

1 **Title**

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3 **Feeding specialisation and longer generation time are**
4 **associated with relatively larger brains in bees**

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7 **Running title:** Brain evolution bees

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9

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34

35 **Abstract**

36 Despite their miniature brains, insects exhibit substantial variation in brain size. Although
37 the functional significance of this variation is increasingly recognized, research on
38 whether differences in insect brain sizes are mainly the result of constraints or selective
39 pressures has hardly been performed. Here, we address this gap by combining prospective
40 and retrospective phylogenetic-based analyses of brain size for a major insect group, bees
41 (superfamily Apoidea). Using a brain dataset of 93 species from North America and
42 Europe, we found that body size was the single best predictor of brain size in bees.
43 However, the analyses also revealed that substantial variation in brain size remained even
44 when adjusting for body size. We consequently asked whether such variation in relative
45 brain size might be explained by adaptive hypotheses. We found that ecologically
46 specialized species with single generations have larger brains —relative to their body
47 size— than generalist or multi-generation species, but we did not find an effect of
48 sociality on relative brain size. Phylogenetic reconstruction further supported the
49 existence of different adaptive optima for relative brain size in lineages differing in
50 feeding specialisation and reproductive strategy. Our findings shed new light on the
51 evolution of the insect brain, highlighting the importance of ecological pressures over
52 social factors and suggesting that these pressures are different from those previously
53 found to influence brain evolution in other taxa.

54 **Introduction**

55 Despite Darwin’s claim that “the brain of an ant is one of the most marvelous atoms of
56 matter in the world” [1], the brain of insects has traditionally been considered too small
57 and simple compared, for instance, to those of vertebrates to provide insight into the
58 evolution of complex brains. Carl von Linné even erroneously pointed to the absence of
59 a real brain as the main characteristic of insects [2]. Only recently it has been broadly
60 recognized that, as in vertebrates, there is great anatomical variation in size and
61 complexity in the brain of other animals, from the 302 neurons of a nematode brain to the
62 complex multi-ganglia brain of an octopus. Evidence has also accumulated that many
63 animal groups, including some molluscs and insects, have evolved integrative processing
64 centers in their brains [3,4].

65

66 The paradigm shift regarding the functional significance of brain size and architecture
67 outside vertebrates has mostly come from research in insects. The insect brain, despite its
68 small size, contains integrative centres—the mushroom bodies—functionally equivalent
69 to the neocortex of mammals and the pallium of birds [4]. These neuropils are responsible
70 for sensory integration [5], discrimination [6], learning, and memory [7,8]. Insects also
71 show advanced cognitive capacities—such as social learning [9], numerosity [10,11] and
72 concept formation [12]—that in the past had only been reported in a few vertebrates.

73

74 Despite progress, it is still a matter of debate whether the same ecological and social
75 pressures hypothesized to have shaped brain size and architecture in vertebrates also
76 apply to the brains of other animals. For instance, although the social brain hypothesis
77 argues that complex social systems should select for enlarged brains [13,14], there are
78 serious doubts that the same logic applies to caste-based societies like eusocial insects
79 [15,16]. This is because division of labour presumably forces individuals to behaviourally
80 specialize in simple tasks [17,18]. In the case of feeding generalization, another popular
81 hypothesis in vertebrates [19,20], the need to discriminate and process a wider variety of
82 resources has been suggested to put higher demands on enlarged brains [21]. In insects,
83 however, specialist pollinators (i.e. oligolectic species) have to discriminate among many
84 stimuli to find suitable flowers, perhaps requiring enhanced learning abilities [22].

85

86 The present study aims to test the major hypotheses proposed to have shaped insect brain
87 evolution, including sociality, feeding specialization, and life history. Among insects,
88 bees (superfamily Apoidea) are particularly suitable for such a study because they have
89 been instrumental in developing modern neuro-ecology [3,23] and because they exhibit
90 substantial variation in life history, sociality, breeding strategies and foraging behaviour
91 [24]. We generated a unique comparative dataset of brain size for 385 specimens from 93
92 bee species to find out that brain size exhibits substantial variation across bee species,
93 even after controlling for allometric effects, and that much of this brain variation is driven
94 by changes in the mushroom bodies. Combined with published information on functional
95 traits and a well-resolved molecular phylogeny based on genetic markers, we then
96 employed prospective phylogenetic-comparative methods and retrospective ancestral
97 state reconstructions to identify which ecological and life history traits that may have
98 significantly shaped the evolution of brain size in insects.

