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Cite this article: Sayol F *et al*. 2020 Feeding specialization and longer generation time are associated with relatively larger brains in bees. *Proc. R. Soc. B* **287**: 20200762.
http://dx.doi.org/10.1098/rspb.2020.0762

Received: 6 April 2020
Accepted: 25 August 2020

Subject Category:
Evolution

Subject Areas:
evolution, cognition

Keywords:
brain evolution, Apoidea, voltinism, lecticity

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Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.5110107.

THE ROYAL SOCIETY PUBLISHING

Feeding specialization and longer generation time are associated with relatively larger brains in bees

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

1. Introduction

Despite Darwin's claim that 'the brain of an ant is one of the most marvellous atoms of matter in the world' [1], the brain of insects has traditionally been considered too small and simple compared, for instance, to those of vertebrates to provide insight into the evolution of complex brains. Carl von Linné even erroneously pointed to the absence of a real brain as the main characteristic of insects [2]. Only recently it has been broadly recognized that, as in vertebrates, there is great anatomical variation in size and complexity in the brain of

other animals, from the 302 neurons of a nematode brain to the complex multi-ganglia brain of an octopus. Evidence has also accumulated that many animal groups, including some molluscs and insects, have evolved integrative processing centres in their brains [3,4].

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

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

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

2. Methods

(a) Brain and body size measurements

We measured brain size as the weight of fixed brains. For this purpose, we collected 385 female specimens from 93 species in various locations from the United States of America, Spain, and the Netherlands. These species represent most major lineages of bees. Because brain size might change plastically between experienced and naïve individuals [25,26], we collected individuals that were foraging in flowers to make sure they had foraging experience. We cut off the heads of each individual collected using a scalpel and stored it in fixative (4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) pH = 7.4). We then extracted the brain from the head capsule on a Petri dish using Dumont #5 forceps, removed the retina from the optic lobes and cleaned the entire brain of all tracheae and fat bodies. Following [27], each brain was then placed on a small piece of Parafilm®. PBS drops were wiped away using finely twisted pieces of Kimwipes® and the brain was weighed within 4 s after the removal of the liquid. In all the specimens, body size was measured as the intertegular span (ITS). ITS is the distance between the two tegulae, small sclerites above the insertion points of the wings, and it is a standard measure of body size in bees because it accurately correlates with body weight [28]. For ITS, measurements were done using a stereomicroscope (magnification 16 x –80 x) with a calibrated ocular micrometre (resolution down to 0.02 mm).

(b) Volumetric measurements of brain components

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

(c) Ecological and sociality traits

We collected species data on diet specialization, sociality, and life history from published sources [33–40], complemented with searches in specialized websites on bee taxonomy and biology such as BWARS (https://www.bwars.com/), Discover Life

