Developmental exposure to pesticide contaminated food impedes bumblebee brain growth predisposing adults to become poorer learners

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Keywords: 3D reconstruction, *Bombus terrestris*; Imidacloprid, micro computed tomography scanning, mushroom body calyces, neonicotinoid, olfactory, segmentation, sublethal

Abstract

Understanding the risk to biodiversity from pesticide exposure is a global priority. For bees, an understudied step in evaluating pesticide risk is understanding how pesticide contaminated foraged food brought back to the colony can affect developing individuals. Provisioning bumblebee colonies with pesticide (neonicotinoid) treated food, we investigated how exposure during two key developmental phases (brood and/or early-adult), impacted brain growth and assessed the consequent effects on adult learning behaviour. Using micro-computed tomography (µCT) scanning and 3D image analysis, we compared brain development for multiple neuropils in workers 3 and 12-days post-emergence. Mushroom body calyces were the neuropils most affected by exposure during either of the developmental phases, with both age cohorts showing smaller structural volumes. Critically, reduced calyces' growth in pesticide exposed workers was associated with lower responsiveness to a sucrose reward and impaired learning performance. Furthermore, the impact from brood exposure appeared irrecoverable despite no exposure during adulthood.

Introduction

Insect pollinator declines are of worldwide concern (Hallmann et al., 2017; Potts et al., 2016; Vanbergen, 2013), and safeguarding this important functional group and ecosystem service provider requires a deep understanding of the driving factors (Gill et al., 2016; Goulson et al., 2015). Social bees, such as bumblebees, honeybees and stingless bees are important insect pollinators, and the threat posed by pesticide exposure is a widespread issue (Brittain and Potts, 2011; Desneux et al., 2007; Woodcock et al., 2017). Pesticide residues have been found inside colonies across the globe (Calatayud-Vernich et al., 2018; Daniele et al., 2018; Mitchell et al., 2017; Mullin et al., 2010; Valdovinos-Flores et al., 2017), raising concerns as to how the prevalence of such chemicals in the environment could affect colony development (Gill et al., 2012; Pohorecka et al., 2017; Whitehorn et al., 2012). For instance, controlled exposure experiments and field studies investigating exposure to neonicotinoid pesticides have reported reduced colony growth and sexual output (Arce et al., 2017; Baron et al., 2017; Gill et al., 2012; Leza et al., 2018; Rundlöf et al., 2015; Tsvetkov et al., 2017; Whitehorn et al., 2012). Such colony level effects are likely to be caused by exposure impairing worker behaviour, with cumulative effects across workers leading to a functionally weakened colony (Bryden et al., 2013; Crall et al., 2018). One possibility is that neonicotinoids, being a neurotoxic pesticide, affect neuronal processes important for cognitive and learning abilities (Decourtye et al., 2004; Siviter et al., 2018b) translating to impaired colony tasks (Feltham et al., 2014; Fischer et al., 2014; Gill and Raine, 2014).

With neurotoxic pesticide residues frequently reported in the pollen and nectar brought back by foragers (Botias et al., 2015; Chauzat et al., 2006; David et al., 2016; Kasiotis et al., 2014; Pohorecka et al., 2012), individuals developing and residing in the colony are likely to be chronically exposed to these compounds (Pohorecka et al., 2017). A possibility, therefore, is that tissue development, such as the central nervous

47 system, is impeded. For example, honeybees reared under sub-optimal environmental conditions have
48 exhibited reduced brain volumetric growth and altered neuronal architecture (Groh et al., 2004; Steijven
49 et al., 2017). Impeded brain development and structural plasticity may impact on behaviours such as
50 learning ability, that require detection, assimilation and processing of sensory input from the environment
51 (Cabirol et al., 2018; Chittka, 2017; Galizia et al., 2011). Knowledge of how pesticide contaminated food
52 inside bee colonies can affect individual physiological development however, is limited (Gregorc et al.,
53 2012; Wu et al., 2012, 2011). Moreover, there has been an urgent call for research linking how potential
54 pesticide induced impairment to brain development can translate to task performance as later adults
55 (Siviter et al., 2018b; Tan et al., 2015; Tomé et al., 2012; Yang et al., 2012). We directly address this call by
56 investigating how developing bumblebees exposed to a neonicotinoid pesticide via treated provisioned
57 food may alter brain development and link this to effects on associative learning behaviour.

58 To experimentally test the effect of pesticide exposure on individual development we needed to first
59 consider the level of developmental plasticity in behaviour and brain growth. In social bees, worker
60 maturation can correlate with stereotyped behavioural changes (Goulson, 2010; Johnson, 2010), and
61 increased brain neuropil volumes (functional structures) (Durst et al., 1994; Galizia et al., 2011; Li et al.,
62 2017; Winnington et al., 1996; Withers et al., 1993). However, it has been recognised that dissecting
63 innate effects of age (experience independent change) from co-varying cumulative increases in sensory
64 input (experience dependent change) on behaviour and brain development is difficult (Fahrbach et al.,
65 1998; Jones et al., 2013; Maleszka et al., 2009; Riveros and Gronenberg, 2010). To distinguish the effects
66 of pesticide exposure from variation caused by other interacting factors, we therefore: i) attempted to
67 standardise experience and sensory input across tested workers, ii) tested workers of controlled age, and
68 iii) compared between young and old age cohorts. Furthermore, by studying two main developmental
69 stages, such as brood (larval & pupal) and early adulthood, here we reveal which development phase is
70 more vulnerable to pesticide exposure, and whether developmental plasticity in bee brains (Farris et al.,

2001; Galizia et al., 2011; Riveros and Gronenberg, 2010; Withers et al., 1995) allows recovery during an unexposed later stage.

Despite the functional importance of non-*Apis* bees (Brittain et al., 2013; Garibaldi et al., 2013; Gill et al., 2016) and possible differences in pesticide sensitivity relative to honeybees (Cresswell et al., 2012; Heard et al., 2017; Piiroinen and Goulson, 2016; Rundlöf et al., 2015; Woodcock et al., 2016), empirical tests on non-*Apis* bees looking at the physiological response to stress, such as postembryonic neuronal development, are limited. Here we studied the response of the bumblebee, *Bombus terrestris,* a species: i) that can be reared under controlled laboratory conditions; ii) for which learning performance assays have been developed (Muth and Leonard, 2019; Riveros and Gronenberg, 2009; Siviter et al., 2018b; Smith and Raine, 2014); iii) individuals can be exposed to pesticides within the social colony environment rather than in isolation (Gill et al., 2012; Maleszka et al., 2009; Whitehorn et al., 2012). Here, we chronically exposed cohorts of workers, reared inside their natal colonies, to a 5ppb concentration of the neonicotinoid imidacloprid via provisioned sugar solution (40% sucrose) during two different developmental stages: a) before and b) after adult eclosion from the pupal case. We investigated how the link between brain growth and learning behaviour may be affected in workers exposed during: brood development (*pre-eclosion*), early adulthood (*post-eclosion*) and both these developmental periods (*continual*), comparing each to unexposed (*control*) workers. For each treatment we tested adult workers at 3 or 12-days after eclosion (Figure 1).

Firstly, we tested worker response to sucrose and then on olfactory associative learning performance using the established proboscis extension reflex (PER) conditioning paradigm (Figure S1) (Bitterman et al., 1983; Giurfa and Sandoz, 2012; Laloi et al., 1999; Riveros and Gronenberg, 2009; Sommerlandt et al., 2014), which has previously been used to test pesticide effects on adult learning in honey bees exposed during the larval stage (Tan et al., 2015; Yang et al., 2012), and bumblebees exposed as adults (Piiroinen

and Goulson, 2016; Stanley et al., 2015; Tison et al., 2017). Secondly, we employed new advances in micro-computed tomography (µCT) scanning and 3D image analysis to explore the brain *in situ* (within headcase) and enable non-destructive volumetric measurements to a standardised voxel size of 4µm (Figure 2) (Baird and Taylor, 2017; Gutiérrez et al., 2018; Ribi et al., 2008; Smith et al., 2016). We segmented five key neuropils: mushroom bodies (associated with learning), antennal lobes (olfaction), optic lobes - medullas and lobulas (vision), and central body (motor function; Table S1). For the mushroom bodies we segmented the two major components, lobes and calyces, to investigate responses of the different functionally multisensory input and output regions, respectively (Fahrbach, 2006; Heisenberg, 2003). Using a sample size exceeding any other study investigating bee brain morphology to date, we present the first comparative study to directly link how chronic pesticide exposure impacts learning performance by affecting bee brain development.

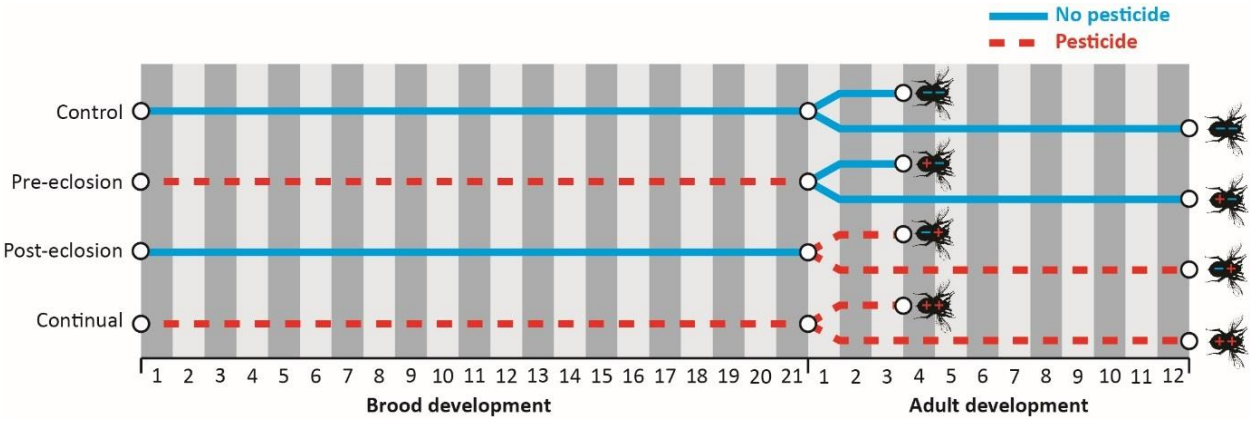


Figure 1. Graphic showing exposure periods for the four colony treatments (*control, pre-eclosion, post eclosion & continual*) and the eight cohorts of individual workers to be tested. Blue solid line represents untreated food (sucrose solution) and red dashed line represents pesticide-treated food. 'Brood development' represents the larval and pupal (brood) stages of workers, with 'Adult development' representing the number of days after eclosion from the pupal case. Individual bee symbols depict removal of controlled aged adult workers at 3 or 12-days after eclosion for behavioural testing followed by decapitation for µCT scanning of the brain.

