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Cumulative Effects of Foraging Behavior and Social Dominance on Brain Development in a Facultatively Social Bee *(Ceratina australensis)*

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Key Words

Mushroom bodies · Bee · Social experience · Brain development · Brain evolution · Foraging · Social dominance · Insect brain · Insect behavior · Evolution

Abstract

In social insects, both task performance (foraging) and dominance are associated with increased brain investment, particularly in the mushroom bodies. Whether and how these factors interact is unknown. Here we present data on a system where task performance and social behavior can be analyzed simultaneously: the small carpenter bee *Ceratina australensis*. We show that foraging and dominance have separate and combined cumulative effects on mushroom body calyx investment. Female *C. australensis* nest solitarily and socially in the same populations at the same time. Social colonies comprise two sisters: the social primary, which monopolizes foraging and reproduction, and the social secondary, which is neither a forager nor reproductive but rather remains at the nest as a guard. We compare the brains of solitary females that forage and reproduce but do not engage in social interactions with those of social individuals while controlling for age, reproductive status, and foraging experience. Mushroom body calyx volume was positively correlated with wing wear, a proxy for foraging experience. We also found that, although total brain volume did not vary among reproductive strategies (solitary vs. social nesters), socially dominant primaries had larger mushroom body calyx volumes (corrected for both brain and body size variation) than solitary females; socially subordinate secondaries (that are neither dominant nor foragers) had the least-developed mushroom body calyces. These data demonstrate that sociality itself does not explain mushroom body volume; however, achieving and maintaining dominance status in a group was associated with mushroom body calyx enlargement. Dominance and foraging effects were cumulative; dominant social primary foragers had larger mushroom body volumes than solitary foragers, and solitary foragers had larger mushroom body volumes than nonforaging social secondary guards. This is the first evidence for cumulative effects on brain development by dominance and task performance.

Introduction

Brain plasticity – changes in brain region size and/or complexity – often accompany changes in environmental experience even in adult animals [Edelman and

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Changeux, 2001; Ricklefs, 2004; Byrne and Bates, 2007; Gronenberg and Riveros, 2009]. Since neural tissue is energetically expensive, it is predicted that brain regions should only enlarge when needed to meet functional demands [Aiello and Wheeler, 1995; Niven and Laughlin, 2008]. Among social insects, individual differences in brain investment patterns are associated with both complex task performance (e.g. foraging behavior) and social dominance [Farris et al., 2001; Molina and O'Donnell, 2007, 2008; Fahrbach and Dobrin, 2009; Riveros and Gronenberg, 2010; Smith et al., 2010; Amador-Vargas et al., 2015]. Whether and how these environmental factors interact in affecting brain development is not known.

The social brain hypothesis posits that social interactions are so cognitively demanding that the social environment selects for enhanced neural development [Humphrey, 1976; Dunbar and Shultz, 2007]. The investment in the mushroom body calyx is of special interest due to its association with sensory integration and learning [Heisenberg, 1998; Strausfeld et al., 2009]. Sensory stimuli and associated learning lead to increased mushroom body development in honey bees [Fahrbach et al., 1995, 1998; Farris et al., 2001]. Mushroom bodies are complex brain structures with separate regions devoted to visual and antennal processing. The evolution of mushroom bodies predated the origin of sociality in Hymenoptera but may facilitate or provide a necessary preadaptation for sociality [Farris and Schulmeister, 2011; reviewed by Lihoreau et al., 2012].

Bees are among the most socially labile organisms, ranging from solitary to an advanced eusocial colony organization [Michener, 1974]. A study on solitary bees, i.e. *Osmia lignaria,* found that adults emerge with neuronal development typical of experienced workers in advanced eusocial insect species, but it also found that the mushroom body neuropil volume further increased with foraging experience [Withers et al., 2007]. These results confirm that mushroom bodies are important for cognitive processes in solitary antecedents, but cannot inform us on the role of social experience on neuropil investment. Smith et al. [2010] examined brain size and development using the facultatively social bee *Megalopta genalis,* a species with both solitary and primitively social colonies in the same populations. Mushroom body size increased with foraging behavior in solitary individuals and workers compared to newly emerged *M. genalis* females, but even greater mushroom body development was found in queens. Smith et al. [2010] attributed the enhanced growth of the mushroom body calyx to maintaining behavioral dominance. Much like obligately eusocial bees, however, *Megalopta* have distinct reproductive (queens that rarely leave the nest) and nonreproductive, foraging (workers) roles and therefore the relative roles of foraging and social experience are not clear.

