A neural correlate of learning fails to predict foraging efficiency in the bumble bee *Bombus terrestris*

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Mushroom bodies (MB) are integrative structures in the insect brain that, in social bees, contribute to both visual and olfactory learning. Changes in the density of presynaptic boutons (or microglomeruli) within the calyx region of the MB have been linked to various aspects of foraging, including forms of learning that are believed to be key in supporting foraging efficiency. Here, we directly tested the relationship between foraging efficiency and microglomerulus density in a bumble bee model, *Bombus terrestris*. We found no evidence for microglomerulus density predicting real-world foraging performance, nor any relationship with foraging experience. Instead, our data suggest a potential nonlinear relationship between an individual's age, which is independent of foraging experience, and microglomerulus density in the lip region of the calyx, which is associated with olfactory processing. Our findings suggest that in real-world scenarios there is no simple direct relationship between microglomerulus density, learning ability and foraging efficiency in bumble bees, highlighting the knowledge gap regarding the relationships between learning abilities, neuroanatomy and foraging efficiency.

The workers of social bees face the challenge of collecting many small nectar and pollen rewards across a relatively vast foraging range. The ability to learn and remember floral characteristics that predict reward, alongside the locations at which rewarding patches have been found and current reward levels available within them, is believed to be integral to the efficient fulfilment of this task (Chittka & Thomson, 2001; Klein et al., 2017). Studies have shown that the microstructure of the mushroom bodies (MB), which are integrative neural structures that are associated with learning and memory abilities, is plastic and reflects aspects of engagement in foraging tasks (Cabirol et al., 2018; Durst et al., 1994; Groh et al., 2012; Ismail et al., 2006; Muenz et al., 2015; Scholl et al., 2014; Withers et al., 1993).

Intrinsic neurons in the MB, the Kenyon cells, connect to dendrites of sensory neurons to form synaptic boutons also known as microglomeruli (MG; Groh & Rössler, 2011). Given that

neurogenesis does not take place in the MB of adult insects (Fahrbach et al., 1995), these structures have been the focus of studies linking MB structural variation to learning performance in bees (Hourcade et al., 2010; Li et al., 2017; Van Nest et al., 2017). Each MG is a synaptic complex that contains a central cholinergic synaptic bouton projecting from the antennal or optic lobes, surrounded by smaller GABAergic or octopaminergic boutons (Frambach et al., 2004).

In honey bees, *Apis mellifera*, where workers exhibit temporal polytheism, the onset of foraging coincides with a decrease in the density of MG in both the collar and lip regions of the MB, which house projections from the optic and olfactory lobes, respectively (Groh et al., 2012). This pruning of projection neuron boutons is not age dependent but is triggered by foraging itself through exposure to light (Scholl et al., 2014). It is accompanied by an increase in Kenyon cell dendrites as well as the overall volume of the MB (Farris et al., 2001; Withers et al., 1993) and has been hypothesized to prime the brain for learning about floral rewards (Cabirol et al., 2018; Farris et al., 2001; Withers et al., 1993). Computational modelling indeed suggests that sparser coding of information within the MB is optimal for updating learned information,

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allowing for the formation of new associations to guide decisions and thus maximize foraging efficiency (Cabirol et al., 2018). MG density subsequently increases again as bees learn about their environment and accumulate foraging experience (Cabirol et al., 2018). Accordingly, learning events such as formation of an associative memory result in an increase in MG density in honey bees (Hourcade et al., 2010) and potentially also in bumble bees, *Bombus* spp. (Li et al., 2017).

These findings and related studies in ants (Stieb et al., 2010) suggest that MB plasticity in social insects may respond to both the need for and the process of learning and memory retrieval, to support efficient foraging (Cabirol et al., 2018; Fahrbach & Van Nest, 2016). Indeed, both longer- and shorter-term memory performance (as captured through laboratory assays) have been found to correlate with foraging efficiency in bumble bees that forage in the real world, although these effects can be specific to particular environments (Pull et al., 2022; Raine & Chittka, 2008). However, no study has yet explored whether this relationship might be reflected in neural structure.

