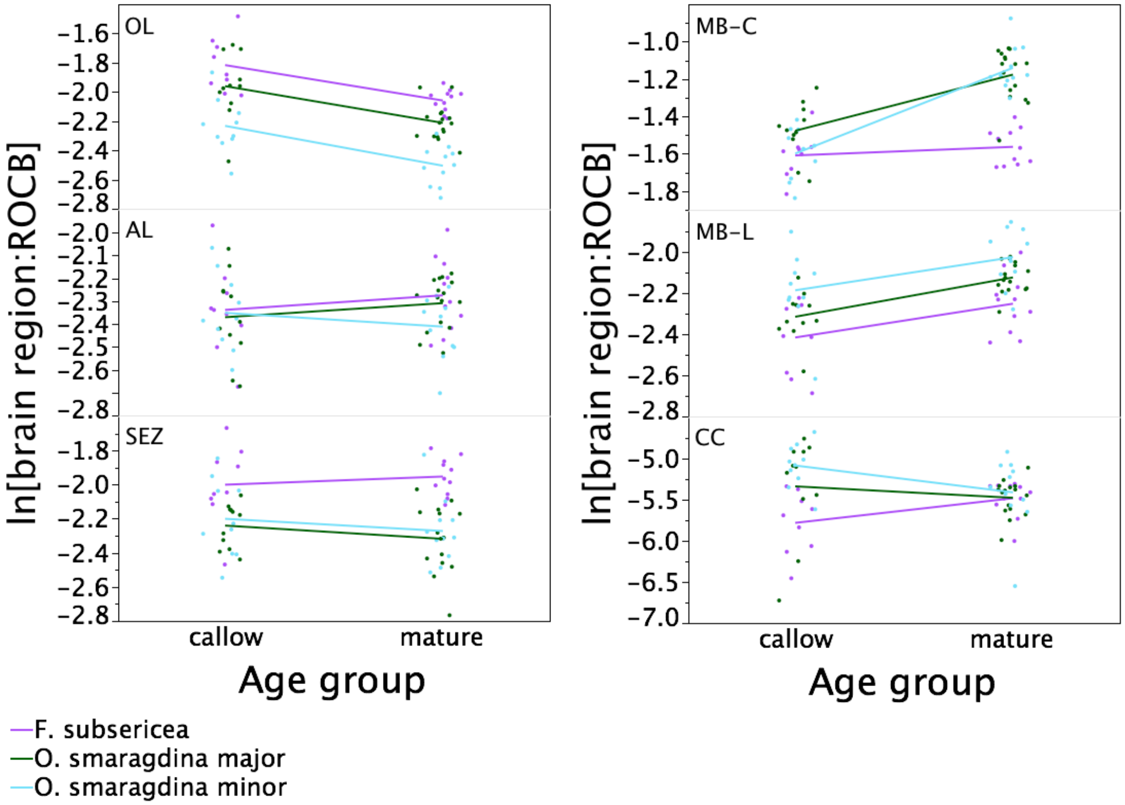

Figure 5.2: Relationship between worker age and brain compartment volumes in *F. subsericea* workers and *O. smaragdina* subcastes. The natural log of the ratio of each brain compartment to the ROCB are presented for callow and mature workers. Lines connect means for each species and subcaste groups and display patterns in brain compartment development. Brain regions: optic lobe (OL), antennal lobe (AL), subesophageal zone (SEZ), mushroom body calyx (MB-C), mushroom body lobes (MB-L), central complex (CC).