To more directly test the relative effects of sociality and foraging experience on brain development, we used the facultatively social small carpenter bee *Ceratina australensis*. This species has sympatric solitary and social life history strategies. Social colonies comprise two sisters: the social primary which monopolizes foraging and reproduction, and the social secondary which is neither forager nor reproductive but rather remains at the nest as a guard [Rehan et al., 2010, 2011, 2014]. In this system we can directly compare the brains of solitary females that forage and reproduce but do not engage in social interactions with those of social individuals while controlling for age, reproductive status, and foraging experience.

The plasticity of insect brain development coupled with the behavioral variation in *C. australensis* allowed us to test four related hypotheses relating to brain size and investment relative to social experience. First, if sociality is more cognitively demanding than solitary living, then all social females should have larger mushroom body calyx volumes than solitary females [Gronenberg and Riveros, 2009]. Second, sociality itself may not be as cognitively demanding as achieving and maintaining dominance status in a group, as found in paper wasps [O'Donnell et al., 2006; Molina and O'Donnell, 2007, 2008; Molina et al., 2009]. If so, we predicted that dominant social primaries should have larger mushroom body calyx volumes than social secondary or solitary females. Third, studies on honeybees and solitary bees suggest that brain volume increases with foraging experience [Fahrbach et al., 2003; Withers et al., 2007]. Under this hypothesis we predicted that both social primary and solitary females (foragers) should have larger mushroom body calyces than secondary females (nonforagers). Fourth, dominance and foraging effects could be compounded or cumulative. If so, we predicted that dominant social primary foragers should have larger mushroom body calyx volumes than solitary foragers, and solitary foragers should have larger mushroom body volumes than nonforaging social secondary guards.

Methods

Field Collections, Classifications, and Measurements

All collections were conducted in Warwick, Qld., Australia (28.24° S, 152.14° E). Natural solitary and social nests were collected from dead broken fennel *(Ferula communis)* stems in the

field at dawn and dusk to ensure that no bees were out foraging. Nests were sealed at the entrance with masking tape and cut at the base of the shoot with pruning shears. Stems were opened in the lab by splitting them lengthwise and the contents were recorded. Nests were categorized based on their contents as per Rehan et al. [2009, 2010]. Predispersal nests contained callow newly emerged adult females prior to departing to found new nests but did not contain pollen provisions or immature offspring (larvae and pupae). Founding nests contained recently dispersed adult females but did not contain pollen masses or eggs. Active brood nests contained actively foraging and reproducing females as evidenced by the presence of pollen masses with eggs. Full brood nests contained adult females guarding their brood and were those in which the cell closest to the nest entrance contained a larva or pupa, suggesting that the female had finished foraging and egg laying. In this species, solitary nests contain a single adult female and social colonies contain two sister cofoundresses [Rehan et al., 2010]. Social nests were conservatively identified as two adult females found within reproductively active (active and full brood) nests [Rehan et al., 2009]. Social nests are formed between two full sisters [Rehan et al., 2014]. Of these two, the social primary female monopolizes reproduction and foraging and the social secondary is nonforaging and nonreproductive [Rehan et al., 2010, 2011].

Based on nest collection, females were assigned to age classes as solitary females from youngest to oldest across the colony cycle as follows: predispersal, founding nest, active brood, and full brood. Solitary females were examined from each of the above-described categories of the colony cycle, each from different nests: six predispersal callow offspring, five adult females from founding nests, five adult females from active brood nests, and six adult females from full brood nests. We examined five social pairs of primary and secondary females from active brood nests. All social primary and secondary females were collected at the active brood stage.

Adult females were measured to quantify body size and dissected to score reproductive status using a dissecting microscope at a ×20 magnification (accuracy ± 0.01 mm). Head width was measured across the widest part of the head to the outer margins of both compound eyes. Head width is linearly correlated with wing length and live weight [Rehan et al., 2010], so head width is a good proxy for body size. In addition, we measured ovarian development and wing wear to determine if reproductive status and foraging effort correlate with brain development to assess the reproductive dominance and foraging experience hypotheses, respectively. Wing wear is a reliable proxy for foraging effort [Cartar, 1992]. Bees with no nicks or tears on the apical margins of both forewings received a score of zero, and bees with the apical margin of both forewings completely worn to shreds received a wing wear score of five. Adult females were dissected to determine their reproductive status. Ovary size was measured as the sum of the lengths of the three largest terminal oocytes (accuracy ± 0.01 mm).

