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Author for correspondence:
Richard J. Gill
e-mail: r.gill@imperial.ac.uk

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Insecticide exposure during brood or early-adult development reduces brain growth and impairs adult learning in bumblebees

Dylan B. Smith[1], Andres N. Arce[1], Ana Ramos Rodrigues[1], Philipp H. Bischoff[1], Daisy Burris[1], Farah Ahmed[2] and Richard J. Gill[1]

[1]Department of Life Sciences, Imperial College London, Silwood Park, Buckhurst Road, Ascot, Berkshire SL5 7PY, UK
[2]Core Research Laboratories, Natural History Museum, Cromwell Road, London SW7 5BD, UK

ANA, 0000-0002-3577-2110; RJG, 0000-0001-9389-1284

For social bees, an understudied step in evaluating pesticide risk is how contaminated food entering colonies affects residing offspring development and maturation. For instance, neurotoxic insecticide compounds in food could affect central nervous system development predisposing individuals to become poorer task performers later-in-life. Studying bumblebee colonies provisioned with neonicotinoid spiked nectar substitute, we measured brain volume and learning behaviour of 3 or 12-day old adults that had experienced in-hive exposure during brood and/or early-stage adult development. Micro-computed tomography scanning and segmentation of multiple brain neuropils showed exposure during either of the developmental stages caused reduced mushroom body calycal growth relative to unexposed workers. Associated with this was a lower probability of responding to a sucrose reward and lower learning performance in an olfactory conditioning test. While calycal volume of control workers positively correlated with learning score, this relationship was absent for exposed workers indicating neuropil functional impairment. Comparison of 3- and 12-day adults exposed during brood development showed a similar degree of reduced calycal volume and impaired behaviour highlighting lasting and irrecoverable effects from exposure despite no adult exposure. Our findings help explain how the onset of pesticide exposure to whole colonies can lead to lag-effects on growth and resultant dysfunction.

1. Introduction

Insect pollinator declines are of worldwide concern [1,2], and safeguarding this important functional group requires a deep understanding of the driving factors [3,4]. Social bees are important insect pollinators, and the threat posed by pesticide exposure is a widespread issue [5,6]. A growing number of studies have highlighted how foragers directly exposed to insecticide compounds can lead to sublethal effects on behaviour (e.g. [7–9]) with possible knock-on effects to colony function [10–12]. However, with insecticide residues detected inside colonies across the globe [13–18], we know less as to how pesticide-contaminated pollen and nectar brought back by foragers [19–24] place developing individuals being reared and residing inside colonies at risk [25–27]. To date, many studies chronically exposing whole colonies to certain insecticides have reported reductions in colony growth to manifest multiple weeks after onset of exposure [11,24,28–31]. A possible mechanistic explanation for this 'lag-effect' is that in-hive exposure is affecting the physiological development of brood and early-stage adults (a.k.a. callows—a cohort representing the future generation of the colony's workforce), predisposing these individuals to exhibit

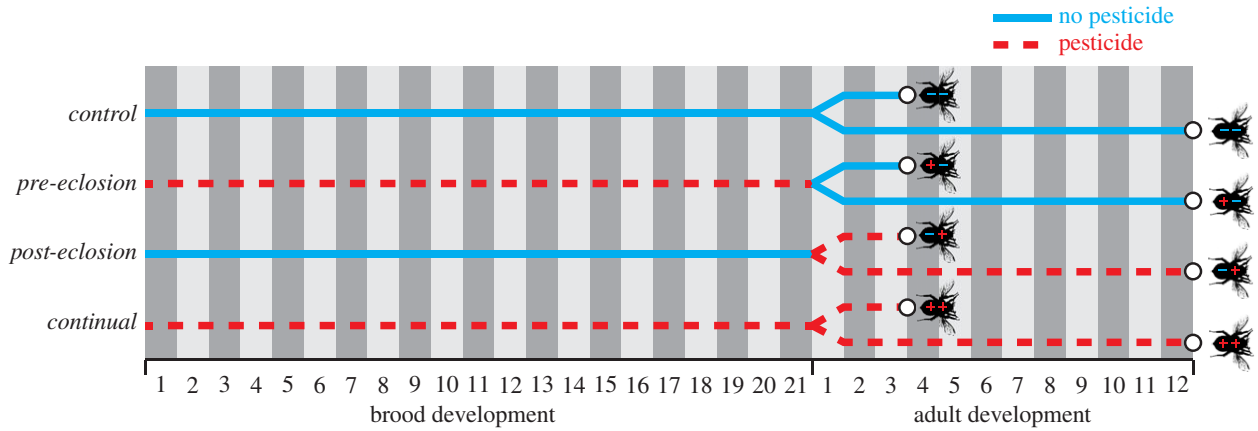

Figure 1. Graphic showing the developmental and exposure periods of individuals inside colonies for the four colony treatments (*control*, *pre-eclosion*, *post-eclosion* and *continual*) and the eight cohorts of workers tested. 'Brood development' represents the larval and pupal stages of workers, with 'Adult development' representing the number of days after eclosion from the pupal case. Colonies were provisioned neonicotinoid untreated/treated sucrose solution with the blue solid line representing worker development in the presence of untreated and red dashed in the presence of treated sucrose solution. Developing brood spend the first *ca* 10 days feeding (direct exposure) and the last *ca* 11 days as non-feeding pupae but may be exposed to residues accumulated inside the pupal case or larval tissue (indirect exposure). White circles and individual bee symbols depict removal of these controlled aged adult workers at 3 or 12-days after eclosion for immediate involvement in the behavioural assay followed by decapitation for µCT scanning of the brain. (Online version in colour.)