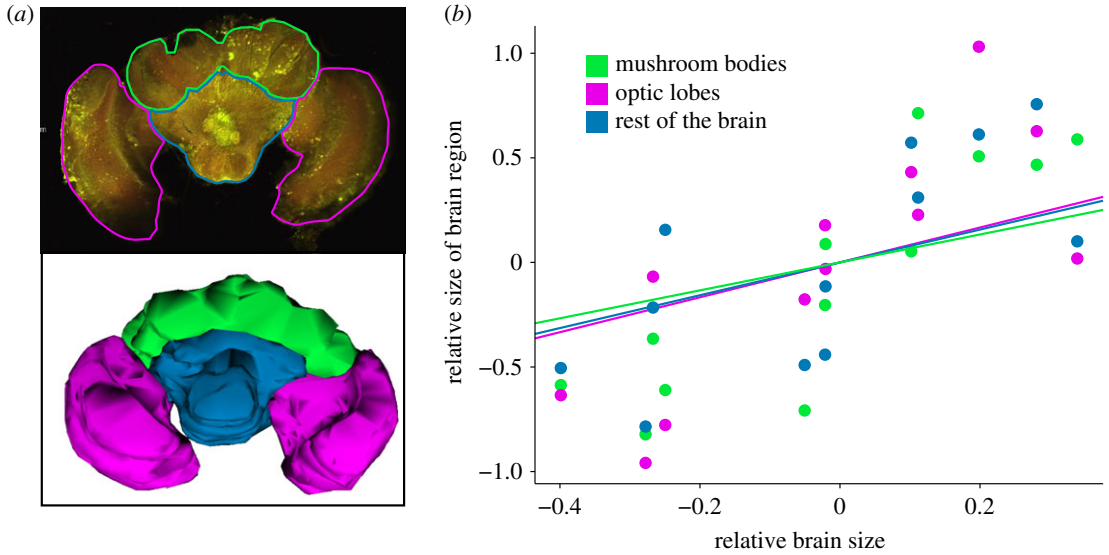

Figure 1. Relation between the relative size of the brain and its main regions. (*a*) An example of a brain image from the confocal microscope from a specimen of *Megachile mucida*, where mushroom bodies, optic lobes, and the rest of the brain were traced in slices and reconstructed in a three-dimensional model. (*b*) The relation between the relative size of the different brain regions in relation to the relative brain size is shown ($N = 12$ species). (Online version in colour.)

(https://www.discoverlife.org/), and WildBienen (http://www.wildbienen.de). Sociality was classified as *eusocial* for the species that live in colonies formed by individuals of the same species, containing castes and division of labour, and *non-eusocial* for the species who breed and live totally independently of other individuals, which also includes species that can build the nest close to other individuals although they breed independently (sometimes referred to as 'communal nesting'). Three of the species could not be unambiguously assigned to a particular category [35]: *Xylocopa virginica* and *Ceratina calcarata* were classified as *non-eusocial*, as they do not show caste differentiation, but they also have some behaviours typical of eusocial species like nest sharing; *Lasioglossum calceatum*, on the other hand, was classified as *eusocial* although it is socially plastic, being eusocial in some regions and solitary in others. Nevertheless, lumping these species with either *eusocial* or *non-eusocial* species did not change the conclusions. Diet specialization was considered as *oligolectic* when a species exclusively uses one plant family to feed its brood, and *polylectic* when several plant families are being used [41]. Finally, because brain size is associated with a slow life-history strategy in vertebrates [20,42,43], we also included voltinism as a proxy for life-history strategy, defined as *univoltine* in species that have a single generation within a year and *multivoltine* for the species that have more than one generation per year. Species exhibiting geographic variation in life history (i.e. being multivoltine in some regions but not in others) were classified as multivoltine.

(d) Construction of the phylogenetic tree

We constructed a phylogenetic tree for the species included in the study, to allow the incorporation of species that are not present in other published phylogenies and that have been sequenced for the purpose of the present study. First, we retrieved existing DNA sequence information from GenBank. For the species that lacked molecular data in GenBank, DNA sequences were generated *de novo* for one mitochondrial and two nuclear markers, commonly used in phylogenetics of bees [44,45]: cytochrome c oxidase subunit 1 (*cox1*), long-wavelength rhodopsin (*LWRho*), and elongation factor-1α (*EF-1α*). In all cases, total genomic DNA was isolated from thoracic muscle or leg tissue of bees previously frozen and pinned (not older than 1–2 years) using the commercial DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions (more information on the molecular markers and DNA sequencing protocols in electronic supplementary material, methods). This resulted in 87 bee species with DNA sequence data available that were aligned using MAFFT v7.310 using an automatic strategy [46]. Given that nuclear protein-coding genes (*LWRho* and *EF-1α*) included multiple intron sequences with poorly aligned regions, we applied Gblocks [47] using relaxed settings [48] to remove such regions. Nucleotide substitution models were estimated by means of PartitionFinder [49] with linked branch lengths, a BIC selection criterion, and a greedy search algorithm. The best substitution models for each partition were the following: GTR + I + G for *cox1*, HKY + I + G for *EF-1a*, and K80 + I + G for *LWRho*. The phylogenetic analysis was performed using MrBayes v.3.2.6 [50] with a clock rate prior fixed to '1', therefore with time units defined as the number of expected substitutions per site. Given that preliminary analyses showed some discrepancies between our tree and the latest trees published using phylogenomic approaches, we decided to constrain some of the phylogenetic relationships to make sure that all genera and subgenera were monophyletic and to make our trees compatible with recent phylogenies based on massive amounts of sequence data [51–53]. The final phylogenetic analysis relied on two independent Bayesian inference runs with four chains each that ran for 25 million generations. After removing the first 10% of generations as burn-in, all parameters sampled in both runs showed highly mixed traces (always with effective sample sizes above 100) and converged into similar posterior estimates with calculated potential scale reduction factors approaching to 1. Finally, six additional species with no molecular data—but for which brain size data and ecology were available—were randomly incorporated within their subgenus. Although there is phylogenetic uncertainty for the placement of these species, we repeated this process in a sample of 100 trees from the posterior distribution, which we used in all subsequent comparative analyses to take this uncertainty into account (see electronic supplementary material, figure S1).