Histology for Brain Anatomy

As the nests were opened, the heads of callow offspring and adult females were detached and stored in fixative (Prefer; Anatech Ltd.). We later removed the mandibles, glossa, and antennae to increase resin infiltration into the head. Head capsules were dehydrated through a series of increasing ethanol concentrations and acetone and then through increasing concentrations of plastic resin [resin composition: 5.5 g of Embed 812 (a mixture of bisphenol A/epichlorohydrin epoxy resin, CAS No. 25068-38-6; epoxy modifier, CAS No. 2425-79-8), 5.7 g of dodecenyl succinic anhydride, 0.65 g of dibutyl phthalate, and 0.31 g of 2, 4, 6-tri(dimethylaminoethyl)phenol]. Individual bee heads were incubated in 0.1 ml of resin in pyramid molds at 60°C for 72 h and then glued to 0.5-ml acrylic cylinders with cyanoacrylate adhesive. Each embedded head was cut along the frontal plane into 14-μm-thick sections using a rotary microtome with disposable steel histology blades. Sections were mounted on gelatin-coated microscope slides, and the tissue was stained with toluidine blue. We cleared the stained sections in a series of increasing ethanol concentrations and cover-slipped under transparent mounting medium.

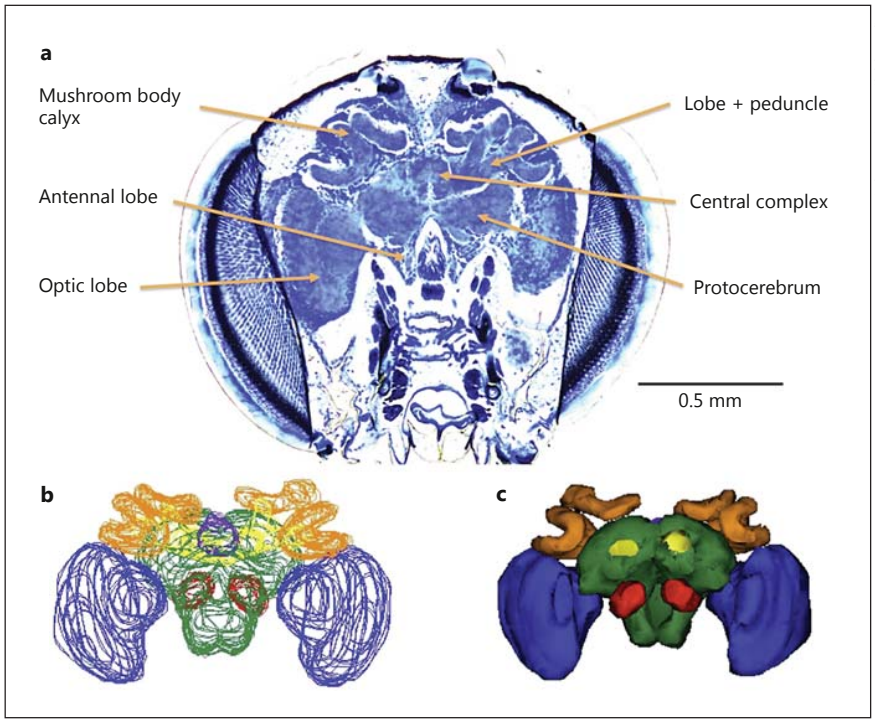
We used a microscope-mounted digital camera to photograph the tissue sections at a resolution of 2,560 by 1,920 pixels using 2.5× microscope objectives (fig. 1a). To estimate the brain structure volume, we outlined target brain regions and quantified the number of image pixels in the structure using IMAGE J digital imaging analysis software (version 1.46; http://rsbweb.nih.gov/ij/), and then we converted the pixel counts to area (in mm^2) using a photograph of a stage micrometer taken at the same resolution and magnification as a size reference. We multiplied the areas by the section thickness (0.014 mm) to yield volume.