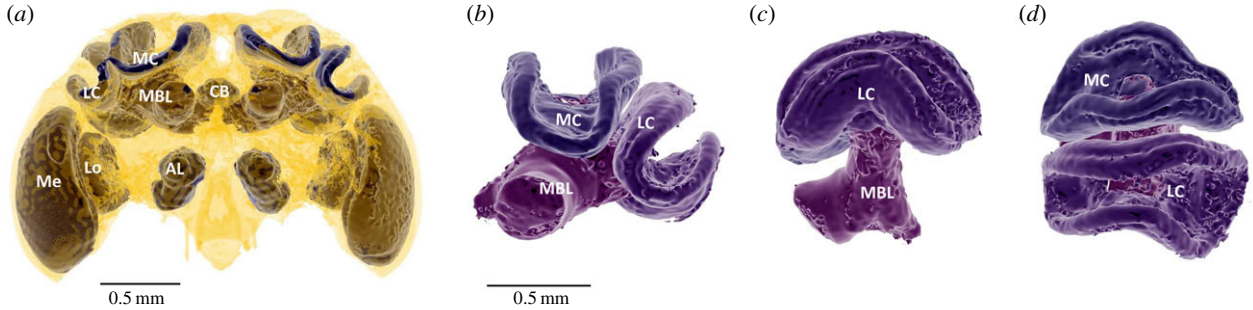

Figure 2. Three-dimensional rendering of one of the studied bumblebee brains using our µCT imaging method. (*a*) Focal neuropils considered in this study are shown in dark purple (optic lobes: medulla (Me); lobula (Lo); antennal lobes (AL); central body (CB); mushroom body: calyces (including lateral calyx (LC) and medial calyx (MC) and lobe (MBL)), surrounded by remaining brain tissue in transparent yellow. (*b–d*) Isolated three-dimensional structure of the mushroom body which has been rotated to show (*b*) frontal, (*c*) lateral and (*d*) dorsal views. (Online version in colour.)

lower performance of tasks important for colony function as older adults [32–34]. In this paper, we test this hypothesis.

Of the neurotoxic insecticides found inside colonies [13,17], many are detected in the food stores and wax that brood (developing larvae and pupae) and residing callows feed-upon and come into contact with. This route of exposure to neurotoxic compounds could potentially affect the developmental plasticity of the central nervous system, with the growth of the brain being at particular risk [35]. Indeed, regions of bee brains are highly plastic early in development [36–38], and volume can increase with task experience [39–42] and correlate with the level of task performance ([43]; but see [44]). Therefore, if pesticide exposure were to affect early brain growth, by for instance reducing brain size, we might predict behavioural task performance, such as learning, to be impaired. However, while recognized as a research priority [32–34,45], no study to date has investigated how pesticide exposure during early-stage development affects brain developmental plasticity and its association with behavioural performance in older adulthood.

Here, we investigated if bumblebees (*Bombus terrestris audax*) developing inside colonies provisioned with imidacloprid (a neonicotinoid pesticide) treated nectar substitute (figure 1), show impaired learning behaviour as adults, and using micro-computed tomography (µCT) scanning [46–49] tested whether this is associated with reduced growth of brain regions during early-stage development (figure 2). Implementing a factorial experiment, we provisioned colonies with treated food at different stages to compare the responses of workers that experienced in-hive exposure during either their brood development stage, early-adult stage or both stages (figure 1). Comparing responses between these three treatments (*pre-eclosion*, *post-eclosion* or *continual* exposure, respectively) relative to unexposed workers (*control*), we investigated which developmental stage was more vulnerable to exposure in terms of later adult performance and physiology. By tracking worker development, we tested two controlled age cohorts of adults at 3 and 12 days old (see electronic supplementary material, figure S1), each of which we attempted to limit variation in prior experience and sensory input. Our comparison of young (3-day) versus older (12-day) workers within and between treatments allowed us to: (i) distinguish the effects of exposure from variation caused by potential innate effects of age (experience independent change); and (ii) test whether developmental plasticity (in behaviour or tissue growth) allows any potential impact from brood exposure to be recovered during the unexposed adult phase.

2. Material and methods

(a) Study system and animal husbandry

Twenty-two *Bombus terrestris audax* colonies were ordered from the commercial supplier Biobest and distributed by Agralan Ltd. Each colony possessed a queen and mean (±s.e.m.) of 14.5 ± 1.1 workers on arrival (electronic supplementary material, table S2) and housed in an aerated plastic box (29 × 22.5 × 13 cm). Throughout the experiment, colonies were kept under a controlled environment (23°C; 60% humidity) red light room and provisioned honeybee collected pollen (distributed by Agralan Ltd) *ad libitum* in a petri-dish and 40/60% sucrose/water solution in a gravity feeder. Food was replenished every 2 days, and feeders thoroughly cleaned prior to refill (electronic supplementary material, table S3). Throughout the experiment, colonies were checked daily for males, gynes or dead individuals which were removed and frozen.

