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Queen dominance may reduce worker mushroom body size in a social bee.

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Data availability.

The data that support the findings of this study are openly available in figshare.com at https://figshare.com/s/2071319e35b38ceecb9c, doi:10.6084/m9.figshare.7927409

Keywords

Brain plasticity, social dominance, Halictidae

Abstract

The mushroom body (MB) is an area of the insect brain involved in learning, memory, and sensory integration. Here we used the sweat bee *Megalopta genalis* (Halictidae) to test for differences between queens and workers in the volume of the MB calyces. We used confocal microscopy to measure the volume of the whole brain, MB calyces, optic lobes and antennal lobes of queens and workers. Queens had larger brains, larger MB calyces and a larger MB calyces:whole brain ratio than workers, suggesting an effect of social dominance in brain development. This could result from social interactions leading to smaller worker MBs, or larger queen MBs. It could also result from other factors, such as differences in age or sensory experience. To test these explanations, we next compared queens and workers to other groups. We compared newly emerged bees, bees reared in isolation for 10 days, bees initiating new observation nests, and bees initiating new natural nests collected from the field to queens and workers. Queens did not differ from these other groups. We suggest that the effects of queen dominance over workers, rather than differences in age, experience, or reproductive status, are responsible for the queen-worker differences we observed. Worker MB development may be affected by queen aggression directly and/or manipulation of larval nutrition, which is provisioned by the queen. We found no consistent differences in the size of antennal lobes or optic lobes associated with differences in age, experience, reproductive status, or social caste.

Introduction

Many animals, including humans, exhibit brain plasticity over the course of their lifetime (May 2011, Nava and Roeder 2011, Harris et al. 2017). Plasticity is widespread even at the adult stage in insects (Fahrbach and Van Nest 2016, Fahrbach et al. 2017, Simoes and Rhiner 2017, Suge et al. 2018). In adult insects, one brain region that exhibits plasticity is the mushroom body (MB). The mushroom bodies support cognitive processes such as sensory integration, learning and memory (Zars 2000, Fahrbach 2006). MBs may increase in volume over time due to dendritic growth (Farris et al. 2001, Seid and Wehner 2009, Muenz et al., 2015). In social insects, patterns of MB development may reflect social roles (Amador-Vargas et al. 2015, O’Donnell and Bulova 2017, O’Donnell et al. 2017). In many primitively social insects reproductives must establish dominance over subordinates and the queens or otherwise dominant individuals have larger MBs than workers or other subordinate individuals (Molina and O’Donnell 2007, 2008, O’Donnell et al. 2007, 2017, Rehan et al. 2015). This is not the case in the honeybees and large-colony ant species, where queens use chemical communication to control worker reproduction, and have smaller MBs than workers (Ehmer and Gronenberg 2004, Julian and Gronenberg 2002, Fahrbach 2006). The queen-worker differences in primitively social species may arise from differences in age, reproductive physiology, experience, or social interactions between the two castes, as all of these factors can affect MB plasticity in the adult brain (reviewed in Fahrbach 2006).

MBs may change with age or reproductive physiology. Honeybee workers exhibit expansion of MB neuropil volume with age during the first week after eclosion (termed 'experience-expectant' plasticity) (Withers et al. 1993, 1995, Durst et al. 1995, Fahrbach et al. 1998). Similar patterns have been found in bumblebees (Jones et al. 2013), wasps (O'Donnell et al. 2007) and ants (Gronenberg et al. 1996, Seid et al. 2005, Seid and Wehner 2009). Because queens are older than workers (who are typically their daughters), queen-worker differences could be related to age. Queens are reproductive, and workers are not, because queens suppress worker ovary development leading to the reproductive division of labor characteristic of eusociality (Spradberry 1991, Michener 1990). Reproductive physiology may also underlie MB differences. In the bee *Ceratina australensis* and the paper wasp *Polistes instabilis,* ovary size correlates with MB volume (Molina and O'Donnell 2007, Rehan et al. 2015). In honeybees, juvenile hormone (JH) affects MB development (Withers et al. 1995), and JH is associated with dominance and reproduction in primitively social insects (Smith et al. 2013, West-Eberhard 1996, Hamilton et al. 2017). Thus, differences in reproductive physiology between queens and workers may underlie MB differences.

MBs may also show experience-dependent plasticity, increasing in volume in response to complex tasks like foraging or other sensory stimuli (Withers et al. 1993, 1995, 2007, Gronenberg et al. 1996, Fahrbach et al. 1998, Farris et al. 2001, Kuhn-Buhlman and Wehner 2006, Ismail et al. 2006, Krofczik et al. 2008, Maleszka et al. 2009, Molina and O'Donnell 2008, Seid and Wehner 2009, Stieb et al. 2010, Jones et al. 2013, Amador-Vargas et al. 2015, Rehan et al. 2015, Montgomery and Merrill 2016, Seid and Junge 2016, Montgomery et al. 2017, van Dijk et al. 2017). Because queens must find and establish a nest, as well as forage for the first brood, they likely have more cumulative sensory experience, and thus larger MBs than workers. Social interactions, rather than sensory experience more generally, may also affect MB development. *Drosophila* reared in social groups had larger MBs than those reared alone (Heisenberg 1995), and socially isolated *Camponotus* ants had smaller MBs than socially integrated ants of the same age (Seid and Junge 2016). If social interactions are important for MB development, then both queens and workers should have larger MBs than pre-social nest foundresses who are not living with another bee.

