

1 **Social complexity influences brain investment and neural operation costs in ants**

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22 cytochrome oxidase

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31 **Abstract:**

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

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## 1 **Introduction**

2           The costs of producing and operating neural tissue needed for socially and ecologically  
3 adaptive behaviour are critically important to brain evolution. Although brains are  
4 metabolically expensive (1-3), brain size has been found to increase with group size in primates  
5 likely due to cognitive challenges associated with more varied and complex interactions and  
6 increased demands of processing social information (4). Eusocial insects such as bees, wasps,  
7 ants, and termites also form large and complex societies, but workers are typically sterile and  
8 their labour benefits the fitness of the colony as a whole rather than individual reproductive  
9 interests. Reproductive competition, mate selection, and pair bonding, which are considered to  
10 be among the primary drivers of brain evolution in vertebrates (4), are thus absent or reduced  
11 in most eusocial insects. Although behavioural challenges confronting social insect workers do  
12 not appear to be similar to those that characterize members of large primate societies, highly  
13 coordinated and integrated worker behaviour in large colonies may nevertheless involve social  
14 selection that affects brain evolution. Our understanding of how colony-level processes  
15 contributing to social complexity impact brain size, structure, and metabolism is very limited  
16 (5-9).

17           In contrast to primate social brain theory, social complexity in insect societies has been  
18 predicted to be associated with a decrease in the size of brains and regions involved in higher-  
19 order processing (7, 10, 11). Large colony size and division of labour (12-14), sophisticated  
20 communication systems that coordinate group behaviour (15-17), emergent collective action  
21 (18, 19), and ecological dominance (20-23) are characteristics of socially complex ant species.  
22 Division of labour is often based on the evolution of morphologically differentiated workers and  
23 age-related schedules of behavioural development, both of which may result in task  
24 specialization (reviewed in 24, 25) and fewer cognitive challenges to individuals. Worker  
25 morphological phenotypes, or subcastes, and worker chronological age are underscored by  
26 mosaics of neuropil volume variation and brain compartment covariance (6, 8) that appear to  
27 reduce brain metabolic costs through adaptive investment in functionally specialized brain

28 regions in accordance with worker social roles (7, 8, 10, 26). Furthermore, self-organization  
29 theory suggests that simple components are sufficient for coordinated worker action and group  
30 decision-making (27, 28) and thus predicts that workers may not require significant cognitive  
31 abilities. Collective intelligence, which may provide fitness benefits by enabling groups of  
32 workers to rapidly render more precise and accurate decisions than those of individuals (27,  
33 29-32), could thus collaterally reduce brain investment in individual workers by lowering  
34 behavioural performance demands (33).

35 To test this hypothesis, we quantified patterns of neural investment in workers by  
36 measuring brain mass and volumes of functionally distinct neuropil regions (Figure 1A,B) to  
37 estimate brain investment (production and development) costs. We measured the activity of  
38 cytochrome oxidase (COX; Figure 1C,D), a catalyst for ATP synthesis and thus an endogenous  
39 metabolic marker (34-37), as a proxy for operation costs in brain regions. We defined  
40 operational metabolic costs as the energy usage needed for neural maintenance and functioning  
41 (3, 38), including but not limited to costs of sustaining membrane resting potential, synthesizing  
42 macromolecules necessary for signalling and recovery (“house-keeping,” [39]), and activating  
43 neural circuitry. We contrasted neuropil investment patterns and metabolic activity in the  
44 brains of workers of the socially complex ant *Oecophylla smaragdina* and the socially basic sister  
45 clade *Formica subsericea*, two species that have significant evolutionary and socioecological  
46 divergence (40). *O. smaragdina* is considered a pinnacle of social complexity in ants (20):  
47 colonies contain as many as ~500,000 workers that show size-based specialization in task  
48 performance. Major workers (average head width [HW] 1.57 mm, range 1.31-1.71 mm) are task  
49 generalists, whereas smaller minor workers (average HW 1.02 mm, range 0.93-1.16 mm)  
50 specialize in brood care within the nest (ESM). We focused our analysis on the more  
51 behaviourally complex major workers, which forage throughout the large arboreal territories  
52 they collectively defend and cooperatively build leaf nests by forming chains with their bodies  
53 to draw leaves together and bind them in place with larval-secreted silk. *F. subsericea* workers  
54 are monomorphic (average HW 1.46 mm, range 1.2-1.7 mm), and in sharp contrast, show