(e) Prospective phylogenetic analyses

Phylogenetically informed general linear models were used in all tests to cope with the non-independence of species due to shared ancestry. The phylogenetic relationships between species were accounted for using the lambda model estimation provided by the 'phylolm' function from the R-package *phylolm* [54]. All

models were repeated for each of the 100 phylogenetic trees and the reported results are the mean values along with the 100 trees. R^2 was assessed using the 'R2.resid' function from R-package *rr2* [55], which computes R^2 for models with auto-correlated errors (i.e. phylogenetic linear models).

We first validated the assumption that our measures of brain size capture changes in functionally relevant brain regions, that is, the mushroom bodies and optical lobes. We modelled the volume of both brain areas as a function of body size (measured as ITS) and found that these areas increase with body size (electronic supplementary material, table S1). We consequently removed these allometric effects by extracting the residuals from a log-log regression of each brain region against body size, using the 'phyl.resid' function form R-package *phytools* [56]. We estimated relative brain size in a similar way and used *phylolm* to test whether variation in relative brain size could be predicted by variation in the relative size of each brain region. We found that relative brain size is positively associated with relative changes in the brain regions analysed (figure 1, electronic supplementary material, tables S2).

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

(f) Retrospective phylogenetic analyses

After identifying voltinism and diet generalization as the most important predictors of brain size variation, we used a retrospective approach to further infer their importance in brain size evolution. First, we used a stochastic character mapping (SCM) approach to reconstruct evolutionary transitions between different combinations of voltinism and diet generalization, as implemented in the 'simmap' function from the R-package *phytools* (Revell, [56]). This method estimates the location of evolutionary transitions between categories on a phylogenetic tree by running simulations that fit the observed characters at the tips. Character histories were inferred using the Markov chain Monte Carlo (MCMC) algorithm and fitting a symmetric model for the transition probability matrix. To minimize the potential effects of uncertainty in both tree topologies and phylogenetic reconstructions from the SCM, we used the 100 phylogenies with 10 simulations for each one, resulting in 1000 phylogenetic reconstructions. To estimate the number of evolutionary transitions between the different character states (i.e. levels of the trait), we used the 'describe.simmap' function over the 1000 trees and estimated mean and confidence interval for each possible transition. The maximum clade credibility tree was used to plot the distribution of inferred characters at each node.

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

3. Results

Our analyses identified body size as the strongest predictor of brain size ($\beta = 2.050 \pm 0.108$; p-value < 0.001; $N = 93$; $R^2 = 0.89$; figure 2*a*). After controlling for allometric effects, however, there is still substantial variation in brain size (figure 2*b*). Our analyses identified two main factors that may account for such brain size variation. When controlling for body size, we found that diet breadth ($\beta = 0.172 \pm 0.079$; p-value = 0.033) and the number of generations per year ($\beta = 0.148 \pm 0.072$; p-value = 0.042; N=93) significantly predicted brain size (electronic supplementary material, table S3). Thus, species that collect pollen from a single family of plants (i.e. oligolectic species) have larger brains than polylectic species (figure 2*c*), and species with a single generation per year (i.e. univoltine) have larger brains than multivoltine species (figure 2*d*). However, our results do not support an effect of sociality on brain size ($\beta = 0.026 \pm 0.086$; p-value = 0.761, figure 2*e*; electronic supplementary material, table S3). We also found an interaction between both factors: oligolectic species are all univoltine and tend to have larger brains than the rest of the species, and polylectic-univoltine species have, in turn, larger brains than polylectic-mutivoltine species (figure 2*f*, electronic supplementary material, table S4).

To further understand brain evolution in bees, we used stochastic character mapping to reconstruct the three combinations of diet specialization and voltinism categories in the bees phylogeny (figure 3*a*). We found that transitions to oligolecty occurred more frequently from polylectic-univoltine species (mean and s.d.: 8.0 ± 3.4) than from polylectic-multivoltine species (3.2 ± 3.0). On the other hand, transitions between multivoltine and univoltine life histories are much more common than transitions from oligolectic to polylectic foraging (figure 3*b*).

