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Social complexity influences brain investment and neural operation costs in ants

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The metabolic expense of producing and operating neural tissue required for adaptive behaviour is considered a significant selective force in brain evolution. In primates, brain size correlates positively with group size, presumably owing to the greater cognitive demands of complex social relationships in large societies. Social complexity in eusocial insects is also associated with large groups, as well as collective intelligence and division of labour among sterile workers. However, superorganism phenotypes may lower cognitive demands on behaviourally specialized workers resulting in selection for decreased brain size and/or energetic costs of brain metabolism. To test this hypothesis, we compared brain investment patterns and cytochrome oxidase (COX) activity, a proxy for ATP usage, in two ant species contrasting in social organization. Socially complex *Oecophylla smaragdina* workers had larger brain size and relative investment in the mushroom bodies (MBs)—higher order sensory processing compartments—than the more socially basic *Formica subsericea* workers. *Oecophylla smaragdina* workers, however, had reduced COX activity in the MBs. Our results suggest that as in primates, ant group size is associated with large brain size. The elevated costs of investment in metabolically expensive brain tissue in the socially complex *O. smaragdina*, however, appear to be offset by decreased energetic costs.

1. Introduction

The costs of producing and operating neural tissue needed for socially and ecologically adaptive behaviour are critically important to brain evolution. Although brains are metabolically expensive [1–3], brain size has been found to increase with group size in primates probably because of cognitive challenges associated with more varied and complex interactions and increased demands of processing social information [4]. Eusocial insects such as bees, wasps, ants and termites also form large and complex societies, but workers are typically sterile and their labour benefits the fitness of the colony as a whole rather than individual reproductive interests. Reproductive competition, mate selection and pair bonding, which are considered to be among the primary drivers of brain evolution in vertebrates [4], are thus absent or reduced in most eusocial insects. Although behavioural challenges confronting social insect workers do not appear to be similar to those that characterize members of large primate societies, highly coordinated and integrated worker behaviour in large colonies may nevertheless involve social selection that affects brain evolution. Our understanding of how colony-level processes contributing to social complexity impact brain size, structure and metabolism is very limited [5–9].

In contrast to primate social brain theory, social complexity in insect societies has been predicted to be associated with a decrease in the size of brains and regions involved in higher order processing [7,10,11]. Large colony size and division of labour [12–14], sophisticated communication systems that coordinate