The nature of social interactions, rather than just social interaction *per se* may also influence MB development. In primitively social insect societies, queens establish social dominance over workers (Michener 1990, Spradberry 1991). This dominance behavior itself, cognitive demands associated with dominance (e.g. Tibbetts et al. 2018), or physiological changes associated with dominance, including increased JH titers and brain amine expression (Hamilton et al. 2017) may also affect MB volume. Paper wasps show a correlation between dominance status and MB size (O'Donnell et al. 2007, Molina and O'Donnell 2007, 2008). In a study controlling for age, Rehan et al. (2015) showed that dominant females had larger MBs, and subordinate females smaller MBs, than solitary nest foundreses, although a study of same-generation paper wasp nest co-foundresses found no difference between dominants and subordinates (Ehmer et al. 2001). Lastly, just as queens are dominant, workers are subordinate and bullied by queen aggressive behaviors which leads to suppressed ovarian development and other physiological effects, including lower levels of JH (Smith et al. 2013, Hamilton et al. 2017). Queen manipulation of worker behavior and reproductive physiology extends to the larval stages as well because it is the foundress queen who controls the larval provisions provided to the developing workers (Michener and Brothers 1974, Kapheim et al. 2011, Kapheim 2017, Lawson et al. 2017). Thus, workers may have smaller MBs as a result of maternal manipulation of nutrition and/or behavioral aggression. As mentioned above, Rehan et al. (2015) showed that subordinate foragers had smaller MBs than solitary nest foundresses; other studies did not distinguish between queens enlarging MBs through dominance versus reducing worker MBs through subordinance. Both the 'enlarged dominant' and 'reduced subordinate' hypotheses posit that these factors influence MB size in addition to the differences in ovarian development that result from queen dominance.

Here we use the facultatively eusocial sweat bee *Megalopta genalis* (Halictidae) to test for queen-worker differences in MB size and other measures of brain volume. Next we test whether these differences arise from workers' MBs being smaller, or queens' being larger. We then use other treatment groups to test the alternative hypotheses outlined above for factors other than social dominance interactions that may lead to queen-worker differences in MB size. *M. genalis* females initiate nests as solitary foundresses by digging tunnels into dead sticks suspended above the ground in vegetation (Wcislo et al. 2004). The first daughter(s) to emerge usually remain in their natal nest as non-reproductive worker(s) (social nests usually have one or two workers). They are smaller than the queen and their younger sisters, which emerge later before dispersing to reproduce, and they are also subject to aggressive dominance from the queen (Smith et al. 2008, 2009, submitted, Kapheim et al. 2011, 2013, 2016). Newly emerged females remain in their natal nest for ~5-7 days before either beginning work as a forager or dispersing to reproduce (Kapheim et al. 2013).

In a previous study on *M. genalis,* Smith et al. (2010) found that newly emerged bees had smaller MBs than queens and solitary reproductives (but not workers). Queens and workers did not differ in MB volume. However, this study did not control for age (bees were of unknown age from field-collected nests), nor did it include any other stages of adult development except for newly emerged females. Here we use known-age queens and workers, newly emerged females, and three other treatment groups to test six non-exclusive hypotheses for queen-worker differences in MB development. To test these hypotheses, we measured the volumes of the MB calyces and whole brain size using unbiased stereological techniques. We also measured the volumes of two sensory neuropils: the antennal lobes (AL, olfactory input center) and optic lobes (OL, visual input center). This let us test whether plasticity in MB calyces was reflected in other brain areas as well, and also to compare whether the sensory neuropils were more responsive to changes in the sensory environment than the MBs. We used bees from six different groups that differed in age and experience: newly-emerged bees, bees kept in sensory and social isolation for 10 days, observation nest foundresses of known age, natural nest foundresses (dispersers) of unknown age who were just beginning a new nest, established queens, and 10-day old workers.

The hypotheses and predictions that we tested are listed below. 1) *Age:* Queens should have larger MBs, and newly emerged bees smaller MBs, than all other groups. 2) *Reproductive status:* Dispersers, observation nest foundresses, and queens are all actively nesting, and thus should have larger MBs than the workers, isolated bees, and newly emerged bees. 3) *Experience:* The queens, which foraged to provision the first worker brood, should have larger MBs, and the newly emerged bees and isolation bees smaller MBs, than the other groups (which have some foraging experience). 4) *Social interactions:* Queens and workers, which are living in a social nest, should have larger MBs than other groups. The predictions are less clear for the dispersers, as they are nesting solitarily, but had social experience with their mother at their natal nest before dispersing. 5) *Enlarged dominant:* Queens should have larger MBs than all other groups if social dominance leads to enlarged MBs. 6) *Reduced subordinate:* If queen aggressive behavior and/or nutritional manipulation of larval nutrition from the queen reduces MB size, the workers should have smaller MBs than all other groups.

Methods

We collected bees at Barro Colorado Island (BCI), Panama (9.1521° N, 79.8465° W), where this species has been studied in detail (Wcislo et al. 2004, Smith et al. 2003, 2008, 2009, 2013, submitted, Kapheim et al. 2011, 2012, 2013, 2016).