99

100 **Methods**

101 *Brain and body size measurements*

102 We measured brain size as the weight of fixed brains. For this purpose, we collected 395
103 female specimens from 93 species in various locations from the United States of America,
104 Spain and the Netherlands. These species represent most major lineages of bees. Because
105 brain size might change plastically between experienced and naïve individuals [25,26],
106 we collected individuals that were foraging in flowers to make sure they were experienced
107 adults. We cut off the heads of each individual collected using a scalpel and stored it in
108 fixative (4% Paraformaldehyde in 0.1 M Phosphate-buffered saline (PBS) pH = 7.4) . We
109 then extracted the brain from the head capsule on a Petri dish using Dumont #5 forceps,
110 removed the retina from the optic lobes and cleaned the entire brain of all tracheae and
111 fat bodies. Following [27], each brain was then placed on a small piece of Parafilm®.
112 PBS drops were wiped away using finely twisted pieces of Kimwipes® and the brain was
113 weighed within 4s after the removal of the liquid. In all the specimens, body size was
114 measured as the intertegular span (ITS). ITS is the distance between the two tegulae, small
115 sclerites above the insertion points of the wings, and it is a standard measure of body size
116 in bees because it accurately correlates with body weight [28]. For ITS, measurements
117 were done using a stereomicroscope (magnification 16x–80x) with a calibrated ocular

118 micrometer (resolution down to 0.02 mm). All data on body and brain measurements can
119 be found in **Supplementary Data file 1**.

120

121 *Volumetric measurements of brain components*

122 Because brains are organized in different functional areas, understanding brain evolution
123 requires to examine its composition [29]. For example, if brains can evolve by the
124 increase of different brain regions in different species, comparisons of whole-brain size
125 may be biologically meaningless. To ensure that this is not the case, we measured the
126 volume of two brain regions —the mushroom bodies and the optic lobes— in a sample
127 of 21 specimens from 12 species. These two brain regions are relevant for their central
128 role in exploration, selective attention, and learning [7,8,30]. The dissected brains of the
129 specimens were stored in fixative overnight at 4°C. Then, they were rinsed briefly three
130 times with 0.1 M PBS pH=7.4, and treated with collagenase (1 mg/ml in PBS) for 10 mins
131 at 38 °C. Brains were then washed three times for 20 minutes with 3% Triton X-100
132 solution in PBS and placed in blocking solution (10% goat serum and 3% Triton X-100
133 solution in PBS) overnight. Subsequently, brains were rinsed briefly with fresh blocking
134 solution and incubated in a primary antibody against synapsin produced in mouse (anti
135 SYNORF1 = 3C11, monoclonal; Developmental Studies Hybridoma Bank, DSHB,
136 University of Iowa) at a 1:50 dilution in blocking solution dilution for 48–72hr. The
137 antibody 3C11 (anti SYNORF1) was deposited to the DSHB by E. Buchner (DSHB
138 Hybridoma Product 3C11 (anti SYNORF1)) [31]. Finally, brain samples were washed
139 three times in 3% Triton X-100 solution in PBS for 10 min and incubated in a secondary
140 antibody anti-mouse produced in goat (Alexa Fluor® 568 antibody, [Product # A-11004](#))
141 at a 1:100 dilution in blocking solution for 24 hours and rinsed three times in PBS for 5
142 min and dehydrated in ethanol series (50%, 70%, 90%, 3x 100%) for 10 min and mounted
143 in glycerol. After the preparation the brains were visualized using an Olympus FV1000
144 Confocal microscope to obtain brain image stacks sampled every 10 µm, resulting in 21-
145 55 slices depending on the individual (with a mean and SD of 37.6±9.2). Volumetric
146 measurements and three-dimensional reconstructions were made by individually tracing
147 the different anatomical structures on each section image from the scope using
148 RECONSTRUCT software [32]. For each bee, we measured the calyces of the mushroom
149 bodies and the optic lobes (without the retina, which were previously removed during
150 dissection). Additionally, we also measured the combined volume of the rest of the brain,

151 which includes the basal peduncles of the mushroom bodies and the antennal lobes (**Fig.**
152 **1 a**). All data on volumetric measurements can be found in **Supplementary Data file 2.**