Here, we directly tested the relationship between foraging efficiency, foraging experience and MG density in the bumble bee, *Bombus terrestris audax*. Unlike honey bees, bumble bees do not exhibit age-based polyethism and, accordingly, MG density reduction occurs earlier in the life cycle than it does in honey bees, in preparation for the commencement of foraging within 2−3 days of emergence from the pupal stage (Kraft et al., 2019). We hypothesize that when sampled mid-life span, MG density may be greater in those bees that have engaged in more foraging trips and in those that foraged more efficiently.

METHODS

Overview

Following a staggered design (Fig. 1), bees of known age with no previous foraging experience were fitted with radiofrequency identification (RFID) chips and allowed to forage freely for 8 days (ca. 40% of foraging life span) using a hole-in-the-wall set-up (Evans et al., 2017; Pull et al., 2022; Raine & Chittka, 2008). All bees originated from laboratory-reared, commercially acquired colonies raised under identical conditions. Foraging efficiency was recorded for each individual as mass of nectar collected/min over multiple recorded foraging trips before quantification of MG density through synapsin-based immunostaining of the lip and collar regions.

Growth Phase

Within each colony, we created a cohort of tagged and RFID-chipped bees of known age and that had never foraged before. Six *B. terrestris audax* colonies in the very early phase of worker number expansion (ca. 1 week following initial worker emergence) were obtained from a commercial supplier (Agralan, Swindon, U.K.). Upon receiving a colony, we standardized the colony size to

20 workers, which we tagged by supergluing (cyanoacrylate, Loctite) plastic numbered discs on the dorsal thorax to identify them as having emerged prearrival. The queen and brood were left undisturbed. Individual age could only be defined for bees that emerged after receipt of the colony from the supplier. To maximize the number of individuals of known age, the colony was allowed to grow in size in the laboratory for 2 weeks. During this growth phase (Fig. 1), the focal colony was kept in a dark room at 24 °C with sugar solution (35% w/w) provided ad libitum and pollen added three times per week. Thrice a week, newly emerged bees were individually tagged (as above) under red light (which bees cannot see) with an RFID chip and a plastic numbered disc on the dorsal part of their thorax (the RFID chip was placed underneath the numbered disc).

Ethical Note

The experiment described here followed the ASAB/ABS Guidelines for the use of animals in research and did not require any licence or permits in the U.K. The colony boxes provided a dark environment, which aimed to replicate the underground conditions preferred by *B. terrestris* colonies in nature. The tags glued on the thorax of the bees did not prevent normal flight behaviour. The colonies were euthanized by freezing them after the experiment was completed.

Foraging Phase

During this phase, tagged and chipped bees were provided with unlimited outdoor access to accumulate foraging experience. We removed access to the sugar solution from colonies 2 days before the end of the growth phase, ensuring that colonies were motivated to forage while still having sufficient stores to avoid starvation. On the first day of the foraging phase, colonies were rehoused under red light into a clean nestbox made of grey Perspex (28 × 16 cm and 10.5 cm high) that opened to a clear Perspex tunnel fitted onto a precision scale (Ohaus Advanced Portable Balance Scout STX; accuracy ± 2 mg) and a directional RFID reader system (MAJA Bundle Bee Identification System iID2000, ISO15693 optimized, Micro-Sensys GmbH, Erfurt, Germany). The 10 cm section of the tunnel that passed over the scale had a false bottom so that bees ran directly across the pan of the scale. This section of the tunnel could be closed on both ends, such that once a focal individual entered this section of the tunnel, they could be trapped to record their weight using the weight averaging function (averaging period = 2 s). In each case, we repeated the measurement three times before releasing the bee. The tunnel was, in turn, connected to a clear plastic tube that gave access to the outdoors through a hole cut in the window of the laboratory. During the foraging phase, the colony was allowed to forage freely on and around our university campus (Egham, Surrey, U.K.), which comprises mixed woodland, parkland and planted ornamental gardens. The campus and private gardens in the surrounding area provided flowering plants to the colonies
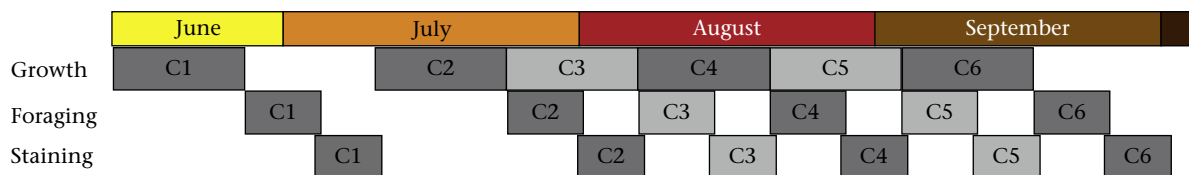