Experimental groups

For this study, we used six experimental groups, similar to the methods of previous studies on this species (Kapheim et al. 2012, Smith et al. 2013). We reared bees from brood cells that we took from field-collected nests at ambient temperature. Developing immatures were checked daily, and newly emerged adults removed each day. Newly emerged bees (N = 4) are females collected upon emergence. Isolated bees (N = 3) were females that were moved to cages (round plastic deli containers 13 cm diameter and 10 cm height) the day of their emergence. Bees were kept in social isolation (one bee per cage) and darkness at ambient temperature with ad-lib food (honey:water:soy-protein powder, 45:45:10 by volume). Other females were placed into standardized observation nests on the day of their emergence, and these nests were then placed in the field. Observation nests consist of a piece of balsa wood with a straight tunnel cut into the middle placed between two sheets of opaque Plexiglas; see Kapheim et al. (2013) and Smith et al. (2013) for more details. Observation nest foundresses (N = 5) are females from these observation nests collected when they have completed an entrance collar for their nest (Smith et al. 2003, 2013). We use this as a proxy for beginning to nest, since the typical first step in nesting, constructing the tunnel, is not necessary in our pre-excavated observation nests. This is the first nest construction step performed by females in observation nests, and distinguishes them from other females who may wait for several days and then abandon the observation nest without nesting. The observation nest foundresses averaged 5.80 ± 4.32 SD days old (range: 2—13) when they were collected. Queen (N = 4) and worker (N = 4) bees were collected from these observation nests 10 days after the emergence of the worker, at which point the worker was foraging to provision the nest. Workers are daughters of the queen who are subject to aggressive dominance from the queen (Kapheim et al. 2016) that suppresses worker ovarian development and reproduction (Smith et al. 2009, 2013, Kapheim et al. 2012). Workers remain in their natal nest, where they forage to provision the queen's offspring. Note that while the workers are similarly aged to the '10-day isolation', 'observation nest foundress', and likely the 'disperser' (below) treatments, the queens are a generation older and averaged 65.25 ± 3.40 days old at collection (range: 62—70). Dispersers (N = 5) are bees collected in the field while initiating a new nest. We first collected sticks that appeared to be suitable nesting substrate, confirmed that they contained no existing nests, and placed them in the freezer (-20 C) for at least 24 hours to ensure that no undetected nests were present. We then placed these sticks in the field and checked them every three days for nesting activity. When a new nest was discovered, we collected it. Dispersers are of unknown age, but given that dispersing females typically leave observation nests ~ 5—7 days after emergence (Kapheim et al. 2013), we assume they are approximately 10 days old. Dispersers had social interactions with their mother (the queen of their natal nest). Newly emerged bees are fed by the queen or other nestmates before they disperse to initiate a new nest or begin foraging flights as a worker (Wcislo and Gonzalez 2006, Kapheim et al. 2016). We assume that dispersers were not subject to aggressive dominance because they left the nest to initiate a new nest and reproduce rather than remain in their natal nest as workers. All bees were collected between 2—30 July, 2016.

Ovarian dissections and size measurements.

We preserved the abdomen of each bee in 70% ethanol at collection and dissected out the ovaries. Ovaries were photographed at 10x magnification through a dissecting microscope and the area of the entire photographed ovary measured using Image J, following methods of previous studies on this species (Smith et al. 2008, 2009). We measured thorax width (intertegular span) of each bee collected with digital calipers as a measure of body size (Cane 1987). Thorax width correlates with both head width ($r^2 = 0.89$) and whole body dry weight ($r^2 = 0.83$) in *M. genalis* (Kapheim et al. 2011).

Brain measurements

We preserved bee heads in 4% paraformaldehyde in phosphate buffered saline (PBS) at collection and stored them at 4°C until dissection. We dissected head capsules in PBS to remove the brain which was immediately placed in glutaraldehyde (2%) for 48 hours, bleached in a formamide solution, and dehydrated in a series of ethanol washes of increasing concentration following McKenzie et al. (2016). Because the fixative and histology methods used here differ from Smith et al. (2010), volume measures are not comparable between the two studies. Prior to imaging, brains were mounted in methyl salicylate. Brains were imaged using an Olympus Fluoview FV1000 confocal microscope using autofluorescence at 10X magnification and a step size of 10 μm (Fig. 1). We calculated volumes of the brain and different neuropils (MB calyces, AL, and OL, including both the lamina and medulla) through tracing and serial reconstruction using the software program Reconstruct (Fiala 2005). We chose these neuropils because they were the ones affected by social status and changes in the sensory environment in previous studies (O'Donnell et al. 2007, 2011, 2013, Molina and O'Donnell 2008, Molina et al. 2009, Rehan et al. 2015). Brain and neuropil volumes were standardized to average body size by calculating a correction factor that was applied to each bee: mean body size of all bees in the study divided by the individual's body size. This correction factor was then multiplied to brain and neuropil volume for each bee, which is referred to as 'size-corrected volumes' below. Ratios for each neuropil: whole brain were calculated for each individual.

Statistical analyses

For queen-worker comparisons, we used a paired t-test to account for the effect of shared nest and developmental history (Kapheim et al. 2016). We tested for differences across all treatment groups using an ANOVA followed by Tukey's posthoc pairwise comparisions.

Results

Body size did not differ between groups ($F_{5,19} = 0.53$, $p = 0.750$). Body size did not correlate with AL:whole brain ratio (r = - 0.05, N = 25, p = 0.807) or MB:whole brain ratio (r = 0.23, N = 25 p = 0.260), but body size did correlate negatively with OL:whole brain ratio (r = - 0.47, N = 25, p = 0.019). Large bees invested relatively less tissue in OLs.

Ovary size differed between groups ($F_{5,19} = 15.21$, $p < 0.001$, Fig 2). Queens and dispersers had enlarged, reproductive ovaries, while the other treatment groups did not (pairwise comparison p values for both queens and dispersers vs. young bees, isolation bees, observation nest bees and workers all ≤ 0.001). Thus, observation nest foundresses were not yet reproductive. Ovary size did not correlate with size corrected MB calyx volume (r = 0.27, N = 25, p = 0.898) or the MB calyces:whole brain ratio (r = -0.14, p = 0.519, N = 25).

Brain differences between queens and workers.