153

154 *Ecological and sociality traits*

155 We collected species data on diet specialization, sociality and life-history from published
156 sources [33–40] and complemented with searches in specialized websites on bee
157 taxonomy and biology such as BWARS (<https://www.bwars.com/>), Discover Life
158 (<https://www.discoverlife.org/>), and WildBienen (<http://www.wildbienen.de>). Sociality
159 was classified as eusocial for the species that live in colonies formed by individuals of
160 the same species, containing castes and division of labour; and *non-eusocial* for the
161 species who breed and live totally independently of other individuals, which also includes
162 species that might build the nest close to other individuals although they breed
163 independently (sometimes referred to as “communal nesting”). Three of the species could
164 not be unambiguously assigned to a particular category [35]: *Xylocopa virginica* and
165 *Ceratina calcarata* were classified as *non-eusocial*, as they do not show caste
166 differentiation, but they also have some behaviours typical of eusocial species like nest
167 sharing; *Lasioglossum calceatum*, on the other hand, was classified as eusocial although
168 it is socially plastic, being eusocial in some regions and solitary in others. Nevertheless,
169 lumping these species with either eusocial or non-eusocial species did not change the
170 conclusions. Diet specialization was considered as *oligolectic* when a species exclusively
171 uses one plant family to feed its brood, and *polylectic* when several plant families are
172 being used [41]. Finally, because brain size is associated with a slow life-history strategy
173 in vertebrates [20,42,43], we also included voltinism as a proxy for life-history strategy,
174 defined as *univoltine* in species that have a single generation within a year and
175 *multivoltine* for the species that have more than one generation per year. Species
176 exhibiting geographic variation in life history (i.e. being multivoltine in some regions but
177 not in others) were classified as multivoltine. All data on species traits can be found in
178 **Supplementary Data file 1.**

179

180 *Construction of the phylogenetic tree*

181 We constructed a phylogenetic tree for the species included in the study, to allow the
182 incorporation of species that are not present in other published phylogenies and that have
183 been sequenced for the purpose of the present study. First, we retrieved existing DNA

184 sequence information from GenBank. For the species that lacked molecular data in
185 GenBank, DNA sequences were generated *de novo* for one mitochondrial and two nuclear
186 markers, commonly used in phylogenetics of bees [44,45]: cytochrome c oxidase subunit
187 1 (*cox1*), long-wavelength rhodopsin (*LWRho*), and elongation factor-1 α (*EF-1 α*). In all
188 cases, total genomic DNA was isolated from thoracic muscle or leg tissue of bees
189 previously frozen and pinned (not older than 1–2 years) using the commercial DNeasy
190 Blood and Tissue Kit (Qiagen) following manufacturer’s instructions (More information
191 on the molecular markers and DNA sequencing protocols in **Supplementary methods**).
192 This resulted in 87 bee species with DNA sequence data available that were aligned using
193 MAFFT v7.310 using an automatic strategy [46]. Given that nuclear protein-coding genes
194 (*LWRho* and *EF-1 α*) included multiple intron sequences with poorly aligned regions, we
195 applied Gblocks [47] using relaxed settings [48] to remove such regions. Nucleotide
196 substitution models were estimated by means of PartitionFinder [49] with linked branch
197 lengths, a BIC selection criterion, and a greedy search algorithm. The best substitution
198 models for each partition were the following: GTR+I+G for *cox1*, HKY+I+G for *EF-1 α*
199 and K80+I+G for *LWRho*. The phylogenetic analysis was performed using MrBayes
200 v3.2.6 [50] with a clock rate prior fixed to “1”, therefore with time units defined as the
201 number of expected substitutions per site. Given that preliminary analyses showed some
202 discrepancies between our tree and the latest trees published using phylogenomic
203 approaches, we decided to constrain some of the phylogenetic relationships to make sure
204 that all genera and subgenera were monophyletic (The list of all constrained nodes is
205 available in the **Supplementary Data file 3**), and to make our trees compatible with
206 recent phylogenies based on massive amounts of sequence data [51–53]. The final
207 phylogenetic analysis relied on two independent Bayesian inference runs with four chains
208 each that ran for 25 million generations. After removing the first 10% of generations as
209 burn-in, all parameters sampled in both runs showed highly mixed traces (always with
210 effective sample sizes above 100) and converged into similar posterior estimates with
211 calculated potential scale reduction factors approaching to 1. Finally, 6 additional species
212 with no molecular data - but for which brain size data and ecology was available - were
213 randomly incorporated within their subgenus. Although there is phylogenetic uncertainty
214 for the placement of these species, we repeated this process in a sample of 100 trees from
215 the posterior distribution, which we used in all subsequent comparative analyses to take
216 this uncertainty into account (See **Figure S1 and Supplementary Data file 4**).

217

218 *Prospective phylogenetic analyses*

219 Phylogenetically-informed general linear models were used in all tests to cope with the
220 non-independence of species due to shared ancestry. The phylogenetic relationships
221 between species were accounted for using the lambda model estimation provided by the
222 “*phylolm*” function from the R-package *phylolm* [54]. All models were repeated for each
223 of the 100 phylogenetic trees and the reported results are the mean values along the 100
224 trees. R^2 was assessed using the ‘R2.resid’ function from R-package *rr2* [55], which
225 computes R^2 for models with auto-correlated errors (i.e. phylogenetic linear models).