Figure 1. Timeline of the experiment. Each colony (C1−6, *N* = 6 colonies) went through a growth phase, during which the workers that were later sampled emerged and were tagged. At the end of the foraging period, foraging bees were sampled and their brain tissues were processed following a standard immunostaining protocol. The time gap between C1 and C2 is due to a colony producing only three workers by the end of the growth phase, which prompted its removal from the study.

throughout the experiment and no additional sugar solution was required to feed the colonies during this phase. Pollen was provided ad libitum throughout to preclude pollen foraging, which typically takes much longer than nectar foraging and is likely subject to different environmental constraints (e.g. humidity, which affects clumping).

Colonies were monitored by a single observer for 6 h (between 0815 and 1630 hours GMT) on 5 different days of the foraging phase, during which time the weight on exit and entry of all foraging workers was recorded (mean of three measurements using the scale's 2 s weight averaging function taken during each tunnel crossing, that is every entry/exit event, which minimizes noise generated by the bees' movement), alongside the time taken for each foraging trip. Foraging efficiency was calculated for each foraging trip as the weight difference between exit and entry, divided by trip duration. We discounted any trips that lasted less than 5 min to exclude trips that involved solely removal of detritus, aborted trips with/without defecation or orientation flights ($N = 17/1339$ trips).

Brain Fixation and Dissection

At the end of the foraging phase, all foragers were sampled upon returning from a foraging trip. The subjects were chilled on ice for 10 min and decapitated using dissecting scissors. The heads were pinned on a dissection plate and submerged in 0.1 M HEPES buffered saline (HBS). We cut a large square window in the front of the head capsule and removed the air sacs around the brain that were accessible through the window, thus exposing the frontal surface of the brain. The heads were then transferred into ice-cold 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS; pH = 7.4) and fixed overnight at 4 °C on an orbital shaker, washed in HBS twice for 5 min, pinned again on a dissection plate and finally submerged in HBS. The compound eyes, ocelli and the remaining air sac membranes at the back of the brain were removed. Finally, the last remaining anchor points of the brain in the head capsule were severed by an incision under the antennal lobes. The free-floating brains were then washed in 0.1 M PBS twice for 5 min at room temperature on an orbital shaker.

Cutting Frozen Sections

The fixed brains were cryoprotected using a graded series of sucrose (10%, 20% and 30%) in 0.1 M PB with 0.005% sodium azide (NaN_3). Each step of the graded series lasted 1 h at room temperature, ensuring that the brains had sunk to the bottom of the vial. The brains were stored in 30% sucrose/phosphate buffer (PB) at 4 °C for 2 days and then embedded in aqueous 20% gelatine solution in stainless steel moulds, oriented in the embedding medium with the frontal side facing down. The moulds were placed on dry ice until the gelatine was uniformly frozen. The gelatine block was fitted to a 'chuck' and left in the cryostat chamber for 15 min to give it time to equilibrate its temperature with the chamber. Sections were cut at −18 °C to a thickness of 30 μm, arranged on Superfrost Plus Adhesion Slides (Thermo Fisher, Waltham, MA, U.S.A.) and stored overnight at 4 °C to let them thaw and dry.