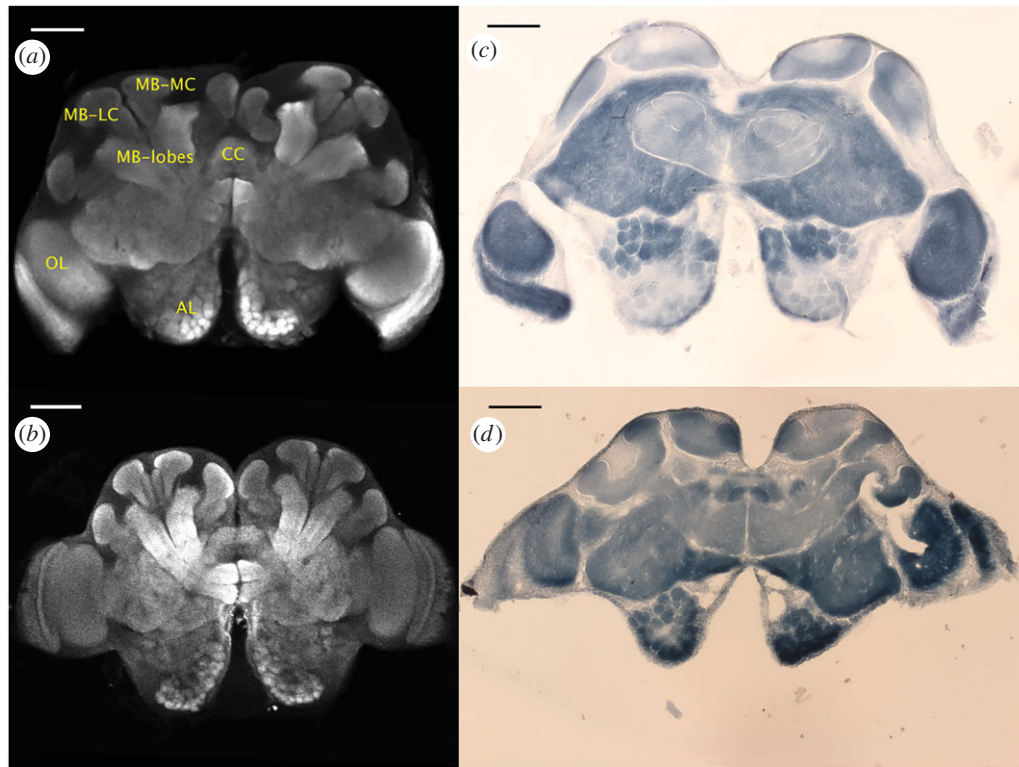


Figure 1. (a) Confocal micrographs of synapsin-labelled brains of an *O. smaragdina* major worker and (b) *F. subsericea* worker. Labels in one hemisphere of (a) demarcate functionally distinct neuropil regions: optic lobes (OL), antennal lobes (AL), mushroom body (MB) lateral and medial calyces (MB-LC and MB-MC, respectively) and peduncle and lobes (MB-lobes), central complex (CC), and the rest of the central brain (unlabelled neuropil). The subesophageal zone (SEZ) is not pictured due to its ventral location with respect to the central brain. Images of (c) *O. smaragdina* major worker and (d) *F. subsericea* worker brain stained for COX. Darker staining indicates greater COX activity. Scale bars, 100 μm . (Online version in colour.)

group behaviour [15–17], emergent collective action [18,19] and ecological dominance [20–23] are characteristics of socially complex ant species. Division of labour is often based on the evolution of morphologically differentiated workers and age-related schedules of behavioural development, both of which may result in task specialization (reviewed in [24,25]) and fewer cognitive challenges to individuals. Worker morphological phenotypes, or subcastes, and worker chronological age are underscored by mosaics of neuropil volume variation and brain compartment covariance [6,8] that appear to reduce brain metabolic costs through adaptive investment in functionally specialized brain regions in accordance with worker social roles [7,8,10,26]. Furthermore, self-organization theory suggests that simple components are sufficient for coordinated worker action and group decision-making [27,28] and thus predicts that workers may not require significant cognitive abilities. Collective intelligence, which may provide fitness benefits by enabling groups of workers to rapidly render more precise and accurate decisions than those of individuals [27,29–32], could thus collaterally reduce brain investment in individual workers by lowering behavioural performance demands [33].

To test this hypothesis, we quantified patterns of neural investment in workers by measuring brain mass and volumes of functionally distinct neuropil regions (figure 1a,b) to estimate brain investment (production and development) costs. We measured the activity of cytochrome oxidase (COX; figure 1c,d), a catalyst for ATP synthesis and thus an endogenous metabolic marker [34–37], as a proxy for operation costs in brain regions. We defined operational metabolic costs as the energy usage needed for neural maintenance and

functioning [3,38], including but not limited to costs of sustaining membrane resting potential, synthesizing macromolecules necessary for signalling and recovery ('housekeeping', [39]), and activating neural circuitry. We contrasted neuropil investment patterns and metabolic activity in the brains of workers of the socially complex ant *Oecophylla smaragdina* and the socially basic sister clade *Formica subsericea*, two species that have significant evolutionary and socioecological divergence [40]. *Oecophylla smaragdina* is considered a pinnacle of social complexity in ants [20]: colonies contain as many as approximately 500 000 workers that show size-based specialization in task performance. Major workers (average head width (HW) 1.57 mm, range 1.31–1.71 mm) are task generalists, whereas smaller minor workers (average HW 1.02 mm, range 0.93–1.16 mm) specialize in brood care within the nest (electronic supplementary material). We focused our analysis on the more behaviourally complex major workers, which forage throughout the large arboreal territories they collectively defend and cooperatively build leaf nests by forming chains with their bodies to draw leaves together and bind them in place with larval-secreted silk. *Formica subsericea* workers are monomorphic (average HW 1.46 mm, range 1.2–1.7 mm), and in sharp contrast, show generalized behavioural repertoires that encompass nursing, foraging and rudimentary nest construction in soil or decayed wood, and live in relatively small colonies (several 100 to approx. 8000 workers). In this socially basic species, cooperation in groups of more than two workers does not enhance overall work effort [41], suggesting that collective capabilities are very limited. The striking differences in colony and worker phenotypes in these species provide an excellent opportunity

to comparatively analyse neurobiological correlates of social complexity and test the hypothesis that increased division of labour through subcaste evolution and a greater reliance on group behaviour are associated with decreased investment and operation costs in the brain and its higher order processing regions.

Based on current theory [7,10,11,42], we predicted that *O. smaragdina* major workers would have reduced total investment in the brain, and specifically, in the mushroom bodies (MBs), a region that functions in sensory integration, learning and memory [43], owing to colony-level division of labour and emergent collective actions that could decrease investment costs. *Oecophylla smaragdina* majors were also predicted to invest substantially in sensory input regions involved in visual navigation and pheromonal coordination integral to territorial defence and foraging. By contrast, *F. subsericea* workers were predicted to invest more in sensory, motor and integrative processing regions and thus larger brains overall because of concurrent cognitive demands for brood care and other intranidal behaviours, as well as foraging and related tasks performed outside the nest. Primary sensory processing regions thus were hypothesized to have similar investment patterns in both species, while investment in the MB was predicted to decrease with increasing division of labour in the more socially complex weaver ants.