226

227 We first validated the assumption that our measures of brain size capture changes in
228 functionally relevant brain regions, that is, the mushroom bodies and optical lobes. We
229 modelled the volume of both brain areas as a function of body size (measured as ITS) and
230 found that these areas increase with body size (**Supplementary Table 1**). We
231 consequently removed these allometric effects by extracting the residuals from a log-log
232 regression of each brain region against body size, using the ‘*phyl.resid*’ function from R-
233 package *phytools* [56]. We estimated relative brain size in a similar way, and used
234 *phylolm* to test whether variation in relative brain size could be predicted by variation in
235 the relative size of each brain region. We found that relative brain size is positively
236 associated with relative changes in the brain regions analyzed (**Fig. 1, Supplementary**
237 **Tables 2**).

238

239 Having confirmed that relative brain size is functionally meaningful, we then used
240 *phylolm* to test the ecological and social hypotheses proposed to account for brain size
241 evolution. We modelled variation in brain size (response variable) as a function of our
242 metrics of sociality, diet generalization, and voltinism (predictors). Body size (measured
243 as ITS) was included as a co-variate in the models to ensure that the effect of the
244 predictors is not confounded by allometric effects.

245

246 *Retrospective phylogenetic analyses*

247 After identifying voltinism and diet generalization as the most important predictors of
248 brain size variation, we used a retrospective approach to further infer their importance in
249 brain size evolution. First, we used a stochastic character mapping (SCM) approach to
250 reconstruct evolutionary transitions between different combinations of voltinism and diet

251 generalization, as implemented in the ‘simmap’ function from the R-package *phytools*
252 (Revell, 2012). This method estimates the location of evolutionary transitions between
253 categories on a phylogenetic tree by running simulations that fit the observed characters
254 at the tips. Character histories were inferred using the MCMC algorithm and fitting a
255 symmetric model for the transition probability matrix. To minimize the potential effects
256 of uncertainty in both tree topologies and phylogenetic reconstructions from the SCM,
257 we used the 100 phylogenies with 10 simulations for each one, resulting in 1000
258 phylogenetic reconstructions. To estimate the number of evolutionary transitions between
259 the different character states (i.e. levels of the trait), we used the ‘describe.simmap’
260 function over the 1000 trees and estimated mean and confidence interval for each possible
261 transition. The maximum clade credibility tree was used to plot the distribution of inferred
262 characters at each node.

263

264 Next, we addressed whether the amount of evolutionary change in brain size was related
265 to changes in selective regimes associated with evolutionary transitions in life history (i.e.
266 from *univoltine* to *multivoltine*, and vice-versa) or diet specialization (i.e. from *oligolectic*
267 to *polylectic*, and vice-versa). Specifically, we used the ancestral state reconstructions to
268 test whether species evolving to a particular ecology and life history were also selected
269 for a different optima in brain size [57]. To avoid allometric effects, we focused on
270 relative brain size estimated by means of the residuals approach previously described. To
271 assess whether relative brain size evolved towards distinct phenotypical optima, we fitted
272 an Ornstein-Uhlenbeck (OU) process emulating selective forces that pull the trait towards
273 an optimal value that is favored by natural selection. We considered two different OU
274 models [57] that include a single phenotypical optimum (OU1 model) or different optima
275 for each character state (OUM model), but a single rate of evolution of the trait. These
276 adaptive models were contrasted with two Brownian motion (BM) models with no
277 optima, but including either a single rate of phenotypic evolution (BM1 model) for all
278 character states or different rates of evolution for each character state (BMS model). A
279 random set of 100 stochastic character maps were analyzed using the R package ‘OUwie’
280 [58] to test which evolutionary model best explains the evolution of brain size under the
281 different character states. To assess the most supported model, we calculated the Akaike
282 weights for each model based on AICc scores [59]. Then, we performed paired t-tests in

283 the results of the 100 trees to assess whether optima and rate estimates were significantly
284 different among character states.