Neuroanatomical Measurements

We estimated the volumes of the following brain subregions: antennal lobes, optic lobes (medulla plus lobula), mushroom body calyces (lip, collar, and basal ring), remaining protocerebrum, central complex, and combined mushroom body lobe and peduncle regions (fig. 1b). Brain regions were identified and delimited as in the studies by Fahrbach et al. [2003] and O'Donnell et al. [2013]. The total volume of each region and the total brain volume were calculated by summation of the brain area across each serial slice. We measured only brain neuropils (regions of dendritic arborization and axonal connections); we did not measure adjacent regions containing the cell bodies (somata) of the brain's intrinsic neurons. Serial images were aligned digitally and 3-D reconstructions (fig. 1c) were made by tracing individual brain regions using RECONSTRUCT software [Fiala, 2005]. Central brain volume (the sum of the volumes of the protocerebrum, central complex, and lobe and peduncle) was used to control for brain size scaling effects [O'Donnell et al., 2013]. All comparisons of antennal lobe, optic lobe (sum of medulla and lobula lobes), and mushroom body calyx volumes are based on central brain scaled measurements.

Statistical Analyses

All statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, Ill., USA). Data were confirmed to have normal distributions and equal variances for ANOVA and regression statistics. We used ANOVA to test for age and social class differences in brain region volumes. Post hoc Tukey HSD tests were applied to analyze significant changes in brain regions associated with age and social class. To determine the effects of foraging experience (as measured by wing wear) and ovarian development on brain size, we performed linear regression tests with Bonferroni's correction to adjust for multiple comparisons. Paired t tests were used for comparisons of social primary and secondary females from the same nest.



Color version available online

Fig. 1. Neuroanatomy of *C. australensis*. **a** Brain subregions quantified in this study. **b** Area of the brain subregions stacked across all 14-µm cross sections. Orange = Mushroom body calyx; red = antennal lobes; blue = optic lobes; green = protocerebrum; purple = central complex; yellow = lobes and peduncle (colors refer to the online version only). **c** 3-D reconstruction of the bee brain by subregion.

Results

Social Class and Age-Related Changes in Brain Volume

Females were indistinguishable in body size regardless of age or social class (ANOVA, $F_{5, 31} = 2.157$, $p = 0.09$). Social primary and secondary females from the same nest were similar in size (paired t test, $t = 1.43$, $P = 0.16$, d.f. = 4, $p = 0.16$). Total brain volume positively scaled with body size (linear regression, $r^2 = 0.348$, $n = 32$, $p = 0.05$; online suppl. fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000381414). We found no effect of age (predispersal, founding nest, active brood, and full brood) or social class (primary and secondary) on body size-corrected total brain volume (ANOVA, $F_{5, 31} = 2.16$, $p = 0.09$; online suppl. fig. S2).

There were no significant differences in the volume of the following brain subregions when correcting for body size among age and social classes: optic lobes ($F_{5, 31} = 1.84$, $p = 0.14$), antennal lobes ($F_{5, 31} = 1.17$, $p = 0.35$), central complex ($F_{5, 31} = 1.05$, $p = 0.41$), lobes and peduncle ($F_{5, 31} = 1.69$, $p = 0.17$), and protocerebrum ($F_{5, 31} = 0.55$, $p = 0.73$). However, there was a significant difference in mushroom body calyx volume relative to central brain volume among female classes ($F_{5, 31} = 18.14$, $p = 0.0001$); post hoc Tukey tests showed that predispersal females had markedly smaller mushroom body calyces than active and full brood females, and social primaries had markedly larger mushroom body calyces than all other female classes. Social secondary females had intermediate mushroom body calyx volumes larger than newly eclosed predispersal females but smaller than solitary active and full brood foragers.

When correcting for central brain (central complex, protocerebrum, and lobes and peduncle) volume, rather than body size-scaled volumes, there were no significant differences observed in antennal lobe ($F_{5, 31} = 1.79$, $p = 0.15$) and optic lobe ($F_{5, 31} = 1.27$, $p = 0.31$) volumes among female classes. There was a significant difference in mushroom body calyx volume among female classes relative to central brain volume ($F_{5, 31} = 10.56$, $p = 0.0001$; fig. 2). Post hoc Tukey tests revealed the same patterns for central brain-corrected mushroom body calyx volume changes as the above uncorrected brain size measures; social primaries had larger mushroom body calyces than all other female classes, social secondary females had intermediary mushroom bodies that were larger than newly eclosed predispersal females but smaller than solitary active and full brood foragers, and the youngest predispersal females had smaller mushroom body calyces than all older female classes (fig. 2).