Each queen was larger-bodied than her worker, but only slightly so (average queen:worker thorax width ratio = 1.040 ± 0.036, range: 1.003—1.076), and this difference was not significant (paired t-test $t_4 = 2.15$, $p = 0.121$). Workers had significantly smaller brains than queens (size-corrected volume paired $t_4 = 3.60$, $p = 0.037$, Fig. 3a). Workers had significantly smaller MBs than queens (size-corrected volume paired $t_4 = 4.97$, $p = 0.016$, Fig. 3b) and significantly smaller OLs than queens (size-corrected volume paired $t_4 = 5.99$, $p = 0.009$, Fig. 3c). There was not a significant queen-worker difference in AL size (size-corrected volume paired $t_4 = 2.37$, $p = 0.098$, Fig. 3d).

Queens also had significantly larger MB calyces then workers when measured as neuropil volume:whole brain volume ratio (paired $t_4 = 5.06$, $p = 0.015$, Fig. 4a). There was no difference between either queen and worker OL:whole brain ratio (paired $t_4 = 1.44$, $p = 0.238$, Fig. 4b) or the AL:whole brain ratio (paired $t_4 = 1.44$, $p = 0.556$, Fig. 4c).

Comparisons across all groups.

Whole brain volume corrected for body size was not significantly different across treatment groups ($F_{5,19} = 2.14$, $p = 0.104$, Table 1), although workers were nearly significantly smaller than newly emerged bees in post-hoc pairwise tests (p = 0.053). Size-corrected MB calyx volume was significantly different across treatment groups ($F_{5,19} = 3.29$, $p = 0.174$, Table 1). Workers had significantly smaller mushroom bodies than newly emerged bees ($p = 0.028$) and isolated bees ($p = 0.037$). The difference between workers and observation nest foundresses was marginally non-significant, with workers again having smaller mushroom bodies ($p = 0.072$).

There was an effect of group on size-corrected optic lobe volume ($F_{5,19} = 3.58$, $p = 0.019$, Table 1). Queens' OL volume was significantly larger than observation nest foundress' OL (p = 0.031). There was no effect of group on size-corrected AL volume ($F_{5,19} = 1.41$, $p = 0.265$, Table 1).

When measured as a ratio of neuropil to whole brain volume, rather than size-corrected volumes, there were also significant differences in MB calyces, and OLs, but not ALs, between groups. MB calyx neuropil:whole brain ratio showed a significant effect of group ($F_{5,19} = 3.97$, $p = 0.012$, Table 1). Worker MB:whole brain ratios were significantly smaller than isolated ($p = 0.015$) and observation nest foundress ($p = 0.021$) bees, and nearly significantly smaller than young bees ($p = 0.061$). There was an effect of group on OL:whole brain volume ($F_{5,19} = 5.19$, $p = 0.004$, Table 1). Queens had significantly higher ratios than newly emerged bees ($p = 0.018$) and observation nest foundresses ($p = 0.016$). Dispersers also had significantly higher ratios than newly emerged bees ($p = 0.044$) and observation nest foundresses ($p = 0.040$). There were not significant differences in the ratio of AL volume to whole brain volume ($F_{5,19} = 2.196$, $p = 0.098$, Table 1).

Discussion

Here we show that workers invest less neural tissue in MB calyces than queens. Comparisons with other groups suggest that workers' MB calyces are relatively reduced, rather than queens' being enlarged. Comparisons with other groups also show that the queen-worker difference is not a result of differences in body size, age, ovarian development, or sensory experience. This suggests that queen dominance behavior and/or maternal manipulation of larval nutrition may affect worker brain morphology.

Body size

Body size did not differ between our treatment groups. In previous studies, we have shown that workers are typically, but not always, smaller than their queens and other reproductive foundresses (Smith et al. 2008, 2009, Kapheim et al. 2012, 2013). In this study, each worker was smaller than her queen, but the differences were slight and not statistically significant. Workers were not smaller than queens in general or dispersers, which differs from previous studies of this species and may be a result of the small sample size of this study (Smith et al. 2008, 2009, Kapheim et al. 2012, 2013). Body size correlated negatively with OL:whole brain ratio, suggesting that bees invest relatively less in OL tissue at larger sizes. We do not know what the effect of increased OL tissue on vision would be, but previous studies on the optics of *M. genalis* suggest that they fly at the limit of their visual abilities (Warrant 2017).

Body size did not correlate with the neuropil:whole brain ratios of MB calyx or AL, suggesting that there is not size-based allometry in these brain areas.

Mushroom Bodies

We made six predictions about MB calyx size variation between groups. First, being dominant would enlarge queens MBs. Second, being subordinate may reduce worker MB calyx volume. Next, we tested whether age, reproductive status, sensory experience, or being part of a social group affected MB calyx size. Workers had smaller MB calyces and MB calyx:brain ratios than all other groups (although not all differences were significant; Figs 3-4, Table 1). The other groups, including quees, did not significantly differ from each other. This suggests that workers' MBs are relatively small, but that queens' MBs are not especially large. Our data did not fit the age prediction because newly emerged bees did not have smaller MB calyces, nor did queens have larger MB calyces than the other groups. It did not fit the reproductive status prediction because the two groups with reproductive ovaries, queens and dispersers, did not have larger MB calyces than the non-reproductive groups, except for workers. This did not fit the sensory experience prediction because newly emerged and isolated bees did not have smaller MB calyces, nor did queens have larger MB calyces than other groups. The results also did not fit the social interactions prediction because workers and queens together did not have larger MB calyces than the other groups.

