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Socially induced brain development in a facultatively eusocial sweat bee *Megalopta genalis* (Halictidae)

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Changes in the relative size of brain regions are often dependent on experience and environmental stimulation, which includes an animal's social environment. Some studies suggest that social interactions are cognitively demanding, and have examined predictions that the evolution of sociality led to the evolution of larger brains. Previous studies have compared species with different social organizations or different groups within obligately social species. Here, we report the first *intraspecific* study to examine how social experience shapes brain volume using a species with facultatively eusocial or solitary behaviour, the sweat bee *Megalopta genalis*. Serial histological sections were used to reconstruct and measure the volume of brain areas of bees behaving as social reproductives, social workers, solitary reproductives or 1-day-old bees that are undifferentiated with respect to the social phenotype. Social reproductives showed increased development of the mushroom body (an area of the insect brain associated with sensory integration and learning) relative to social workers and solitary reproductives. The gross neuroanatomy of young bees is developmentally similar to the advanced eusocial species previously studied, despite vast differences in colony size and social organization. Our results suggest that the transition from solitary to social behaviour is associated with modified brain development, and that maintaining dominance, rather than sociality *per se*, leads to increased mushroom body development, even in the smallest social groups possible (i.e. groups with two bees). Such results suggest that capabilities to navigate the complexities of social life may be a factor shaping brain evolution in some social insects, as for some vertebrates.

Keywords: brain organization; social evolution; social brain; Machiavellian intelligence; neural plasticity; mushroom bodies

1. INTRODUCTION

Understanding how selection helps shape the mosaic nature of brain evolution is a major challenge in evolutionary biology (e.g. Edelman & Changeux 2001; Ricklefs 2004; Goodson *et al.* 2005; Healy & Rowe 2007). Neural tissue is energetically expensive, and thus specific brain regions should enlarge only when needed to meet functional demands (Niven & Laughlin 2008). In some taxa, social interactions are hypothesized to be so cognitively demanding that the social environment selects for enhanced neural development (the 'social brain hypothesis'; Humphrey 1976; Adolphs 2001; Goodson *et al.* 2005; Byrne & Bates 2007*a*; Dunbar & Shultz 2007; Gronenberg & Riveros 2009). Studies supporting the social brain hypothesis typically rely on comparative analyses across taxa with varying social systems, while using statistical methods to control for phylogenetic effects (e.g. Dunbar & Shultz 2007; Pérez-Barbería *et al.* 2007). It is often problematic, however, to compare social behaviour across species because of the lack of uniform behavioural metrics or biologically meaningful definitions of social complexity (Byrne & Bates 2007*b*; Healy & Rowe 2007). It also may be problematic to apply the hypothesis to the many social insects, such as ants and honeybees, in which individual decision-making is relatively simplified and social complexity arises via self-organization, rather than via increasingly sophisticated individual behaviour (see Gronenberg & Riveros 2009 and references therein). Here, we report the first *intraspecific* test of the effect of sociality on brain size, which allows us to isolate and directly examine the effects of social behaviour on brain development by using a facultatively social or solitary sweat bee, *Megalopta genalis* (Hymenoptera: Halictidae).

A number of studies exploring relative brain size and social behaviour in insects have focused on the *corpora pedunculata*, or mushroom bodies (MBs), a region of the arthropod brain associated with multi-sensory integration, memory and learning that is capable of structural plasticity during adult life (Heisenberg 1998; Menzel & Giurfa 2001; Fahrbach 2006; Withers *et al.* 2007; Strausfeld *et al.* 2009). MB morphology represents a combination of evolved developmental patterns and individual life experience (Heisenberg *et al.* 1995; Fahrbach 2006). For instance, when individuals of many social insect species switch from performing tasks within a confined nest to foraging in a complex environment, with additional sensory stimuli and navigation