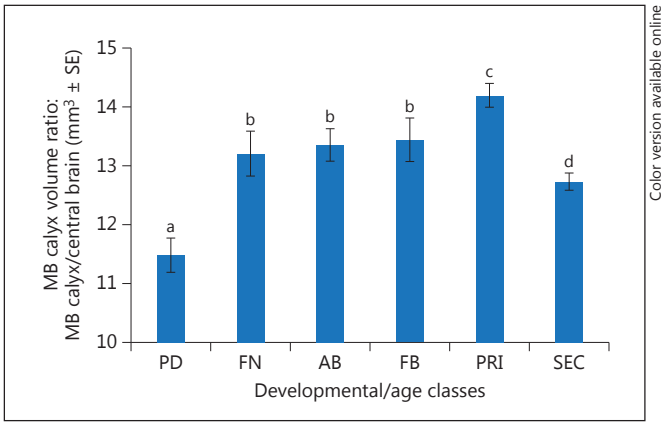


Fig. 2. Differences in mushroom body (MB) calyx volume corrected for central brain volume between female nesting classes in *C. australensis*. Solitary females in order of age from youngest to oldest: predispersal (PD), founding nest (FN), active brood (AB), and full brood (FB); social females age-matched from active brood nests: social primary (PRI), social secondary (SEC). Different letters denote significant differences between classes ($p < 0.05$); classes marked with the same letter are not significantly different from each other (Tukey's HSD post hoc analysis following ANOVA).

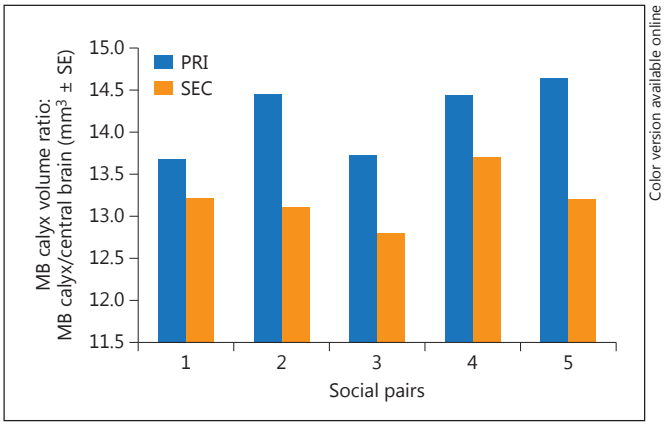


Fig. 3. Mushroom body (MB) calyx volume of paired same-age, full sisters for five social colonies of *C. australensis*. Each pair consists of primary (PRI) and secondary (SEC) females. Social primary and secondary females from the same nest were consistently different in mushroom body calyx volume (paired t test, $t = 5.33$, d.f. = 4, $p = 0.006$).

Social primary and secondary females from the same nest were consistently different in terms of mushroom body calyx volume (paired t test, $t = 5.33$, d.f. = 4, $p = 0.006$; fig. 3). Social primaries had on average a 10% larger mushroom body calyx volume than social secondaries from the same nest.

Physiological Predictors of Mushroom Body Calyx Development

Ovary size differed among groups ($F_{5, 31} = 14.63$, $p = 0.001$). Tukey post hoc pairwise comparisons revealed that social primaries and solitary females from active brood nests had greater ovarian development than callow females from predispersal nests, solitary founding nest females, and social secondary females (online suppl. fig. S3). Likewise, wing wear differed significantly among groups ($F_{5, 31} = 50.22$, $p < 0.0001$). Tukey post hoc pairwise comparisons revealed that social primaries and solitary females from active brood and full brood nests had greater wing wear than callow females from predispersal nests, solitary founding nest, and social secondary females (online suppl. fig. S4).

Mushroom body calyx volume scaled positively but not significantly with ovarian development (linear regression, $r^2 = 0.13$, $n = 32$, Bonferroni-corrected $p = 0.08$; fig. 4). However, there was a strong positive correlation between wing wear score and mushroom body calyx volume (linear regression, $r^2 = 0.35$, $n = 32$, Bonferroni-corrected $p = 0.001$; fig. 5).