Our MB data suggest that a combination of reduced nutrition during development and/or behavioral dominance after emergence lead to reduced neural investment in worker MB calyces relative to other bees of the same age and size that are either experimentally (the observation nest foundresses and isolated bees) or naturally (the dispersers) free from queen control. High dominance status, rates of aggression, and enlarged ovaries are associated with larger MB calyces in paper wasps (Molina and O'Donnell 2007, 2008, O'Donnell et al. 2007, 2017), and socially dominant females of the bee *Ceratina australensis* also have larger MB calyces than their subordinate sisters (Rehan et al. 2015). Rehan et al. (2015) showed that queens had larger MBs than solitary reproductives, and that workers had smaller MBs than solitary reproductives. The latter result is similar to the queen-worker differences we report here, although in our study queens did not differ from the solitary nest foundress groups. In the related sweat bee *Augochlorella aurata,* early season nest foundresses (collected before the emergence of the worker brood) had larger MB calyces than workers from social nests (Pahlke et al. submitted). Previous authors interpreted these results in terms of increased cognitive demand associated with dominance and/or the older age of the queens (Molina and O'Donnell 2007, 2008, O'Donnell et al. 2007, 2017, Smith et al. 2010, Rehan et al. 2015) or the increased larval nutrition provided to future queens to survive overwintering diapause in temperate climates (Pahlke et al. submitted). However our results here suggest that workers' MB development may be suppressed by queen dominance, rather than queen's MBs being enlarged, since workers' MB calyx volume, both absolutely and as a ratio of whole brain volume, was significantly smaller than all other groups except dispersers, while queens' MB calyx volume was not significantly larger than any other group except workers.

How might maternal manipulation affect MB development? We do not know whether it is behavioral dominance, maternal manipulation of nutrition or a combination of the two that leads to the reduction of worker MB calyx size. Previous work on this species shows that caste has morphological and physiological components: workers are smaller than queens (Smith et al. 2008, 2009, Kapheim et al. 2013) due to reduced larval nutrition (Kapheim et al. 2011), although the queen-worker size differences were greater in those studies than we found here. Queens aggressively dominate workers (Kapheim et al. 2016, Smith et al. submitted). This results in physiological changes: workers have reduced ovaries, lower vitellogenin (the egg precursor protein, Vg) titers, and lower juvenile hormone (JH) levels than queens and both observation nest foundresses and natural dispersers (Smith et al. 2009, 2013, Kapheim et al. 2012). JH is associated with, but not required for, MB expansion in honeybees (Withers et al. 1995, Fahrbach et al. 2003). However, in the studies cited above (Kapheim et al. 2012, Smith et al. 2013), newly emerged and socially isolated bees also had reduced ovaries, Vg, and JH, yet those same groups showed greater MB development than workers in this study, which suggests that variation in MB development is not driven directly by these physiological variables. It may be the aggressive dominance behavior of queens toward their workers itself that affects MB calyx size in workers. In the ant *Diacamma,* aggression toward workers reduced brain dopamine levels (Shimoji et al. 2017), which may influence brain volume (Taylor et al. 1992). Larval thermal stress can affect MB development in *Drosphila* (Wang et al. 2007), and stress generally affects brain structure and function across animals (Lupien et al. 2009). The reduced MB investment in workers may be a response to the behavioral stress of queen aggression, a factor to which no other group in the study was subjected.

Another explanation for workers' smaller MB calyces, and whole brains as well, is that larval nutrition may influence neural development. Larval nutrition affects brain morphology in honeybees, with queens having larger and more rapidly growing brains in the larval stage (Moda et al. 2013), and reduced larval nutrition results in smaller MB calyces at emergence in workers (Steijven et al. 2017), but this has not been studied in primitively eusocial groups. Variation in larval nutrition can affect reproductive physiology and behavior in other sweat bee species (Richards and Packer 1994, Brand and Chapuisat 2012) as well as other species of primitively eusocial insects (Judd et al. 2015, Lawson et al. 2016, 2017, Kapheim 2017). Our previous work on this species suggests that queens manipulate larval pollen resources to create small, subordinate worker daughters (Smith et al. 2008, 2009, Kapheim et al. 2011, 2013). However our study suggests that effects on worker brain morphology result from more subtle nutritional variation than just reduced quantity, as workers were not significantly smaller than the other groups. On the one hand, the lack of queen-worker body size difference is likely an artifact of the small sample size of this study, given the ubiquity of this difference in previous studies (Smith et al. 2008, 2009, Kapheim et al. 2013). But on the other hand, it allows us to see that the dramatic MB calyx differences of this study are apparently related to being a subordinate worker, rather than just a small bee. Richards and Packer (1994) showed that offspring of different castes but similar body size differed in the relative amounts of sugar and protein in their larval provisions in the sweat bee *Halictus ligatus*. Future studies coupling nutritional manipulation with brain measurements, and the interaction of larval nutrition and adult experience of aggression, would be productive.

Our study found no effect of age on MB development. The lack of difference between newly emerged bees and other groups contrasts with a previous study on this species which showed that newly emerged bees had significantly smaller MB calyces than queens, but not workers (newly emerged bees' AL and OL volumes were also lower in the previous study) (Smith et al. 2010). Previous studies on honeybees, bumblebees, the bee *C. australensis,* the paper wasp *Mischocyttarus mastigophorus,* and multiple species of ants also showed increases in MB calyx volume during the first week after emergence (Gronenberg et al. 1996, Fahrbach et al. 1998, 2003, Seid et al. 2005, Seid and Wehner 2009, O'Donnell et al. 2007, Rehan et al. 2015, Seid and Junge 2016). The solitary bee *Osmia lignaria* did not show such an increase but that is complicated by the fact that *O. lignaria* overwinters in their nest as an adult; MB expansion may have occurred prior to leaving the nest (Withers et al. 2007). Our contrasting results with our previous study on *M. genalis* may also result from the limited sample sizes in each study (Smith et al. 2010).