We found evidence that these evolutionary transitions influenced brain size evolution. By fitting different evolutionary models, we show that the model that better describes changes in relative brain size is an adaptive process with multiple evolutionary optima (OUM model; the average difference in AICc with the second-best model $\delta = 8.21$, electronic supplementary material, table S5). The estimated optima corroborate that oligolectic species tend to have relatively larger brains than polylectic species (figure 3*c*). Among the 100 tree reconstructions, the optimum for relative brain size in oligolectic species is higher than the optima for both polylectic-univoltine species (paired *t*-test with a mean difference of 0.23, p-value < 0.001) and polylectic-multivoltine species (paired *t*-test with a mean difference of 0.36, p-value < 0.001).

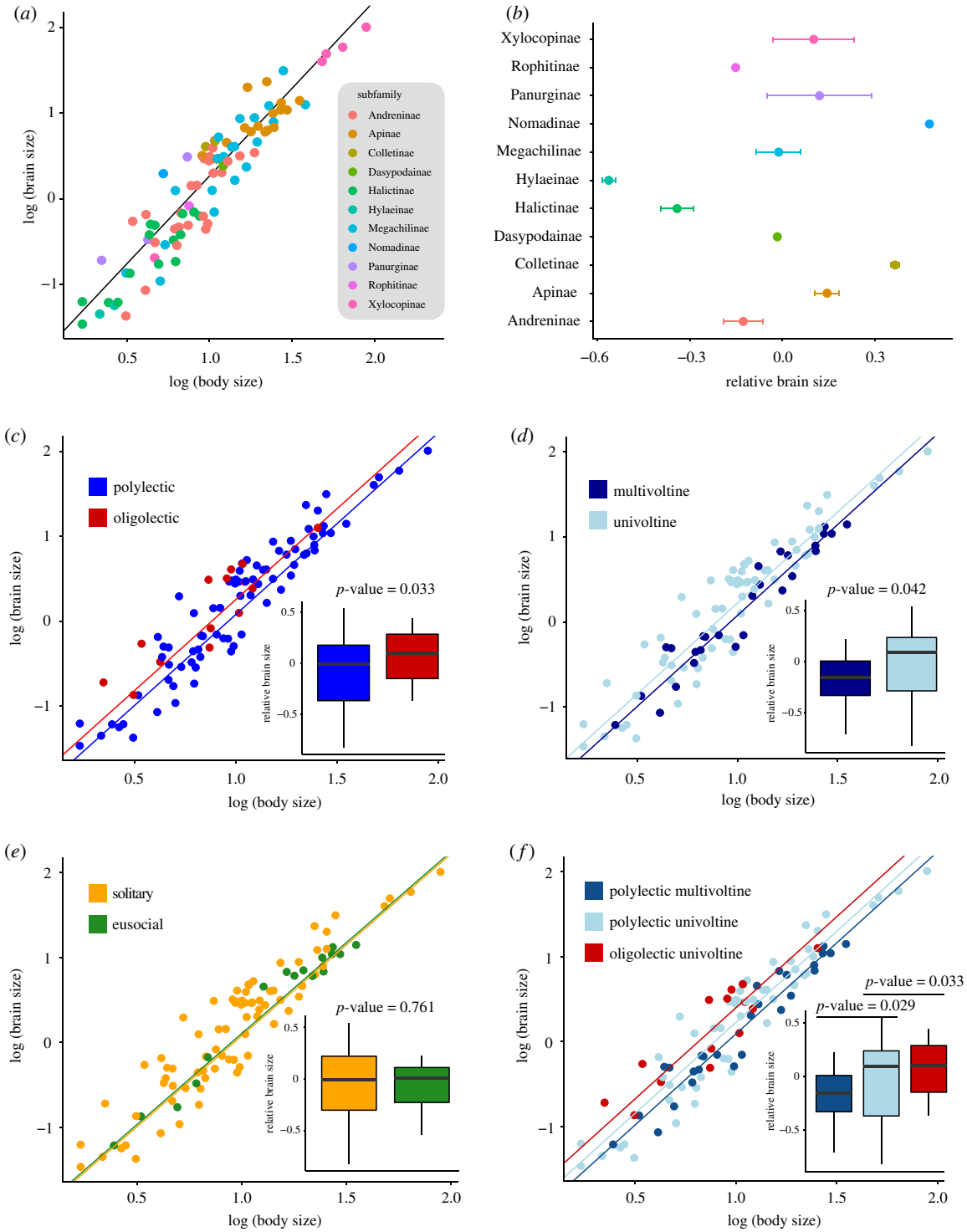

Figure 2. The effect of feeding specialization and voltinism on relative brain size. The relation between body and brain size is shown for 93 bee species from different subfamilies (*a*), with some groups having consistently larger or smaller brains than expected by their body size, represented as residuals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