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requirements (Capaldi *et al.* 2000; Menzel & Giurfa 2001; Seid & Wehner 2009), the neuropil (N) of the MB increases in volume, while Kenyon cell body (K) volume decreases, resulting in a dramatic increase in the N : K ratio (honeybees: Withers *et al.* 1993, 1995; Durst *et al.* 1994; Fahrbach *et al.* 1995; Farris *et al.* 2001; Ismail *et al.* 2006; ants: Gronenberg *et al.* 1996; Kühn-Bühlmann & Wehner 2006; wasps: O'Donnell *et al.* 2004, 2006). Much of this change is developmentally programmed to coincide with the onset of foraging, demonstrating an evolved response to the predictable cognitive demands of foraging. Individual foraging experience further changes the relative proportions of N and K relative to age-matched caged controls, suggesting that cognitive experience also shapes neuroanatomy (Withers *et al.* 1993, 2007; Heisenberg *et al.* 1995; Fahrbach *et al.* 1998; Farris *et al.* 2001; Fahrbach 2006; Ismail *et al.* 2006; Kühn-Bühlmann & Wehner 2006).

Relative to solitary nest-building bees and wasps (e.g. Wcislo *et al.* 1993; O'Neill 2001), social insects in small colonies face additional cognitive demands that arise from sociality *per se*, such as recognizing individuals or kin (e.g. Fletcher & Michener 1987; Tibbetts 2002; D'Ettorre & Heinze 2005) and assessing dominance relationships (e.g. Arneson & Wcislo 2003; Bhadra & Gadagkar 2008). Recent studies have shown a social component to MB plasticity in two species of obligately social paper wasps (Vespidae) with small colonies, which are the only two species studied to date in which dominance is maintained by individual aggressive interactions rather than by pheromones (O'Donnell *et al.* 2006; Molina & O'Donnell 2007, 2008; Molina *et al.* 2009). Ehmer *et al.* (2001), in contrast, did not find a difference in MB volume between dominant and subordinate wasps of *Polistes dominulus* wasps, but they did not measure MB subregions.

While structural plasticity in the MB is well studied, no study has directly tested how sociality shapes brain size, although sociality has long been hypothesized to lead to an increase in MB volume (for a review of earlier literature, see Howse & Williams 1969; Howse 1974). All but one of the bees, ants and wasps for which brain development has been studied to date are obligately eusocial species, precluding social versus solitary comparisons. A solitary bee (*Osmia lignaria*) apparently follows a different trajectory of brain development than the social species (Withers *et al.* 2007; §4), but its lineage (Megachilidae) has never given rise to eusocial forms (Michener 2007). *Osmia lignaria* adults emerge from their natal cells with a neuroanatomical structure typical of experienced workers of highly social insect species, though MB neuropil volume further increases with foraging experience, similar to honeybees (Withers *et al.* 2007). Comparative interpretations are speculative because MB development has not been studied in other solitary bees. To avoid these problems and directly test the effect of sociality, we used a facultatively social sweat bee, *M. genalis* (Halictidae), which can nest either socially or solitarily (Wcislo *et al.* 2004; Smith *et al.* 2007, 2009), to directly compare the brains of social and solitary individuals within the same species.

Megalopta genalis societies typically consist of a queen and one worker (Wcislo *et al.* 2004; Smith *et al.* 2007, 2009), and individual behaviour is not simplified relative to solitary bees, unlike many other social insects. Despite the smallest colony size possible (i.e. two-bee groups), social nests exhibit the same behaviours that characterize eusocial insects: reproductive division of labour such that queens reproduce and rarely leave the nest, while workers forage for pollen and nectar, and tend to have slender, undeveloped ovaries (Wcislo & Gonzalez 2006; Smith *et al.* 2008, 2009). All nests are independently founded, so even queens have experience foraging when they are raising their first brood. Young bees then emerge into the nest, and at around one week of age they either disperse or begin foraging.

The flexibility of *M. genalis* social behaviour, coupled with the structural plasticity of the insect brain, allows us to test three hypotheses relating sociality to brain size and development. A number of previous studies have focused on the N : K ratio (see above), so we tested for an effect on it to compare with earlier work, despite the lack of any functional interpretation of this ratio. We also analysed volumes of individual brain regions. First, if sociality is more cognitively demanding than solitary living, social females should have higher N : K ratios, or larger absolute MB neuropil volume, than solitary reproductives. Second, being part of a group in itself may not influence brain plasticity so much as the demands of achieving and maintaining high dominance status, as suggested by the studies of small-colony paper wasps (O'Donnell *et al.* 2006; Molina & O'Donnell 2007, 2008; Molina *et al.* 2009). If so, only social queens should have a larger N : K ratio and/or MB neuropil volume than solitary reproductives, while that of social workers should be comparable to solitary bees. Third, Withers *et al.* (2007) proposed that the massive MB reorganization shortly after adult emergence seen in social insects, but not in the solitary *O. lignaria*, may be restricted to social species. We tested for such a developmental pattern in *M. genalis* by comparing recently emerged females to experienced social and solitary females. We also measured ovary size to test whether ovarian development correlates with MB development, and measured the volume of peripheral sensory processing areas of the brain to test whether volume differences between groups are restricted to the MB or whether they characterize other brain regions as well.