Antibody Incubation and Mounting

The slides were placed on a hot plate for approximately 5 s to melt the gelatine before being rehydrated for 10 min in 0.1 M PB in a Coplin jar. All subsequent washes were done in 0.1 M PBS with 0.2% Triton X-100 (PBSTx). Slides were rinsed in PBSTx and preincubated with 5% normal goat serum in PBSTx (NGS/ PBSTx) for 45 min. Slide edges were framed with a hydrophobic pen (Advanced PAP Pen, Sigma-Aldrich, St Louis, MI, U.S.A.) around the edges, covered with 200 μl of antisynapsin primary antibody 1:50 in NGS/PBSTx (3C11 monoclonal mouse anti-SYNORF1, Klagges et al., 1996; Developmental Studies Hybridoma Bank, University of Iowa, U.S.A.), for 2.5 h in a moisture chamber.

The slides were briefly rinsed in PBSTx, washed four times in PBSTx for 10 min and then incubated with Cy3-conjugated affinity-purified goat antimouse IgG (H+L) polyclonal antibody (1:100; ThermoFisher, cat. no. A10521) and Alexa 488-conjugated phalloidin (1:200; ThermoFisher, cat. no. A12379) in NGS/ PBSTx. Incubation was as for the primary antibody but protected from direct light with an aluminium foil cover. After 1.5 h, slides were rinsed in PBSTx before being washed four times in PBSTx for 10 min and mounted in 90% glycerol/PB containing 3% n-propyl gallate as antifading agent.

Confocal Microscopy and Microglomerulus Density Measurement

Stacks of confocal images of the MB were captured on an Olympus FV-10 laser scanning confocal microscope (Olympus Corporation, Tokyo, Japan) with a 60× oil immersion objective (Olympus UPLSAPO 60XO, NA = 1.35) and z-spacing of 0.41 μm. For each subject, we chose four contiguous physical 30 μm sections. We randomly assigned one of the four calices to each physical section so that each of the left lateral, left medial, right medial and right lateral calyx were scanned once per individual. The stacks were analysed with the software ImageJ (Bourne, 2010). We used a random offset grid to place three cubes (8.2 × 8.2 × 8.2 μm) in the dense collar region and three cubes in the lip region of the calyx (Fig. 2). We then manually counted the presynaptic boutons contained in the cubes to quantify their density. In summary, for each bee, we calculated MG density by inspecting ca. 100 stacked images per cube, across 12 cubes that spanned the calices.
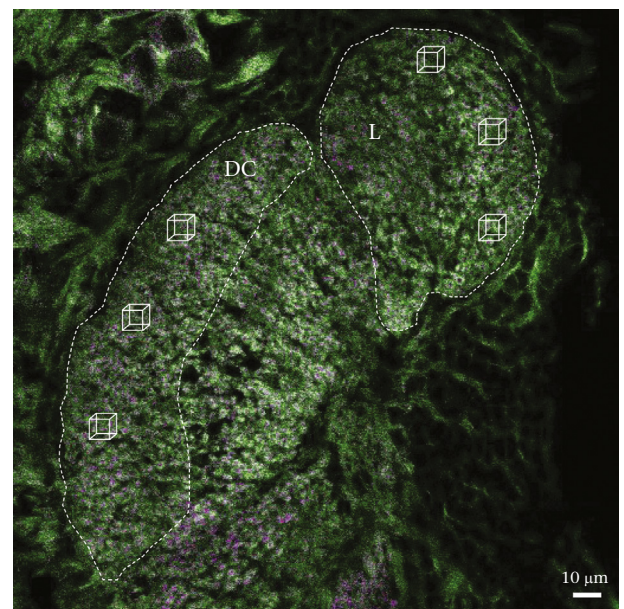

Figure 2. Single optical section of a cryosection of the mushroom body calyx of *Bombus terrestris* stained with antisynapsin antibody (magenta) and f-actin (green). Dashed lines delineate the two subregions of the calyx analysed (DC: dense collar; L: lip). Three cubes, each 8.2 × 8.2 × 8.2 μm, were randomly placed in the DC and L regions to quantify microglomerulus density.