(b) Experimental set-up

In-hive exposure to developing individuals was achieved by spiking the sucrose solution provisioned to each colony with 5 parts per billion (ppb) imidacloprid (see electronic supplementary material for preparation). This is a neonicotinoid that: (i) is used across the globe [13,50–52]; (ii) targets nACh receptors found in insect brains [53,54]; (iii) has been shown to affect bee foraging and navigation reliant on aspects of learning behaviour [7–9]; (iv) is at a concentration approximating that found in pollen and nectar of crop and wild flowers that bees forage on [20,55,56]. Colonies were assigned to one of four treatments which determined the two periods of time (phase I or II) that colonies were provisioned treated and/or untreated sucrose (exposure): *control* = phases I and II both unexposed ($n = 5$ colonies); *pre-eclosion* = phase I exposed, phase II unexposed ($n = 6$); *post-eclosion* = phase I unexposed, phase II exposed ($n = 6$); *continual* = phases I and II exposed ($n = 5$). Phase I started 2 days after colonies arrived and lasted for a period of 21 days as this approximates the development time from an egg or very small larva to adult eclosion [57–59] (electronic supplementary material, figure S1). During phase I (figure 1; electronic supplementary material, figure S1), we conducted daily checks of all newly eclosed bees and marked each using a white paint pen (uni Posca, PC.5M 1.8–2.5 mm) so we could later distinguish these workers (not-tested in this study) from the workers eclosing after day 21 (individuals we did test in this study). Phase I colony exposure was thus what we used to investigate the effects of exposure during brood development. Phase II started on day 22 and ended on day 45 when the experiment stopped and colonies were frozen, and was the period allowing us to investigate the effects of exposure during early-adult development (up to 3 and 12 days old in this study). For the first 11 days of phase II, we checked daily for callow workers (adults recently eclosed from the pupal case) and attached a unique numbered Opalith tag using superglue. This tagging period (days 22–33; electronic supplementary material, table S4) approximates the time of pupal development in which individuals evacuate their gut and stop feeding [59]. This means that, for example, any worker eclosing on day 22 will have been directly feeding as larvae on treated/untreated sucrose during the first *ca* 10 days of phase I, with any worker eclosing on day 33 having fed as larvae in the last 10 days of phase I. This staggered design allowed us to standardized pesticide exposure as best possible while providing us with appropriate sample sizes to test our hypotheses.

For each tagging day, we took the total number of workers per colony that had eclosed on that day and randomly assigned half to the 3-day cohort and remaining half to the 12-day cohort. Using tag ID we could, therefore, remove adult workers for testing 3 or 12 days later accordingly without bias. Brain and behavioural development has been reported to occur both during brood and early-adult stages [38,60].

(c) Assessing olfactory learning performance using proboscis extension reflex conditioning

Proboscis extension reflex (PER) conditioning paradigm [61] is established as an appropriate discerning test for assessing pesticide effects on learning behaviour in bumblebees exposed as adults [62–64], and here we adapted it from a reported bumblebee set-up [62]. On removal from the colony, workers were taken to a neighbouring laboratory, cooled on ice and harnessed (13.00–14.00) using a modified 2 ml centrifuge tube and split pin yoke, under natural light and left to settle for 2 h (electronic supplementary material, figure S2). All bees were then fed 40% sucrose solution to satiety using a Gilmont® syringe by presenting droplets directly to the mouthparts. The bee was then taken to a separate controlled environment room and left for 17 h (overnight and under identical conditions as the rearing room with all further testing also done under red light). For unknown reasons, 24 workers did not survive overnight and so were excluded from the analyses (see electronic supplementary material, table S4).

Prior to the learning assay, between 08.00 and 09.00, we tested whether harnessed workers (total = 413; *control* = 110; *pre-eclosion* = 116; *post-eclosion* = 108; *continual* = 79; electronic supplementary material, table S4) showed a PER in response to their antenna being touched by a 50% sucrose solution droplet (‘*responsive test*’; electronic supplementary material, figure S2). Immediately after and 15 min before starting the PER test, each bee was fed a small droplet (0.8 µl) of 40% sucrose solution for motivation (electronic supplementary material, figure S2). PER conditioning was conducted in front of a filtered ventilation system (Expo Drills & Tools AB500 Extractor fan), preventing the odour coming into contact with neighbouring harnessed bees. Each bee was initially conditioned by exposure to clean air for 5 s, followed by scented air for 10 s. A harnessed bee was positioned 3 cm away from a glass odour tube, with airflow delivered at a constant rate of 80 ml s^{-1} (Tetra APS – 100) channelled through either a ‘clean’ unscented odour tube or diverted through a ‘scented’ odour tube containing a piece of filter paper (5 × 20 mm) impregnated with 1 µl of lemon essential oil (Naturally Thinking Ltd). Airflow diversion was controlled by a solenoid valve (Nass Magnet 108-030-0257 24vAC/12vDC) connected to a Raspberry Pi 2 (Model B) computer ensuring standardized exposure to clean and scented air volume. To develop an association between the lemon odour and reward, the bee’s antennae was touched with a droplet of 0.8 µl of 50% sucrose 6 s into the 10 s odour delivery phase and allowed the bee to feed.

Following trial 1, the odour and reward presentation sequence was repeated to each adult an additional nine times. The inter-trial interval per individual was 10 min allowing PER testing in batches of up to 20 workers. We waited 15 s after the odour and reward presentation sequence before moving to the next individual [65], recording if each bee showed a PER to the odour stimulus prior or after the reward and defining as a learnt or non-learnt response, respectively. This provided a tally of learnt responses achieved by each worker over the nine trials. Bees were excluded if they responded to the initial conditioning trial (trial 1) before presenting the reward ($n = 24$). Bees were removed from testing and categorized as a non-learner if over three consecutive trials no PER (did not feed) was exhibited even after the reward was provided.