2. MATERIAL AND METHODS

(a) *Collections, observations and measurements*

All collections and observations were performed on Barro Colorado Island, Republic of Panama. We modified natural solitary and social nests that were already established with multiple, sealed, provisioned brood cells for observations (for methods, see Smith *et al.* 2008), and left them in their natural locations. Adult females were individually marked with white paint on the thorax. Social nests were filmed with a camcorder under infrared light during the approximately 2 h a day (1 h each before sunrise and after sunset, respectively) when bees forage (Wcislo *et al.* 2004; Kelber *et al.* 2006). These recordings allowed us to determine which bees were queens and workers (Smith *et al.* 2008, 2009). Solitary and social nesting are distinct behavioural strategies, rather than different points on the same developmental trajectory (Smith *et al.* 2007, 2009). To distinguish solitary reproductives from social queens waiting for offspring to emerge, we monitored single-bee nests for at least five weeks (the time needed for offspring to complete

development) after they were modified. To obtain young bees, we collected nests from the field and left brood cells in the dark at ambient temperature. We checked cells daily, and collected all newly emerged adults. These 1-day-old bees are referred to as 'young bees' below. We do not know ages of the other bees. Social queens are older than their workers because the latter are the foundresses' daughters. Social queens and solitary reproductives come from the same cohort of bees and establish nests at the same times of year (Wcislo *et al.* 2004; Smith *et al.* 2007), so there is no reason to expect any systematic bias in age of one class or the other. Each class consists of adult females in post-emergent nests; among post-emergent nests, older nests are not more likely to be social than are younger nests (Smith *et al.* 2007; K. M. Kapheim, A. R. Smith & W. T. Wcislow 2008, unpublished data).

For each female, the head was removed for processing and placed into a fixative (below). The metasoma was placed into Ringer's solution and stored in a refrigerator for no more than 24 h before dissections to measure ovary size. To measure ovary size, we dissected the bees' metasomas by removing the tergites and exposing the ovaries. We photographed the ovaries dorsally at 20× magnification through a dissecting microscope with a digital camera at 2272 × 1720 pixels resolution. As a metric of ovary size, we calculated total ovary area from the digital photographs using Adobe Photoshop 6.0, calibrated with similarly produced digital photographs of a stage micrometer as described in Smith *et al.* (2008, 2009). We report the mean of the left and right ovary areas as 'ovary size'. Bees were dissected, so we could not measure their dry weight. As a metric of body size, we measured inter-tegular (thorax) width using an ocular micrometer at 20× magnification. Inter-tegular distance is an excellent predictor of body size, measured as dry weight, in bees (Cane 1987) and in this population of *M. genalis* (Kapheim *et al.* submitted).

We collected six 1-day-old bees, six pairs of queens and workers and nine solitary reproductives between 14 May and 10 July 2008. Two of the social nests contained a third young female that did not forage. These females may have been future reproductives that had not yet dispersed (Smith *et al.* 2008, 2009), but we had no knowledge of their behaviour, so we excluded them.

(b) *Histology*

Upon collection, the head was immediately detached, the mandibles and glossa were removed to improve resin infiltration and the head was placed into and stored in an aldehyde-based fixative (Prefer, Anatech Ltd). We later removed most of the compound and simple eyes, antennae and pieces of the cephalic cuticle to improve infiltration. Brains were then prepared for embedding in epon by dehydrating them in 2,2-dimethoxiproan followed by 100 per cent acetone. The brains were then incubated in epon/acetone solution for 3–24 h followed by two 100 per cent epon incubations for 3 h each, and then placed in Beem capsules in 100 per cent epon and cured overnight at 60°C. Embedded brains were serial-sectioned using disposable steel knives at a thickness of 10 μm on a Microm 355s microtome. Serial sections were placed on glass slides, stained with Ricardson's stain (1% methylene blue in 1% borax with 1% azure) and then photographed and viewed using a Nikon Coolpix 8700 attached to a Nikon E600 microscope. Serial images were aligned digitally using Reconstruct software (Fiala 2005)