285

286

287 **Results**

288 Our analyses identify body size as the strongest predictor of brain size ($\beta = 2.050 \pm$
289 0.108 ; p -value < 0.001 ; $N = 93$; $R^2 = 0.89$; **Fig. 2a**). After controlling for allometric
290 effects, however, there is still substantial variation in brain size (**Fig. 2b**). Our analyses
291 identify two main factors that may account for such brain size variation. When
292 controlling for body size, we find that diet breadth ($\beta = 0.172 \pm 0.079$; p -value = 0.033)
293 and number of generations per year ($\beta = 0.148 \pm 0.072$; p -value = 0.042; $N=93$)
294 significantly predicts brain size (**Supplementary Table 3**). Thus, species that collect
295 pollen from a single family of plants (i.e. oligolectic species) have larger brains than
296 polylectic species (**Fig 2c**), and species with a single generation per the year (i.e.
297 univoltine) have larger brains than multivoltine species (**Fig 2d**). However, our results
298 do not support an effect of sociality on brain size ($\beta = 0.026 \pm 0.086$; p -value = 0.761,
299 **Fig 2e; Supplementary Table 3**). We also find an interaction between both factors:
300 oligolectic species are all univoltine and tend to have larger brains than the rest of
301 species, and polylectic-univoltine species have, in turn, larger brains than polylectic-
302 multivoltine species (**Fig. 2f, Supplementary Table 4**).

303

304 To further understand brain evolution in bees, we used stochastic character mapping to
305 reconstruct the three combinations of diet specialization and voltinism categories in the
306 bees phylogeny (**Fig. 3a**). We find that transitions to oligolecty occurred more frequently
307 from polylectic-univoltine species (Mean and SD: 8.0 ± 3.4) than from polylectic-
308 multivoltine species (3.2 ± 3.0). On the other hand, transitions between multivoltine and
309 univoltine life-histories are much more common than transitions from oligolectic to
310 polylectic foraging (**Fig. 3b**).

311

312 We finally ask whether and how these evolutionary transitions influenced brain size
313 evolution. By fitting different evolutionary models, we show that the model that better
314 describes changes in relative brain size is an adaptive process with multiple evolutionary
315 optima (OUM model; average difference in AICc with the second-best model $\delta = 8.21$,

316 **Supplementary Table 5**). The estimated optima corroborate that oligolectic species tend
317 to have relatively larger brains than polylectic species (**Fig. 3c**). Among the 100 tree
318 reconstructions, the optimum for relative brain size in oligolectic species is higher than
319 the optima for both polylectic univoltine species (paired *t*-test with mean difference of
320 0.23, *p*-value < 0.001) and polylectic multivoltine species (paired *t*-test with mean
321 difference of 0.36, *p*-value < 0.001). In addition, polylectic univoltine species have a
322 larger relative brain optimum than polylectic multivoltine species (paired *t*-test with mean
323 difference of 0.14, *p*-value < 0.001).

324

325

326 **Discussion**

327 By means of an enlarged comparative dataset of bee brain sizes, our results confirm
328 previous findings that brain size is allometrically correlated with body size in bumblebees
329 [60], and shows that body size as the main predictor of brain size may be a general rule
330 among animals [61]. Our results also contribute to clarify the biological meaning of brain
331 size variation in bees [60,62], showing that larger brains are the result of concerted
332 increases in mushroom bodies and optical lobes [62].

333

334 After removing the effect of body size, however, we still find substantial variation in brain
335 size (and brain areas), with some bee species having brains consistently larger than
336 expected by their body size while others having brains that are smaller than expected.
337 When looking at which factors might select for variation in relative brain size, we found
338 that variation in relative brain size is primarily explained by ecological and life history
339 pressures rather than by the social context.

340

341 In vertebrates, the social brain hypothesis suggests that sociality is a main selective
342 pressure for the evolution of larger brains [13]. This hypothesis remains however
343 controversial because while some studies found that social species have relatively bigger
344 brains [13,14,63], others reported no relationship between brain size and measures of
345 sociality [64–66]. These contrasting results may in part arise because variation in sociality
346 can also be achieved by altering receptor expression with little to no effect of brain size,
347 as shown in microtine voles [67] and estrildid finches [68]. However, another possibility
348 is that sociality is a wide concept, involving several aspects (i.e. group size, social-

349 bonding, or social learning) that could differently select for brain size. Our work on a
350 wide array of bee species allowed us to test whether a specific aspect of the social
351 spectrum—the division of labour in eusocial species—can explain brain size variation.
352 Unlike previous work in wasps [15,16], our results do not support an effect of sociality
353 on brain size, suggesting an independent evolution of eusocial life and brain increase in
354 bees.