Following the PER assay, bees in their harness were placed on ice to immobilize the bee and allow swift decapitation using a disposable surgery scalpel. Heads were immediately submerged in a 70/30% ethanol/de-ionized water solution in separate 1.5 ml centrifuge tubes and stored at 5°C. Thorax inter-tegula distance of the remaining bodies were measured as a proxy for body size [66] by taking the mean of two repeat measurements using a digital calliper (Workzone® 150 mm, accuracy 0.01 mm).

(d) Micro-computed tomography scanning

Micro-computed tomography scanning enables us to accurately and non-destructively investigate the brain tissue *in situ* (within headcase) at high resolution [46–49]. Headcase preparation followed precisely the published protocol by Smith *et al*. [47] with the internal soft brain tissue being stained for 7 days with phosphotungstic acid before being CT scanned at a voxel size of 3.5–4 µm using a Nikon Metrology HMX ST 225 system (Nikon Metrology, Tring, UK). Raw µCT data for each brain scan was reconstructed using CTPro 2.1 software (Nikon Metrology, Tring, UK) and processed using VG Studio Max 2.1 (Volume Graphics GmbH, Heidelberg, Germany). Each three-dimensional reconstructed scan was re-oriented to the same optimum plane-of-view for visualization, and for each neuropil, we re-sliced into a new series of two-dimensional images. For each sample, scan images were exported as 8-bit BMP image series at a standardized voxel size of 4 µm. In total, 92 worker brains were µCT scanned and based on staining quality and the success of segmenting both left and right structures per individual, we considered the mushroom bodies for 78, central body for 88, antennal lobes for 89, medullas for 71 and lobulas for 71 workers (electronic supplementary material, table S5).

(e) Neuropil volume measurements

We analysed how the volumetric growth of six different functional components (neuropils) of the brain (see electronic supplementary material, table S1 for assigned functions) responded to exposure and reveal how an individual worker's learning performance is predicted by volume of the mushroom body calyces and how neonicotinoid exposure influences this relationship.

Segmentation and volume analysis of brain neuropils was undertaken using SPIERS 2.20 software. For segmentation, scan slices were converted to binary threshold images adjusted to achieve an optimum ratio of active white pixels comprising the structure of interest. Looped splines were placed around the active pixels at regular five slice intervals and then interpolated across all slices between intervals to define each structure for three-dimensional reconstruction and volumetric calculation (full segmentation protocol reported in Smith *et al*. [47]). Neuropil absolute volume was calculated using the voxel count function in SPIERS Edit, with relative volume calculated by dividing absolute volume by the worker's inter-tegula width. Volumes of the left and right paired structures per neuropil (except central body) were summed so a single value could be used in the analyses.

(f) Data analysis

Statistical analyses were conducted in R v. 3.5.1 (R Development Core Team 2018) using RStudio v. 1.1.463, with models implemented using lme4 package [67]. For all models, we included *treatment* as a fixed categorical factor. Bee body size (*ITD*) was considered a continuous variable and *colony* as a random factor in our models if inclusion increased the fit of the model (model comparisons assessed by AIC comparison) otherwise they were not retained. For responsiveness and learning, data were analysed using the proportion of individuals showing a response with a generalized linear model (glm) using a binomial distribution and included the categorical variable *age* (3 or 12-day) as an additional fixed factor with *age*treatment* interaction term removed as it showed no significant effect. For looking at the proportion of learners by trial we used a linear mixed-effects model (lmer) in which treatment consisted of two categories, *control* workers and pesticide workers (pooled from all three pesticide treatments because of the reduced sample sizes from the negative effects of exposure, see Results). We considered a second-order polynomial fit for *trial* number and individual *ID* as a random factor. For relative neuropil volumes, we used a linear mixed-effects model (lmer) that included *age*, *ITD* and *colony* (random effect). We used a binomial generalized linear model (glm) to analyse how calycal volume—by analysing the *calycal volume*treatment* interaction-influenced learning score, as a proportion of the maximum learning score.

3. Results

(a) Did pesticide exposure affect the proportion of workers responding to a sucrose reward prior to the learning test?

When considering the 413 harnessed workers across all treatments, we found that a significantly higher proportion of 12-day compared to 3-day workers responded (GLM: *age*: $z = -4.10$, $p < 0.001$). This effect of age enhancement did not differ between treatments as no *age*treatment* effect was detected (supported by overlapping CIs in figure 3 and the interaction term not being retained in the model; electronic supplementary material, table S5). However, when we then focused on the effect of treatment independently, we found consistent negative model estimates for all three pesticide treatments relative to *control*, and detected a significantly lower proportion of responsive workers from *post-eclosion* and *continual* exposed colonies ($z = -2.53$, $p = 0.011$ and $z = -2.40$, $p = 0.016$; figure 3; electronic supplementary material, table S6 for *post hoc* Tukey tests). Worker size had no significant effect ($z = 1.41$, $p = 0.16$).

(b) Did pesticide exposure affect learning performance?