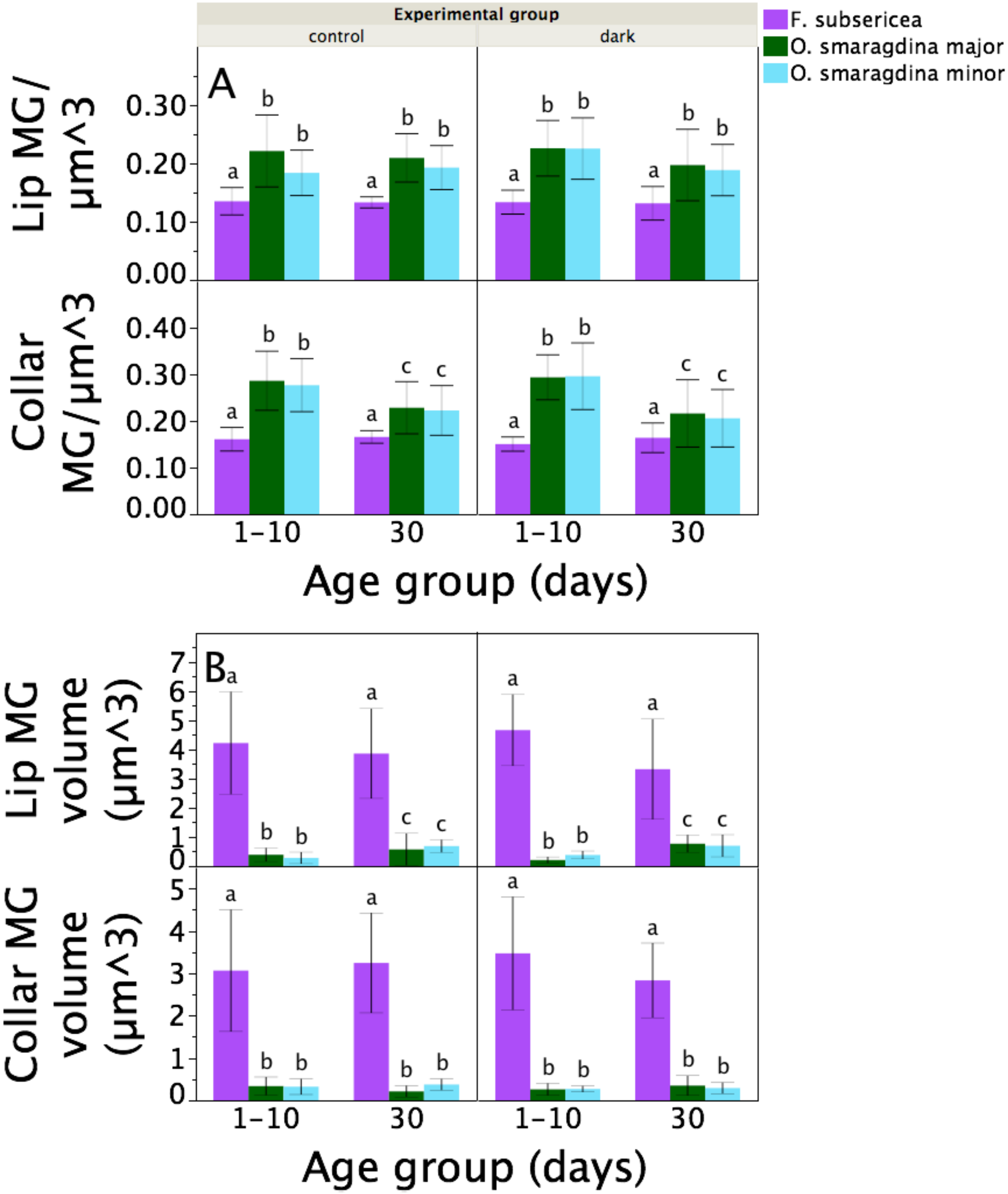

Figure 5.3: Mean MG lip and collar density (A) and MG volume in the lip and collar (B) are presented for *F. subsericea* workers and *O. smaragina* major and minor workers.

Different letters above bars indicate significant differences between groups. Error bars represent standard deviation.