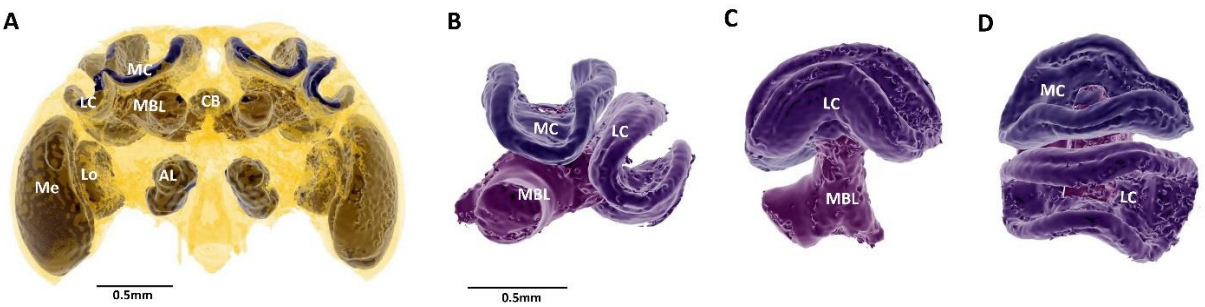


Figure 2. 3D rendering of one of the studied bumblebee brains using the µCT imaging method. a, Focal neuropils considered in this study shown in dark purple (optic lobes: medulla (Me), lobula (Lo); antennal lobes (AL); central body (CB); mushroom body: calyces (including lateral calyx (LC) & medial calyx (MC) and lobes (MBL)), surrounded by remaining brain tissue in transparent yellow. **b-d,** Isolated 3D structure of the mushroom body which has been rotated to show **b**, frontal, **c**, lateral, and **d**, dorsal views.

Results

Responsiveness

Prior to the learning assay, we confirmed whether harnessed workers (n=413; Table S2) showed a PER in response to their antenna being touched by a 50% sucrose solution droplet (Figure S1). We found a significantly higher proportion of 12-day compared to 3-day workers responded (GLM: *age*: z=-4.10, p<0.001) which was consistent across treatments as evidenced by no *age*treatment* effect and the interaction term not being retained in the model (Table S3). We also 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 & z=-2.40, p=0.016; Figure 3).

Learning

For the responsive workers (n=181; Table S2), we tested each worker's ability to learn to associate an odour with a sucrose reward by demonstrating a PER response over ten consecutive trials (Figure S1; see methods). Firstly, we categorised workers as either those exhibiting at least one response as 'learners' and those showing no learnt response as non-learners. Whilst our model showed a positive estimate for the effect of *age*, unlike responsiveness we did not detect a significant increase which was consistent across treatments as evidenced by no *age*treatment* effect and the interaction term not being retained in the model (Table S3). However, we again detected a strong effect of pesticide exposure relative to the *control*, with each treatment showing a significantly lower proportion of learners (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 3b).

For all individuals classed as learners, we then looked at how the proportion of learnt responses changed over the successive trials (analysis considered trials 2-10, as by definition a naïve worker cannot learn on the first trial). However, because of the strong negative effect of pesticide exposure on passing the responsiveness stage and proportion of learners, sample sizes for each pesticide treatment were significantly reduced. Therefore, given the similarity in responses across pesticide treatments, we pooled all workers from these three treatments and compared them to *control* whilst not distinguishing between 3-and 12-day workers. From this analysis we found that the proportion of responses increased over the trials (GLM polynomial: p^1: t=14.26, p<0.001). This relationship, however, was non-linear and the incremental proportion decreased in rate over the consecutive trials (p^2: t=-2.48, p=0.014; Table S3). This was primarily driven by the significant negative effect of pesticide exposure (t=-2.04, p=0.046), with workers from exposed colonies showing a distinctly lower proportion of learnt responses in the latter few trials relative to *control* (Figure 4).

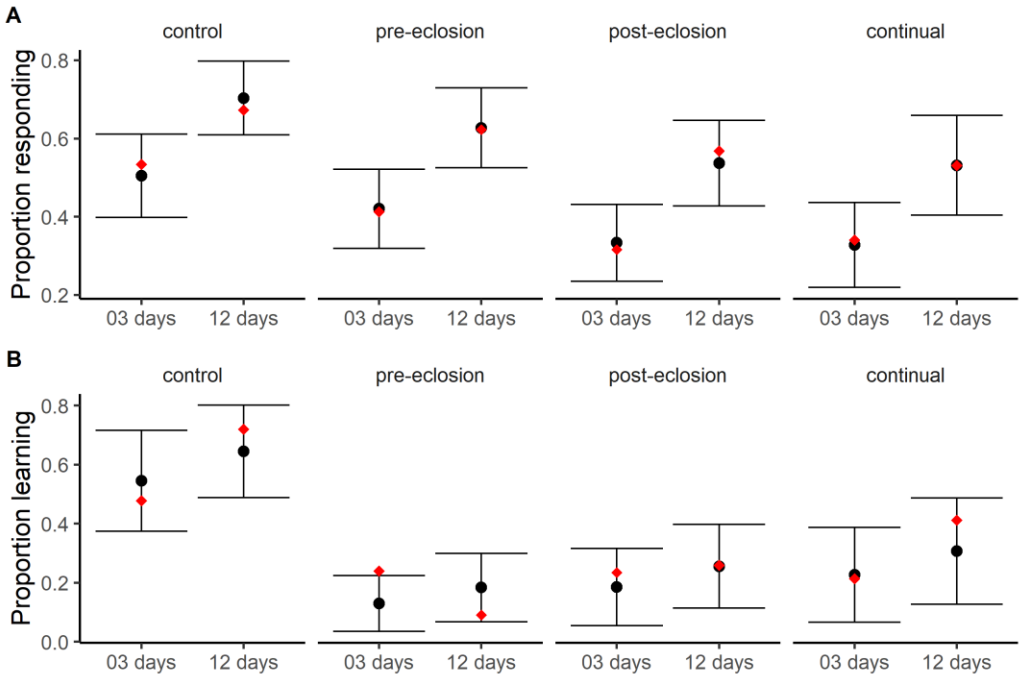
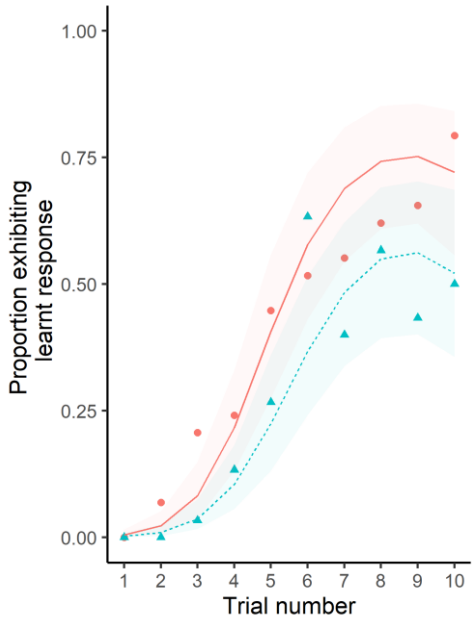


Figure 3. Proportion of responsive workers and proportion demonstrating an olfactory associative learning response using proboscis extension reflex (PER) conditioning between treatments. A, Workers exhibiting a PER response when touching the antennae with a sucrose solution droplet prior to the PER conditioning trials; **B,** Learners (workers exhibiting at least one learnt response during the PER conditioning trials). Intersecting circular point represents the estimated model mean taken from back-transformation of the model (binomial GLM) with bars depicting the associated ±95% confidence limits. Red diamond corresponds to the mean value taken from the raw response data.



165

166 **Figure 4. Proportion of workers by trial exhibiting an olfactory conditioned learnt response.** Workers
167 from all three pesticide treatments were pooled (blue triangles; n=30 workers) and compared against
168 *control* workers (red circles; n=29), with both age cohorts aggregated per treatment. Lines (blue dashed =
169 pesticide treatment; red solid = control) represent the binomial model (LMER polynomial) estimates over
170 the consecutive trials.

171

172 *Brain neuropil volumes*

173 Focusing first on the mushroom body calyces, relative volumes were significantly smaller in workers from
174 all three pesticide exposure treatments compared to *control* (*pre-eclosion*: t=-2.41, p=0.049; *post-*
175 *eclosion*: t=-3.83, p<0.01; *continual*: t=-2.90, p=0.021; Table S4-5). This was consistent for both 3 and 12-
176 day workers as evidenced by no effect of *age*treatment* and the interaction term not being retained in
177 the model (Table S5). Focusing second on the relative volume of the mushroom body lobes, we again
178 found negative model estimates for all three pesticide treatments relative to the *control*, however unlike
179 the calyces none of these comparisons were detected as significantly lower (Table S5). Analysis of the four
180 other segmented neuropils (central body, antennal lobes, lobulas and medullas) further showed that

181 workers from pesticide treated colonies showed no significant volumetric differences relative to *control,*
182 although we did find consistent negative model estimates for the antennal lobes across all pesticide
183 treatments (Table S6).