2. Material and methods

(a) Colony collection and maintenance

Oecophylla smaragdina nests were collected from savannah woodlands on the James Cook University campus (19°19'40.3" S, 146°45'31.3" E) in Townsville, Queensland, Australia. Workers collected for brain volume and body mass analyses were obtained directly from the field. For other analyses, colonies of several hundred *O. smaragdina* workers were transported to Boston University. Samples from workers in queenless nests were processed shortly after collection. *Formica subsericea* queen-right colonies of several dozen workers were collected in temperate mixed-hardwood forests in Hammond Woods (42°19'37.1" N, 71°10'26.8" W) and the Middlesex Fells Reservation (42°27'18.3" N, 71°06'30.1" W) in Massachusetts, USA. All colonies were housed in Fluon®-lined plastic boxes and fed carbohydrates (1:3 honey water or 1 M sugar water) and insect prey ad libitum every other day. Colonies were maintained on a 12 L:12 D cycle at 55% humidity and 25.5°C.

(b) Brain and body mass scaling

Brain scaling was determined by measuring the brain and body mass of fully pigmented, mature workers of both species. We used *O. smaragdina* brain mass data from Kamhi *et al.* [44], using major and minor workers from two colonies ($n = 41$). Brains were dissected in insect Ringer's [45] after HW was measured at the widest point of the head capsule, excluding the eyes. Brains were placed in a tin capsule of known weight, dried at 55°C, and weighed at 48 h. Following the same protocol, *F. subsericea* worker brains from three colonies ($n = 27$) were measured. Brain mass and HW displayed a significant linear correlation (*O. smaragdina*: brain mass = $(30.43 \times \text{HW}) + 14.83$; $F_{1,39} = 12.22$, $R^2 = 0.24$; $p < 0.0183$; *F. subsericea*: brain mass = $(24.72 \times \text{HW}) + 7.62$; $F_{1,26} = 6.43$, $R^2 = 0.71$; $p < 0.001$). Body masses were obtained by placing whole, intact bodies in tin capsules of known weight. The same drying and weighing protocol was used as noted above. Three *O. smaragdina* colonies ($n = 296$) and four *F. subsericea* colonies ($n = 52$) were used. Brain weight

measurements were acquired during prior neural studies; brain and body mass therefore were quantified in separate individuals. Body mass and HW also showed a highly significant linear correlation (*O. smaragdina*: body mass = $(4.18 \times \text{HW}) - 3.68$; $F_{1,294} = 2010.88$, $R^2 = 0.87$; $p < 0.001$; *F. subsericea*: body mass = $(9.65 \times \text{HW}) - 8.15$; $F_{1,50} = 110.12$, $R^2 = 0.69$; $p < 0.001$). Using the equations describing the correlation between body mass and HW, we estimated body mass for workers of known brain mass for each species. Standardized major axis regression was used to compare scaling of species and subcaste measurements with the program (S)MATR v. 2.0 [46]. Between-group effects were tested using ANCOVA [6]. Analyses were performed using JMP Pro v. 11 statistical software unless otherwise specified.

(c) Immunohistochemical and statistical analysis of neuropil investment

Fully pigmented, mature *O. smaragdina* major worker ($n = 16$) and *F. subsericea* worker ($n = 11$) brains from four colonies for each species were dissected in HEPES buffer and tissue was fixed in zinc 4% paraformaldehyde. Whole brains were processed according to a modified protocol based on Muscedere *et al.* [6] to visualize the pre-synaptic protein synapsin (3C11, anti-SYNORF1, DSHB; detailed methods in the electronic supplementary material). Brains were imaged using a confocal microscope (Zeiss LSM 710) with a 10× objective and optically sectioned at 12.34 µm intervals. Brains of *F. subsericea* workers were optically sectioned at 3.1 µm intervals using an Olympus FluoView 1 confocal microscope with a 10× objective.

Brain images on confocal scans were traced blind using AMIRA v. 3.1 to measure the volume of the following functionally distinct neuropil regions (figure 1a,b): the lobula and medulla of the optic lobe (OL, visual processing), antennal lobe (AL, olfactory input [47]), subesophageal zone (SEZ, mandible and mouthpart function [48]), MB (higher order regions of sensory input, learning and memory, partitioned into the lateral and medial calyces (MB-LC and MB-MC, respectively) and the peduncle and lobes (MB-lobes) [43]), the central complex (CC, visual and motor aspects of navigation, sensory integration and learning [49]) and the remainder of the undifferentiated central brain (ROCB). The MB-LC and MB-MC were measured separately due to possible functional differences [50]. For bilateral structures, one hemisphere selected at random was measured; for regions located along the brain midline (SEZ and CC) the whole structure was measured.