Statistical Analysis

Data were analysed using the lme4 and mgcv R-packages (Bates et al., 2014; Wood, 2017) in R version 4.1.2 (R Core Team, 2021). We performed two main analyses (see below) and within each we analysed MG density in the collar and the lip region separately.

Over the course of the experiment, we recorded foraging efficiency for 2396 foraging trips made by 169 workers. Since not all bees survived until the end of the experiment, we obtained MG density estimates for 65 of these bees, which contributed 1339 foraging trips. During data exploration, we removed one very young bee that was detected as an outlier because it had emerged during (rather than prior to) the foraging period. Consequently, 64 bees contributed to the analysis. We also inspected the RFID data set (using an R script) to remove any instances where a bee passed only one of the two RFID readers, indicating that it did not continue to enter/exit the colony, but instead turned around.

We first explored whether MG density in our sampled bees was predicted by age, size or foraging experience (total time spent outside of the nest during the foraging phase, based on RFID readings). Data exploration revealed a potential nonlinear effect of age on MG density for measurements from the lip region but not from the collar region. We thus used a generalized additive mixed model (GAMM) to analyse the data for the lip region and a linear mixed model (LMM) for the collar region. In both cases, the response variable was log transformed to reduce the skew of the data, continuous predictors were scaled and 'Colony' was included as a random intercept. We established the importance of each predictor (fixed factor) by removing it from the full model and evaluating the change in Akaike information criterion (AIC) relative to the full model (Burnham & Anderson, 2002). A predictor was retained if the full model showed improved fit compared to the simpler model (ΔAIC > 2). Interactions between predictors were not included in the model to avoid overparameterization considering the sample size. Finally, data exploration revealed a positive correlation between MG density in the collar and the lip region ($r =$ 0.62). We chose to analyse the two regions separately because the differences in their function are well documented (Fahrbach & Van Nest, 2016).

We then tested the a priori hypothesis that MG density predicts foraging efficiency. Because we expected foraging efficiency to increase with foraging experience (Pull et al., 2022) and had repeated measures of foraging efficiency for each bee, we created an initial LMM with foraging efficiency (mg/min) as the response variable. The fixed effects were: (1) age on sampling, (2) size, (3) Julian date of initial release and (4) foraging experience (number of foraging trips performed so far). Because each bee performed multiple foraging bouts, bee identity was included as a random intercept, nested within colony. Foraging efficiency ($N = 64$) was transformed to improve model fit using ordered quantile normalization with the BestNormalize function (Peterson & Cavanaugh, 2019). We then tested whether adding MG density (collar or lip) improved the model, based on the change in AIC value. As above, we assessed the importance of a predictor based on the change in AIC value achieved by adding (MG density) or removing (all other predictors) fixed factors from the model.

RESULTS

Predicting MG Density

For the collar region of the MB, we found that neither age, size, total foraging experience nor Julian date of release predicted MG density. In each case, the full model (which contained all predictors) did not perform significantly better than simpler models that excluded the single predictors (ΔAIC compared to full model <2 in all cases, Table 1). For the lip region, we identified a nonlinear relationship between age at sampling and MG density (Fig. 3a). A nonlinear GAMM thus provided a better fit to the data than a linear model (ΔAIC = 39.11) and the model containing all predictors

Table 1
Response variable: microglomerulus density in the collar region

Variable removed	ΔAIC
Age sampled	−1.26
Julian date released	−1.95
Mass	−1.99
Total time out of nest	−1.99

Model type: LMER. Full model: log(Density in collar) ~ Age sampled + Julian date released + Mass + Total time out of nest + (1|Colony). All continuous variables are scaled. AIC: Akaike information criterion.