Many studies have shown experience-dependent plasticity of the MB calyces in Hymenoptera (Withers et al. 1993, 1995, 2007, Gronenberg et al. 1996, Fahrbach et al. 1998, Farris et al. 2001, Kuhn-Buhlman and Wehner 2006, Maleszka et al. 2009, Molina and O'Donnell 2008, Stieb et al. 2010, Jones et al. 2013, Amador-Vargas et al. 2015, Rehan et al. 2015), other insects (Montgomery and Merrill 2016, Motgomery et al. 2017, van Dijk et al. 2017) and even a spider (Stafstrom et al. 2017). Fahrbach et al. (2003) showed that honeybee foragers that spend the winter in the hive without foraging had similar MB development to other foragers of younger age but similar experience. Seid and Junge (2016) showed that socially isolated ants had smaller MBs than same-age ants in their natural social group. This suggests that age-MB volume associations after the first week or so of adult life result from increased experience rather than age per se. Yet in our study, the one group that was both markedly older and more experienced, the queens, did not have larger MB calyces than any of the younger, less experienced groups, except for their workers. Future studies of known-age nests coupled with observations of foraging trips could explicitly measure experience-dependent MB plasticity.

Optic lobes and antennal lobes

We predicted OLs and ALs to increase with sensory experience. Our results were mixed (Figs 3, 4), and given the small sample sizes of each group, our data have little power to partition the different influences on sensory neuropil development. However, the sensory neuropils were not as dramatically reduced in workers relative to queens as were the MB calyces, especially when measured relative to whole brain size (Fig 4). This suggests that the effects of queen manipulation are stronger on the MB calyces than the sensory neuropils.

Conclusions

The most dramatic result of our study was that workers had smaller MB calyces than queens, but other groups in the study did not. This suggests that the queen-worker differences were not a result of age, sensory environment, experience, or reproductive status, because other groups in the study differed from queens in at least one of each of those variables. Workers were the only group subject to queen control. This may have taken the form of manipulation of developmental nutrition (Kapheim et al. 2011) and aggressive behavioral dominance (Kapheim et al. 2016, Smith et al. submitted). Future studies partitioning these two types of manipulation will be useful for understanding social influences on brain development.

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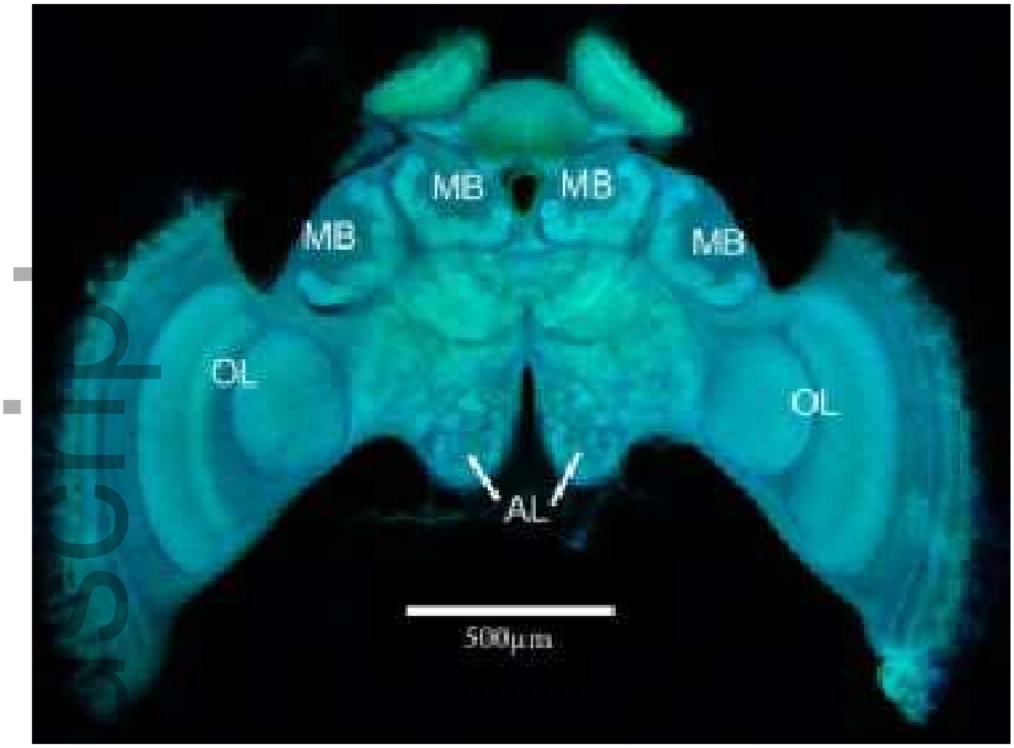
		New	Dispersers	Obs. Nest	Isolated	Queens	Workers
SC Whole brain, mm^3	Mean	0.420	0.384	0.382	0.399	0.392	0.333
	SD	0.041	0.058	0.030	0.019	0.044	0.019
SC MB calyces, mm^3	Mean	0.025	0.021	0.023	0.025	0.021	0.015
	SD	0.006	0.005	0.003	0.002	0.004	0.002
SC Optic lobes, mm^3	Mean	0.075	0.082	0.068	0.076	0.086	0.069
	SD	0.009	0.011	0.005	0.011	0.007	0.002
SC Antennal lobes, mm^3	Mean	0.015	0.015	0.015	0.014	0.017	0.013
	SD	0.001	0.002	0.001	0.001	0.002	0.003
MB: Whole brain ratio	Mean	0.059	0.055	0.060	0.063	0.053	0.045
	SD	0.008	0.006	0.007	0.006	0.007	0.005
OL: Whole brain ratio	Mean	0.178	0.215	0.179	0.189	0.222	0.207
	SD	0.018	0.018	0.011	0.024	0.023	0.005
AL: Whole brain ratio	Mean	0.035	0.039	0.039	0.034	0.043	0.040
	SD	0.004	0.003	0.003	0.002	0.003	0.007

Table 1. Means and standard deviations (SD) for size corrected (SC) volumes and neuropil:whole brain ratios for each group of bees used in the study. See text for explanations of treatment groups and statistical tests of pairwise comparisons.