355

356 Rather than sociality, we find that brain size variation in bees is better explained by
357 differences in foraging ecology, supporting previous claims that the ecology of animals
358 have great influence on brain size evolution [66,69,70]. Theory suggests that generalist
359 foragers should need a wider range of behavioural skills (and hence a larger brain) to find
360 and extract resources [71], an idea that receives strong support in birds [72,73]. In insects,
361 the scarce evidence available so far also shows that generalist-feeding beetles have more
362 complex mushroom bodies [21]. Instead, our analyses suggest that species with narrower
363 floral preferences (i.e. oligolectic species) have relatively larger brains than more
364 generalist species.

365

366 Why then do more specialized bees have bigger brains? This may have to do with the
367 cognitive challenges faced by bee species looking for pollen [74]. As central place
368 foragers, bees rely on finding suitable resources and remembering their location in the
369 landscape [75,76]. Floral characteristics are frequently used by bees to discriminate the
370 most rewarding resources [77], and may thus have been key in shaping the evolution of
371 cognitive adaptations [78]. As oligolectic species use a narrower spectrum of all floral
372 resources available in an area, they might have increased benefits for enhanced cognition
373 (i.e. navigation skills) to discriminate and remember their target resources. Indeed, there
374 is evidence that oligolectic bees are more efficient at exploiting flower resources and
375 moving between flowers [79], but it is still an open question whether they have better
376 learning abilities or memory. Nevertheless, even generalist bees always forage in flowers
377 and despite variation in flower morphology they do not display a wide variety of foraging
378 techniques [80,81]. Instead, in vertebrates a typical generalist species can use resources
379 as different as seeds, fruits and insects, which require completely different handling
380 techniques [73]. Thus, it may be that the theory that generalist foragers need larger brains
381 is less relevant in bees than in vertebrates and other species groups. Finally, other aspects
382 of the niche, like habitat use breadth [82], might influence brain size evolution in bees.

383 Therefore, incorporating such additional traits into future macroevolutionary analysis
384 might help to fully understand the relationship between niche breadth and brain size in
385 bees.

386

387 The possession of a relatively large brain may not only confer cognitive benefits but also
388 entails costs as well. In vertebrates, a short developmental period is expected to constrain
389 the evolution of large brains [83–85]. In bees, species with single generations (i.e.
390 univoltine species) might have short flying periods, but have a higher latency to reproduce
391 (i.e. a longer time for development) than multivoltine species [86]. In line with this, we
392 find that multivoltine species have relatively smaller brains than species with single
393 generations, suggesting that life history can also constraint the evolution of brain size in
394 insects.

395

396 With the objective of better understanding how ecological and life-history traits influence
397 brain size evolution, we reconstructed the evolutionary history of diet specialization and
398 voltinism and fitted a variety of evolutionary models to describe subsequent changes in
399 the evolution of the brain. The strength of this analysis, in comparison to phylogenetic
400 regressions, is that it takes evolutionary time into account and fit phenotypic evolution as
401 a function of the time spent in each selective regime (i.e. the ecological condition that
402 select for a particular phenotype). Here we found more support for an OU model of
403 evolution, where each ecological category selects for a different brain size optimum rather
404 than promoting different evolutionary rates of phenotypic evolution (i.e. BM model). Our
405 results also show that transitions to oligolectic foraging have evolved several times across
406 the phylogeny of bees and that these changes have involved an increase in relative brain
407 size. In addition, transitions between multivoltine and univoltine species occur repeatedly
408 across the phylogenetic tree, involving a decrease in the relative brain size optima for
409 multivoltine species.

410

411 Although we explored several main hypotheses frequently studied in brain size evolution
412 (i.e., ecology [65,69,73], sociality [13,14,63,64,66] and life-history [20,42,43,84]), other
413 factors might also be relevant in the evolution of the insect brain. For instance, across the
414 Hymenoptera, parasitoidism (i.e. laying eggs in or on a host species and larvae, causing
415 their death) has been suggested to be a precursor for the evolution of elaborate cognition
416 [87]. Although we did not have any parasitoid species represented in our sample, we did

417 have two kleptoparasitic species that lay the eggs in another insects' nest to steal their
418 provision. The sample size is too small to draw conclusions, yet we note that the two
419 species have contrasting brain sizes, with *Nomada* having one of the largest relative brain
420 sizes whereas *Stelis* has one of the smallest. These differences suggest that either
421 divergent parasitic strategies might select for contrasting brain architectures or that other
422 factors are behind such differences. Although there is evidence that this species might
423 exhibit slightly distinct parasitic strategies [88], we need data on additional parasitic
424 lineages, such as those on the genus *Sphcodes*, to distinguish between these
425 explanations.