Discussion

We used the unique social structure and division of labor patterns of the small carpenter bee *C. australensis* (Apidae: Xylocopinae) to test the relative effects of task performance and social interactions on brain development. In *C. australensis,* social secondaries do not forage and therefore this study uniquely addresses the relative roles of foraging experience, dominance status and sociality within one species as all three phenotypes are represented in solitary, primary, and secondary females, respectively. We showed evidence for independent and cumulative effects of both factors in promoting increased mushroom body calyx development. Carpenter bees represent an independent origin of eusociality from halictine and apine bees [Rehan et al., 2012] and therefore provide important information on the phylogenetic conservation and social influences of brain volume and neuropil developmental processes.

The social brain hypothesis predicts that social females should have larger brains than solitary females [Hum-

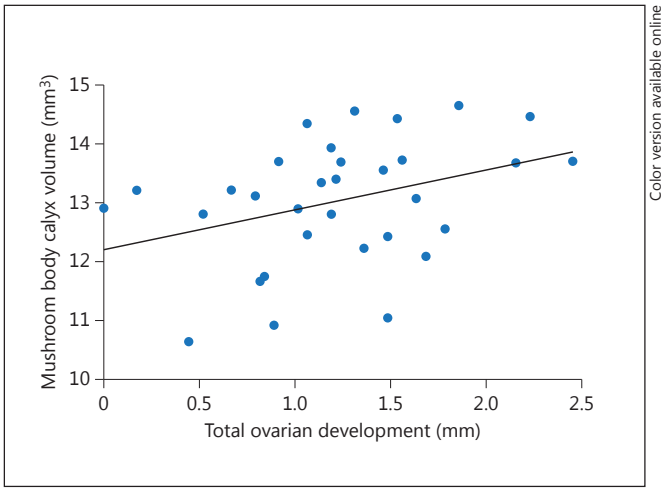


Fig. 4. Mushroom body calyx volume as a function of ovarian development. The line represents the linear regression best fit. Mushroom body calyx volume scaled positively, but not significantly, with ovarian development (linear regression, $r^2 = 0.13$, n = 32, Bonferroni-corrected p = 0.08).

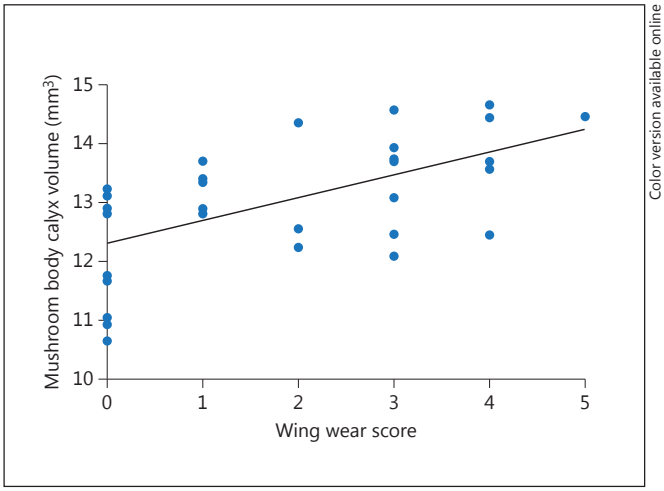


Fig. 5. Mushroom body calyx volume as a function of wing wear score. Females wear their wings with foraging effort. There was a strong positive correlation between wing wear score and mushroom body calyx volume (linear regression, $r^2 = 0.35$, n = 32, Bonferroni-corrected p = 0.001).

phrey, 1976; Dunbar and Shultz, 2007]. We found that, although the total brain volume did not vary among reproductive strategies (social vs. solitary nests), social primaries had larger mushroom body calyces than solitary females, and social secondaries had the least-developed mushroom body calyces. These data show that sociality by itself does not explain the mushroom body calyx volume, similar to findings in halictine bees [Smith et al., 2010]. Living in a social group might not be simply more cognitively demanding since individuals in these groups have very different social experiences and roles within the colony.