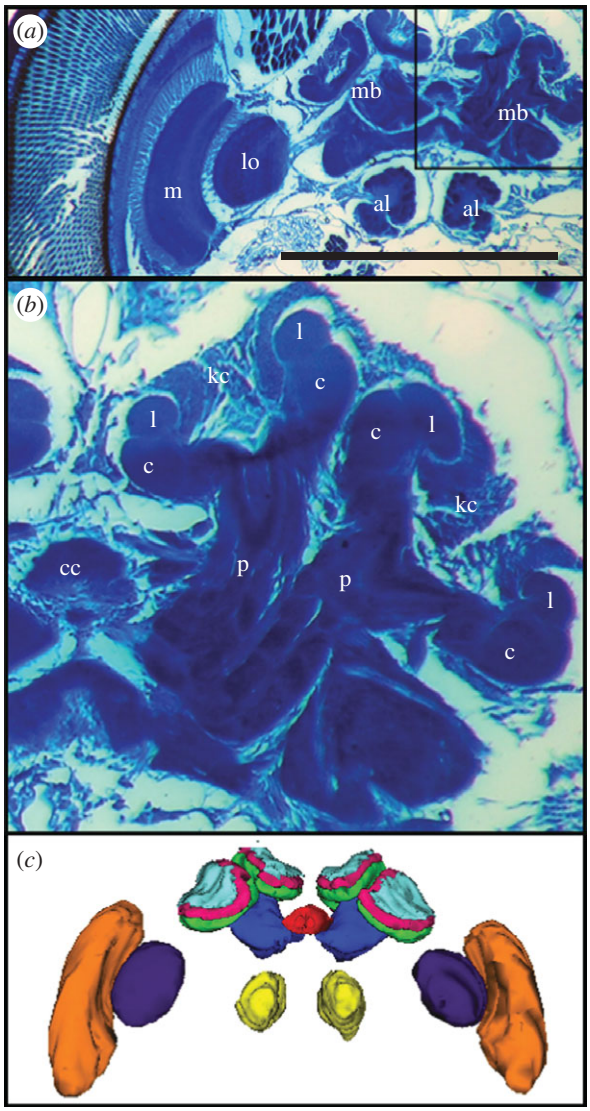

Figure 1. (*a*) Section of *M. genalis* brain including the left compound eye, medulla (m), lobulla (lo), both mushroom bodies (mb) and antennal lobes (al). Scale bar, 1 mm. The box in the upper right is enlarged in (*b*) to show the central complex (cc) and subunits of the mushroom body: peduncle (p), collar (c) lip (l) and Kenyon cell bodies (kc). (*c*) Three-dimensional reconstruction of the *M. genalis* brain from serial histological sections. The medulla is orange, lobulla purple, antennal lobe yellow, peduncle royal blue, central complex red, collar green, lip pink and Kenyon cells sky blue.

and the magnification was determined using a calibration slide at a resolution of 0.01 mm. Volumetric measurements and three-dimensional reconstructions were made by individually tracing the neuroanatomical structures on individual sections using Reconstruct software. For each bee, we measured total brain volume, as well as the volumes of the following: the antennal lobes; the MB lateral and medial calyces separately, distinguishing the collar (including the basal ring), the lip and the Kenyon cell bodies, and the peduncle of the MB, including the medial and vertical lobes; the medulla and lobulla of the optic lobes; and the central complex, including the ellipsoid body, the superior arch and the fan-shaped body, but not the paired noduli or protocerebral bridge (figure 1).

For all areas except the central complex, we averaged the values from the left and right hemispheres. To control for any

effects of body size, we used the percentage of total brain volume for each brain subregion, as well as absolute volume, for our analyses.

(c) *Statistics*

All statistics were performed in SPSS 17.0.