426

427 Our study represents one of the first attempts to use a phylogenetic comparative
428 framework to test the brain size evolution hypothesis in bees. For our set of 93 bee
429 species, sociality was unable to explain the observed variation in brain size. Instead,
430 specialization in foraging breadth, together with single annual generations is associated
431 with relatively larger brains. These findings not only reveal several evolutionary pressures
432 that shaped brain size evolution in bees, but also challenge the generality of important
433 hypotheses for brain size evolution that have been developed from a vertebrate
434 perspective.

435

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442

443 **Competing interests**

444 The authors declare no competing interests.

445

446 **Data availability**

447 All the datasets generated during this research can be found in Dryad
448 (doi:10.5061/dryad.3xsj3txd9). Newly determined DNA sequences have been deposited
449 in GenBank under accession numbers MN917434-MN917452, MN928991-MN929009,
450 and MN938935-MN938953.

451

452 **Author contributions**

453 FS, IB, and DS designed the study. FS, MAC, MAS, JG, IR and IB collected the data.

454 AA and DSM generated new DNA sequences and JGP built the phylogenetic trees. FS

455 analysed the data. FS wrote the paper, with input from all authors.

456

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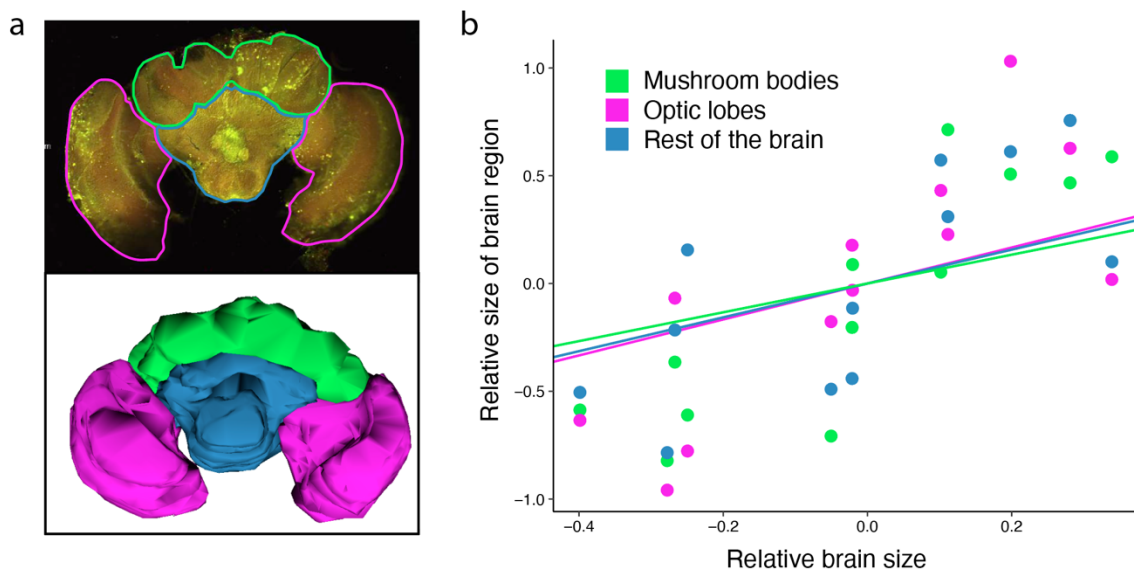
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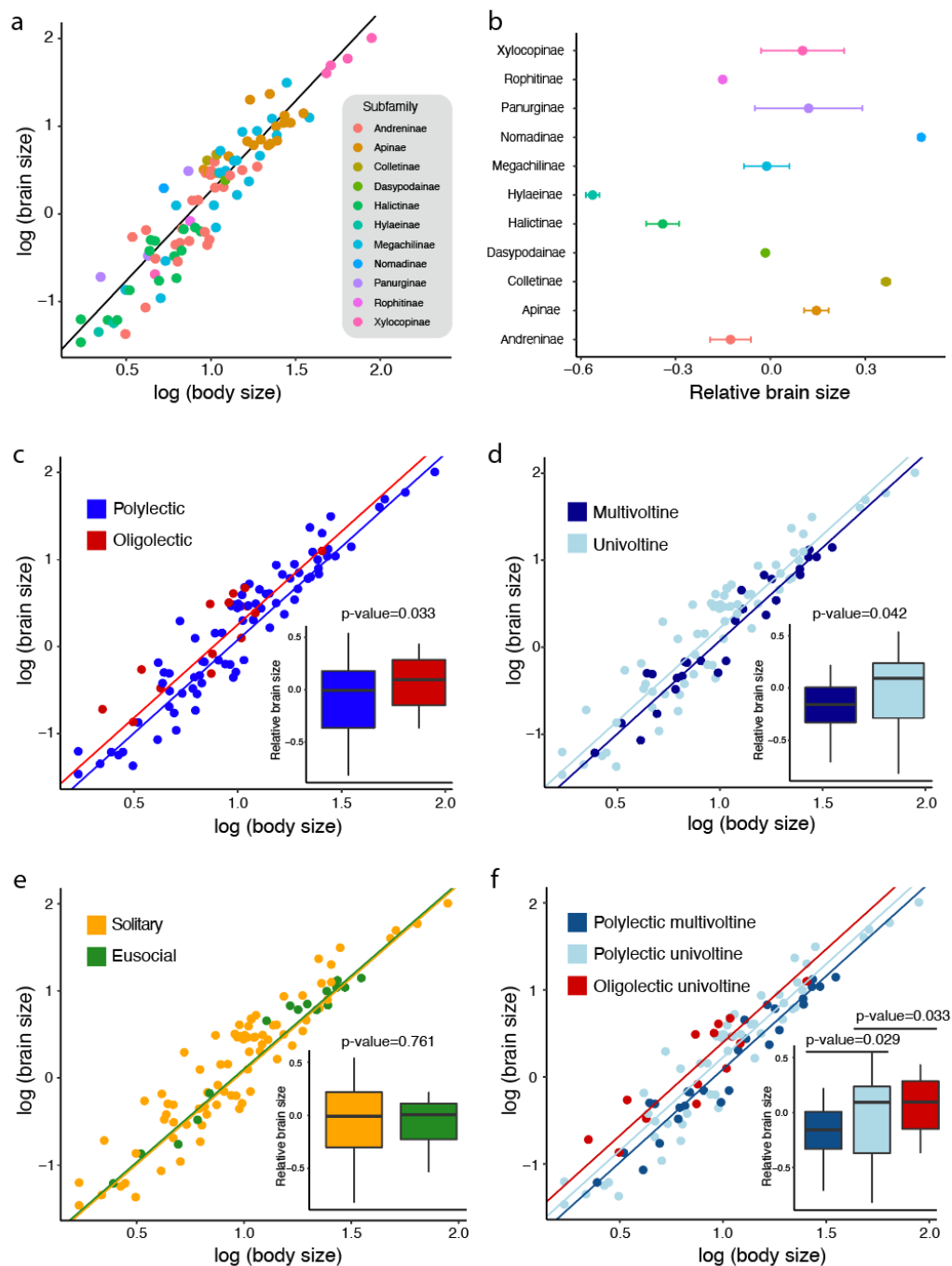
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698 **Figure 1. Relation between the relative size of the brain and its main regions. (a)** An
699 example of a brain image from the confocal microscope from a specimen of *Megachile*
700 *mucida* is shown, where mushroom bodies, optic lobes and the rest of the brain were
701 traced in slices and reconstructed in a 3D model. (b) The relation between the relative
702 size of the different brain regions in relation to the relative brain size is shown (N=12
703 species).
704
705