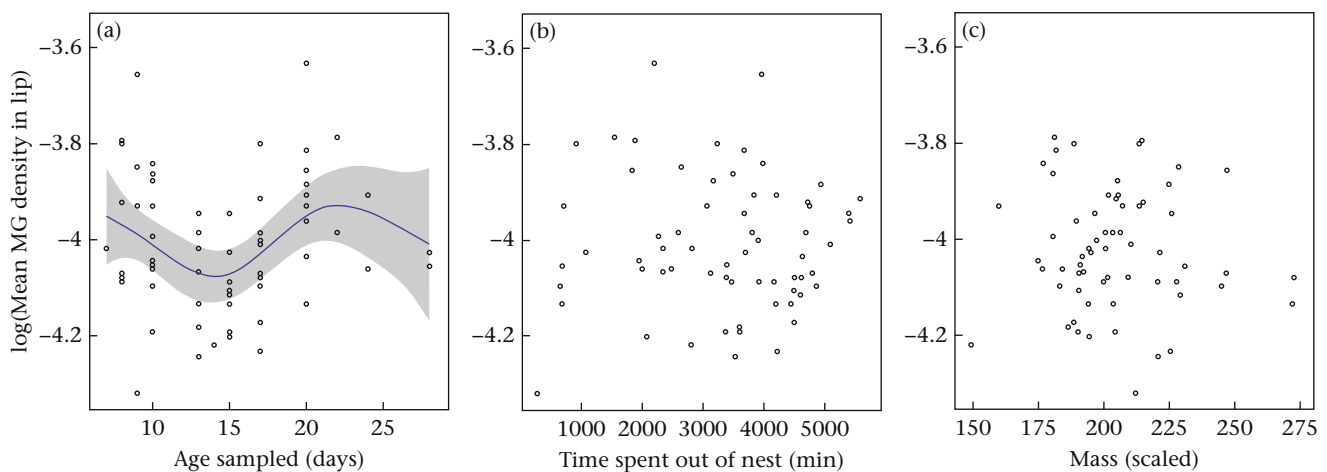**Figure 3.** Exploratory analysis of microglomerulus (MG) density (MG/μm^3) in the lip region of the mushroom body calyx of *Bombus terrestris* workers that had experienced 1 week of foraging in the wild, immediately before sampling, as a function of (a) age at sampling, (b) the amount of time that a forager had spent outside the nest (as measured by RFID readings) and (c) body mass. Blue line with 95% confidence interval, CI (grey band) in (a) indicates a nonlinear relationship between age at sampling and MG density in the lip region.

performed better than a simpler but otherwise identical model that did not contain age (ΔAIC = 5.39). For all the other predictors, the full model showed no improvement over simpler alternatives that did not contain the predictor of interest (ΔAIC < 2 in all cases; Table 2).

Predicting Foraging Efficiency

We found no evidence to support our a priori hypothesis that bees with higher sampled MG density may forage more efficiently, since adding MG density to a model containing all other predictors did not improve fit (Δ < 2 for both lip and collar, Fig. 4a and b). Individual foraging efficiency increased as bees became more experienced foragers: the full model performed better than a simpler alternative where 'number of foraging trips performed so far' was removed (ΔAIC = 124.606, Fig. 4c). Age at release, body size and date of release had no effect on foraging efficiency (ΔAIC < 2 in all cases, Table 3).

DISCUSSION

Our results did not reveal any association between MG density and foraging efficiency in bumble bees. Previous work has shown that MG density in the MB changes in response to learning events (Hourcade et al., 2010; Li et al., 2017), predicts learning ability (Cabirol et al., 2018; Li et al., 2017) and coincides with the onset of foraging (Kraft et al., 2019; Muenz et al., 2015). However, our results

suggest that these changes, if they are related to learning ability or the experience of learning, do not produce an effect large enough to be detected among potential sources of noise within the confines of our protocol. Instead, we found that the number of foraging trips performed prior to a focal trip was the only predictor of foraging efficiency within our test cohort. In other words, in line with previous findings (Pull et al., 2022), foraging efficiency improved with experience as bees learned about their environment.