55 generalized behavioural repertoires that encompass nursing, foraging, and rudimentary nest  
56 construction in soil or decayed wood, and live in relatively small colonies (several 100 to  
57 ~8,000 workers). In this socially basic species, cooperation in groups of more than two workers  
58 does not enhance overall work effort (41), suggesting that collective capabilities are very  
59 limited. The striking differences in colony and worker phenotypes in these species provide an  
60 excellent opportunity to comparatively analyse neurobiological correlates of social complexity  
61 and test the hypothesis that increased division of labour through subcaste evolution and a  
62 greater reliance on group behaviour are associated with decreased investment and operation  
63 costs in the brain and its higher-order processing regions.

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

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## 77 **Methods**

### 78 *Colony collection and maintenance*

79         *O. smaragdina* nests were collected from savannah woodlands on the James Cook  
80 University campus (19°19'40.3"S, 146°45'31.3"E) in Townsville, Queensland, Australia. Workers  
81 collected for brain volume and body mass analyses were obtained directly from the field. For

82 other analyses, colonies of several hundred *O. smaragdina* workers were transported to Boston  
83 University. Samples from workers in queenless nests were processed shortly after collection. *F.*  
84 *subsericea* queenright colonies of several dozen workers were collected in temperate mixed-  
85 hardwood forests in Hammond Woods (42°19'37.1"N, 71°10'26.8"W) and the Middlesex Fells  
86 Reservation (42°27'18.3"N, 71°06'30.1"W) in Massachusetts, USA. All colonies were housed in  
87 Fluon®-lined plastic boxes and fed carbohydrates (1:3 honey water or 1M sugar water) and  
88 insect prey ad libitum every other day. Colonies were maintained on a 12:12 light dark cycle at  
89 55% humidity and 25.5°C.

90

### 91 *Brain and body mass scaling*

92 Brain scaling was determined by measuring the brain and body mass of fully pigmented,  
93 mature workers of both species. We used *O. smaragdina* brain mass data from Kamhi et al. (44),  
94 using major and minor workers from two colonies (n=41). Brains were dissected in Insect  
95 Ringer's (45) after head width was measured at the widest point of the head capsule, excluding  
96 the eyes. Brains were placed in a tin capsule of known weight, dried at 55°C, and weighed at 48  
97 hours. Following the same protocol, *F. subsericea* worker brains from three colonies (n=27)  
98 were measured. Brain mass and head width (HW) displayed a significant linear correlation (*O.*  
99 *smaragdina*: brain mass = [30.43 x HW] + 14.83;  $F_{1,39} = 12.22$ ,  $R^2=0.24$ ;  $p<0.0183$ ; *F. subsericea*:  
100 brain mass = [24.72 x HW] + 7.62;  $F_{1,26} = 6.43$ ,  $R^2=0.71$ ;  $p<0.001$ ). Body masses were obtained by  
101 placing whole, intact bodies in tin capsules of known weight. The same drying and weighing  
102 protocol was used as noted above. Three *O. smaragdina* colonies (n=296) and four *F. subsericea*  
103 colonies (n=52) were used. Brain weight measurements were acquired during prior neural  
104 studies; brain and body mass therefore were quantified in separate individuals. Body mass and  
105 HW also showed a highly significant linear correlation (*O. smaragdina*: body mass = [4.18 x HW]  
106 - 3.68;  $F_{1,294} = 2010.88$ ,  $R^2=0.87$ ;  $p<0.001$ ; *F. subsericea*: body mass = [9.65 x HW] - 8.15;  $F_{1,50} =$   
107 110.12,  $R^2=0.69$ ;  $p<0.001$ ). Using the equations describing the correlation between body mass  
108 and HW, we estimated body mass for workers of known brain mass for each species.