Relative volumes were calculated by dividing the volume of the region of interest by the total brain volume. The ROCB was used as a baseline measurement for relative brain compartment comparisons because of its multifunctionality. Compartments were compared using repeated measures ANOVA with mixed models, with the eight brain regions as the within-subjects factor and species as the between-subjects factor. The effect of colony was not significant; therefore, colonies were combined for analyses. Student's *t*-tests were performed for post-hoc comparisons between groups (species) and brain regions. Additional analyses were performed to include *O. smaragdina* minor workers in comparisons of brain region volumes (see the electronic supplementary material). Analyses were performed using JMP Pro v. 11 statistical software.

(d) Histochemical and statistical analysis of brain operation

The COX staining provides a qualitative neuroanatomical approach to mapping the presence or the absence of prominent COX activity in brain circuits (e.g. [51]). Histochemical measurements of COX also provide information about the metabolic

status of the brain and metabolic differences in discrete brain regions [52]. Most of the energy provided by COX is required for ion pumps that maintain neuronal membrane balances and thus allow neuronal information processing in the form of graded potentials and action potentials [34]. Fully pigmented mature workers of each species were analysed for COX activity. Two colonies of *O. smaragdina* ($n = 10$) and three colonies of *F. subsericea* were used ($n = 10$). Brains were dissected in HEPES buffer and fixed in 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS) for 40 min. Following PBS washes, tissue was sectioned at 30 μm on a cryostat and incubated in cytochrome *c* and diaminobenzidine solutions for visualizing COX activity (detailed protocol in the electronic supplementary material). Sections were imaged with a 10 \times objective using a Canon Rebel t3i with an adaptor for an Olympus BX40 light microscope.

Images were quantified using IMAGEJ 1.48s. Whole brain sections were first outlined to determine minimum and maximum intensity owing to variability in overall staining among sections. The COX activity is concentrated in neuropil [34]; neuropils measured for volume analyses were also measured for COX activity. Individual brain regions of each section were delimited and average staining intensity was recorded. Similar to volume analyses, whole measurements of the SEZ and CC were made while all other brain regions were measured in one hemisphere selected at random. Staining intensity of each brain region in a section was scaled to the range of staining intensity in that section. Regions were measured in 1–6 sections, as available. The COX activity per unit area for each brain region was calculated by averaging the scaled intensities of each compartment across sections. We performed repeated measures ANOVA with mixed models using the eight brain regions as within-subjects factors and species as the between-subjects factor. No differences were found between brains of workers from queenright and queenless *O. smaragdina* colonies or between *F. subsericea* colonies; therefore, colonies were combined for analyses. Additional analyses including *O. smaragdina* minors were performed to compare COX activity as well as COX activity scaled to both absolute and relative brain region volumes between both *O. smaragdina* subcastes and *F. subsericea* workers (see the electronic supplementary material). Student's *t*-tests were performed for post-hoc comparisons between groups and brain regions. JMP Pro v. 11 statistical software was used for all statistical analyses.

3. Results

(a) Relationships between brain and body mass

Workers in both species had a common brain mass to HW scaling slope not statistically different from 1 (mean slope 1.13, range 0.95–1.37; $\chi^2 = 1.98$, $p = 0.16$). There was a significant grade shift ($W^2 = 104.27$, $p < 0.001$) and *x*-axis shift ($W^2 = 5.38$, $p < 0.05$) indicating that *O. smaragdina* workers of both subcastes have significantly larger brains than *F. subsericea* workers when scaled for body size ($F_1 = 78.22$, $p < 0.001$). There was insufficient statistical evidence to support a common slope between species with respect to body mass to HW scaling ($p < 0.05$); further group comparisons therefore could not be performed. A statistically significant linear relationship of brain mass to estimated body mass was found for both species (*O. smaragdina*: brain mass = $(7.28 \times \text{body mass}) + 41.63$; $F_{1,39} = 12.22$, $R^2 = 0.24$; $p < 0.01$ (brain mass: average 55.73 μg , range 33–93.5 μg ; body mass: average 1.67 mg, range 0.23–4.45 mg); *F. subsericea*: brain mass = $(2.56 \times \text{body mass}) + 28.50$; $F_{1,25} = 60.43$, $R^2 = 0.71$; $p < 0.001$ (brain mass: average 43.28 μg , range 33–51 μg ; body mass: average 5.34 mg, range 2.20–

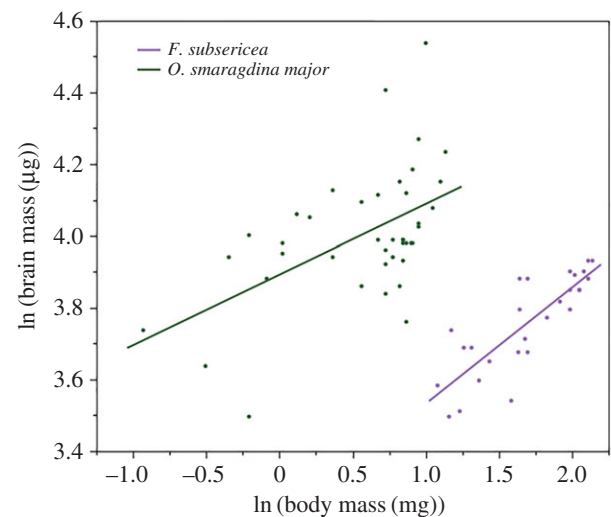


Figure 2. Regression of brain mass scaled to estimated body mass for *F. subsericea* and both *O. smaragdina* subcastes. (Online version in colour.)