Our analysis identified a potential nonlinear effect of age on MG density in the lip region of the MB, whereby MG density decreased until ca. 15 days of age, but returned to similar levels in older bees. Previous work also suggests that MG density is high on emergence and decreases quickly (Kraft et al., 2019), although note that our study did not include very young bees at an equivalent stage, because all sampled individuals had foraged. We were cautious in any interpretation because this nonlinear relationship was identified post hoc, and the pattern should be further explored through an inference-based approach (Tredennick et al., 2021). MG absolute number (but not always density) in honey bees follows a similar pattern to some extent (Cabirol et al., 2018), but MB plasticity in honey bees is to some extent driven by the onset of foraging rather than ageing itself (Ismail et al., 2006); age-based polyethism means that the two variables are correlated in this species. In bumble bees, we found no evidence that foraging experience, which was independent of age in our study, predicted MG density. The findings from our study and others (Pull et al., 2022) suggest that MG density may be fairly robust to the influence of exposure to the visual and olfactory cues of the real-world foraging environment in bumble bees.

Our experimental set-up allowed us to directly test the relationship between foraging efficiency, as the fitness-relevant potential product of learning, and MG density. We hypothesized that if MG density either responds to (Hourcade et al., 2010; Li et al., 2017) or promotes (Cabirol et al., 2018; Fahrbach & Van Nest, 2016; Li et al., 2017) learning about the environment, variation in MG density might predict variation in foraging efficiency, but this was not the case. However, a recent study by Pull et al. (2022) that used an experimental set-up and location almost identical to ours found that the relationship between a cognitive ability (specifically a short-term form of memory, as assayed through a radial arm maze) and foraging efficiency is not straightforward, and can vary across the year. We tested our bees in the height of the U.K. summer, when

Table 2
Response variable: microglomerulus density in the lip region

Variable removed	ΔAIC
S (age sampled)	+5.39
Julian date released	−0.35
Mass	−1.44
Total time out of nest	−1.59

Model type: GAMM. Full model: log(Density in collar) ~ s(Age sampled)* + Julian date released + Mass + Total time out of nest + s(Colony, type = 're')**. All continuous variables are scaled. *s() = modelled as a nonlinear smoother. ** s(type = 're') indicates modelled as a random effect within the GAMM framework. AIC: Akaike information criterion.
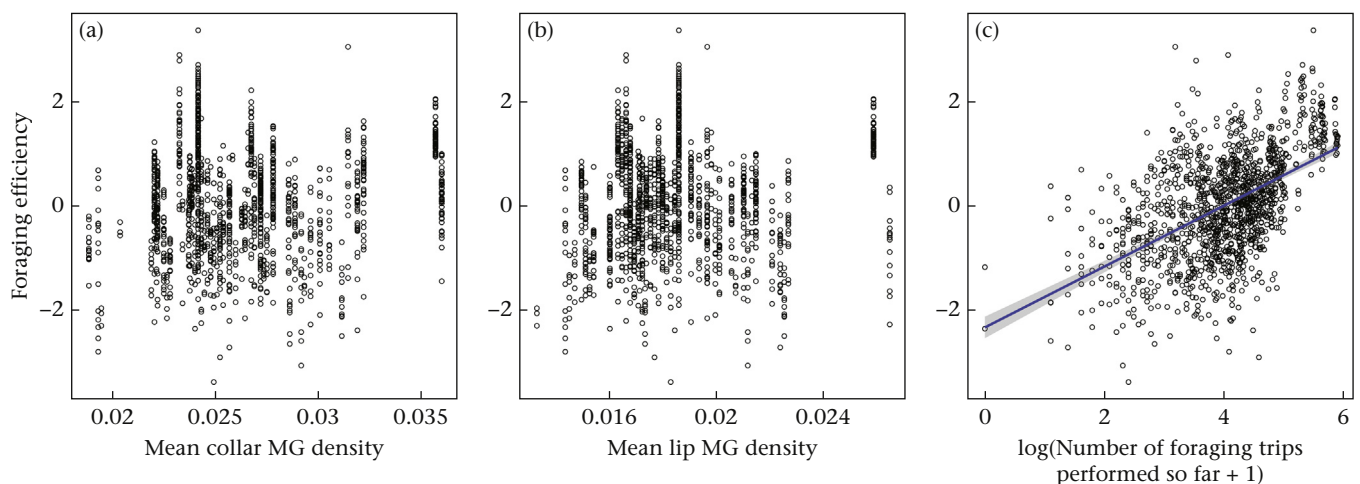