For responsive workers, we analysed the proportion of workers exhibiting at least one learnt response over the 10 trials ($n = 181$ tested in PER assay; electronic supplementary material, figure S2 and table S4). Our model showed a positive estimate for the effect of *age*, but unlike responsiveness, no significant increase was detected which was consistent across treatments as evidenced by no *age*treatment* effect and the interaction term not being retained in the model (electronic supplementary material, table S5). However, we detected for each exposure treatment a significantly lower proportion of learners relative to the *control* (GLM: *pre-eclosion*: $z = -4.38$, $p < 0.001$; *post-eclosion*: $z = -3.49$, $p < 0.001$; *continual*: $z = -2.78$, $p < 0.01$; figure 3*b*; see electronic supplementary material, table S6 for *post hoc* Tukey tests), and worker size had a significant positive effect on the probability of being a learner ($z = 2.31$, $p = 0.021$).

Across all learners, we then looked at the proportion of learnt responses over the successive trials (trials 2–10 considered; by definition a naive worker cannot learn on the first trial). The strong negative effect of pesticide exposure discussed above heavily reduced sample sizes per pesticide treatment (*pre-eclosion* = 9, *post-eclosion* = 11, *continual* = 10), therefore, we pooled workers from the three treatments ($n = 30$ exposed workers) and compared them to *control* workers ($n = 29$) while not distinguishing between 3- and 12-day cohorts. The proportion of learnt responses increased over the trials (GLM polynomial: p^1: $t = 14.26$, $p < 0.001$) with the incremental proportion decreasing in rate over the consecutive trials (p^2: $t = -2.48$, $p = 0.014$; electronic supplementary material, table S5) driven primarily by the significant negative effect of pesticide exposure ($t = -2.04$, $p = 0.046$) in which exposed workers

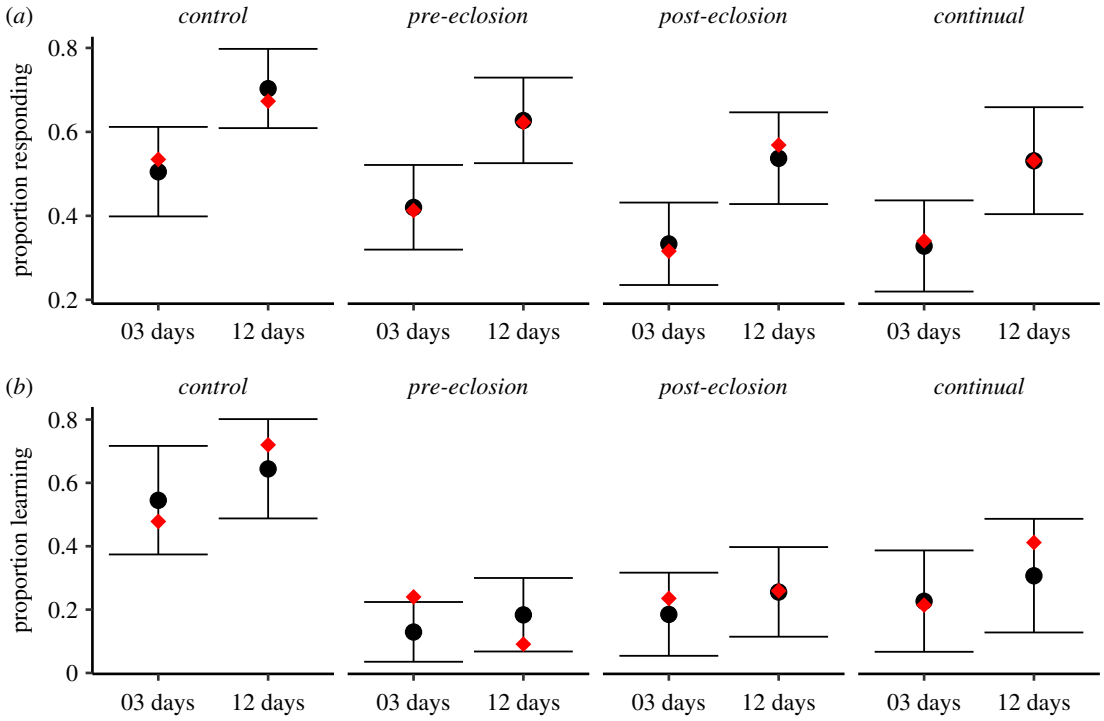

Figure 3. Proportion of (*a*) responsive workers and (*b*) learners between treatments. Intersecting circular points represent estimated model mean taken from model back-transformation (binomial GLM) with bars depicting associated ±95% confidence limits. Red diamond corresponds to the mean value taken from the raw response data. Sample sizes of 3- and 12-day worker cohorts for 'responsive': *control* = 58 and 52, *pre-eclosion* = 63 and 53; *post-eclosion* = 57 and 51; *continual* = 47 and 32; 'learners': *control* = 23 and 25, *pre-eclosion* = 25 and 33; *post-eclosion* = 17 and 27; *continual* = 14 and 17. (Online version in colour.)

showed a lower proportion of learnt responses in the latter few trials relative to *control* (figures 4 and 5).

(c) Did pesticide exposure affect brain neuropil volumes?

Focusing first on the mushroom body calyces, relative volumes were significantly smaller in workers from all three pesticide exposure treatments relative to *control* (*pre-eclosion*: $t = -2.41$, $p = 0.049$; *post-eclosion*: $t = -3.83$, $p < 0.01$; *continual*: $t = -2.90$, $p = 0.021$; figure 5; electronic supplementary material, tables S7, S8). This was consistent for 3- and 12-day workers as evidenced by no effect of *age*treatment* and the interaction term not being retained in the model (electronic supplementary material, table S8; see table S9 for *post hoc* Tukey tests). For the relative volume of the mushroom body lobes, we found negative model estimates for all three pesticide treatments relative to the *control*, however, unlike the calyces, we did not detect these comparisons as significantly lower (figure 5; electronic supplementary material, table S8). Analysis of the four other segmented neuropils (central body, antennal lobes, lobulas and medullas) showed workers from pesticide-treated colonies possessed no significant volumetric differences relative to *control*, although we did find consistent negative model estimates for the antennal lobes across all pesticide treatments (electronic supplementary material, table S10).