109 Standardized major axis regression was used to compare scaling of species and subcaste  
110 measurements with the program (S)MATR v. 2.0 (46). Between-group effects were tested using  
111 ANCOVA (6). Analyses were performed using JMP Pro 11 statistical software unless otherwise  
112 specified.

113

#### 114 *Immunohistochemical and statistical analysis of neuropil investment*

115 Fully pigmented, mature *O. smaragdina* major workers (n=16) and *F. subsericea*  
116 workers (n=11) brains from four colonies for each species were dissected in HEPES buffer and  
117 tissue was fixed in Zinc 4% paraformaldehyde. Whole brains were processed according to a  
118 modified protocol based on Muscedere et al. (6) to visualize the pre-synaptic protein synapsin  
119 (3C11, anti-SYNORF1, DSHB; detailed methods in supplemental information). Brains were  
120 imaged using a confocal microscope (Zeiss LSM 710) with a 10x objective and optically  
121 sectioned at 12.34  $\mu\text{m}$  intervals. Brains of *F. subsericea* workers were optically sectioned at 3.1  
122  $\mu\text{m}$  intervals using an Olympus FluoView 1 confocal microscope with a 10x objective.

123 Brain images on confocal scans were traced blind using Amira (v3.1) to measure the  
124 volume of the following functionally distinct neuropil regions (Figure 1A,B): the lobula and  
125 medulla of the optic lobe (OL, visual processing), antennal lobe (AL, olfactory input [47]),  
126 subesophageal zone (SEZ, mandible and mouthpart function [48]), mushroom body (MB,  
127 higher order regions of sensory input, learning, and memory, partitioned into the lateral and  
128 medial calyces [MB-LC and MB-MC, respectively] and the peduncle and lobes [MB-lobes] [43]),  
129 the central complex (CC, visual and motor aspects of navigation, sensory integration, and  
130 learning [49]), and the remainder of the undifferentiated central brain (ROCB). The MB-LC and  
131 MB-MC were measured separately due to possible functional differences (50). For bilateral  
132 structures, one hemisphere selected at random was measured; for regions located along the  
133 brain midline (SEZ and CC) the whole structure was measured.

134 Relative volumes were calculated by dividing the volume of the region of interest by  
135 the total brain volume. The ROCB was used as a baseline measurement for relative brain

136 compartment comparisons because of its multifunctionality. Compartments were compared  
137 using repeated measures ANOVA with mixed models, with the eight brain regions as the  
138 within-subjects factor and species as the between-subjects factor. The effect of colony was not  
139 significant; therefore, colonies were combined for analyses. Student's t-tests were performed  
140 for post-hoc comparisons between groups (species) and brain regions. Additional analyses  
141 were performed to include *O. smaragdina* minor workers in comparisons of brain region  
142 volumes (see ESM). Analyses were performed using JMP Pro 11 statistical software.

143

#### 144 *Histochemical and statistical analysis of brain operation*

145         The COX staining provides a qualitative neuroanatomical approach to mapping the  
146 presence or absence of prominent COX activity in brain circuits (e.g. 51). Histochemical  
147 measurements of COX also provide information about the metabolic status of the brain and  
148 metabolic differences in discrete brain regions (52). Most of the energy provided by cytochrome  
149 oxidase is required for ion pumps that maintain neuronal membrane balances and thus allow  
150 neuronal information processing in the form of graded potentials and action potentials (34).  
151 Fully pigmented mature workers of each species were analysed for COX activity. Two colonies  
152 of *O. smaragdina* (n=10) and three colonies of *F. subsericea* were used (n=10). Brains were  
153 dissected in HEPES buffer and fixed in 4% paraformaldehyde in 0.01M PBS for 40 minutes.  
154 Following PBS washes, tissue was sectioned at 30µm on a cryostat and incubated in cytochrome  
155 c and diaminobenzidine solutions for visualizing COX activity (detailed protocol in ESM).  
156 Sections were imaged with a 10x objective using a Canon Rebel t3i with an adaptor for an  
157 Olympus BX40 light microscope.