3. RESULTS

(a) *Body size and brain volume*

Queens and solitary females were larger than workers (also Smith *et al.* 2008, 2009), but owing to a large worker in one of the six social nests, the between-group difference in body size was not significant (one-way ANOVA, $F_{3,23} = 1.58$, $p = 0.2$). There was no significant association between body size and brain volume (linear regression, $n = 27$, $r^2 = 0.11$, $p = 0.09$). Body size correlated negatively with Kenyon cell volume across all bees ($n = 27$, Pearson's $r = -0.56$, Bonferroni-corrected $p = 0.015$), but not with any other brain region volume.

(b) *Developmental effects on brain volume*

ANOVAs on brain region volume showed a significant effect of group on each brain region (figure 2; electronic supplementary material, table S1; one-way ANOVAs: central complex, $F_{3,23} = 6.31$, $p = 0.003$; antennal lobes, $F_{3,23} = 3.83$, $p = 0.03$; Kenyon cell bodies, $F_{3,23} = 4.33$, $p = 0.02$; optic lobes, $F_{3,23} = 5.08$, $p = 0.01$; MB neuropil, $F_{3,23} = 5.01$, $p = 0.01$). These effects appear to be driven entirely by differences between young bees and the other groups. Tukey's *post hoc* pairwise comparisons revealed no differences between queens, workers or solitary reproductives for any brain region. Nearly all comparisons with the 1-day-old bees, however, were significant (electronic supplementary material, table S1). Young bees had significantly greater Kenyon cell volume, and smaller volume for all other brain regions, relative to older bees (figure 2; electronic supplementary material, table S1).

ANOVAs for absolute volumes (not proportional to total brain volume) across groups are significant only for Kenyon cell body volume ($F_{3,23} = 19.34$, $p < 0.001$) and MB neuropil volume ($F_{3,23} = 4.07$, $p = 0.02$). As with the previous analysis, these results are apparently driven by differences between young bees and the other groups. Tukey's *post hoc* pairwise comparisons revealed significant Kenyon cell body volume differences between young bees and the other three groups ($p < 0.001$ for all), as well as a significant MB neuropil difference with solitary females ($p = 0.01$) and a marginally non-significant difference with queens ($p = 0.08$; electronic supplementary material, table S1).

(c) *Effect of behavioural group on MB development*

There were no significant differences between social (queens and workers) and solitary bees' MB neuropil or Kenyon cell body volumes measured as a percentage of total brain volume. Kenyon cell body volume (but not MB neuropil) differed between social and solitary females when analysed as absolute volume (social mean = 22.68 ± 0.91 μm^3, solitary mean = 26.00 ± 1.16 μm^3; $t_{19} = 2.29$, $p = 0.03$). There were no significant differences between reproductive (queens and solitary reproductives) and non-reproductive (workers) females in MB neuropil or Kenyon cell body volume, whether measured as a percentage of total brain area or absolute volume.

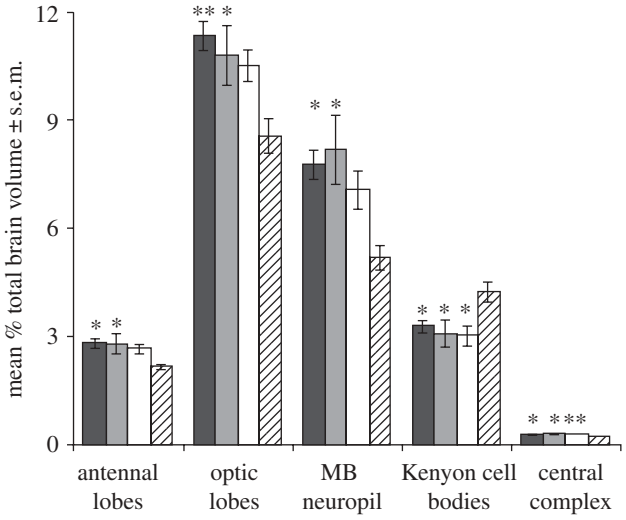

Figure 2. Mean percentage (±s.e. of the mean) of total brain volume for each brain region. Asterisks indicate values significantly different from young, 1-day-old bees (Tukey's HSD pairwise comparisons). One, two and three asterisks indicate *p*-values <0.05, <0.005 and <0.0005, respectively. Black bars, solitary; grey bars, queen; white bars, worker; striped bars, young.