In addition, polylectic-univoltine species have a larger relative brain optimum than polylectic-multivoltine species (paired *t*-test with a mean difference of 0.14, p-value < 0.001).

4. Discussion

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

After removing the effect of body size, however, we still find substantial variation in brain size (and brain areas), with some

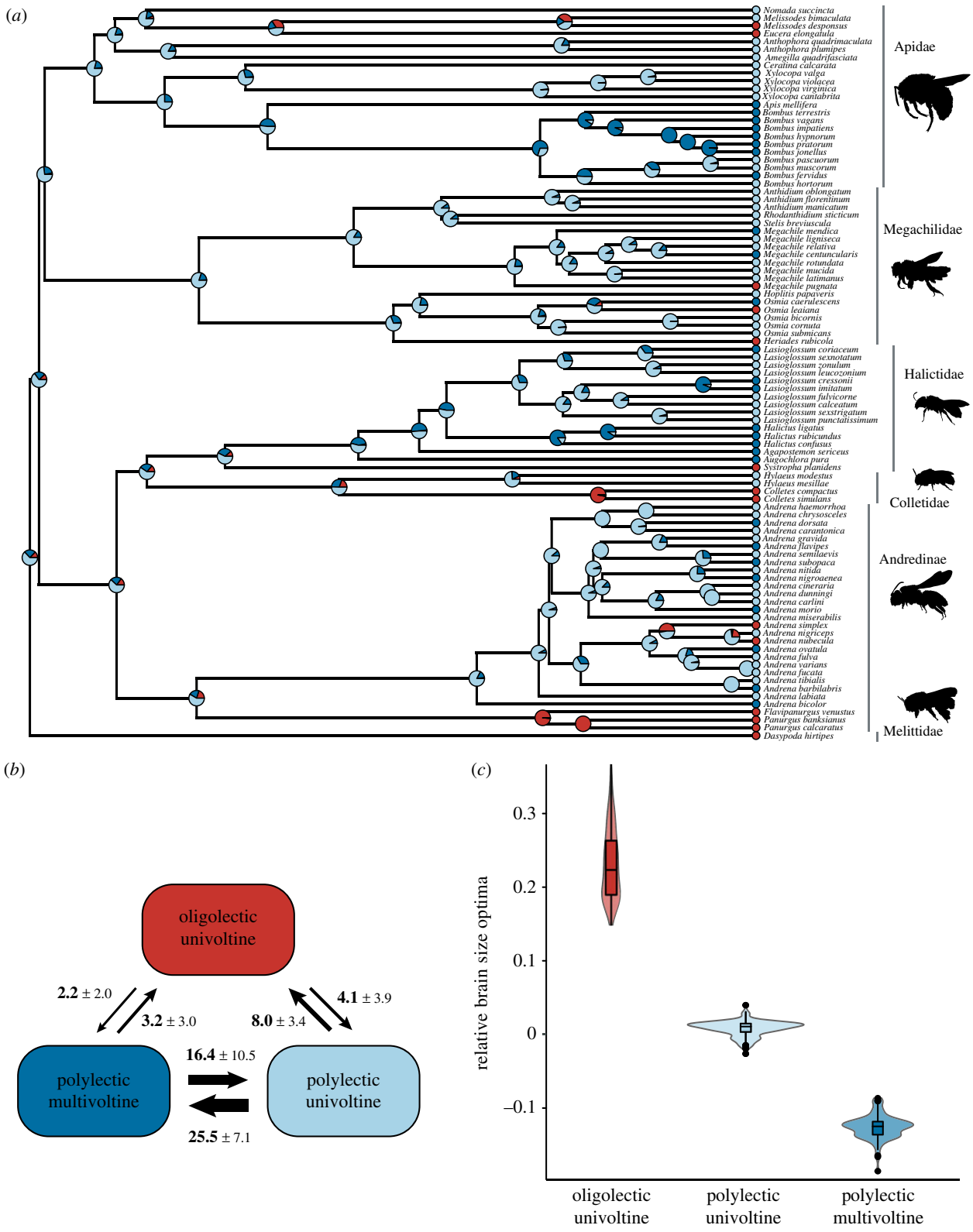

Figure 3. Ancestral reconstructions of ecological and life-history strategies and the evolution of brain size. We reconstructed different trait categories representing different combinations of ecological and life-history strategies: oligolectic species (red), polylectic-multivoltine species (dark blue), and polylectic-univoltine species (light blue). A summary of the estimated probability of ancestral states at each node along 1000 reconstructions is shown (*a*), as well as the average number of transitions (mean and 95% CI) between trait categories (*b*). These reconstructions were used to model how the character states influence the evolution of relative brain size. The estimated relative brain size optima for each trait category are shown for the best evolutionary model (OUM) along 100 trees, using boxplots showing 2.5, 25, 50, 75, and 97.5 percentiles, as well as the whole distribution of parameter estimates (*c*). Bee silhouettes are available at http://phylopic.org/. (Online version in colour.)

bee species having brains consistently larger than expected by their body size while others have brains that are smaller than expected. When looking at which factors might select for variation in relative brain size, we found that variation in relative brain size is primarily explained by ecological and life-history pressures rather than by the social context.

The social brain hypothesis suggests that social interactions are the main selective pressure for the evolution of

larger brains [13]. This hypothesis remains, however, controversial because while some studies found that social species have relatively bigger brains [13,14,63], others reported no relationship between brain size and measures of sociality [64–66]. These contrasting results may, in part, arise because variation in sociality can also be achieved by altering receptor expression with little to no effect of brain size, as shown in microtine voles [67] and estrildid finches [68]. However, another possibility is that sociality is a wide concept, involving several aspects (i.e. group size, social-bonding, or social learning) that could differently select for brain size. Our work on a wide array of bee species allowed us to test whether a specific aspect of the social spectrum—the division of labour in eusocial species—can explain brain size variation. Unlike previous work in wasps [15,16], our results do not support and effect of sociality on brain size, suggesting an independent evolution of eusocial life and brain increase in bees.