9.38 mg)), and they shared a common slope that was significantly different from 1 (mean slope 0.38, range 0.32–0.46; $\chi^2 = 78.01$, $p < 0.001$; figure 2). There was a significant grade shift between species ($W^2 = 187.74$, $p < 0.001$) and a significant shift along the *x*-axis ($W^2 = 5.55$, $p < 0.05$), indicating that there were species differences in both brain and body mass. Between-group analyses showed that *O. smaragdina* workers had significantly greater brain mass for their body size than *F. subsericea* workers ($F_1 = 93.19$, $p < 0.001$).

(b) Neuropil investment patterns

Oecophylla smaragdina majors showed significantly greater investment in all brain compartments, excluding the ROCB, relative to the size of the whole brain than *F. subsericea* workers (repeated measures ANOVA, $F_{1,25.37} = 7.83$, $p < 0.01$). There was a significant interaction of species by brain region ($F_{6,150.7} = 31.12$, $p < 0.001$; figure 3a; electronic supplementary material, table S2). *Oecophylla smaragdina* majors had proportionally larger MB calyces than *F. subsericea* workers after accounting for larger brain size of *O. smaragdina* majors (post-hoc student's *t*-tests, MB-LC: $t_{150.36} = 7.30$, $p < 0.001$; MB-MC: $t_{150.36} = 7.99$, $p < 0.001$). The MB-lobes were also significantly larger in *O. smaragdina* majors than *F. subsericea* workers ($t_{150.36} = 2.70$, $p < 0.01$). *Formica subsericea* workers had significantly larger OL ($t_{150.36} = 3.45$, $p < 0.001$). There was no difference in relative AL volume between species ($t_{150.36} = 0.80$, $p = 0.42$). The SEZ was relatively larger in *F. subsericea* than in *O. smaragdina* majors ($t_{150.36} = 8.26$, $p < 0.001$). There were no significant differences between species in relative CC volume ($t_{150.36} = 0.01$, $p = 0.99$).

(c) Patterns of brain COX activity

Oecophylla smaragdina major workers had lower total brain COX activity than *F. subsericea* workers although this effect was not statistically significant (repeated measures ANOVA, $F_{1,18} = 3.80$, $p = 0.07$). A significant interaction effect was found for species and regional COX activity ($F_{7,126} = 3.46$, $p < 0.01$; figure 3b; electronic supplementary material, table S1). *Oecophylla smaragdina* major workers had significantly lower COX activity than *F. subsericea* workers in both the MB-LC (post-hoc student's *t*-test, $t_{51.76} = 3.54$,

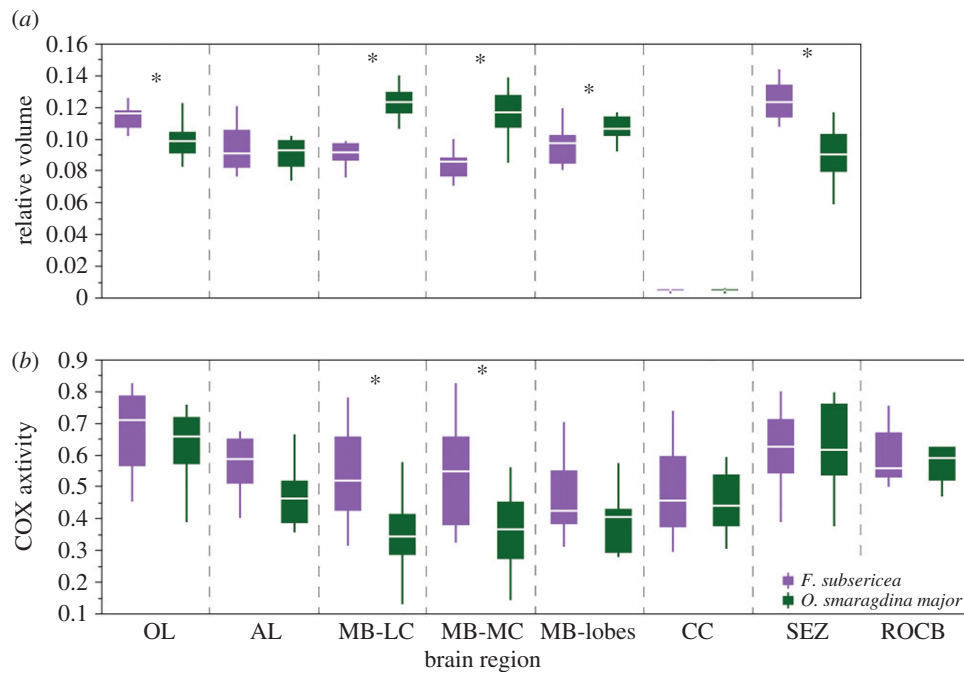


Figure 3. Box plots for each functionally distinct brain region for *O. smaragdina* major and *F. subsericea* monomorphic workers of (a) relative brain region volumes and (b) COX normalized by unit volume. Asterisks indicate statistical significance. (Online version in colour.)