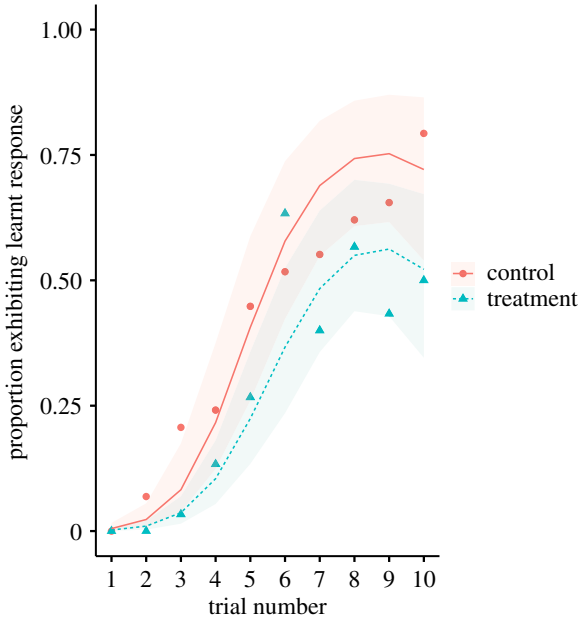

Figure 4. Proportion of workers by trial exhibiting a learnt response. Workers from all three pesticide treatments were pooled (blue triangles; $n = 30$ workers) and compared against *control* workers (red circles; $n = 29$), with both age cohorts aggregated per treatment. Lines (blue dashed = pesticide treatment; red solid = control) represent the binomial model (LMER polynomial) estimates over the consecutive trials and shaded areas represent the 95% confidence intervals. (Online version in colour.)

(d) Are bigger mushroom body calyces associated with higher learning scores and how does pesticide exposure affect this relationship?

For each responsive worker starting the PER learning assay that had their mushroom body successfully segmented, we investigated how the total number of demonstrated learnt responses (*learning score*) was associated with relative calycal volume (predictor). As for the learning-by-trial data, workers from the three pesticide exposure treatments were pooled and compared to *control* workers when not distinguishing age. We found a significant positive association between relative calycal volume and learning score ($t = 4.51$, $p < 0.001$; figure 6; electronic supplementary material, figure S3), but this

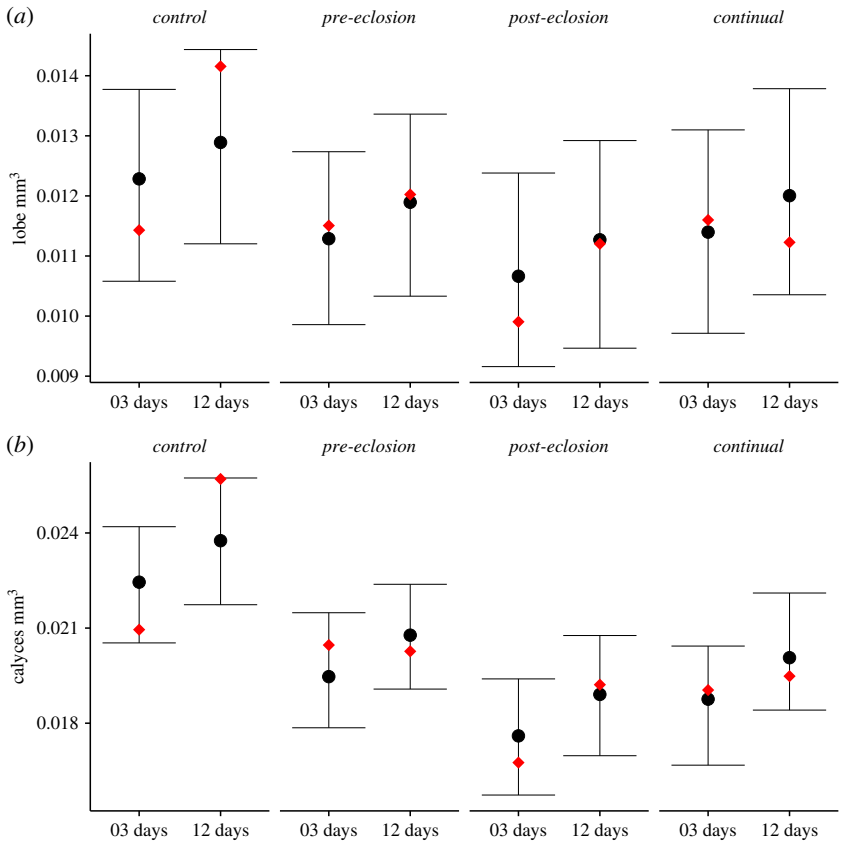

Figure 5. Relative volumes of bumblebee worker mushroom body (*a*) calyces, (*b*) lobes. Intersecting circular point represents the estimated model mean taken from model back-transformation (lmer) with bars depicting associated ±95% confidence limits. Red diamond corresponds to the mean value taken from the raw response data. Sample sizes of 3- and 12-day worker cohorts for: 'lobes': *control* = 10 and 10, *pre-eclosion* = 11 and 11; *post-eclosion* = 13 and 11; *continual* = 13 and 9; 'calyces': *control* = 9 and 8, *pre-eclosion* = 11 and 11; *post-eclosion* = 10 and 10; *continual* = 11 and 8. (Online version in colour.)

relationship was driven by *control* workers as pesticide exposed workers showed no clear relationship as supported by a significant negative *volume*treatment* interaction ($t = -3.96$, $p < 0.001$; electronic supplementary material, tables S11, S12).