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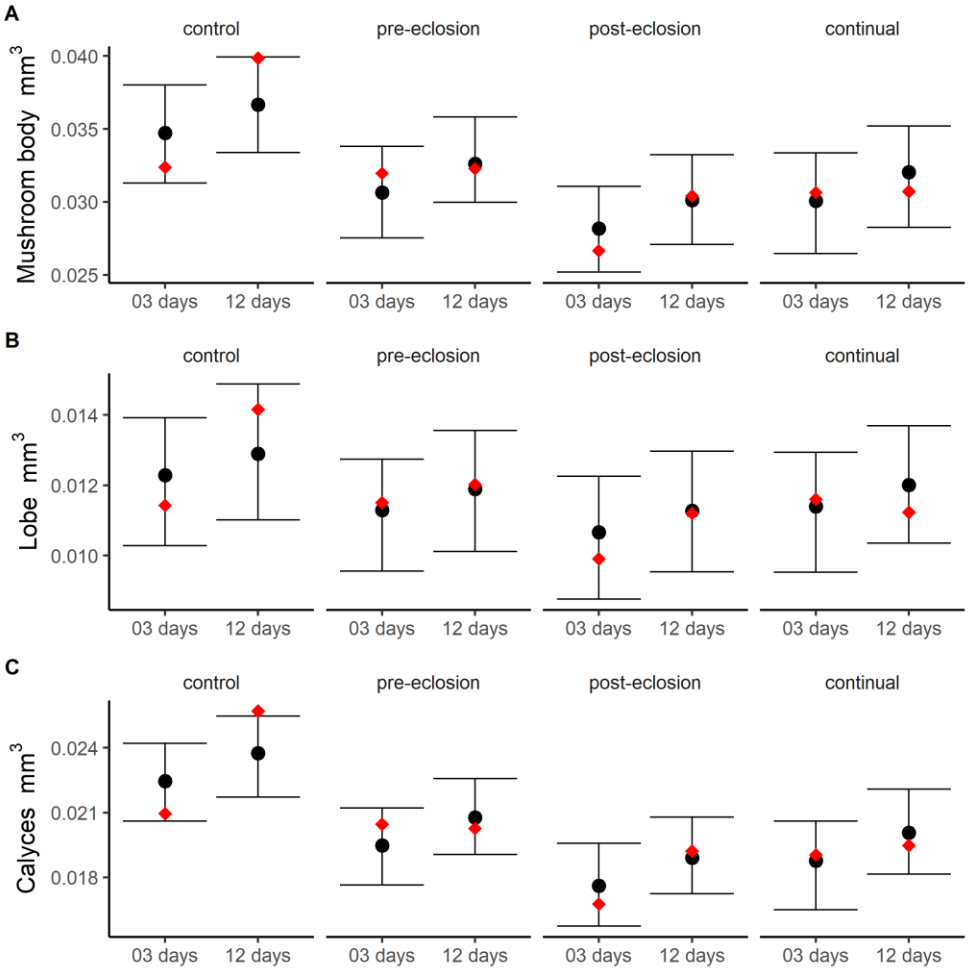

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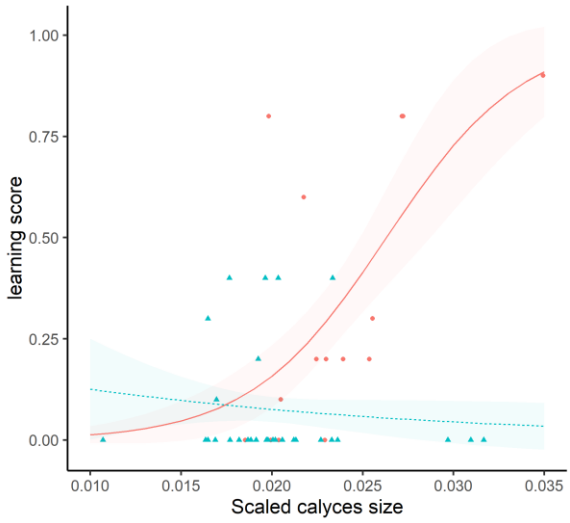
186 **Figure 5. Volumes for A) whole mushroom body, B) mushroom body calyces and C) mushroom body**
187 **lobes of bumblebee workers.** These represent volumes relative to the body size of the worker (absolute
188 volume divided by body size). Intersecting circular point represents the estimated model mean taken from
189 back-transformation of the model (LMER) with bars depicting the associated ±95% confidence limits. Red
190 diamond corresponds to the mean value taken from the raw response data.

191

192 *Relationship between mushroom body calyces volume and learning score*

193 For each responsive worker that started the PER conditioned learning assay and for which we had the
194 volume of their mushroom body calyces, we took the total number of demonstrated learnt responses
195 ('*learning score*') and investigated the association with relative calyces' volume as the predictor variable
196 (given this was the neuropil most affected from exposure). As we did for the learning-by-trial data, we
197 pooled all workers from the three pesticide exposure treatments and compared their scores to that of
198 *control* workers when no distinguishing age. From this, we found a significant positive association
199 between relative volume of the calyces and learning score ($t=4.51$, $p<0.001$; Figure 6; Figure S2), but this
200 relationship was driven by *control* workers in which larger calyces equated to higher learning score.
201 Pesticide exposed workers, in contrast, showed no clear relationship as supported by the significant
202 negative *volume*treatment* interaction ($t=-3.96$, $p<0.001$; Table S7-8). This finding reveals that impaired
203 functioning of the mushroom body in workers from pesticide exposed colonies is not only from reduced
204 volumetric growth, but presumably also from affected physiological composition of the tissue.

205



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207 **Figure 6. Relative volume of mushroom body calyces plotted against learning score.** Calyces volume
208 predicts learning score for *control* workers but not pesticide exposed workers. Workers from all three

209 pesticide treatments were pooled (blue triangles) and compared against *control* workers (red circles), with
210 fitted lines (blue dashed = pesticide treatment; red solid = control) representing binomial model (GLM)
211 estimates.

212

213 **Discussion**

214 Our study reveals that worker bumblebees exposed to a neurotoxic pesticide, a neonicotinoid, can affect
215 the developmental plasticity of the brain with reduced volumetric growth manifested not only from
216 exposure as an adult but also during brood development. The effects on adult behaviour and brain
217 physiology from brood exposure (*pre-eclosion*) appeared irrecoverable despite no experimental provision
218 of pesticide treated food during the 12-days of adulthood. Critically, impeded growth of the mushroom
219 body calyces of worker brains from pesticide exposure, was associated with functional impairment as
220 evidenced by reduced responsiveness and poorer olfactory learning behaviour.

221 *Pesticide exposure during early development affected responsiveness and learning*

222 Neonicotinoid exposure as an adult (*post-eclosion* & *continual*) reduced the proportion of workers
223 responding to a sucrose droplet prior to the PER assay, and reduced olfactory learning performance during
224 the PER assay. These findings contribute to previous studies reporting adult neonicotinoid exposure
225 negatively affecting aspects of responsiveness in honeybees (Aliouane et al., 2009; Démares et al., 2018,
226 2016) and learning in bumblebees (Stanley et al., 2015). However, a key novelty of our study is that we
227 could compare responses from chronic exposure between age cohorts. Firstly, this revealed that young
228 (3-day) and older (12-day) workers from *post-eclosion* and *continual* exposure colonies were similarly
229 affected despite differences in the number of days of adult exposure. Additionally, despite *pre-eclosion* 3-
230 day adults being exposed for up to 3-weeks during brood development, compared to only three days of
231 exposure for 3-day adults from *post-eclosion* colonies, the degree of impaired learning was again similar.

Together these findings highlight the first 72 hours of adulthood to be critical in behavioural development, and reveals a susceptible developmental window to environmental stress (in this case pesticide exposure) (Sandrock et al., 2014; Wu et al., 2011), reiterating the importance of considering different life-stages when assessing pesticide risks.

Secondly, workers exposed during brood development (*pre-eclosion*), that received no or substantially lower exposure as an adult, exhibited impaired learning performance at a similar reduced level as adult only exposed workers (*post-eclosion*). This indicates a lag-effect from brood exposure on adult learning, highlighting the importance of considering delayed effects of pesticide exposure; a view reinforced by other studies on honeybees (*Apis cerana & A. mellifera*) and a stingless bee (*Melipona quadrifasciata anthidioides*) reporting larvae reared under topical or oral neonicotinoid exposure exhibited negative effects on adult learning and motor function (Tan et al., 2015; Tomé et al., 2012; Yang et al., 2012). More importantly, with 3- and 12-day adults from *pre-eclosion* colonies exhibiting a similar level of impaired learning performance, this reveals that the effects from brood exposure appear irrecoverable even as an unexposed adult.

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

Focusing on the mushroom body calyces, 12-day adult workers from all pesticide exposure treatments possessed smaller mushroom body calyces relative to *control* colonies, and we even found differences in 3-day adult workers from *post-eclosion* and *continual* exposed colonies. With rates of mushroom body development in bumblebees considered to be at its highest during the first 72 hours of adulthood (Jones et al., 2013; Riveros and Gronenberg, 2010), this may explain why in our experiment an effect on calyces volume was detected in just 3-days of adulthood. Furthermore, our finding that 3-day adults from both

254 *pre-eclosion* and *post-eclosion* exposed colonies showed similar reductions in mushroom body volumes,
255 reiterates the apparent vulnerability of brain development during the first 72 hours.

256 Average volume reductions of the mushroom body calyces and lobes showed a strikingly mirrored pattern
257 to the reduced proportion of learners in each respective pesticide treatment. More tellingly, when relative
258 volume of the calyces was plotted against each respective worker's learning score, bigger relative brain
259 size equated to better learning performance in *control* workers, but this relationship was not found for
260 pesticde exposed workers. Despite some *control* and pesticide exposure workers possessing similar
261 relative mushroom body volumes, pesticide exposure workers demonstrated a lower learning score
262 indicating impaired neuronal functioning of this brain region. For workers from *post-eclosion* and
263 *continual* exposed colonies this effect could be explained by sublethal neonicotinoid concentration
264 affecting neuronal signalling given its role as a nAChE receptor agonist (Palmer et al., 2013). However,
265 with *pre-eclosion* workers also being affected, this indicates that neonicotinoid exposure might be
266 affecting synaptic development (i.e. proliferation & dendritic outgrowth) in the calyces. Indeed, reduced
267 microglomeruli density has been shown to occur in neonicotinoid exposed honeybees (Peng and Yang,
268 2016), and density of these structures is correlated with increased learning and memory in bees (Hourcade
269 et al., 2010; Li et al., 2017). Furthermore, the reduced learning performance in *pre-eclosion* workers in our
270 study could stem from impeded neurogenesis where neuronal precursor cells are in some way prevented
271 from giving rise to Kenyon cells in the mushroom bodies, which in honeybees occurs during development
272 before eclosion from the pupal case (Fahrbach et al., 1995; Farris et al., 1999). Alternatively, the size of
273 Kenyon cells could be affected, as has been shown from exposure experiments on bumblebee cell cultures
274 (Wilson et al., 2013).