$p < 0.001$) and MB-MC ($t_{51.76} = 3.13$, $p < 0.01$). *Oecophylla smaragdina* major workers had lower COX activity than *F. subsericea* workers in the ALs ($t_{51.76} = 1.89$, $p = 0.06$) but this difference was not significant. All other brain regions had similar COX activity levels when compared across species (all $p > 0.10$). No significant difference between *O. smaragdina* major and *F. subsericea* workers in the MB-LC or MB-MC were found when COX activity was scaled to either absolute or relative neuropil volume (all $p > 0.2$; details in the electronic supplementary material).

4. Discussion

(a) Social brain theory and ant brain evolution

Our study suggests that brain investment and operations costs, estimated from tissue volumes and COX activity, respectively, are associated with colony size and the complexity of social organization with respect to worker social roles and variation in their cognitive demands, as well as the emergent behavioural capabilities of coordinated worker groups. Contrary to the hypothesis that greater social complexity correlates with smaller brains and lower investment in higher order processing regions [7,10,11], our results show that *O. smaragdina* major workers had significantly larger brains and MBs than *F. subsericea* workers, although they are nearly identical in body size. The MB calyx COX activity in *O. smaragdina* majors was significantly lower, suggesting that selection for lower operation costs may compensate for higher neural production costs potentially associated with large colony size and complexity and/or related variation in worker cognitive demands.

Social brain theory predicts a positive correlation between group size and individual brain size [53]. Ecological factors such as diet and energy intake and community-level interactions that vary among species, may also contribute to differences in brain size [33]. Additionally, brain size

comparisons could be affected by whether the species studied have ancestral or derived neural traits. Perhaps because of these multiple influences on brain size evolution, support for this hypothesis in ants is mixed. Worker brain size in fungus-growing ants decreased in larger colonies of monomorphic species, but brain size and its covariation with some brain regions, colony size and task specialization is inconsistent and thus appears to be complex [11]. Similar to our findings, a significant positive relationship between colony size and individual worker brain mass was found in the ant genus *Cataglyphis* [54]; in this case, brain size does not appear to be an adaptive response to the cognitive demands associated with interspecific variation in foraging range size and related navigational challenges. Larger colonies, rather, may have more resources available to support larger worker brains and increased social interactions have been hypothesized to be the driving force of brain evolution in this genus [54].

In vertebrates, large group size positively correlates with brain size, hypothetically owing to the higher level of cognition required for individualized social interactions [53], mate choice [4] and parental care [55]. Although some eusocial insects may have social life histories and colony structures generating cognitive demands similar to those of vertebrates [7], it appears difficult to directly apply the social brain hypothesis to ants using the same behavioural variables [9] because workers are often sterile. The relative demands of alloparental care in ants and parental care in other eusocial insects are probably similar but are not known. However, ant workers do not appear to be able to recognize individuals [24,56] and associated social selection is therefore unlikely. Recognition *per se* may not demand extensive higher order processing, but the recall of long-term relationships and interaction outcomes that are dependent on recognition are thought to require greater cognitive capability [57,58]. However, no neuroanatomical or physiological evidence exists suggesting which brain components or circuits might be involved in such processing in insects. Nevertheless, cuticular

hydrocarbons function in nestmate [59–61] and social role [62] discrimination; these recognition mechanisms may be more highly developed in socially complex species because advanced division of labour can generate morphologically or behaviourally variable worker groups and thus require greater discriminatory capabilities to support colony-level functions. The neural mechanisms underlying these social processes and others remain unclear [63], and it is therefore difficult to determine their precise role in brain evolution.

(b) Colony-level social organization and brain evolution

We considered that brain size would be relatively small in *O. smaragdina* due to what we assume are lower cognitive demands placed on morphologically differentiated and behaviourally specialized workers that function as components of a complex system. Neurometabolic costs that may limit investment in total brain size of individual workers and cognitive processing centres could also be lowered if behaviourally ‘simple’ workers are sufficient to assemble collectively functioning groups whose capabilities exceed those of individual workers. Alternatively, behavioural algorithms coordinating collective action may be simple, but the underlying neural mechanisms of individual action could be sophisticated. For example, weaver ant nest construction is governed by a positive-feedback system [64]; *O. smaragdina* have a relatively large number of chemical communication systems involving tactile and pheromonal cues [17], which also may be involved in this coordinated action (J. F. Kamhi, J. F. A. Traniello 2016, which also may be involved in this coordinated action). Collective actions of this type may thus require substantial sensory perception and processing and perhaps more neural tissue and/or specialized circuitry, even if decisions are made at the level of the group.

