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Summary

The volumes of brains and major brain regions were compared between European (EHB) and African (AHB) honey bee workers. The brain volume was not significantly different between the two bee races. The overall composition of major brain regions appeared similar except for the lobes of the mushroom bodies, which were significantly larger in EHB. Discriminant analysis indicated that brains from EHB could be distinguished from those of AHB based on the volumes of the central body together with either the mushroom body lobe or the mushroom body calyx. Whether learning and memory capacities differ between AHB and EHB based on the size of mushroom body lobes and whether the differences are adaptive due to the environments where EHB and AHB originated are discussed.

Introduction

African honey bees (Apis mellifera scutellata) (AHB) differ from European honey bees (Apis mellifera ligustica L.) (EHB) in both behavioural and physiological attributes (see Schneider et al., 2004). The selection pressures exerted on AHB and EHB from the geographic areas where they have established have shaped the foraging priorities of the two populations. EHB is a temperate adapted bee selected to store large amounts of resources so the colony can survive during periods of confinement such as in the winter (Winston, 1987). Management of EHB by beekeepers has further selected for bees that produce large amounts of surplus honey. AHB are tropically adapted and follow changing floral resources by absconding and re-establishing new nests (Winston, 1992). AHB foragers have a greater tendency to collect pollen.
and rear brood compared to EHB which recruit proportionately larger foraging populations and collect and store large amounts of honey (Pesante et al., 1987; Schneider and McNally, 1992). Because AHB convert food resources to brood more readily than EHB, swarming rates in AHB are also higher (Schneider, 1995; Rubink et al., 1996).

Associative learning is an essential component of foraging behaviour in honey bees, as they must remember attractive food sources (e.g., associate a flower's high nectar yield with its odour and/or colour or pattern). Likewise, learning is crucial for a forager's navigation towards food sources and back to the nest. These and other advanced cognitive abilities of honey bees have been well documented (Erber et al., 1980; Bitterman et al., 1983; Srinivasan et al., 1994; Giurfa et al., 1999; reviewed by Menzel and Giurfa, 2006) and they require a neural substrate (the brain) able to integrate many different sensory cues and generate the appropriate behavioural output.

The brains of honey bees are large and relatively accessible and were well described anatomically more than 100 years ago (Kenyon, 1896). They have become a model system in invertebrate brain research, and much is known about the function of particular brain components (Homberg, 1984; Gronenberg, 1987; Hertel, 1987; Galizia et al., 1999; Hammer, 1993). The mushroom bodies, a brain centre involved in learning, memory (Erber et al., 1980) and multimodal sensory integration (reviewed by Strausfeld et al., 1998), have received particular attention in honey bees (Gronenberg, 1987; Withers et al., 1993; Grünewald, 1999; Farris et al., 2001; Malun et al., 2002; Strausfeld, 2002).

Studies of the honey bee brain have been performed on EHB, as is true for most behavioural studies. While the overall behaviours of AHB are well known, particularly at the colony level, few studies exist that quantitatively describe individual AHB behaviours under reproducible conditions that allow direct comparison with EHB (Abramson et al., 1997, Pankiw and Rubik, 2002). Basically nothing is known about the brains of AHB, or how they differ from those of EHB. Given the pronounced behavioural differences between the two honey bee races, one might expect to also find some differences regarding their brain structures. The purpose of our study was to compare overall brain volume and the volumes of individual brain components between EHB and AHB foragers. The current study is a first approach to look for brain variation at the gross anatomical level (as opposed to the cellular level) that might underlie the known behavioural differences.

**Preparation of brains**

Worker honey bees were captured leaving colonies during periods of high foraging activity. Since the bees were captured at random, their age, foraging history and whether they were pollen, nectar or water collectors are not known (a potential source of the variation found across the morphometric brain data). Bees were chilled on ice and weighed after the abdomen was removed to minimize variation from differences in volume of fluid in the crop. Hence, what is referred to as 'body weight' comprises only the weight of the bees' head and thorax. We weighed 34 AHB and 47 EHB.

Brain dissections were performed by removing the head of the bee and imbedding it in dental wax. The head capsule was opened frontally and the brain dissected while immersed in a solution of 4% formaldehyde in phosphate buffer (pH 6.8) that served as a fixative. The brain remained in the fixative for 3 hours. Brains were rinsed in buffer three times and then stained in 1% aqueous osmiumtetroxide solution for 2 hours at 4°C and for one additional hour at room temperature. The brains were rinsed in water, dehydrated, plastic-embedded (Fluka, Durecupan, ACM) and polymerized at 65°C. Brains were sectioned on a sliding microtome (Reichert OmE; Austria) at 15μm thickness, and then mounted on slides.