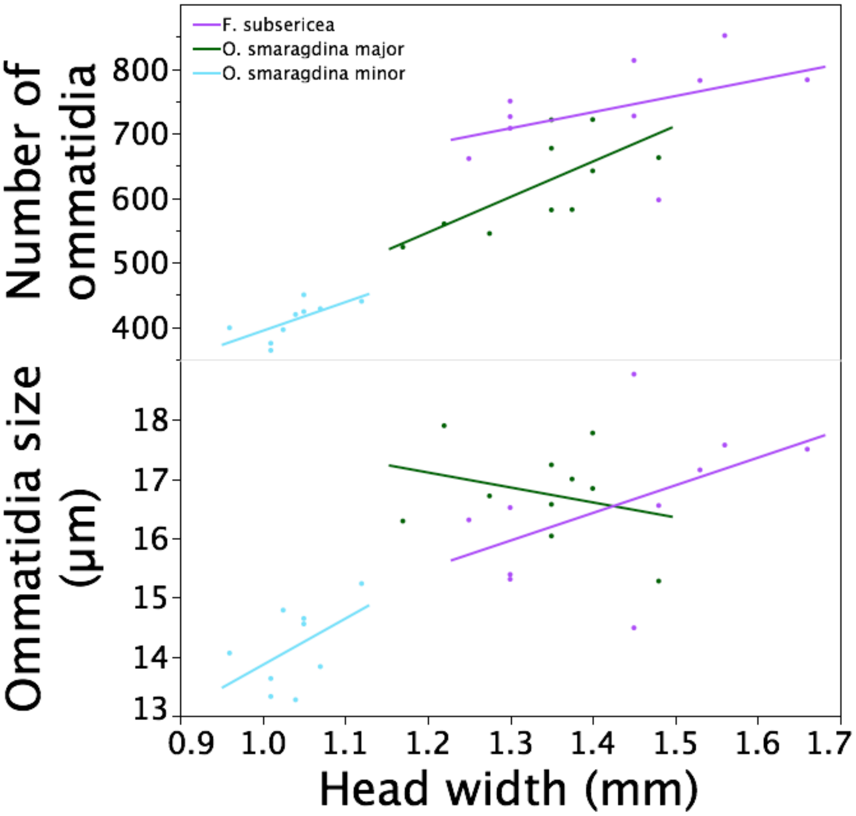

Figure 5.4: Ommatidia number and size scaled to head width for *F. subsericea* workers and *O. smaragina* major and minor workers. Lines represent best-fit regression lines for each species and subcaste group.

CHAPTER 6: CONCLUSIONS

Organizational complexity in eusocial insects is associated with large colony size, worker division of labor, and emergent collective action, all of which are instrumental to ecological success. Cognitive challenges correlated with increased social complexity are difficult to precisely specify, yet are considered significant influences on worker brain evolution. Moreover, the social brain hypothesis, which was developed in reference to vertebrates, does not appear to be directly applicable to eusocial insects due to selection acting at the level of both the individual and society, and perhaps due to brain miniaturization, which can be extreme. Integrating vertebrate social brain theory with sociobiological concepts generates a revised social brain hypothesis that incorporates the kin-selected traits of eusocial insects and ecological drivers of brain evolution. This "eusocial brain" hypothesis predicts smaller brain size with increasing division of labor and thus enhanced social complexity (Gronenberg and Riveros, 2009). Metabolic costs of producing and operating the brain and its functional compartments are predicted to correspond to the cognitive requirements of worker task performance that vary interspecifically and change with behavioral development. Brain region size would thus be expected to be optimized to maintain sufficient cognitive ability in light of the high metabolic costs of neural tissue. Alternatively, in an ecological challenges hypothesis, brain size and compartment size should correspond to the cognitive demands of worker behavioral repertoire independent of degree of social complexity. Through comparative neurochemical and neuroanatomical studies this dissertation is based on research that integrated social brain theory, neuroecology, sociobiology, and brain miniaturization theory.

The social brain hypothesis does not posit specific predictions as to how the expression and action of biogenic amines such as serotonin, dopamine, and octopamine are associated with the evolution of social complexity. However, based on comparative analyses in vertebrates, neurochemical receptors appear to be highly conserved in regions associated with social behavior, whereas the location of neurochemical production is more plastic (O'Connell and Hofmann, 2012). Extending the social brain hypothesis to biogenic amines, I expect that maintaining synapses responsive to ecological and social demands likely incurs high metabolic costs (Niven and Laughlin, 2008). Thus, specialized behavioral repertoires and biogenic amine circuitry and titers that tune efficient responses in minute amounts of brain tissue could reduce metabolic costs. My research supports this hypothesis by showing that the action of a specific biogenic amine, octopamine, was positively correlated with worker morphological subcaste-related territorial aggression and causally modulated this social behavior in *Oecophylla smaragdina* (Kamhi et al., 2015). *O. smaragdina* major workers that specialize in colony defense appear to have octopaminergic circuitry that is adapted to respond aggressively to non-nestmates, whereas octopamine titers are reduced in minor workers.

While *O. smaragdina* and *Formica subsericea* vary substantially in their level of social complexity, the differences in worker behavioral repertoire render comparisons involving social brain evolution more challenging. Additionally, it is difficult to interpret how components of social complexity affect the worker brain because we have a very limited understanding of the circuitry of the ant brain as well as the cognitive demands associated with worker task performance, worker interactions, and the genesis of emergent collective intelligence. However, the results presented here appear to support a combination of the eusocial brain and ecological challenges hypotheses and demonstrate that components of social complexity, including division of labor, large colony size, and collective action, may have varying effects on brain evolution. Collective intelligence may decrease brain metabolic activity through reduced individual cognitive requirements for cooperative decision-making. Workers also invest in neural tissue according to the cognitive demands of worker behavioral repertoires. Consistent with the social brain hypothesis as defined in vertebrates, large colony size is associated with greater brain size and more neural plasticity. Both group and individual level cognitive demands therefore may drive social brain evolution.