We used COX staining, which has resulted in path-breaking discoveries in invertebrate and vertebrate brain research [51,65,66], as a proxy for operation costs in worker brains. Given the strong evolutionary conservation of this enzyme [35], the temporal and spatial activity profiles and distribution of COX in the brains of animals in diverse taxa is most probably similar. Indeed, patterns of COX activity in different honeybee [67,68] and fly [69] brain compartments resemble those we present here for ants. *Oecophylla smaragdina* major workers tended to have lower total brain metabolic activity than *F. subsericea* workers, as revealed by COX analyses. This pattern was most notable in the MB, and the AL also showed this trend. Other factors such as neuron size and number and the density and size of synapses [70–73] may influence brain metabolism. The number of neurons in the MB and AL appear to be similar in our study species; *O. smaragdina* majors, however, have larger MB neurons (J. F. Kamhi, J. F. A. Traniello 2016, unpublished data). This appears counterintuitive, although neurons may be optimized to reduce ATP consumption [74] and thus COX activity, which is linked to neural activation [35] and not necessarily directly correlated with neuron size. Behavioural phenotype also may be related to brain metabolic activity [75]. The variation in total brain metabolic activity in our study species indicates that *F. subsericea* worker task performance within and outside the nest may contribute to increased brain operation costs. Interspecific overlap in behavioural repertoire and general neurobiological requirements, however, may be high in *O. smaragdina* majors and *F. subsericea* workers. To examine variation in task specialization in our comparisons, we performed additional analyses that included both

O. smaragdina majors and specialist minor workers. Results showed that *O. smaragdina* majors had lower MB calyx and AL operation costs than minors and minors had similar brain and MB calyx operation costs as *F. subsericea* workers (electronic supplementary material, figure S1 and table S1). By accounting for variation in brain region size between species and subcaste in metabolic activity, we infer that the larger MB size in *O. smaragdina* majors could be compensated for in part by lower operations costs because MB COX activity scaled to absolute and relative volume revealed no significant species differences (electronic supplementary material, figure S2).

Our COX staining results can be interpreted in respect to the time course of translation and transcription of the enzyme subunits in neurons [35], supporting the inferences we draw from our data. COX precursor subunits may be present within distal mitochondria and can be activated upon demand when neurons are more active for shorter periods (roughly 5 h [35]). Downregulation as a consequence of strongly reduced neuronal activity, by contrast, requires approximately 2 days [76]. Therefore, COX is not well suited to visualize short-term changes in neuronal activity [77], but serves as an appropriate proxy for neuronal operation costs. We assume that activity variation across the brains of workers we sampled (figure 3) may to some degree represent individual differences in COX activity, hence differences in neuronal electrical activity, perhaps reflecting worker experience and stress during sampling. However, we compare long-term overall differences across different brain regions and between two species. Average COX activity does not vary significantly on a short-term basis because it relies on the transport of mitochondria from cell bodies [35]. In most invertebrates, including ants, neuronal cell bodies are located in the brain’s cell body rind, relatively far from the neuropil. Although dramatic changes in COX activity can result from artificially reduced or increased long-term neuronal activity, such changes are temporally slow, in the order of hours or days to weeks rather than minutes [76,77]. Long durations of time are thus required to modify the overall COX activity of brain regions. We therefore are confident that our average COX activity measures represent ‘standard’ activity conditions of the respective brain regions and species and are not influenced by minor handling effects or other immediate stimuli that workers may have been exposed to before their brains were dissected and fixed.

One prominent distinguishing characteristic of *O. smaragdina* majors is their engagement in collective actions that serve important colony functions in nest construction, territory maintenance and cooperative foraging. Although the association of neural circuits and such emergent colony-level behaviours is unknown [78], our data suggest that *O. smaragdina* majors have larger brains than *F. subsericea* workers, indicating more circuitry, yet the collective action pre-eminent in the organization of work by *O. smaragdina* majors could decrease neural operation costs. Brain metabolic costs can be reduced by decreasing redundancy in neural circuitry, leading to a lower signal-to-noise ratio [71]. In the context of worker collective action, any resulting decrease in worker processing capability may not have a colony-level cost because of cooperation: groups of workers acting in concert may be able to compensate for potential cognitive deficiencies in individual workers [18]. Our results suggest that the relationship between behavioural ability and brain size may be complex and manifest in multiple ways [79–81]. We hypothesize that

controlling behavioural responses by collective cognition may be less energetically expensive than the cost of producing a brain capable of generating greater individual behavioural performance capabilities.