706 **Figure 2. The effect of feeding specialization and voltinism on relative brain size.**
 707 The relation between body and brain size is shown for 93 bee species from different
 708 subfamilies (a), with some groups having consistently larger or smaller brains than
 709 expected by their body size, represented as residuals from the log-log regression between
 710 brain and body size (b). Differences in relative brain size due to ecology, life-history and
 711 sociality are also shown (c-f). Both diet generalization (c) and voltinism (d) have an effect
 712 on relative brain size, whereas sociality does not (e). A categorical trait resulting from the
 713 combination between diet generalization and voltinism (note that not all combinations
 714 exist) also shows the same result (f). The scatterplot shows the difference in absolute brain
 715 size intercept between oligolectic and polylectic bees, whereas the boxplot shows the
 716 differences in relative brain size (residuals from body size).



717 **Figure 3. Ancestral reconstructions of ecological and life history strategies and the**
 718 **evolution of brain size.** We reconstructed different trait categories representing different
 719 combinations of ecological and life history strategies: Oligolectic species (red), polylectic
 720 multivoltine species (dark blue) and polylectic univoltine species (light blue). A summary
 721 of the estimated probability of ancestral states at each node along 1000 reconstructions is
 722 shown (a), as well as the average number of transitions (mean and 95% C.I.) between
 723 trait categories (b). These reconstructions were used to model how the character states
 724 influence the evolution of relative brain size. The estimated relative brain size optima for
 725 each trait category are shown for the best evolutionary model (OUM) along 100 trees,
 726 using boxplots showing 2.5, 25, 50, 75 and 97.5 percentiles, as well as the whole
 727 distribution of parameter estimates (c).