We found that social primaries had larger mushroom body calyces than solitary and secondary females; furthermore, nest-matched social pairs revealed that primary females consistently had larger mushroom body calyces than secondary females (fig. 3). Achieving and maintaining dominance status in a group has been associated with mushroom body calyx enlargement in paper wasps [O'Donnell et al., 2006; Molina and O'Donnell, 2007, 2008; Molina et al., 2009]. Dominance hierarchies may result from social aggression and fighting [Michener, 1974]. In other primitively social bees and wasps, manipulation of subordinate helpers by dominant reproductives is often attributed to age- and size-based social hierarchies [Alexander, 1974; Hogendoorn and Velthuis, 1999]. In this species, we have no evidence of these predictors, as females are full sisters, with no consistent differences in body size, morphology, or fat body size [Rehan et al., 2010, 2011, 2014]. Physical aggression has never been observed between cohabiting reproductive females in any *Ceratina* species studied to date [Sakagami and Maeta, 1977, 1995], although there is evidence for aggression in postreproductive assemblages [Rehan and Richards, 2013]. Brain gene expression data from *C. calcarata* suggests that aromatic biosynthesis and pheromone binding might be foundational to reproductive dominance [Rehan et al., 2014]. Although the mechanisms of social dominance are not known for *C. australensis,* the neuroanatomy data presented here suggest that social dominance in addition to reproductive and foraging behavior is cognitively demanding.

The foraging experience hypothesis predicts that social primary and solitary females should have larger mushroom body calyces than secondary females. Mushroom body calyx volume increased with greater foraging experience in both social primary and solitary females. We found a strong correlation between mushroom body calyx volume and wing wear in adult females (fig. 5). Wing wear is a good indicator of lifetime foraging experience in this and other bee species [Cartar, 1992; Rehan et al., 2010]. Social secondaries do not forage and showed intermediate mushroom body calyx enlargement between that of newly eclosed predispersal females and for-

aging solitary and social primary females. These data are consistent with previous findings in solitary, facultatively social, and obligately eusocial bees [Fahrbach et al., 2003; Withers et al., 2007; Smith et al. 2010]. Regardless of social experience or social complexity, central place foraging is cognitively demanding and requires advanced memory and learning processes [reviewed by Lihoreau et al., 2012]. If enhanced sensory processing is a necessary precondition to central place foraging, then it is perhaps not surprising to find a strong correlation between foraging experience and mushroom body calyx volume.

Mushroom body calyx volume increased with foraging and social experience, but mushroom body calyx volume did not decrease in full brood females that had ceased foraging for the season (fig. 2). Mushroom body volume is thought to be a brain anatomy metric of increased memory and learning capacity processes; however, this may not always reflect current task performance demands. Honeybee workers enlarged their mushroom body calyces when transitioning from nurse to forager behavior, but reversion back to nurse behavior did not decrease the mushroom body calyx size [Fahrbach et al., 2003]. Likewise, we found that mushroom bodies scaled positively but not significantly with ovarian development. Again, mushroom body development may be related to ovary development but may not track it closely when ovaries regress. Unlike wing wear, which accumulates with age, ovaries are small in predispersal and founding nest females prior to the active brood rearing period and quickly resorb during the full brood stage after reproduction [Rehan et al., 2010; online suppl. fig. S3]. Although ovarian development is a good indicator of reproductive dominance in active brood nests, the dynamics of ovary development may preclude simple correlations between ovaries and brain architecture in *Ceratina* bees.

Lastly, we found that dominance and foraging effects are cumulative; dominant social primary foragers had larger mushroom body calyx volumes than solitary foragers, and solitary foragers had larger mushroom body calyx volumes than nonforaging social secondary guards. This is the first evidence for cumulative effects on brain development between dominance and task performance. In *C. australensis,* we were able to directly compare the brains of solitary females that forage and reproduce but do not engage in social interactions with those of social individuals while controlling for age, reproductive status, and foraging experience [Rehan et al., 2010, 2011, 2014]. This species is amenable to future studies directly testing the effects of sociality and foraging experience on brain development.

Future Directions

Facultatively social bees have been rarely studied by neuroecologists but offer great insights into the relative roles of phylogenetic history and social experience on brain investment and development pathways. *Ceratina* are an emerging model system for understanding the evolutionary development of neuroanatomy and social complexity as species of this genus range the full solitary to eusocial spectrum [Michener, 1985; Rehan et al., 2012]. Future studies on incipiently social and other facultatively social bees are much needed to better understand the relevance of the social brain and dominance hierarchy hypotheses for the evolutionary development of neural processes.

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