4. Discussion

In-hive exposure to imidacloprid-spiked nectar substitute caused workers to exhibit impeded developmental plasticity. The degree of impact from exposure during brood development appeared comparatively as detrimental as exposure during adulthood, with workers exhibiting lower responsiveness to the presentation of a sucrose reward and impaired learning performance coupled with possessing smaller mushroom body calyces. Furthermore, investigation of the relationship between each worker's respective calycal volume and learning performance revealed that while 'bigger is better' for *control* workers this positive relationship (i.e. larger calyces improves learning) was absent for pesticide exposed workers indicating functionally impaired mushroom bodies from exposure.

(a) Pesticide exposure during early development affected responsiveness and learning

Our findings of an impact on learning performance from direct adult neonicotinoid exposure conforms with previous studies showing negative effects on learning in honeybees [68–70] and bumblebees [62]. However, our study design allowed us to compare responses between young and older adults receiving different chronic exposure histories. Three- and 12-day workers from *post-eclosion* and *continual* exposure colonies (only adult stage exposed) showed a similar level of impaired behaviours despite differences in exposure length. Similarly, although 3-day adults from *pre-eclosion* colonies were exposed for up to two weeks during brood development compared to just 3 days for *post-eclosion*, learning appeared impaired to the same degree. Together these findings highlight that the first 72 h of adulthood must be important in behavioural development but also represents a susceptible developmental window to insecticide exposure [26,71] showing the importance of considering different life-stages when assessing pesticide risk.

Workers exposed during brood development (*pre-eclosion*) exhibited impaired learning performance at a similar reduced level as adult only exposed workers (*post-eclosion*) indicating a lag-effect from brood exposure on adult learning. Consideration of delayed effects of pesticide exposure is thus important when assessing pesticide risk. Indeed, studies on honeybees (*Apis cerana* and *A. mellifera*) and a stingless bee (*Melipona quadrifasciata anthidioides*) reported adult workers showing negative effects on adult learning and motor function when reared as larvae under topical or oral exposure to a neonicotinoid [32–34]. Our findings further show that comparison of 3- and 12-day adults from *pre-eclosion* colonies (exposed only as brood) exhibited a similar level of impaired learning performance showing that no recovery could be made during the 9-day interim period.

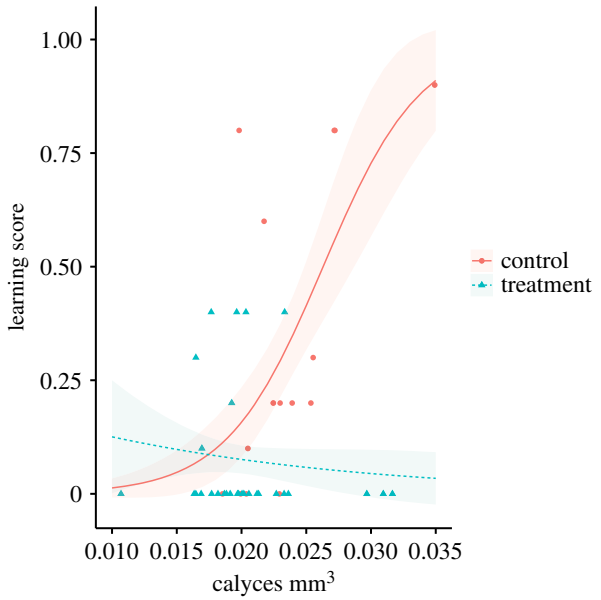

Figure 6. Relative mushroom body calycal volume plotted against the respective worker's learning score. Workers from all three pesticide treatments were pooled (blue triangles, $n = 29$; *pre-eclosion* = 11, *post-eclosion* = 12, *continual* = 6) and compared against *control* workers (red circles, $n = 15$), with fitted lines (blue dashed = pesticide treatment; red solid = control) representing binomial model (GLM) estimates and shaded areas representing the 95% confidence intervals. (Online version in colour.)

(b) Impaired learning in pesticide exposed workers was associated with reduced volumetric growth of the mushroom body calyces

Exposure at either stage of development was associated with workers possessing smaller mushroom body calyces. A focus on 3-day adults revealed that *post-eclosion* and *continual* exposed workers showed reduced volumes relative to *control* workers, highlighting that in just 3 days of adult exposure a key brain region was unable to grow as normal. With mushroom body growth rate in bumblebees considered to be highest during the first 72 h of adulthood [36,38], our findings indicate this to be a developmental period particularly susceptible to stress.

The average volume reductions of the mushroom body calyces and lobes showed a strikingly mirrored pattern to the reduced proportion of learners in each respective pesticide treatment. More tellingly, for workers in which we could pair relative volume with learning score, we found that bigger did equate to better learning performance for *control* workers. However, such a positive relationship was not the case for imdacloprid exposed workers, indicating impaired neuronal functioning of this brain region. While our study cannot explicitly test whether reduced calycal volume is the direct cause of lowered learning—for example, behaviour could be affected if exposure were to affect immune pathways [72] or demotivate individuals during harnessing prior to the PER assay (although similar learning performance between *pre-* and *post-eclosion* bees would not support this view), our findings do support for our clear *a priori* hypothesis that effects of exposure on the brain during early-stage development would be associated with impacts on behavioural performance.