158         Images were quantified using ImageJ 1.48s. Whole brain sections were first outlined to  
159 determine minimum and maximum intensity due to variability in overall staining among  
160 sections. The COX activity is concentrated in neuropil (34); neuropils measured for volume  
161 analyses were also measured for COX activity. Individual brain regions of each section were  
162 delimited and average staining intensity was recorded. Similar to volume analyses, whole



163 measurements of the SEZ and CC were made while all other brain regions were measured in  
164 one hemisphere selected at random. Staining intensity of each brain region in a section was  
165 scaled to the range of staining intensity in that section. Regions were measured in 1 – 6  
166 sections, as available. The COX activity per unit area for each brain region was calculated by  
167 averaging the scaled intensities of each compartment across sections. We performed repeated  
168 measures ANOVA with mixed models using the eight brain regions as within-subjects factors  
169 and species as the between-subjects factor. No differences were found between brains of  
170 workers from queenright and queenless *O. smaragdina* colonies of between *F. subsericea*  
171 colonies; therefore colonies were combined for analyses. Additional analyses including *O.*  
172 *smaragdina* minors were performed to compare COX activity as well as both absolute and  
173 relative brain region volumes between both *O. smaragdina* subcastes and *F. subsericea* workers  
174 (see ESM). Absolute volumes were used in COX scaling analyses. Student's t-tests were  
175 performed for post-hoc comparisons between groups and brain regions. JMP Pro 11 statistical  
176 software was used for all statistical analyses.

177

## 178 **Results**

### 179 *Relationships between brain and body mass*

180 Workers in both species had a common brain mass to head width (HW) scaling slope not  
181 statistically different from 1 (mean slope 1.13, range 0.95 – 1.37;  $\chi^2 = 1.98$ ,  $p=0.16$ ). There was a  
182 significant grade shift ( $W^2 = 104.27$ ,  $p<0.001$ ) and x-axis shift ( $W^2 = 5.38$ ,  $p<0.05$ ) indicating that  
183 *O. smaragdina* workers of both subcastes have significantly larger brains than *F. subsericea*  
184 workers when scaled for body size ( $F_1=78.22$ ,  $p<0.001$ ). There was insufficient statistical  
185 evidence to support a common slope between species with respect to body mass to HW scaling  
186 ( $P<0.05$ ); further group comparisons therefore could not be performed. A statistically  
187 significant linear relationship of brain mass to estimated body mass was found for both species  
188 (*O. smaragdina*: brain mass = [7.28 x body mass] + 41.63;  $F_{1,39} = 12.22$ ,  $R^2=0.24$ ;  $p<0.01$  [brain  
189 mass: average 55.73 $\mu$ g, range 33 – 93.5 $\mu$ g; body mass: average 1.67mg, range, 0.23 – 4.45mg]; *F.*

190 *subsericea*: brain mass = [2.56 x body mass] + 28.50;  $F_{1,25} = 60.43$ ,  $R^2=0.71$ ;  $p<0.001$  [brain mass:  
191 average 43.28 $\mu$ g, range 33 – 51 $\mu$ g; body mass: average 5.34mg, range, 2.20 – 9.38mg]), and they  
192 shared a common slope that was significantly different from 1 (mean slope 0.38, range 0.32 –  
193 0.46;  $\chi^2 = 78.01$ ,  $p<0.001$ ; Figure 2). There was a significant grade shift between species ( $W^2 =$   
194 187.74,  $p<0.001$ ) and a significant shift along the x-axis ( $W^2 = 5.55$ ,  $p<0.05$ ), indicating that  
195 there were species differences in both brain and body mass. Between-group analyses showed  
196 that *O. smaragdina* workers had significantly greater brain mass for their body size than *F.*  
197 *subsericea* workers ( $F_1=93.19$ ,  $p<0.001$ ).