Figure 4. Foraging efficiency (transformed using the function BestNormalize) of 64 workers, over multiple trips per bee, in relation to (a) mean microglomerulus (MG) density (MG/μm^3) in the dense collar region; (b) mean MG density (MG/μm^3) in the lip region; (c) foraging experience. The blue line with 95% confidence interval (grey band) in (c) indicates the linear relationship between number of foraging trips performed so far (log transformed) and foraging efficiency.

Table 3
Response variable: foraging efficiency

Variable	Removed or added?	ΔAIC
Age	Removed	−1.758
Julian date released	Removed	−1.812
Mass	Removed	−1.91
Number of foraging trips performed so far	Removed	+124.606
Microglomerulus density (collar)	Added	+1.52
Microglomerulus density (lip)	Added	+ 0.322

Model: Foraging efficiency* ~ scaled(Age) + scaled(Julian date released) + scaled(Mass) + log(Number of foraging trips performed so far + 1) + (1|Colony/ ID). * transformed using ordered quantile normalization with the BestNormalize function Peterson & Cavanaugh, 2019). AIC: Akaike information criterion.

food availability is low (Pull et al., 2022) and performance in an associative learning task has been previously shown to predict foraging efficiency, at least at the colony level (Raine & Chittka, 2008). However, future studies could fruitfully explore how the relationship between learning ability and foraging efficiency changes depending on ecological conditions, and particularly whether MG density varies between bees exposed to complex, rich spring environments compared with those foraging in the summer dearth.

Another explanation for our results could be that cognitive abilities play little role in foraging under harsher ecological conditions where food is scarce and exploratory activity would be of greater importance (Pasquier & Grüter, 2016). Fidelity to a route, which requires learning, decreases the likelihood of discovering a new, more profitable food source by chance, and might conceivably be detrimental in a poor and changing environment, although this has not been tested. Furthermore, a recent study failed to find neural correlates between MB extrinsic neurons' activity and exploratory behaviour in bumble bees (Jin et al., 2020) which suggest exploratory behaviour is unlikely to produce the repetitive stimulation necessary to cause an increase in synaptic density. Nevertheless, exploration is expected to lead to substantial energetic costs that colonies might find harder to balance in a poorer environment. Similarly, increased cognitive abilities also come at a cost and have been shown to trade off with survival (Mery & Kawecki, 2005). Further research is therefore needed to determine the trade-offs involved with exploratory active alone and in comparison with those of cognitive abilities. Overall, our experiment highlights the gaps in knowledge on foraging and its relationship with cognition and neuroanatomy.

Author Contributions

Christopher D. Pull: Writing — review & editing, Writing — original draft, Methodology. **Ellouise Leadbeater:** Writing — review & editing, Writing — original draft, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Grégoire Pasquier:** Writing — review & editing, Writing — original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Swidbert R. Ott:** Writing — review & editing, Writing — original draft, Methodology.

Data Availability

The data set used for this study is publicly available at https:// doi.org/10.5281/zenodo.13952787.

Declaration of interest

None.

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