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

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

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

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

Although we explored several main hypotheses frequently studied in brain size evolution (i.e. ecology [65,69,73], sociality [13,14,63,64,66] and life history [20,42,43,84]), other factors might also be relevant in the evolution of the insect brain. For instance, across the Hymenoptera, parasitoidism (i.e. laying eggs in or on a host species and larvae, causing their death) has been suggested to be a precursor for the evolution of elaborate cognition [87]. Although we did not have any parasitoid species represented in our sample, we did have two kleptoparasitic species that lay the eggs in another insects' nest to steal their provision. The sample size is too small to draw conclusions, yet we note that the two species have contrasting brain sizes, with *Nomada* having one of the largest relative brain sizes whereas *Stelis* has one of the smallest. These differences suggest that either divergent parasitic strategies might select for contrasting brain architectures or that other factors are behind such differences. Although there is evidence that *Nomada* and *Stelis* exhibit slightly distinct parasitic strategies [88], we need data on additional parasitic lineages, such as those on the genus *Sphecodes*, to distinguish between the above explanations.

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

Data accessibility. All the datasets on species traits, list of constrained nodes, and distribution of phylogenetic trees are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.3xsj3txd9 [89]. Newly determined DNA sequences have been deposited in GenBank under accession numbers MN917434–MN917452, MN928991–MN929009, and MN938935–MN938953.

Authors' contributions. F.S., I.B., and D.S. designed the study. F.S., M.A.C., M.A.S., J.G., I.R., and I.B. collected the data. A.A. and D.S.M. generated new DNA sequences and J.G.P. built the phylogenetic trees. F.S. analysed the data. F.S. wrote the paper, with input from all authors.

Competing interests. We declare we have no competing interests.

Acknowledgements. This work was supported by funds from the Ministry of Science, Innovation and Universities—Spanish government (CGL2013-47448-P and CGL2017-90033-P) to D.S. and I.B. F.S. was supported by funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska–Curie grant agreement no. 838998.

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