198

### 199 *Neuropil investment patterns*

200 *O. smaragdina* majors showed significantly greater investment in all brain  
201 compartments, excluding the ROCB, relative to the size of the whole brain than *F. subsericea*  
202 workers (Repeated measures ANOVA,  $F_{1,25.37}=7.83$ ,  $p<0.01$ ). There was a significant interaction  
203 of species by brain region ( $F_{6,150.7}=31.12$ ,  $p<0.001$ ; Figure 3A; Table S2). *O. smaragdina* majors  
204 had proportionally larger MB calyces than *F. subsericea* workers after accounting for larger  
205 brain size of *O. smaragdina* majors (Post-hoc Student's T-tests, MB-LC:  $t_{150.36}=7.30$ ,  $p<0.001$ ; MB-  
206 MC:  $t_{150.36}=7.99$ ,  $p<0.001$ ). The MB-lobes were also significantly larger in *O. smaragdina* majors  
207 than *F. subsericea* workers ( $t_{150.36}=2.70$ ,  $p<0.01$ ). *F. subsericea* workers had significantly larger  
208 OL ( $t_{150.36}=3.45$ ,  $p<0.001$ ). There was no difference in relative AL volume between species  
209 ( $t_{150.36}=0.80$ ,  $p=0.42$ ). The SEZ was relatively larger in *F. subsericea* than in *O. smaragdina* majors  
210 ( $t_{150.36}=8.26$ ,  $p<0.001$ ). There were no significant differences between species in relative CC  
211 volume ( $t_{150.36}=0.01$ ,  $p=0.99$ ). No significant difference between *O. smaragdina* major and *F.*  
212 *subsericea* workers in the MB-LC or MB-MC were found when COX activity was scaled to either  
213 absolute or relative neuropil volume (all  $p>0.2$ ; details in ESM).

214

### 215 *Patterns of brain COX activity*

216 *O. smaragdina* major workers had lower total brain COX activity than *F. subsericea*  
217 workers although this effect was not statistically significant (Repeated measures ANOVA,  
218  $F_{1,18}=3.80$ ,  $p=0.07$ ). A significant interaction effect was found for species and regional COX  
219 activity ( $F_{7,126}=3.46$ ,  $p<0.01$ ; Figure 3B, Table S1). *O. smaragdina* major workers had  
220 significantly lower COX activity than *F. subsericea* workers in both the MB-LC (Post-hoc  
221 Student's T-test,  $t_{51.76}=3.54$ ,  $p<0.001$ ) and MB-MC ( $t_{51.76}=3.13$ ,  $p<0.01$ ). *O. smaragdina* major  
222 workers had lower COX activity than *F. subsericea* workers in the ALs ( $t_{51.76}=1.89$ ,  $p=0.06$ ) but  
223 this difference was not significant. All other brain regions had similar COX activity levels when  
224 compared across species (all  $p>0.10$ ).

225

## 226 **Discussion**

227

### 228 *Social brain theory and ant brain evolution*

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

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

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

255 In vertebrates, large group size positively correlates with brain size, hypothetically due  
256 to the higher level of cognition required for individualized social interactions (53), mate choice  
257 (4), and parental care (55). Although some eusocial insects may have social life histories and  
258 colony structures generating cognitive demands similar to those of vertebrates (7), it appears  
259 difficult to directly apply the social brain hypothesis to ants using the same behavioural  
260 variables (9) because workers are often sterile. The relative demands of alloparental care in  
261 ants and parental care in other eusocial insects are likely similar but are not known. However,  
262 ant workers do not appear to be able to recognize individuals (24, 56) and associated social  
263 selection is therefore unlikely. Recognition *per se* may not demand extensive higher-order  
264 processing, but the recall of long-term relationships and interaction outcomes that are  
265 dependent on recognition are thought to require greater cognitive capability (57, 58). However,  
266 no neuroanatomical or physiological evidence exists suggesting which brain components or  
267 circuits might be involved in such processing in insects. Nevertheless, cuticular hydrocarbons  
268 function in nestmate (59-61) and social role (62) discrimination; these recognition mechanisms  
269 may be more highly developed in socially complex species because advanced division of labour

270 can generate morphologically or behaviourally variable worker groups and thus require greater  
271 discriminatory capabilities to support colony-level functions. The neural mechanisms  
272 underlying these social processes and others remain unclear (63), and it is therefore difficult to  
273 determine their precise role in brain evolution.