275 *Mushroom body calyces were disproportionately affected over the other neuropils*

276 The effect of neonicotinoid exposure was primarily localized to the mushroom body, and even within this
277 structure was manifested more heavily in the calyces than the lobes. Localised variation in plasticity has
278 been shown in bumblebees where foraging experience increased medial but not lateral calyx volume
279 (Riveros and Gronenberg, 2010). The calyces act as multisensory processors fed by afferent neurons,
280 whereas the lobes predominantly function as output regions with efferent neurons, which could explain
281 why calyx volumetric variation is more tightly associated with our measure of learning performance.
282 Antennal lobes are involved in detecting and processing olfactory information (Galizia et al., 1999;
283 Hansson and Anton, 2002; Sachse et al., 1999), developmentally plastic during early adulthood (Jones et
284 al., 2013; Riveros and Gronenberg, 2010), and exhibit reduced neuronal function under nicotinic agonists
285 (Andrione et al., 2016; Barbara et al., 2008; Thany and Gauthier, 2005). Considering pesticide exposed
286 workers performed worse in the olfactory conditioning, we might therefore expect a pattern of impeded
287 growth in the antennal lobes similar to that found for the mushroom body calyces. Whilst in support of
288 this view we found consistent negative model estimates for all three pesticide treatments, unlike the
289 calyces our analysis did not detect a significant effect. Furthermore, we found no consistent reduction in
290 volume of the optic lobes (medullas & lobulas) or central body for pesticide exposed workers.

291 Possible explanations for the disproportionate effect of neonicotinoid exposure on the mushroom bodies
292 may include: i) nACh receptors targeted by neonicotinoids are found in the highest density in the
293 Kenyon cells of the mushroom bodies (Galizia et al., 2011) and so could affect Kenyon cell
294 proliferation leading to volumetric reductions; ii) The mushroom bodies, in particular the calyces, of
295 social insects have consistently been reported to be highly plastic structures due to their role in learning
296 and memory development as early adults (Cabirol et al., 2018; Farris et al., 2001; Heisenberg, 2003; Jones
297 et al., 2013; Kühn-Bühlmann and Wehner, 2006; Riveros and Gronenberg, 2010). The large amount of
298 neuronal development and re-organisation therefore increases the risk of neurotoxic exposure interfering
299 with this process; iii) Our experimental setup was stimulus deprived and not void, therefore whilst

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 (Fahrbach et al., 1998; Jones et al., 2013); iv) the change in growth of non-mushroom body neuropils was simply too subtle for our µCT technology and/or sample sizes to detect.

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

Our study reared workers under a stimulus deprived environment, therefore a positive effect of age is indicative of experience independent age-enhanced learning. For both our measures of responsiveness and learning we found positive estimates for age, with 12-day workers performing better on average than 3-day. This finding contrasts with previous bumblebee studies reporting no effect of age on aspects of learning ability (Riveros and Gronenberg, 2009; Smith and Raine, 2014), but these were carried out in foraging arenas whereby prior experiences could not be fully controlled. Indeed, to our knowledge, there is a lack of studies looking to identify innate age-related growth on bumblebee behaviour as well as on brain growth. Only one histological study on the bumblebee *Bombus impatiens* by Jones and colleagues has shown age-dependent volumetric growth in brain neuropils separate form environmental stimuli (Jones et al., 2013). Their findings suggested c.10% increase in the mushroom body calyces and lobes which interestingly is around half the increase we found in our *control* workers (just over 20%), a difference that perhaps stems from variation in methodological approaches, sample sizes (lower in Jones et al.) or taxonomic variation. However, together this evidence supports an innate increase in neuropil volume over the first 12-days of adulthood, which presumably is important to prepare workers for the complex colony tasks required at this age (Maleszka et al., 2009).

Implications of our findings for social insects

Our findings that early exposure effects later adult behaviour provides a mechanistic explanation for why reduced colony growth is often detected 2-3 weeks after onset of neonicotinoid exposure (Arce et al., 2017; Gill et al., 2012; Rundlöf et al., 2015; Siviter et al., 2018a; Tsvetkov et al., 2017; Whitehorn et al., 2012), and why reduced colony productivity has been correlated with neonicotinoid treated neighbouring fields (Rundlöf et al., 2015; Woodcock et al., 2016). With eusocial bee colonies having overlapping generations, colonies are reliant on newly emerging cohorts of workers to be effective task performers. 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 (Bryden et al., 2013). Our results suggest that even if workers were to delay undertaking a task, such as foraging, in attempt to developmentally recover, this strategy may be futile given we saw little adult recovery from 3 to 12 days of adulthood from *pre-eclosion* colonies. Importantly, these effects are unlikely to be exclusively applicable to neonicotinoids as a multitude of other neurotoxic pesticides including the possible neonicotinoid replacements, sulfoxamines and butenolides (Siviter et al., 2018a; Tosi and Nieh, 2019), are likely to end-up inside bee colonies with the potential to influence tissue development in reared bees.

Materials & methods

Animal Husbandry

Twenty-two *Bombus terrestris audax* colonies were delivered by a commercial supplier (Agralan Ltd), with colonies possessing a queen and mean (±s.e.m.) of 14.5 ± 1.1 workers on arrival (Table S9) and housed in an aerated plastic box (29 x 22.5 x 13 cm). All colonies were moved to a controlled environment (23°C; 60% humidity) red light room where they remained for the duration of the experiment. Throughout the experiment, colonies were provisioned untreated honeybee collected pollen (supplied by a commercial

supplier Agralan Ltd) ad-libitum in a petri dish, and 40/60% sucrose/water solution in a gravity feeder. Food was replenished every two days, and feeders thoroughly cleaned prior to refill (Table S10 for colony consumption). During Phase I (days 1-21; Figure 1; Figure S3), we conducted daily checks of all newly eclosed bees and marked each using a white paint pen (uni Posca, PC.5M 1.8-2.5mm), allowing us to distinguish between newly eclosed workers during Phase II (day 22 onwards) from eclosed workers before this. Colonies were checked daily for males, gynes or dead individuals which were removed and frozen at -20°C.

Experimental setup

On arrival, colonies were randomly assigned to the four treatments, with no significant difference in the number of workers between treatments (ANOVA: $F_{3,22}$=1.04, p=0.40). Mean worker thorax width was similar between treatments (LMM, p>0.07) with *control* being 4.23mm (range=3.29-5.17), *pre-eclosion* 4.16mm (3.09-5.12), *post-eclosion* 4.28mm (3.14-5.63) and *continual* 4.33mm (3.36-5.34). Monitoring overall development of workers in colonies, we implemented a fully factorial design with our colony treatments comprising a combination of two exposure phases: Phase I encompassing the majority of brood (larval & pupal) development period and Phase II comprising the early adult development period (up to 12 days). Phase I exposure period started two days after colonies arrived and lasted for 21 days approximating development time from an egg or very small larva to adult eclosion (Alford, 1975; Cnaani et al., 2002; Duchateau and Velthuis, 1988). This ensured that all sampled adults will have been exposed/unexposed in a standardised manner during the vast majority of brood development (Figure S3). On the 22nd day Phase II started, during which we checked daily for callow workers (adults recently eclosed from their pupal case) and tagged each with a unique numbered Opalith tag using superglue. On tagging, we randomly assigned half of the workers per colony per day to the 3-day cohort and remaining half to the 12-day cohort, with tag ID used to correctly remove for testing 3 or 12 days later. Tagging period lasted

11 days to provide us with a high number of workers to test (Table S2). This window of opportunity approximates the minimum time of pupal development, in which pupae evacuate their gut and stop feeding (Cnaani et al., 2002), allowing us to standardised pesticide exposure as best possible across all tested workers. Adult workers aged 3 and 12-days after eclosion were chosen to be tested as brain development has been reported to occur both during brood and early adult stages (Farris et al., 2001; Jones et al., 2013).

We applied four treatments to colonies: *control* = phases I & II unexposed (n=5 colonies); *pre-eclosion* = phase I exposed to Imidacloprid, phase II unexposed (n = 6); *post-eclosion* = phase I unexposed, phase II exposed to Imidacloprid (n = 6); *continual* = phases I & II exposed to Imidacloprid (n = 5). The neonicotinoid imidacloprid was used as: i) it is widely used across the globe (Casida, 2018; Cressey, 2017; Mitchell et al., 2017; Zhang, 2018); ii) it targets nAChE receptors found in insect brains (Jeschke and Nauen, 2008; Palmer et al., 2013); iii) exposure has been shown to affect bee foraging and navigation known to be reliant on learning ability and working memory (Feltham et al., 2014; Fischer et al., 2014; Gill and Raine, 2014; Samuelson et al., 2016; Stanley and Raine, 2016). The imidacloprid treated sucrose solution provisioned to the colony was made from a primary stock solution (1mg/ml) consisting of 100mg of imidacloprid (powder; grade: PESTANAL®, analytical standard; brand: Fluka) dissolved in 100ml of acetone. An aliquot was then added to a 40/60% sucrose/water solution to produce a 5ppb imidacloprid solution of required volume. A *control* sucrose solution was made by repeating this process but a same aliquot volume of pure acetone.