Theories of brain evolution in eusocial insects need to be modified to consider both colony-level, or social selection, and individual selection for ecological function. Specifying the exact contribution of either selective agent is difficult because worker behavior is in both cases predicted to be designed to improve colony fitness. Colony-level cognitive challenges appear to be driving overall brain architecture and neural development. My results demonstrated that *O. smaragdina* worker subcastes have similar patterns of brain size and developmental neuroplasticity whereas there were striking differences between *O. smaragdina* and *F. subsericea* workers. Patterns of metabolic activity, brain compartment size, and neurochemical action suggest that these neurobiological characteristics are driven by the cognitive demands associated with individual worker task performance. Metabolic activity and brain region and neurochemical circuitry development appear to be more plastic because there is significant variation between *O. smaragdina* worker subcastes as well as differences between *O. smaragdina* and *F. subsericea* workers. Models of social brain hypothesis should therefore take into account both social and individual selection for brain evolution to accurately address the ecological demands of social complexity in ants.

Future directions

My dissertation provides insight into how neuroanatomy, neurometabolic activity, and neurochemistry correspond to division of labor, behavioral development, and indirectly, emergent collective action. My results offer a framework for further experimental and comparative analyses to explore the relationship between neurobiology and task performance in the context of social complexity. I discuss two opportunities for future research that would aid in developing a cohesive model of eusocial brain evolution in ants.

Neuromodulation of social behavior

Biogenic amines play an important role in modulating a variety of social behaviors from nestmate recognition and territorial defense to subcaste- and age-related as well as reproductive division of labor. It is therefore no surprise that studies of neuromodulation in ants are increasing in an attempt to fill the numerous gaps in our understanding of the regulation of complex social behavior. Apparent contradictions, however, have arisen from results in different species using different behavioral assays. In Chapter 2, several avenues of future research were discussed that would help clarify the role of biogenic amines in social behavior in ants and other eusocial insects (Kamhi and Traniello, 2013). Here, I will focus on a synthesis that follows from the work presented in Chapter 3 (Kamhi et al., 2015).

Division of labor among morphologically distinct worker subcastes is a hallmark of social complexity in ants, and has been shown to be epigenetically regulated (Bonasio et al., 2012) and lead to both neuroanatomical (Muscedere and Traniello, 2012) and neurochemical (Kamhi et al., 2015) phenotypic variation. To date, the genomes for eight ants have been sequenced (Bonasio et al., 2010, Nygaard et al., 2011, Smith et al., 2011a, Smith et al., 2011b, Suen et al., 2011, Wurm et al., 2011, Oxley et al., 2014). However, thus far in social insects, genomics and biogenic amine studies have been bridged only in the honey bee (Hauser et al., 2006). Expanding this work in ants would open the door for rigorous studies of subcaste variation in gene expression of biogenic amine receptors and monoamine synthesis. To better understand the neurochemical mechanisms underlying subcaste-related division of labor, future studies should also explore how neuroanatomical variation among subcastes alters biogenic amine production, action, and receptor expression. Receptors are likely expressed ubiquitously throughout the brain (Schafer and Rehder, 1989, Blenau and Thamm, 2011, Sinakevitch et al., 2011), and changes in brain region size that may affect synaptic connectivity (Chittka and Niven, 2009) and receptor expression could be associated with task performance (Reim and Scheiner, 2014). Additionally, studies should aim to causally demonstrate how biogenic amine titers and action affects subcaste-related behavioral output. Exploring the role of biogenic amines in division of labor will provide a better understanding of one of the key components of social complexity and aid in developing hypotheses for how biogenic amines may adaptively serve social organization.

Comparative neurobiological studies of eusocial brain evolution

My results showed that distinct patterns of brain organization correspond to degree of social complexity in *O. smaragdina* and *F. subsericea* colonies. Whether these findings can be used in a generalized model of macroevolutionary neuroanatomical and neurochemical phylogenetic comparisons across all ant subfamilies remains to be explored. There is a wide range of social organization found in ants, ranging from slavemaker ants (e.g. *Polyergus*), which are highly specialized for raiding nests to capture brood from host colonies that then provide the labor force for the parasitic colony (Topoff et al., 1989, O'Donnell et al., 2007), to queenless ants (e.g. *Streblognathus peetersi*) that do not have epigenetically determined reproductive division of labor and are behaviorally versatile, with the ability to be both reproductively dominant and behave as workers (Ware et al., 1990). Additionally, it is not known whether other species with emergent collective action, such as leaf cutter ants (e.g. *Atta cephalotes*) that maintain an agricultural system, show similar neural characteristics to *O. smaragdina*. Moreover, as previously mentioned, there is currently no hypothesis of how biogenic amines reflect social complexity. Adding neurochemical analyses to phylogenetic comparisons would provide a framework for developing and testing theories of how biogenic amine expression and action may correspond to colony attributes associated with social complexity. Examining the neurobiological correlates of social behavior in phylogenetically closely related species with a wide variety of social systems across many ant subfamilies is crucial for developing a comprehensive theory of social brain evolution in ants.

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