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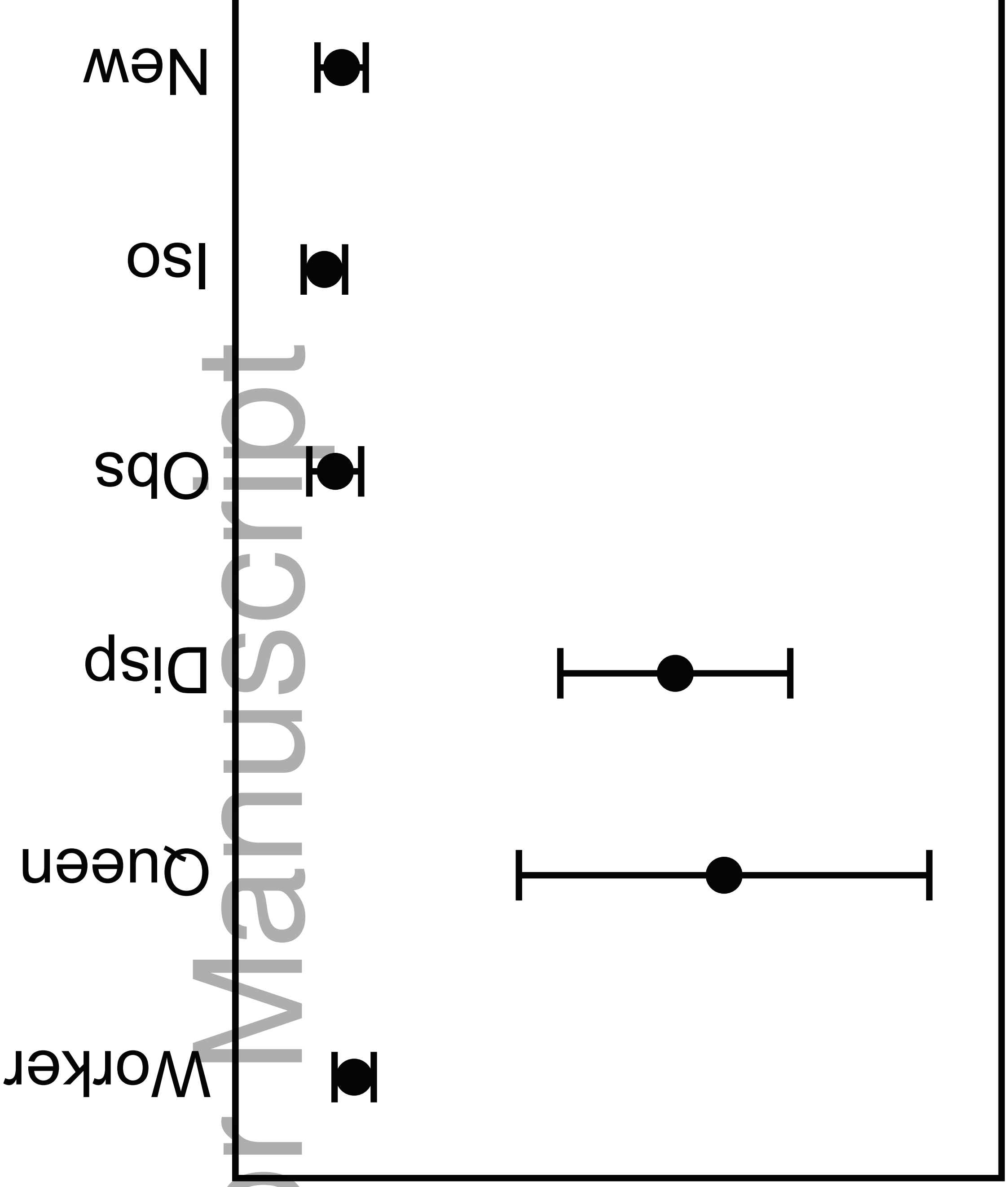