Outlines of the brains and brain components were drawn from the sections using images generated by a microprojector (Ken-A-Vision; Kansas City, MO). The drawings did not include the retina and the outermost visual processing centre, the lamina, which were often incomplete after dissection. The following regions were morphometrically analyzed (outlined in Fig. 1a): the optic lobes (visual centres) medulla and lobula; the glomeruli and central neuropil of the antennal lobes (olfactory centres); the central body (the smallest of the brain compartments examined); and the mushroom bodies and their sub-compartments (the calyx and the lobes). The mushroom bodies comprise another component, the peduncle, which is difficult to discriminate from the lobes, so in our measurements has not been differentiated from the lobes proper. Hence, 'lobes' here refers to the sum of both mushroom body peduncle and lobes.

Brain drawings were scanned to create computer images that were measured using the Photoshop (Adobe) pixel counting routine. Every second section of each brain was drawn and measured. While this approach is not based on random samples, it yields results as accurate as the 'Cavalieri' method (Mares et al., 2005), a commonly used randomized morphometric technique (Gundersen and Jensen, 1987; Michael and Cruz-Orive, 1988).

Volumes were calculated by multiplying the area measurements by the section thickness and number of sections (Mares et al., 2005). Relative volumes of brain components were calculated and compared by dividing the respective volume (e.g., the medulla volume) by the overall brain volume.Brains of 10 EHB and 10 AHB captured while leaving the hive were sampled and compared in this way.

**Statistical analysis**

Total volume of brains and individual brain regions of African and European worker bees were compared using Student's t-tests. Discriminant function analysis was used to determine the set of brain measures (treated as independent variables) that allowed for the best discrimination between EHB and AHB (Sokal and Rohlf, 1995). Statistical analysis was performed with JMP IN 5.1.2 software (SAS, Cary, North Carolina, USA).

**Materials and Methods**

**Sources of European and African worker honey bees**

EHB were selected from colonies headed by commercially reared *Apis mellifera ligustica* queens (Big Island Queens in Captain Cook, Hawaii) mated in Hawaii. AHB populations are not present in Hawaii. AHB colonies were collected from swarms in Tucson, Arizona. Southern Arizona has a resident feral population of AHB (Rabe et al., 2005) that originated from the northward expansion of African bee populations originally introduced into Brazil (Schneider et al., 2004). Africanization was confirmed using mitochondrial DNA and morphometric analyses (Rinderer et al., 1993, Nielsen et al., 2000).

**Preparation of brains**

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Brains and brain components in African and European honey bees

Brain size and brain size
AHB weighed significantly less than EHB in our sample (AHB = 47.8 mg, EHB = 51.6 mg; t = 3.875; n = 81; p < 0.0002). Average brain volumes (measured for 10 bees each) were estimated to be 1.18 + 0.17 mm³ for EHB and 1.06 + 0.074 mm³ for AHB, the difference being significant (t = 2.02; n = 20; p = 0.033). When comparing relative brain sizes (brain volume divided by the respective body mass), the average proportion of the worker bees’ body weight comprising brain tissue did not differ between EHB and AHB (EHB = 0.022, AHB = 0.023, t = -0.26; d.f = 17; p = 0.80). In contrast, when comparing the brains of only those EHB and AHB whose body weight overlapped (the six smallest EHB and the five largest AHB), we found a trend for the AHB to have smaller brains than EHB (EHB = 1.21 + 0.22 mm³ and AHB = 1.049 + 0.06 mm³; t = 1.77; n = 11; p < 0.064). This comparison is, however, difficult to interpret because of the small sample size.

Brain components
Because of the differences in overall brain size within and across groups, we standardized the size of individual brain regions by dividing them by the respective overall brain volume. The proportion of each brain region relative to the total brain volume did not differ between AHB and EHB with the exception of the mushroom body lobes which occupied a significantly larger proportion of the EHB brain than the AHB (t = 2.01; d.f = 17; p = 0.033) (Fig. 1b). In addition, a trend existed for the medulla to be relatively larger in EHB (0.193 ± 0.0105) than in AHB (0.181 ± 0.021; t=1.62; d.f. = 17; p = 0.06).
Discriminant analysis

Despite absolute differences in average brain and body size, the relative brain compositions of AHB and EHB were similar. There was no single brain parameter that would allow EHB and AHB to be reliably distinguished. There were, however, combinations of parameters that together allowed for the best discrimination between the two races. The three most decisive parameters were the volumes of the mushroom body calyces, the mushroom body lobes and the central body (all relative to the respective overall brain volume). The central body together with either the mushroom body lobe or the mushroom body calyx were sufficient to distinguish EHB from AHB with 95% confidence (Fig. 1c, d). As the mushroom body components (calyx and lobes) showed opposite tendencies (calyx larger in AHB, lobes larger in EHB), the entire mushroom body turned out not to be a distinctive feature as the opposing tendencies (calyx vs. mushroom body lobes) cancelled each other out. Regarding brain structures other than the mushroom body (Fig. 1c, d), at least three brain components were required to distinguish between the two bee races (e.g., antennal lobe, medulla and central body, or mushroom body, medulla and antennal lobe; or other permutations of these four major brain components; see Fig. 1e).