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

The proboscis extension reflex (PER) conditioning paradigm we implemented was adapted from a previously reported setup on bumblebees (Stanley et al., 2015). On removal from the colony, workers were harnessed (between 13:00-14:00) using a modified 2ml centrifuge tube and a split pin yoke, under

392 natural light in the lab and left for 2hrs to settle (Figure S1 for harness setup). All bees were then fed to
393 satiety using a Gilmont® syringe to present 40% sucrose solution droplets directly to the mouthparts and
394 left for 18 hours (overnight) in a separate controlled environment room under identical conditions as the
395 rearing room. For unknown reasons, 24 workers did not survive overnight and were excluded from any
396 data analysis. Between 08:00-09:00 the PER testing began on the remaining bees (n=413) by first testing
397 their PER responsiveness to a 50% sucrose solution. Immediately after, each bee was fed a small droplet
398 (0.8µl) of the sucrose solution for motivation 15 minutes before the start of the PER test (Figure S1).
399 PER conditioning was conducted in front of a filtered ventilation system (Expo Drills & Tools AB500
400 Extractor fan), preventing the odour coming in to contact with neighbouring harnessed bees. Each bee
401 was initially conditioned by exposure to clean air for 5 seconds, followed by scented air for 10 seconds. A
402 harnessed bee was positioned 3 cm away from a glass odour tube, with the airflow delivered at a constant
403 rate of 80 ml/second (Tetra APS – 100), which was channelled through either a 'clean' unscented odour
404 tube or diverted through a 'scented' odour tube containing a piece of filter paper (5 x 20 mm) impregnated
405 with 1 µl of lemon essential oil (Naturally Thinking Ltd.). Airflow between the clean and scented tube was
406 controlled by a solenoid valve (Nass Magnet 108-030-0257 24vAC/12vDC) connected to a Raspberry Pi 2
407 (Model B) computer to ensure each bee was exposed to a consistent amount of clean and scented air. To
408 develop an association between the lemon odour and the reward, we touched the bee's antennae with a
409 droplet of 0.8 µl of 50% sucrose 6 seconds into the 10 second odour delivery phase and allowed the bee
410 to feed.
411 Following trial 1, the odour and reward presentation sequence was repeated to each adult an additional
412 nine times. The inter-trial interval (ITI) per individual was 10 minutes allowing us to conduct the PER
413 testing in batches of up to 20 workers. We waited 15 seconds after the odour and reward presentation
414 sequence before moving to the next individual (Smith and Burden, 2014). We recorded whether the bees
415 showed a PER to the odour stimulus prior to or after the reward, which were defined as a learnt or non-

learnt response respectively. This provided a number of learnt responses achieved by each worker over the nine trials, enabling us to estimate the probability per trial of workers demonstrating a learnt response for each treatment. If a bee responded to the initial conditioning trial (trial 1) before the reward had been presented (n=24) the individual was excluded from the experimental analyses. If a bee showed no PER (did not feed) even after the reward was provided, and exhibited this over the next three consecutive trials, the individual was removed from testing from that point and categorised as a non-learner.

Micro-CT scanning

Linking variation in learning to differences in brain growth requires high-resolution imaging technology that can explore minute changes to soft tissue. Using traditional histological methods would have been technically challenging as it relies on physical extraction from the headcase followed by tissue fixation, dehydration, embedding and sectioning (Simmons and Swanson, 2009) increasing the risk of destructive sampling. Potentially this could have caused a greater change to neuropil volume than the experimental treatment itself. We attempted to overcome such challenges by using micro-computed tomography (µ-CT) scanning.

Following the PER assay, bees were humanely sacrificed by swiftly decapitating the live individual using a disposable surgery scalpel and heads immediately fully submerged in a 70/30% ethanol/de-ionised water solution in separate 1.5ml centrifuge tubes and stored at 5°C. Preparation of the heads followed precisely the published protocol by Smith et al. (2016) with the soft brain tissue being stained for seven days with phosphotungstic acid (PTA) 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). The staining and scanning methodology we employed has been shown to give us confidence in the accuracy of our measurements of these complex neuropil structures (Smith et al., 2016). The 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 3D reconstructed scan was then re-oriented to the same optimum plane-of-view for visualization, and for the neuropils in question we re-sliced into a new series of 2D 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, but based on staining quality and that both left and right structures could be segmented (including both medial and lateral calyx for the mushroom body calyces) we successfully segmented the mushroom bodies for 78 workers, central body for 88, antennal lobes for 89, medullas for 71 and lobulas for 71 (Table S4). For the purposes of comparing relative volumes (absolute volume divided by ITD to correct for body size), bees were originally sampled to have a balanced representation across treatments and age but blind of learning performance.

Neuropil volume measurements

Segmentation and volume analysis of brain structures was carried out using the software SPIERS 2.20 (Serial Paleontological Image Editing and Rendering System). For segmentation, scan slices were converted to binary threshold images (of white active pixels and black inactive pixels) adjusted to achieve an optimum ratio of active white pixels that comprise the structure of interest, and inactive black pixels for the surrounding tissue. For each component structure, looped splines were placed around the active pixels at regular five slice intervals which were then interpolated across all slices between the intervals, so that each structure could be defined as an independent object for 3D reconstruction and volumetric calculation (for full segmentation protocol see Smith et al. 2016). This soft tissue segmentation protocol has been shown to provide repeatable and precise volumetric measurements of morphological structures of the bumblebee brain. To calculate absolute volume of each structure we used the voxel count function in SPIERS Edit, with relative volume calculated by dividing by the inter-tegula width (standard proxy for body size (Cane, 1987)). Inter-tegula width was measured using digital callipers (Workzone®) with the mean of two repeated measurements used. A single value was used in our analyses for each of the

464 mushroom bodies, antennal lobes, lobulas and medullas by summing the volume of the left and right
465 paired structures.
466
467 ***Data Analysis***
468 Statistical analyses were conducted in R version 3.5.1 (R Development Core Team 2018) using RStudio
469 version 1.1.463, with models implemented using the lme4 package (Bates et al., 2015). For all models we
470 included *treatment* as a fixed categorical factor. We considered measure of bee body size (*ITD*) as a
471 continuous variable and *colony* as a random factor in our models if inclusion increased the fit of the model
472 (model comparisons were assessed by comparing the AIC) otherwise they were not retained. For
473 responsiveness and learning the data was analysed using the proportion of individuals showing a response
474 with a generalized linear model (glm) using a binomial distribution and included the categorical variable
475 *age* (3 or 12-day) as an additional fixed factor with *age* x *treatment* interaction term removed as it showed
476 no significant effect. For looking at the proportion of learners by trial we used a linear mixed effects model
477 (lmer) in which treatment consisted of two categories, *control* workers and pesticide workers (pooled
478 from all three pesticide treatments due to low sample sizes per treatment). We considered a second order
479 polynomial fit for *trial* number and individual *ID* as a random factor. For relative neuropil volumes we used
480 a linear mixed-effects model (LMER) that included *age, ITD* and *colony* (random effect). We used a
481 binomial generalised linear model (glm) to analyse how calyces' volume influenced the learning score, as
482 a proportion of the maximum learning score that could be achieved using calyces volume to analyse the
483 *calyces volume* x *treatment* interaction on score.
484
485 **Author Contributions**

486 RJG conceived the project; DBS & RJG designed the experiment; DBS, ARR & PHB conducted the
487 experiment; DBS & FA carried out the µCT scanning; DBS & DB reconstructed and segmented the brains;

ANA, DBS & RJG performed data analyses; DBS & RJG wrote the manuscript and ANA provided critical feedback.

Acknowledgements

We thank Russell Garwood for help using SPIERS software, Dan Sykes and Amin Garbout for help with the µCT scanning protocol, Paul Beasley for technical support, Alfredo Sánchez Tójar and Peter Graystock for advice on da ta analysis, and Richard Abel, Mark Brown, Inti-Pedroso, Nigel Raine and Seirian Sumner for advice on pilot work. This work was supported by NERC grants (NE/L00755X/1 & NE/P012574/1) awarded to RJG which funded ANA and ARR. DBS's PhD was supported by a NERC funded SSCP DTP scholarship in affiliation with the Grantham Institute at Imperial College London. RJG is supported by Imperial College's Grand Challenges in Ecosystems and the Environment initiative.

Supplementary Figures

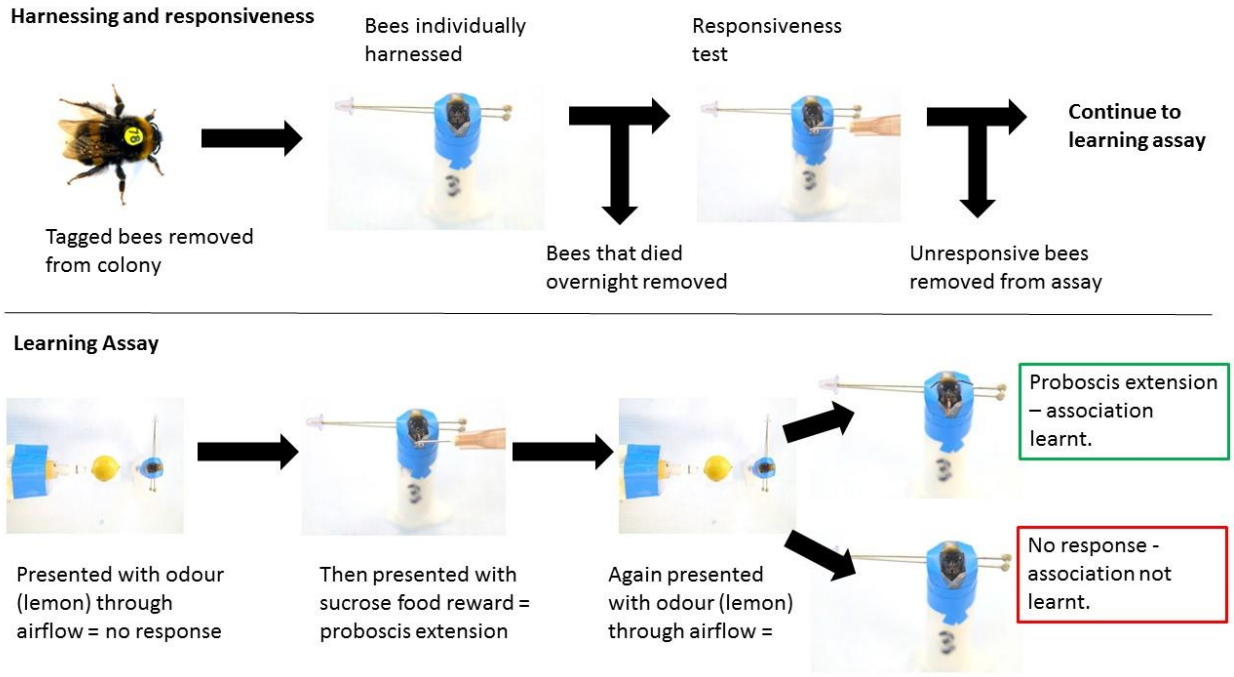


Figure S1. Proboscis extension reflex assay setup and step-by-step guide. Individuals were placed inside a plastic test tube and the tube was placed on ice for 10 minutes. Individuals were then harnessed in modified 2ml centrifuge tubes and a split pin yoke held them in place with electrical tape (blue). Harnessed bees were always placed the same distance from the air flow odour source and an extractor fan was mounted behind to remove excess odour.