With neonicotinoids acting as nACh receptor agonists [54] calycal functional impairment does make sense for adult exposed workers (*post-eclosion* and *continual*). However, with brood exposed workers (*pre-eclosion*) also being affected this indicates exposure to be affecting synaptic development and proliferation in the calyces. For instance, microglomeruli configuration is considered to be associated with learning and memory in bees [73–76], and reduced density from neonicotinoid exposure has been reported in honeybees [35]. It could also stem from impeded neurogenesis where neuronal precursor cells were prevented from giving rise to mushroom body Kenyon cells, which in honeybees occurs during development pre-eclosion [77,78], or reduced Kenyon cell size as shown in neonicotinoid exposure experiments on bumblebee cell cultures [79].

(c) Mushroom body calyces were disproportionately affected over the other neuropils

Mushroom body calyces are multisensory processors fed by afferent neurons, in contrast to the lobes that predominantly function as output regions with efferent neurons [36]. This functional difference could explain why calycal volumetric variation was more tightly associated with our measure of learning performance and seemingly more vulnerable to exposure when considering plasticity. But why was little effect also observed in the other neuropils? For instance, antennal lobes are involved in detecting and processing olfactory information (electronic supplementary material, table S1), are developmentally plastic during early adulthood [36,38], and exhibit reduced neuronal function under nicotinic agonists [80,81]. Considering exposed workers performed worse in the olfactory conditioning assay, we might have expected a pattern of impeded growth in the antennal lobes, but our analysis did not detect the negative model estimates as significant. Possible explanations for the disproportionate effect on the mushroom bodies include: (i) nACh receptors targeted by neonicotinoids are found in high density in the mushroom body Kenyon cells [82], and if Kenyon cell proliferation was affected by exposure this could lead to neuropil volumetric reductions; (ii) the high degree of mushroom body plasticity compared to other neuropils [36,38,60,83] requires a high level of neurogenesis and organization possibly increasing the risk of neurotoxic exposure interfering with this process; (iii) developing adult workers were stimulus deprived but not void, therefore, while mushroom body volumetric increase is likely to be more experience independent than dependent, we could not rule out investment in olfactory processing to compensate for a lack of visual information [38,39]; (iv) consistent volumetric reductions in non-mushroom body neuropils did occur as a result of exposure but we did not have the power to detect these small effect sizes or the resolution of our µCT technology was too low.

(d) Improved behavioural performance and mushroom body growth with age independent of experience

Previous bumblebee studies have reported no effect of age on aspects of learning ability [84,85], although these were carried out in foraging arenas whereby prior experiences were not controlled. Having kept our colonies under a stimulus deprived environment and compared between controlled age cohorts, we, in contrast, do provide evidence of a positive effect of *B. terrestris* age on responsiveness and learning indicative of experience independent age enhancement. In

parallel, we show brain volume to increase which to our knowledge has only been shown once before in a histological study on the bumblebee *Bombus impatiens* by Jones *et al*. [38]. Jones' findings suggested *ca* 10% increase in mushroom body volume between workers of similar ages used in our study compare to just over 20% in our study. This difference perhaps stems from variation in methodological approaches, sample sizes (higher in our study) or taxonomic variation, but together supports an innate increase in neuropil volume over the first 12-days of adulthood.

(e) Conclusions

Our findings of early exposure affecting later adult behaviour can provide an explanation for why reduced colony growth has been detected two to three weeks after the onset of neonicotinoid exposure in previous studies [11,24,28–31]. If future generations of workers are predisposed to be inefficient functioning cohorts, this could lead to a density-dependent build-up of colony-level impairment increasing the risk of colony collapse [12]. Our results suggest that even if newly eclosed workers were to delay the age at which they start any specific task performance, such a strategy could be futile given we saw a little adult recovery in behaviour from 3 to 12 days of adulthood from *pre-eclosion* colonies. Our method of provisioning colonies with a treated nectar substitute may also represent a conservative level of exposure given that developing brood are more dependent on pollen for tissue growth than adults, and that concentrations of neonicotinoid residues in pollen are typically higher than found in nectar [20,55,56]. Future work could also look to compare responses between insect species that progressively feed their offspring during development, such as bumblebees, and those that mass provision offspring, such as pollen and nectar packages in the cells of solitary bees [71]. Importantly, our findings are unlikely to be exclusively applicable to: (i) workers, as newly reared males and queens are also at risk with possible implications for mating and hibernation; (ii) neonicotinoids, as many neurotoxic pesticides including cholinergic insecticides (e.g. sulfoxamines, butenolides) can build up inside bee colonies and induce sublethal effects on individual and colony-level traits [31,86].

Data accessibility. Raw data and scripts used for statistical analysis and figure production are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.63xsj3tzp [87].

Authors' contributions. R.J.G. conceived the project; D.B.S. and R.J.G. designed the experiment; D.B.S., A.R.R. and P.H.B. conducted the experiment; D.B.S. and F.A. carried out the µCT scanning; D.B.S. and D.B. reconstructed and segmented the brains; A.N.A. and R.J.G. performed the data analyses; D.B.S., A.N.A. and R.J.G. wrote the manuscript.

Competing interests. We declare we have no competing interests

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