Discussion

We showed that overall brain size correlates with body size and found no statistically significant differences between EHB and AHB in brain size when corrected for body size (but see below). While the overall brain composition was similar in the two bee races, the mushroom body lobes were significantly smaller in AHB and particular combinations of brain components (most notable the central body and the mushroom body calyx and lobes) allowed discrimination of AHB from EHB brains. In addition, weak trends existed suggesting that AHB have smaller medulles and slightly smaller overall brain sizes than EHB, although verification of this latter trend requires a larger sample size.

Our results confirm the general difference in body weight known to exist between EHB and AHB (Michelette and Engels, 1995). The estimates of brain volume in EHB are very similar to those previously reported by Withers et al., (1993). A positive correlation between body and brain size has long been established for vertebrates (reviewed by Striedter, 2005) and has recently been found to exist in insects such as bumble bees (Mares et al., 2005) and desert ants (Wehner et al., 2007). AHB brains would therefore be expected to be slightly smaller than EHB brains because of the (likewise small) difference in average body size. However, Mares et al. (2005) failed to establish such a correlation for honey bees. A large sample size is probably needed to determine if a correlation exists for honey bees because their overall variation in body size is small and because volumetric brain measurements are more error-prone than linear measurements such as head or thorax width, wing length or body weight.

We found significant differences in the volume of the mushroom body lobes between EHB and AHB. However, we did not know the age or foraging experience of the workers we collected, and experience and age are known to correlate with the size of certain brain components. The volume of a few particular glomeruli in the antennal lobes increases when workers begin to forage (Winnington et al., 1996; Brown et al., 2003), but the overall antennal lobe volume appears to stay constant. The volume of the mushroom body calyces increases as worker bees shift from tasks inside the hive to foraging (Withers et al., 1993; Durst et al., 1994; Farris et al., 2001). More importantly in the current context, the volume of the mushroom body calyx neuropil also becomes larger with foraging experience (Farris et al., 2001). As our sample probably comprised foragers differing in their age and experience, this might contribute to the variation we found for mushroom body size. However, unlike the calyces, previous studies have not suggested that the mushroom body lobe volume correlates with foraging experience. The only significant differences we found between EHB and AHB brains were in the mushroom body lobe size. We therefore suggest that our results indeed show race specific differences rather than differences in foraging experience. Before it is certain though, that differences do exist between EHB and AHB mushroom body lobes, additional samples from different populations will be needed to determine if our findings can be repeated.

Mushroom bodies play a role in odour learning in both honey bees and Drosophila (Menzel et al., 1974; Erber et al., 1980; Heisenberg et al., 1985; deBelle and Heisenberg, 1994; Hammer and Menzel, 1998; Akalal et al., 2006). In Drosophila, the mushroom body lobes in particular are involved in memory formation (Zars et al., 2000; Riemensperger et al., 2005). Our finding that the mushroom body lobes are smaller in AHB might therefore suggest a poorer performance of AHB in learning and memory tasks. While there is limited information about the learning and memory abilities of AHB, a study by Abramson et al. (1997) suggests that EHB are indeed better learners than AHB in olfactory tasks. We also have preliminary evidence that EHB are faster learners than AHB (M. Couvillon, G. DeGrandi-Hoffman, and W. Gronenberg, in preparation), suggesting that differences between EHB and AHB mushroom body lobes indeed translate into differences in olfactory learning.

Assuming that these anatomical differences are adaptive, they might be related to differences in the selection pressures that shaped the two races of bees. Unlike the AHB, EHB evolved in a commercial environment. To survive they must forage efficiently to secure sufficient resources to feed and heat the colony during a 3–4 month period in the winter. Larger mushroom bodies might make EHB foragers more adept at learning and remembering the location of distant or widely dispersed nectar sources than AHB. The EHB used in this study were from commercial queen producers that select for (among other criteria) honey production. Thus, any differences in brain composition particularly in regions associated with foraging that might exist between EHB and AHB due to the geographic regions, climate and resource availability where the bees evolved, might be amplified in commercial EHB lines.

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