274

#### 275 *Colony-level social organization and brain evolution*

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

289 We used COX staining, which has resulted in path-breaking discoveries in invertebrate  
290 and vertebrate brain research (51, 65, 66), as a proxy for operation costs in worker brains.  
291 Given the strong evolutionary conservation of this enzyme (35), the temporal and spatial  
292 activity profiles and distribution of COX in the brains of animals in diverse taxa is most likely  
293 similar. Indeed, patterns of COX activity in different honey bee (67, 68) and fly (69) brain  
294 compartments resemble those we present here for ants. *O. smaragdina* major workers tended to  
295 have lower total brain metabolic activity than *F. subsericea* workers, as revealed by COX  
296 analyses. This pattern was most noticeable in the MB, and the AL also showed this trend. Other

297 factors such as neuron size and number and the density and size of synapses (70-73) may  
298 influence brain metabolism. The number of neurons in the MB and AL appear to be similar in  
299 our study species; *O. smaragdina* majors, however, have larger MB neurons (unpub. data). This  
300 appears counterintuitive, although neurons may be optimized to reduce ATP consumption (74)  
301 and thus COX activity, which is linked to neural activation (35) and not necessarily directly  
302 correlated with neuron size. Behavioural phenotype also may be related to brain metabolic  
303 activity (75). The variation in total brain metabolic activity in our study species indicates that *F.*  
304 *subsericea* worker task performance within and outside the nest may contribute to increased  
305 brain operation costs. Interspecific overlap in behavioural repertoire and general  
306 neurobiological requirements, however, may be high in *O. smaragdina* majors and *F. subsericea*  
307 workers. To examine variation in task specialization in our comparisons, we performed  
308 additional analyses that included both *O. smaragdina* majors and specialist minor workers.  
309 Results showed that *O. smaragdina* majors had lower MB calyx and AL operation costs than  
310 minors and minors had similar brain and MB calyx operation costs as *F. subsericea* workers  
311 (Figure S1; Table S1). By accounting for variation in brain region size between species and  
312 subcaste in metabolic activity, we infer that the larger MB size in *O. smaragdina* majors could be  
313 compensated for in part by lower operations costs because MB COX activity scaled to absolute  
314 and relative volume revealed no significant species differences (Figure S2).

315 Our COX staining results can be interpreted in respect to the time course of translation  
316 and transcription of the enzyme subunits in neurons (35), supporting the inferences we draw  
317 from our data. COX precursor subunits may be present within distal mitochondria and can be  
318 activated upon demand when neurons are more active for shorter periods (roughly five hours  
319 [35]). Down regulation as a consequence of strongly reduced neuronal activity, in contrast,  
320 requires approximately two days (76). Therefore, COX is not well suited to visualize short-term  
321 changes in neuronal activity (77), but serves as an appropriate proxy for neuronal operation  
322 costs. We assume that activity variation across the brains of workers we sampled (Figure 3)  
323 may to some degree represent individual differences in COX activity, hence differences in

324 neuronal electrical activity, perhaps reflecting worker experience and stress during sampling.  
325 However, we compare long-term overall differences across different brain regions and between  
326 two species. Average COX activity does not vary significantly on a short-term basis because it  
327 relies on the transport of mitochondria from cell bodies (35). In most invertebrates, including  
328 ants, neuronal cell bodies are located in the brain's cell body rind, relatively far from the  
329 neuropil. Although dramatic changes in COX activity can result from artificially reduced or  
330 increased long-term neuronal activity, such changes are temporally slow, on the order of hours  
331 or days to weeks rather than minutes (76, 77). Long durations of time are thus required to  
332 modify the overall COX activity of brain regions. We therefore are confident that our average  
333 COX activity measures represent 'standard' activity conditions of the respective brain regions  
334 and species and are not influenced by minor handling effects or other immediate stimuli that  
335 workers may have been exposed to before their brains were dissected and fixed.