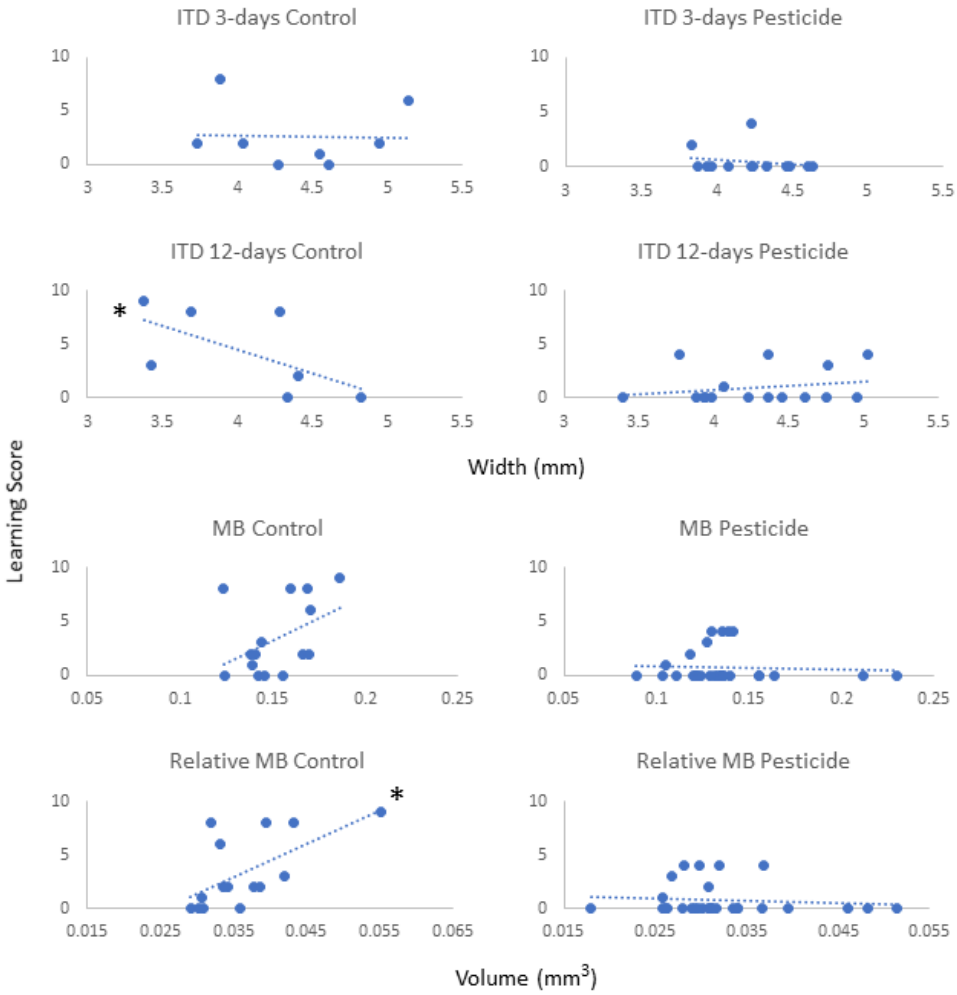
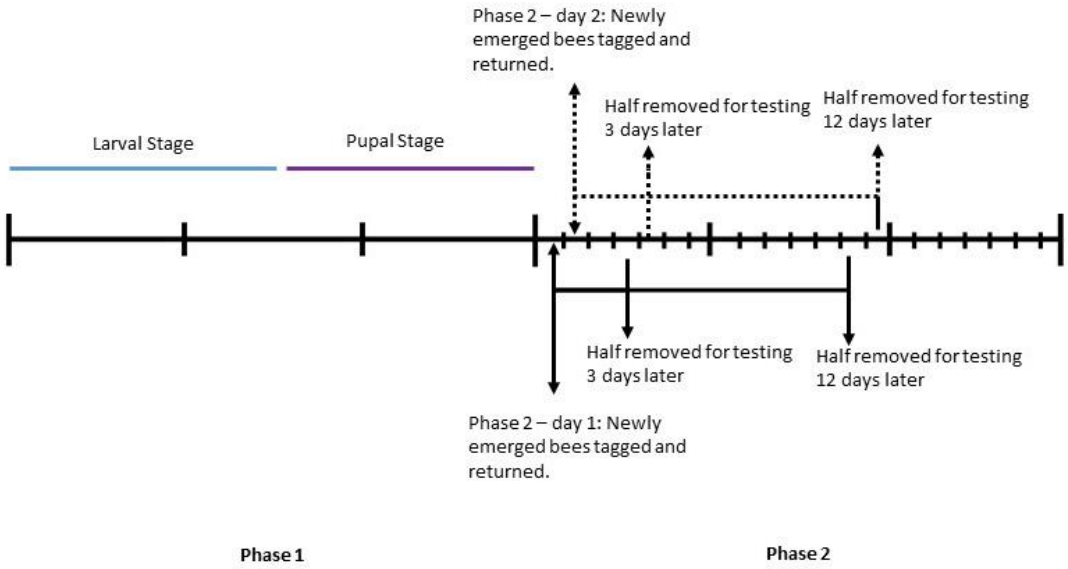


Figure S2. Predictor variables individual body size and mushroom body (MB) volumes were plotted against respective learning scores. The effect of width (mm) of the intertegula distance (ITD; proxy for body size) for 3-day and 12-day adult workers are shown. The absolute and relative (corrected for body size) whole mushroom body volumes (mm^3), representing the combined volumes of the left and right hemispheres, for all workers regardless of age are shown. Filled circles represent the raw data with a dashed trend line fitted. Asterisks denote a significant negative or positive relationship (α=0.05) based on a Spearman's Rank correlation analysis (detailed in Table S8).

519



520

521 **Figure S3. Sampling for 3 and 12-day adult cohorts.** During Phase I: all newly eclosed bees were marked
522 using a white paint pen, but this also represents the developmental time of workers with our sampled
523 bees having been larvae (blue) and pupae (purple) during this 21 day development period. Phase II: for 11
524 days, colonies were checked daily with any newly eclosed workers being tagged and returned to their
525 natal colony with half randomly assigned to a 3-day cohort and the other half a 12-days cohort. The
526 respective cohorts were then removed 3 days or 12 days later. This continual tagging and sampling over
527 the 11 day period provided us with a large number of workers to test (here we provide an example for
528 the first two days for demonstration purposes).

529

530

Supplementary Tables

Table S1 Informed from honeybee studies, our study focused on five key neuropils considered to be involved in the following primary functional roles.

Structure	Function	References
Mushroom Bodies	Associated with higher cognition and learning with the processing of multimodal sensory information. Possess distinct sub-compartments each with functional specialisation.	(Durst et al., 1994; Fahrbach, 2006; Farris et al., 2001; Hammer and Menzel, 1995; Heisenberg, 2003, 1998; Mobbs, 2006; Zars, 2000)
Antennal Lobes	Principle olfactory centre associated with processing of chemical stimuli.	(Hansson and Anton, 2002)
Medullas & Lobulas (optic lobes)	Processing of visual information	(Dyer et al., 2011; Lotto and Chittka, 2005; A. C. Paulk et al., 2009; Angelique C. Paulk et al., 2009; Paulk et al., 2008; Pfeiffer and Homberg, 2013)
Central Body (a.k.a. fan shaped body)	Considered involved in locomotion and orientation	(Li et al., 2009; Pfeiffer and Homberg, 2013; Strauss, 2002)

Table S2. Number of 3- and 12-day adult workers from each colony that were prepared for tested using our PER setup. 'Tagged' = number of newly emerged workers that were tagged with a unique colour and numbered Opalith tag; 'Survived to Harnessing' = number of tagged workers survived to 3- or 12-days post-emergence and could be harnessed for the PER assay; 'Harnessed & survived overnight' = number of workers that were harnessed but also were alive inside the harness the next day (n=24 died overnight); 'Responsive' = number of workers that exhibited a PER response on touching the antenna with a 50% sucrose solution droplet; 'Considered for PER assay' = the number of workers remaining once any workers showing a PER to the lemon odour and before the sucrose provision on the 1st trial were removed; 'Learner' = number of workers that showed at least one olfactory conditioned PER response over the ten trials.