MB neuropil to Kenyon cell body volume ratio (N : K) was significantly different among groups (figure 3; one-way ANOVA, $F_{3,23} = 66.81$, $p < 0.001$). Tukey's HSD *post hoc* pairwise comparisons showed that young bees significantly differed from each of the other three groups ($p < 0.001$ for all comparisons). There were no significant differences between solitary reproductives and social workers. Queens' N : K ratios were significantly larger than solitary reproductives' ($p = 0.049$). While queens' ratios were marginally not significantly larger than workers' as a behavioural class ($p = 0.054$), they were significantly larger when compared with *their* workers (paired $t = 3.17$, d.f. = 5, $p = 0.025$). Some previous studies analysed only the calyx (lip + collar + basal ring) : Kenyon cell (C : K) ratio, rather than the entire neuropil (e.g. Molina & O'Donnell 2008 and references therein). Analysing our data this way yielded results similar to the N : K comparison (one-way ANOVA, $F = _{3,23}$, $p < 0.001$; Tukey's HSD *post hoc* pairwise comparisons for young bees, $p < 0.001$ for all comparisons; queens versus solitary, $p = 0.02$; queens versus workers, $p = 0.12$; queens versus workers, paired $t = 2.50$, d.f. = 5, $p = 0.05$). Analysing the lip or collar + basal ring separately did not change the results.

(d) *Ovary size and brain volume*

Ovary size differed between adult groups, excluding young bees because they have only small, incipient ovaries (ANOVA $F_{2,17} = 4.75$, $p = 0.02$). Ovary size was not correlated with total brain volume or the volume of any single brain region, whether measured as a bivariate correlation or a partial correlation controlling for behavioural group, after Bonferroni corrections for multiple comparisons. Likewise, there was no significant effect of ovary size on either C : K or N : K ratios, either as a bivariate or partial correlation ($p > 0.3$).

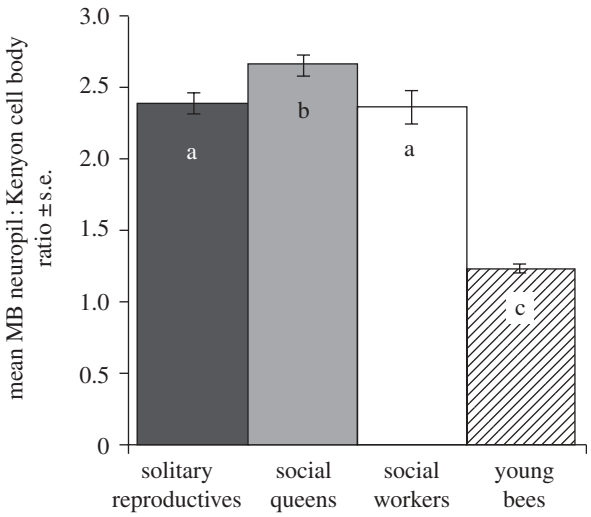

Figure 3. MB neuropil : Kenyon cell body ratio for each group. Bars with different letters are significantly different from each other; see text for statistics.

4. DISCUSSION

Numerous previous studies have shown that the increased sensory stimuli and presumed cognitive demands of foraging promote MB development in many social insects (§1). Here, we show that sociality, in addition to foraging experience, is associated with increased MB development in social reproductives (queens). The social brain hypothesis posits that the cognitive challenges of group living are specifically *social* ones (Gronenberg & Riveros 2009), which therefore demand increased neural capacity analogous to the differential demands for neural processing in simple versus complex foraging environments (Bernays & Wcislo 1994; Farris & Roberts 2005). Alternatively, it may be that there are no social challenges *per se*, but that increased interactions and sensory stimuli and associated learning lead to increased MB development. Heisenberg *et al.* (1995) showed that female *Drosophila* reared in dense larval cultures had up to 20 times more Kenyon cell fibres than flies reared in low-density cultures. In *M. genalis*, the lack of differences among the three adult groups in the peripheral and central areas responsible for processing chemosensory input (antennal lobe and MB lip, respectively) and visual input (optic lobe and MB collar, respectively) suggest that the MB differences are not due to differing sensory inputs between groups. The difference between *M. genalis* workers and queens suggests that social status, rather than just group membership, matters, although this interpretation may be confounded by age. This is consistent with both intra- and interspecific comparisons in paper wasps (Vespidae): in small-colony species, in which behavioural dominance is established through individual aggressive interactions, queens had larger MBs than workers, but not in related species with pheromone-based control (Molina *et al.* 2009). Thus, *M. genalis*, living in the smallest possible social groups with only one or a few other individuals to keep in mind, show neuroanatomical effects of sociality consistent with obligately social species living in much larger and putatively more complex societies. This observation suggests that some aspect of dominance behaviour, rather than general behavioural interactions and contact rates, shapes brain size variation in small-colony social insects.