(c) Worker neuroecology

Additional analyses of relative brain region volumes that include *O. smaragdina* minors elucidate how brain region investment corresponds to variation in behavioural repertoire and associated information-processing needs. Our results suggest that cognitive demands faced by workers may affect sensory processing region volumes (electronic supplementary material, figure S3 and table S2). For example, *O. smaragdina* majors visually navigate and chemically orient between nests and worksites in large territories and *F. subsericea* workers appear to primarily rely on visual orientation during foraging [20,82,83]. Accordingly, OL volume was greater in *F. subsericea* and *O. smaragdina* major workers than minors, whose social role appears to be limited to caring for brood within leaf nests.

Selection in socially basic monomorphic species such as *F. subsericea* appears to have favoured diverse worker task repertoires [84,85], leading to more totipotent and plastic task performance in different sensory environments inside and outside the nest. *Formica subsericea* workers were therefore predicted to invest more in the MB in response to increased needs for greater higher-order processing ability. Our results did not support this hypothesis: both *O. smaragdina* majors, which are task generalists, and specialist minors had significantly larger MB volume than *F. subsericea*, suggesting that behavioural plasticity may not affect MB circuitry and modify neuropil volume, or *O. smaragdina* workers may in fact be more behaviourally flexible than predicted and thus require greater higher order processing ability than *F. subsericea*. *Oecophylla smaragdina* MBs may undergo experience-dependent growth [86] that is absent or less substantial in *F. subsericea*. Additional comparisons between ant sister clade species that contrast in division of labour and other elements of social structure are crucial to fully understand the relationship between behavioural specialization and MB investment and identifying macroevolutionary patterns of brain evolution.

Organization and development of the MB in relation to behavioural plasticity could underlie sensory processing requirements for task performance. Enhanced visual input, rather than social complexity, has been suggested to be associated with increased MB calyx size [9], but our findings do not corroborate this prediction. In our study species, OL size, a correlate of input into the MB calyces [87], did not appear to directly influence MB calyx volume. Our findings alternatively suggest that worker behavioural repertoire in the context of colony organization is associated with MB size. *Oecophylla smaragdina* major worker behaviour, for example, may require significant sensory integration, thus contributing to MB calyx size. Weaver ants mark territories with colony-specific secretions; major workers collectively defend their territory and fight more intensively when they recognize their colony's scent [88]. This scaling of aggression to social context requires perception of a chemical signature probably acquired during development as well as higher order processing and neural signalling between brain compartments to control motor patterns of fighting under different social conditions. Further neuroecological studies are needed to determine the extent of

behavioural and neural plasticity and sensory integration in relation to MB circuitry.

5. Conclusion

Our data are, to our knowledge, the first to demonstrate an association between brain size and brain region volume, COX activity and behaviour in relation to the level of complexity of a society. Previous studies have suggested that larger colony size is associated with greater task specialization and lower brain investment [7–11], which should reduce brain metabolic cost. However, our comparative study of formicine ant sister clades suggests that components of social complexity such as division of labour, large colony size and collective intelligence may be among the driving forces of brain evolution and could have compensatory effects. Consistent with the social brain hypothesis, the *O. smaragdina* colony phenotype appears to be associated with the requirement for greater MB and total brain investment in individual workers, indicating that integrative processing is important to living in large and more complex social groups. The greater food intake probably required for producing this tissue may be possible through living in large, cooperative colonies [61]. However, increased brain volumes do not seem to be associated with higher metabolic costs; rather this greater investment may be compensated for by lower COX activity, an estimate of operational metabolic costs, in weaver ant major workers. Collective intelligence, which is associated with large colony size and socially advanced colony organization, may be one mechanism enabling ant colonies to conserve metabolic investment in the brain, particularly in the MB. The size of primary and higher order sensory input regions appears to reflect sensory requirements of worker task performance, thus providing a neuroecological basis for patterns of neuropil investment. Individual action in the context of division of labour and the ecology of individual task performance may also drive the evolution of information-processing circuitry, represented in brain mass, compartment size and ATP usage.

Ethics. Animals were collected in Australia in accordance with permits WITK09412611 and WITK14544014 and exported with Wildlife Trade permits WT2012-4106, PWS2013-AU-000415, and PWS2014-AU-001493. Animals were imported into the USA and contained according to the conditions of USDA permit P526P-12-04067 and cultured under containment conditions simulating a tropical environment. Experiments minimized pain and used universally accepted methods of euthanizing ants.

Data accessibility. The datasets supporting this article have been uploaded to Dryad (<http://dx.doi.org/10.5061/dryad.39gb2>) [89].

Authors' contributions. J.F.K., W.G. and J.F.A.T. co-conceived of this study; J.F.K. performed experiments; J.F.K. and S.K.A.R. analysed results; J.F.K. and J.F.A.T. drafted the manuscript, and all authors contributed to the design of the study and gave final approval for publication.

Competing interests. There are no competing interests.

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