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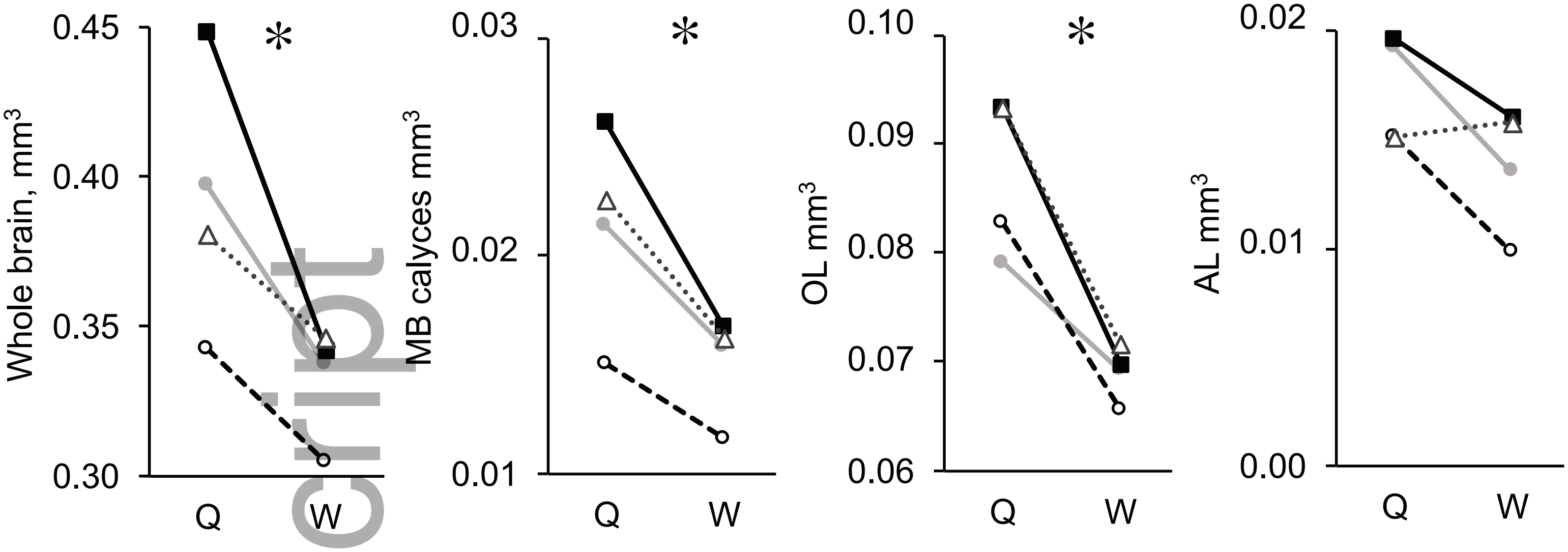

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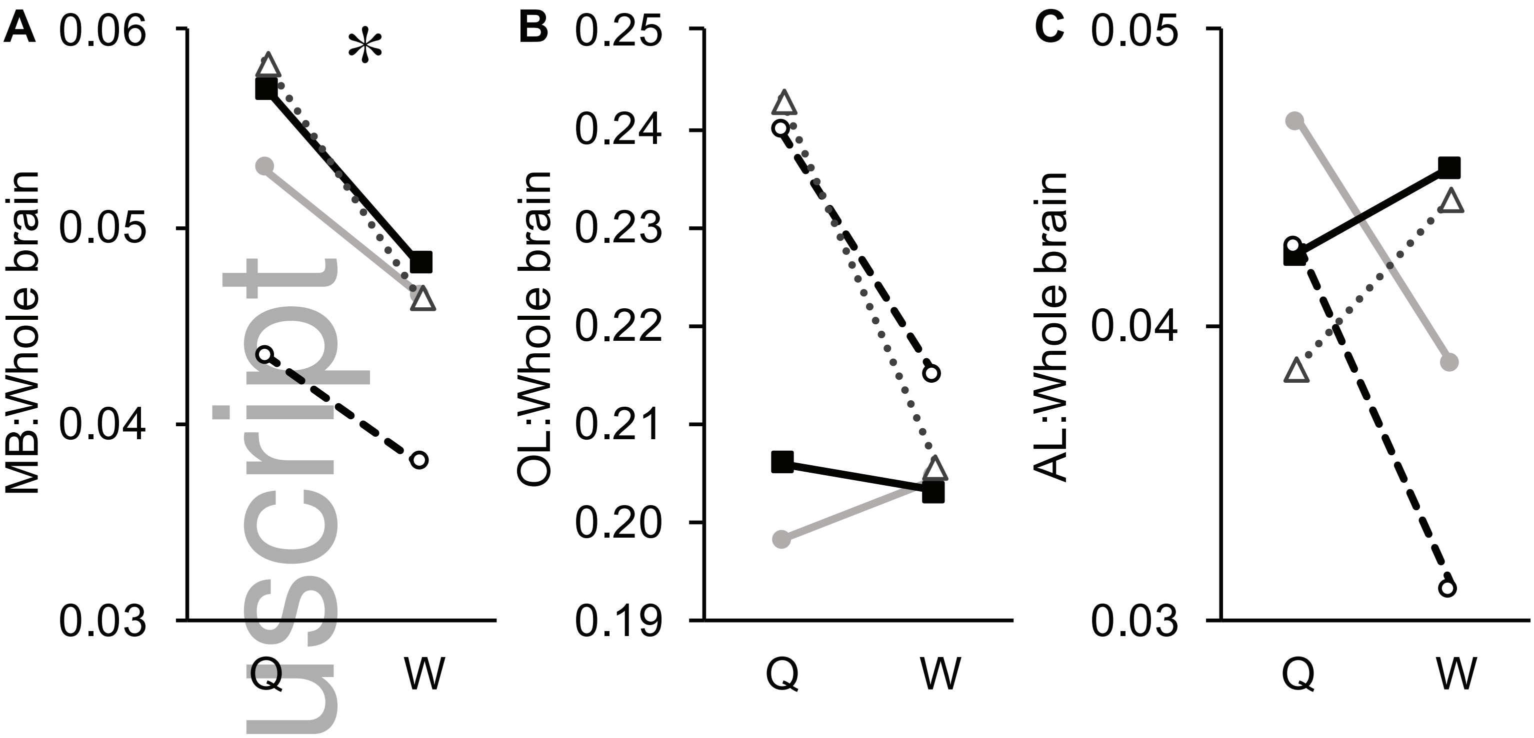

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