336         One prominent distinguishing characteristic of *O. smaragdina* majors is their  
337 engagement in collective actions that serve important colony functions in nest construction,  
338 territory maintenance and cooperative foraging. Although the association of neural circuits and  
339 such emergent colony-level behaviours is unknown (78), our data suggest that *O. smaragdina*  
340 majors have larger brains than *F. subsericea* workers, indicating more circuitry, yet the  
341 collective action preeminent in the organization of work by *O. smaragdina* majors could  
342 decrease neural operation costs. Brain metabolic costs can be reduced by decreasing  
343 redundancy in neural circuitry, leading to a lower signal-to-noise ratio (71). In the context of  
344 worker collective action, any resulting decrease in worker processing capability may not have a  
345 colony-level cost because of cooperation: groups of workers acting in concert may be able to  
346 compensate for potential cognitive deficiencies in individual workers (18). Our results suggest  
347 that the relationship between behavioural ability and brain size may be complex and manifest  
348 in multiple ways (79-81). We hypothesize that controlling behavioural responses by collective  
349 cognition may be less energetically expensive than the cost of producing a brain capable of  
350 generating greater individual behavioural performance capabilities.

351

352 *Worker neuroecology*

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

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

376 Organization and development of the MB in relation to behavioural plasticity could  
377 underlie sensory processing requirements for task performance. Enhanced visual input, rather



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

391

### 392 *Conclusion*

393 Our data are the first to demonstrate an association between brain size and brain region  
394 volume, COX activity, and behaviour in relation to the level of complexity of a society. Previous  
395 studies have suggested that larger colony size is associated with greater task specialization and  
396 lower brain investment (7, 11), which should reduce brain metabolic cost. However, our  
397 comparative study of formicine ant sister clades suggests that components of social complexity  
398 such as division of labour, large colony size, and collective intelligence may be among the  
399 driving forces of brain evolution and could have compensatory effects. Consistent with the  
400 social brain hypothesis, the *O. smaragdina* colony phenotype appears to be associated with the  
401 requirement for greater MB and total brain investment in individual workers, indicating that  
402 integrative processing is important to living in large and more complex social groups. The  
403 greater food intake likely required for producing this tissue may be possible through living in  
404 large, cooperative colonies (33). However, increased brain volumes do not seem to be

405 associated with higher metabolic costs; rather this greater investment may be compensated for  
406 by lower COX activity, an estimate of operational metabolic costs, in weaver ant major workers.  
407 Collective intelligence, which is associated with large colony size and socially advanced colony  
408 organization, may be one mechanism enabling ant colonies to conserve metabolic investment in  
409 the brain, particularly in the MB. The size of primary and higher-order sensory input regions  
410 appears to reflect sensory requirements of worker task performance, thus providing a  
411 neuroecological basis for patterns of neuropil investment. Individual action in the context of  
412 division of labour and the ecology of individual task performance may also drive the evolution  
413 of information-processing circuitry, represented in brain mass, compartment size, and ATP  
414 usage.

415

416

417 **Ethics** Animals were collected in Australia in accordance with permits WITK09412611 and  
418 WITK14544014 and exported with Wildlife Trade permits WT2012-4106, PWS2013-AU-  
419 000415, and PWS2014-AU-001493. Animals were imported into the US and contained  
420 according to the conditions of USDA permit P526P-12-04067 and cultured under containment  
421 conditions simulating a tropical environment. Experiments minimized pain and used  
422 universally accepted methods of euthanizing ants.

423

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

426

427 **Competing interests** There are no competing interests.

428

429 **Authors' contributions** JFK, WG, and JFAT co-conceived of this study, JFK performed  
430 experiments, JFK and SKAR analysed results, JFK and JFAT drafted the manuscript, and all  
431 authors contributed to the design of the study and gave final approval for publication.

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