The gross developmental pattern of *M. genalis* MB is strikingly similar to both small- and large-colony obligately eusocial bees, wasps and ants in displaying large decreases in Kenyon cell body volume early in adult life and modest increases in neuropil volume with age and experience (Fahrbach 2006; O'Donnell *et al.* 2006). In fact, regardless of any functional interpretation, the N : K ratios of newly emerged and mature (both solitary and social) *M. genalis* are nearly identical to those of honeybees, despite the massive differences in colony size, social organization and phyletic distance between the two species (§3 and Withers *et al.* 1993). Patterns of brain development in *M. genalis* and obligately social Hymenoptera may be an evolved characteristic of sociality—even facultative sociality, such as seen in *Megalopta*. Brain development of the only solitary bee or wasp studied to date, the orchard bee *O. lignaria*, differs from social species: there is a slight expansion of MB neuropil with foraging experience, but Kenyon cell body volume does not decrease with age (Withers *et al.* 2007). Unlike *Megalopta* and other eusocial bees and wasps, *O. lignaria* forage immediately after emergence, and may emerge 'pre-wired' (Withers *et al.* 2007), but the latter also eclose in the autumn and overwinter as adults in sealed natal cells, so MB reorganization characteristic of ageing may take place after eclosion, but before spring emergence. Developmental studies of additional solitary species, especially those in which daughters remain at the nest before dispersing (e.g. Wcislo *et al.* 1993; Wcislo 1997), are necessary to ascertain the extent to which patterns of brain development between solitary and social species are associated with behavioural differences linked to sociality or with delayed dispersal or other traits.

The social brain hypothesis has been invoked to explain some patterns of brain size variation in vertebrates (e.g. Byrne & Bates 2007*a*,*b*; Healy & Rowe 2007; Dunbar & Schultz 2007). Applying this hypothesis to insect societies in general may be problematic because insect sociality spans a wider spectrum of social organization than for vertebrates. For instance, the extreme behavioural specialization seen in individuals of some highly eusocial species hypothetically may impose *less* neural demand on any individual relative to a smaller colony with a more labile dominance hierarchy and behaviourally totipotent individuals (Gronenberg & Riveros 2009). Thus, MB volume may be likely to increase at the transition from solitary to social behaviour in small groups, but decrease at the transition to advanced eusociality with specialized worker subcastes (Gronenberg & Riveros 2009). Direct tests of the basic prediction of the social brain hypothesis—that the specific cognitive challenges imposed by sociality should be reflected in brain organization—have faced several difficulties, which can be overcome by intraspecific studies with insects. One problem involves quantifying social complexity, or measuring behavioural repertoires, across different taxa (de Waal & Tyack 2003; Byrne & Bates 2007*b*; Dunbar & Schultz 2007; for critiques see Eberhard 2007; Healy & Rowe 2007). Intraspecific comparisons obviate this difficulty. A second difficulty involves assigning specific cognitive challenges to specific areas of the brain, coupled with the assumption that specific areas are primarily shaped by one function

(Healy & Rowe 2007). The relatively specific functions of different lobes in the insect brain facilitate tests of whether brain morphology reflects presumed cognitive challenges (e.g. Julian & Gronenberg 2002; Gronenberg *et al*. 2007; Molina *et al*. 2009).

Our neuroanatomical studies indicate that the relative size of the brain of *M. genalis* is dependent on experience and environmental stimulation, including the social environment, as for some social vertebrates. Facultatively social species like *M. genalis* can be used as a powerful tool to understand how sociality shapes brain evolution, because they enable comparisons between social and solitary behaviour, without species differences as a confounding factor.

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