Treatment	Colony	Tagged		Survived to harnessing		Harnessed & survived overnight		Responsive		Considered for PER assay		Learner	
		3	12	3	12	3	12	3	12	3	12	3	12
control	1	16	29	15	29	13	11	9	8	7	5	5	4
control	2	18	40	17	40	16	17	9	9	8	7	3	7
control	3	13	22	13	21	12	10	6	6	4	5	2	1
control	13	18	22	18	21	17	13	7	12	4	8	1	6
control	14	0	2	0	2	0	1	0	0	0	0	0	0
		65	**115**	**63**	**113**	**58**	**52**	**31**	**35**	**23**	**25**	**11**	**18**
pre-eclosion	4	9	7	9	7	9	5	4	2	4	2	1	0
pre-eclosion	5	21	26	21	25	20	19	10	11	10	11	4	1
pre-eclosion	6	9	4	8	4	8	3	3	1	3	1	1	0
pre-eclosion	15	17	24	16	21	16	15	5	11	4	11	0	1
pre-eclosion	16	6	12	6	12	6	8	2	6	2	6	0	1
pre-eclosion	17	4	4	4	4	4	3	2	2	2	2	0	0
		66	**77**	**64**	**73**	**63**	**53**	**26**	**33**	**25**	**33**	**6**	**3**
post-eclosion	7	4	2	4	2	4	2	1	0	1	0	0	0
post-eclosion	8	14	28	14	27	11	12	5	7	5	7	2	1
post-eclosion	9	11	11	11	11	11	7	5	4	4	4	2	1
post-eclosion	18	19	28	19	26	18	16	2	8	2	7	0	1
post-eclosion	19	11	17	11	15	9	9	3	8	3	7	0	4
post-eclosion	20	4	9	4	6	4	5	2	2	2	2	0	0
		63	**95**	**63**	**87**	**57**	**51**	**18**	**29**	**17**	**27**	**4**	**7**
continual	10	15	39	15	35	11	10	4	2	3	2	0	0
continual	11	13	16	13	15	12	8	5	2	4	2	1	1
continual	12	6	4	5	4	5	2	3	2	3	2	2	2
continual	21	14	26	13	25	13	8	3	8	3	8	0	3
continual	23	6	18	6	17	6	4	1	3	1	3	0	1
		54	**103**	**52**	**96**	**47**	**32**	**16**	**17**	**14**	**17**	**3**	**7**
	Totals	**248**	**390**	**242**	**369**	**225**	**188**	**91**	**114**	**79**	**102**	**24**	**35**
			638		**611**		**413**		**205**		**181**		**59**

Table S3. Statistical comparisons of responsiveness and learning. **a-b)** statistical outputs from binomial Generalized Linear Model in R (GLM) when analysing the proportion of workers that showed a PER response to a sucrose droplet prior to undertaking the learning assay, and the proportion of workers showing at least one olfactory conditioned PER learnt response during trials 2-10 of the learning assay. Exposure treatments are comparisons to *control* workers ('intercept') with *age* (3 versus 12-day workers) and ITD (inter-tegula distance which is a proxy for body size) considered. For both models the interaction term between pesticide treatment and age was removed as no significant effect could be detected. **c)** statistical output from a binomial Linear Mixed Effects Model in R (LMER) when analysing the proportion of workers that showed a PER response over the PER assay trials. For this model all workers from the three pesticide exposure treatments were pooled to compare one cohort against *control* workers (intercept). A 2nd order polynomial relationship was found to be the best fit to the data. Significant differences (α=0.05) are highlighted in bold.

a) Responsive				
GLM(responsive(y/n) ~ treatment + age + ITD, family = binomial				
	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-1.279	0.959	-1.333	0.182
pre-eclosion	-0.342	0.276	-1.242	0.214
post-eclosion	-0.714	0.282	-2.529	**0.011**
continual	-0.737	0.307	-2.400	**0.016**
age	0.842	0.205	4.101	**<0.001**
ITD	0.306	0.218	1.406	0.160
b) Learners				
GLM(learner(y/n) ~ treatment + age + ITD, family = binomial				
(Intercept)	-3.865	1.820	-2.123	0.034
pre-eclosion	-2.086	0.476	-4.382	**<0.001**
post-eclosion	-1.663	0.476	-3.490	**<0.001**
continual	-1.409	0.507	-2.778	**0.005**
age	0.413	0.360	1.147	0.251
ITD	0.953	0.413	2.308	**0.021**
c) Learners by trial				
LMER(learner(y/n) ~ treatment + poly(trial,2) + (1|worker_id), family = binomial				
(Intercept)	-0.889	0.308	-2.887	0.004
pesticide_treatments	-0.861	0.413	-2.083	**0.037**
poly(trial, 2)1	48.427	5.398	8.972	**<0.001**
poly(trial, 2)2	-18.909	3.965	-4.769	**<0.001**

Table S4. Average volumes (mm^3) of segmented brain neuropils using µCT scanning for each experimental treatment and age cohort. Mean (± s.e.m.) values are based on workers across all colonies and represent relative volumes (absolute volume divided by workers size). The percentage difference (% diff.) of the mean of each pesticide treatment relative to the control group is provided with negative values showing smaller and positive values showing larger average volumes. Based on the quality of staining and scanning, sample sizes (n) for each neuropil were limited to those that had both structures from the left and right brain hemispheres successfully segmented. (N.B. for the *central body* one individual (18G) was removed from the analysis as it represented an extreme outlier likely caused by a segmentation error).

treatment	age	n	mean	s.e.m.	% diff.	n	mean	s.e.m.	% diff.
		total mushroom bodies				***mushroom body lobes***			
control	3	9	0.1413	0.0057		10	0.0496	0.0013	
pre-eclosion	3	11	0.1402	0.0094	-0.8	11	0.0504	0.0039	1.6
post-eclosion	3	10	0.1166	0.0051	-17.5	13	0.0453	0.0022	-8.7
continual	3	11	0.1306	0.0105	-7.5	13	0.0485	0.0032	-2.3
control	12	8	0.1607	0.0050		10	0.0569	0.0017	
pre-eclosion	12	11	0.1307	0.0076	-18.7	11	0.0487	0.0039	-14.3
post-eclosion	12	10	0.1287	0.0079	-19.9	11	0.0480	0.0029	-15.7
continual	12	8	0.1346	0.0065	-16.2	9	0.0486	0.0026	-14.5
		mushroom body calyces							
control	3	9	0.0916	0.0045					
pre-eclosion	3	11	0.0897	0.0037	-2.0				
post-eclosion	3	10	0.0733	0.0056	-20.0				
continual	3	11	0.0813	0.0040	-11.3				
control	12	8	0.1040	0.0030					
pre-eclosion	12	11	0.0819	0.0050	-21.2				
post-eclosion	12	10	0.0813	0.0069	-21.8				
continual	12	8	0.0852	0.0039	-18.0				
		central body				***antennal lobes***			
control	3	10	0.000597	0.000031		10	0.00522	0.00020	
pre-eclosion	3	12	0.000555	0.000040	-7.0	12	0.00509	0.00020	-2.4
post-eclosion	3	13	0.000582	0.000041	-2.6	13	0.00418	0.00013	-20.0
continual	3	14	0.000586	0.000029	-1.9	14	0.00479	0.00019	-8.3
control	12	10	0.000645	0.000046		10	0.00590	0.00035	
pre-eclosion	12	9	0.000599	0.000514	-10.1	10	0.00581	0.00070	-1.5
post-eclosion	12	11	0.000584	0.000067	-9.5	11	0.00516	0.00036	-12.6
continual	12	9	0.000607	0.000023	-5.9	9	0.00467	0.00018	-20.9
		lobulas				***medullas***			
control	3	7	0.00816	0.00035		7	0.0252	0.0009	
pre-eclosion	3	11	0.00866	0.00175	6.1	11	0.0261	0.0013	3.7
post-eclosion	3	10	0.00844	0.00083	3.4	10	0.0262	0.0011	4.0
continual	3	8	0.00933	0.00199	14.4	8	0.0293	0.0029	16.5
control	12	9	0.00938	0.00115		9	0.0280	0.0014	
pre-eclosion	12	10	0.00981	0.00197	4.6	10	0.0275	0.0020	-1.8
post-eclosion	12	8	0.00970	0.00137	3.4	8	0.0305	0.0015	9.0
continual	12	8	0.00906	0.00078	-3.4	8	0.0268	0.0009	-4.5

578 **Table S5. Statistical comparisons of mushroom body relative volumes**. Statistical outputs from a
579 Generalized Mixed Effects Model in R (LMER) for the combined volumes of the left and right **a.** whole
580 mushroom bodies, **b.** the medial and lateral calyces of the mushroom body together, **c.** lobes of the
581 mushroom body for only those individuals that also had the calyxes segmented (n=78), d. the lobes of the
582 mushroom body for any individual that had them segmented (n=88). Exposure treatments are
583 comparisons to *control* workers ('intercept') with *age* (3 versus 12-day workers) and ITD (inter-tegula
584 distance - proxy for body size) considered. For both models the interaction term between pesticide
585 treatment and age was removed as no significant effect could be detected. Significant differences (α=0.05)
586 highlighted in bold black.

587

a) Total Mushroom Bodies (n=78)					
LMER(relativeMB ~ treatment + age + size + (1|colony)					
	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	0.058	0.008	43.244	7.732	<0.001
pre-eclosion	-0.004	0.002	5.845	-1.822	0.120
post-eclosion	-0.007	0.002	6.166	-2.873	**0.027**
continual	-0.005	0.002	6.348	-2.032	0.086
age	0.002	0.001	67.913	1.473	0.145
ITD	-0.006	0.002	49.038	-3.246	**0.002**
b) Total Mushroom Body Calyces (n=78)					
LMER(relativeMBCalyces ~ treatment + age + size + (1|colony)					
(Intercept)	0.035	0.005	40.979	7.735	<0.001
pre-eclosion	-0.003	0.001	6.637	-2.409	**0.049**
post-eclosion	-0.005	0.001	7.212	-3.828	**0.006**
continual	-0.004	0.001	7.626	-2.896	**0.021**
age	0.001	0.001	69.152	1.565	0.122
ITD	-0.003	0.001	44.961	-2.919	**0.005**
c) Total Mushroom Body Lobes (n=78)					
LMER(relativeMBLobes ~ treatment + age + size + (1|colony)					
(Intercept)	0.024	0.003	65.458	8.182	<0.001
pre-eclosion	-0.001	0.001	6.440	-0.890	0.406
post-eclosion	-0.001	0.001	6.184	-1.181	0.281
continual	-0.001	0.001	6.350	-0.936	0.383
age	0.001	0.000	74.263	1.137	0.259
ITD	-0.003	0.001	76.887	-4.285	**<0.001**
d) Total Mushroom Body Lobes (n=88)					
LMER(relativeMBLobes ~ treatment + age + size + (1|colony)					
(Intercept)	0.024	0.003	53.979	7.295	<0.001
pre-eclosion	-0.001	0.001	5.995	-0.865	0.421
post-eclosion	-0.002	0.001	6.217	-1.387	0.213
continual	-0.001	0.001	6.261	-0.757	0.476
age	0.001	0.001	66.485	1.145	0.256
ITD	-0.003	0.001	63.112	-3.661	**<0.001**

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589 **Table S6. Statistical comparisons of the relative volumes of the central body, antennal lobes, lobulas**
590 **and medullas**. Statistical outputs are for the combined volumes of the left and right (except the central
591 body), from a Generalized Mixed Effects Model in R (LMER). Exposure treatments are comparisons to
592 *control* workers ('intercept') with *age* (3 versus 12-day workers) and ITD (inter-tegula distance which is a
593 proxy for body size) considered. For both models the interaction term between pesticide treatment and
594 age was removed as no significant effect could be detected. Significant differences (α=0.05) highlighted
595 in bold black.
596

Central Body n=88					
lmer - central Body ~ treatment + age + size + (1|colony)					
	Estimate	**s.e.**	**df**	**t**	**p**
(Intercept)	0.000573	0.000176	64.7	3.26	0.002
Pre-eclosion	0.000074	0.000073	5.9	1.01	0.351
Post-eclosion	-0.000002	0.000072	5.6	-0.03	0.976
Continual	0.000001	0.000072	5.7	0.02	0.985
age	0.000027	0.000028	74.4	0.96	0.339
size	-0.000102	0.000038	78.4	-2.67	**0.009**
Antennal Lobes n=89					
lmer - antennal lobes ~ treatment + age + size + (1|colony)					
(Intercept)	0.003658	0.000320	63.6	11.42	<0.001
Pre-eclosion	-0.000034	0.000133	5.5	-0.26	0.807
Post-eclosion	-0.000208	0.000130	5.3	-1.60	0.168
Continual	-0.000176	0.000131	5.3	-1.35	0.232
age	0.000101	0.000051	73.9	1.98	0.051
size	-0.000553	0.000070	77.9	-7.96	**<0.001**
Lobulas n=71					
lmer - lobulas ~ treatment + age + size + (1|colony)					
(Intercept)	0.004984	0.000493	47.1	10.11	<0.001
Pre-eclosion	0.000052	0.000140	6.4	0.37	0.723
Post-eclosion	0.000034	0.000142	7.3	0.24	0.818
Continual	0.000057	0.000146	7.4	0.39	0.706
age	0.000192	0.000083	61.5	2.30	**0.025**
size	-0.000693	0.000108	52.2	-6.44	**<0.001**
Medullas n=71					
lmer - medullas ~ treatment + age + size + (1|colony)					
(Intercept)	0.014020	0.001639	42.7	8.55	<0.001
Pre-eclosion	-0.000094	0.000425	6.7	-0.22	0.832
Post-eclosion	0.000295	0.000435	8.2	0.68	0.517
Continual	0.000208	0.000447	8.0	0.46	0.655
age	0.000369	0.000287	62.5	1.29	0.203
size	-0.001826	0.000360	46.9	-5.08	**<0.001**

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Table S7. Statistical output from a binomial generalised linear model in R (GLM) when analysing the final learning score achieved by each worker by the end of PER assay trials. For this model all workers from the three pesticide exposure treatments were pooled to compare one cohort against *control* workers (intercept). Significant differences (α=0.05) are highlighted in bold.

glm(learning_score ~ relativeMBCalyces*treatment, family = binomial)				
	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-6.992	1.416	-4.939	<0.001
calyces	265.823	58.981	4.507	**<0.001**
pesticide	5.611	1.802	3.113	**0.002**
calyces*pesticide	-321.899	81.268	-3.961	**<0.001**

Table S8. Spearman's rank correlations to investigate the relationship between the response variable learning score and the predictor variables body size (ITD) or mushroom body volume. Row 1 is intertegula distance (ITD; proxy for body size) for workers regardless of age; Rows 2 & 3 are ITDs for 3 and 12-day workers (respectively); Rows 4-5 is the absolute volume of the whole mushroom bodies (left and right combined), and relative volume of the whole mushroom bodies (left and right combined) corrected for body size, respectively; Rows 6-9 also show absolute and relative volumes for the calyces and lobes separately (left and right combined). Significant differences (α=0.05) are highlighted in bold red, with near significant differences (α=0.1) in bold black.

Row	MB Component	Control		Pesticide	
		SR	p	SR	p
1	**ITD**	-0.509	**0.053**	0.020	0.919
2	**ITD 3-Day**	-0.123	0.772	-0.314	0.295
3	**ITD 12-Day**	-0.818	**0.024**	0.171	0.527
4	**MB**	0.403	0.136	-0.030	0.879
5	**Relative MB**	0.690	**0.004**	-0.126	0.516
6	**MB Calyces**	0.354	0.195	-0.047	0.807
7	**Relative MB Calyces**	0.622	**0.013**	-0.130	0.503
8	**MB Lobes**	0.579	**0.024**	0.007	0.972
9	**Relative MB Lobes**	0.633	**0.011**	-0.098	0.612

619 **Table S9. Colony census at start of the experiment, and at the end of each phase.** All workers
620 eclosing during Phase II were tagged with a unique colour and numbered Opalith tag so the age of
621 each worker was known.

622

colony#	treatment	starting # workers	# workers eclosed	
			end of phase I	end of phase II (i.e. tagged)
1	*Control*	25	27	45
2	*Control*	17	48	58
3	*Control*	20	26	35
13	*Control*	10	28	40
14	*Control*	17	12	2
4	*Pre-eclosion*	9	17	16
5	*Pre-eclosion*	24	23	47
6	*Pre-eclosion*	18	36	13
15	*Pre-eclosion*	16	36	41
16	*Pre-eclosion*	11	19	18
17	*Pre-eclosion*	8	24	8
7	*Post-eclosion*	12	41	6
8	*Post-eclosion*	20	54	42
9	*Post-eclosion*	20	34	22
18	*Post-eclosion*	15	24	47
19	*Post-eclosion*	12	41	28
20	*Post-eclosion*	7	33	13
10	*Continual*	12	51	54
11	*Continual*	14	42	29
12	*Continual*	17	20	10
21	*Continual*	13	38	40
22	*Continual*	6	24	24

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Table S10. Colony daily sucrose consumption (ml) as the experiment progressed (days 1-43). Of the total provisioned sucrose (1,110ml per colony), *control, pre-eclosion, post-eclosion* and *continual* treatments consumed a median (IQR) of 54 (50-56), 47 (37-57), 61 (48-69) and 51 (35-63) % respectively.

Treatment	Colony #	Consumption (ml) per day																					
		1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	41	43
Control	**1**	9	15	25	15	18	21	20	22	28	34	15	40	30	35	37	37	31	36	35	33	33	37
Control	**2**	14	20	29	17	20	20	29	28	27	33	13	28	28	30	30	34	26	32	35	38	37	35
Control	**3**	10	10	19	14	20	20	21	22	23	30	14	30	28	38	37	38	30	37	35	37	39	39
Pre-eclosion	**4**	7	10	13	10	13	14	15	12	16	19	9	20	17	20	16	17	16	21	21	22	25	26
Pre-eclosion	**5**	13	12	24	18	24	22	20	20	23	26	12	27	25	30	31	32	28	32	30	33	35	35
Pre-eclosion	**6**	25	21	31	24	33	33	31	31	29	39	14	41	34	39	37	40	28	35	37	35	37	45
Post-eclosion	**7**	13	10	29	13	17	18	16	17	20	26	12	26	37	45	46	43	36	43	48	48	54	59
Post-eclosion	**8**	29	22	32	21	24	24	29	23	30	37	14	30	34	39	39	38	32	42	57	42	45	52
Post-eclosion	**9**	18	17	30	23	28	28	35	39	35	39	21	46	42	48	53	52	36	46	52	48	55	55
continual	**10**	19	18	25	20	22	25	27	28	33	35	13	35	35	40	42	46	27	40	36	40	40	35
continual	**11**	20	15	22	24	25	25	28	26	28	32	15	35	35	39	36	46	36	38	35	35	33	32
continual	**12**	5	15	17	12	15	15	21	16	18	25	11	24	1	29	25	28	25	31	33	36	40	41
Control	**13**	17	17	17	23	21	25	21	26	24	28	15	32	30	36	33	35	38	40	39	40	40	41
Control	**14**	15	16	14	17	13	15	16	20	16	21	12	25	23	34	30	31	29	33	33	35	32	34
Pre-eclosion	**15**	17	19	23	26	25	29	24	29	25	29	17	37	28	33	31	31	30	30	26	31	29	31
Pre-eclosion	**16**	17	18	14	23	17	22	18	22	20	24	14	30	27	29	25	25	23	23	22	24	23	23
Pre-eclosion	**17**	13	14	11	19	16	21	19	22	18	20	11	25	21	23	23	23	23	23	21	21	17	19
Post-eclosion	**18**	15	15	15	22	18	22	29	30	19	24	14	28	23	27	36	32	30	28	32	25	27	27
Post-eclosion	**19**	23	23	23	29	25	31	26	36	34	36	21	38	33	38	29	34	34	36	36	34	34	33
Post-eclosion	**20**	15	15	20	26	20	23	22	26	21	26	13	23	22	26	25	29	30	31	28	27	26	26
Continual	**21**	18	19	19	26	25	29	29	37	30	39	21	42	35	39	41	43	45	46	46	47	45	46
Continual	**22**	13	13	10	13	10	12	9	10	7	10	4	10	7	12	12	12	12	13	15	16	9	11
Continual	**23**	14	15	15	21	13	16	16	19	16	19	12	24	22	25	25	25	23	25	24	25	24	26

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