

1 **Potential costs of learning have no detectable impact on reproductive success for bumblebees**

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11

12 **Abstract**

13 Development and maintenance of neural architecture supporting learning and memory, as well as
14 active storage of learnt information, are predicted to carry significant energetic costs. Here we explore
15 whether the demands of learning about fitness-relevant stimuli come at a cost to the reproductive
16 success of an insect model. Bumblebee (*Bombus* spp.) queens found nests alone, and early in the
17 colony cycle must invest simultaneously in learning about rewarding floral resources and colony
18 founding. We exposed queens emerging from diapause to a six-day reversal learning task, and
19 compared colony founding success of these individuals to two control groups that did not learn but
20 received equal food. Additionally, we limited carbohydrate intake in half of queens across all
21 treatments. Nutritionally stressed queens had a lower probability of egg-laying, delayed nest initiation
22 and lower offspring production compared with nutritionally unrestricted queens, suggesting resource
23 availability at this life-cycle stage is crucial to founding successful colonies and population growth.
24 Nonetheless, potential energetic demands of learning did not reduce colony founding success in a
25 laboratory set-up, even in nutritionally stressed groups. Our findings provide a rare real-world test of
26 the costs associated with learning, and suggest they do not affect reproductive potential in bumblebee
27 queens.

28

29 **Keywords**

30 *Bombus terrestris*, Cognition, Fitness, Memory, Pollinator, Trade-off.

31 Introduction

32

33 Learning and memory are cognitive traits that allow animals to acquire, retain and recall information
34 about their environments (Shettleworth, 2010) to make decisions that may increase fitness and
35 survival prospects (e.g., Dukas & Bernays, 2000; Maille & Schradin, 2016; Shaw et al., 2019;
36 Sonnenberg et al., 2019), but are also associated with physiological costs. These are generally grouped
37 into costs that are constitutive or induced (Burns, Foucaud & Mery, 2010). Constitutive costs describe
38 evolutionary costs associated with maintaining neural infrastructure, and are paid by an individual
39 irrespective of whether this infrastructure is put to use (Aiello & Wheeler, 1995; Niven & Laughlin,
40 2008). For example, in *Drosophila melanogaster*, individuals bred from high-learning lines that do not
41 undergo any learning trials show decreased survival probability and larval competitive ability,
42 compared with control lines, suggesting that evolutionary investment in learning ability comes at a
43 cost (Burger et al., 2008; Mery & Kawecki, 2003). Conversely, the active processes of learning and
44 memory formation also consumes energy, resulting in proximate trade-offs with other traits that are
45 also energetically expensive (induced costs; Mery & Kawecki, 2004). Here, we investigate the induced
46 costs of memory formation in a social insect model, the bumblebee *Bombus terrestris*, at a stage in
47 the lifecycle when energy budget requirements are particularly high.

48

49 Increased energetic requirements during learning and memory formation may be driven by structural
50 and molecular changes that occur in the brain. Formation of long-term memories requires *de novo*
51 protein synthesis (Menzel, 2012; Tully et al., 1994), and previous work has also identified changes to
52 neural structures that occur during learning and memory formation (Cabirol et al., 2018; Hourcade et
53 al., 2010; Li et al., 2017). This reconfiguration process is likely to be costly (Niven, 2016), and in fruit
54 flies (*D. melanogaster*) neurons in the mushroom bodies (part of the brain involved in learning and
55 memory in insects) show increased energy consumption following long-term memory formation
56 (Plaçais et al., 2017). Accordingly, classical conditioning is associated with a subsequent increase in
57 sucrose consumption (Plaçais et al., 2017). These energetic costs may reduce available energy budgets
58 for investment in other processes, since elicitation of long-term memory formation has been shown
59 to reduce survival and egg-laying in the same species (Mery & Kawecki, 2004; Mery & Kawecki, 2005).
60 In honeybees (*Apis mellifera*), associative learning trials have been shown to be followed by lower
61 levels of trehalose (a precursor to glucose) in haemolymph, and again, with reduced survival
62 (Jaumann, Scudelari & Naug, 2013). Proximate trade-offs have also been identified between learning
63 and immune system activation (Alghamdi et al., 2008; Mallon, Brockmann & Schmid-Hempel, 2003).

64 Potential costs are likely to have relatively greater impacts on individuals at vulnerable stages during
65 the life-cycle. Bumblebees are annual eusocial insects in which a colony is founded by a single queen
66 in the spring (Goulson, 2010). When mated queens emerge from diapause, they are effectively solitary
67 individuals until their colony is founded, and must therefore perform all tasks that will later be taken
68 on by workers, in addition to nest-searching, nest-building and reproduction (Riveros & Gronenberg,
69 2009). This includes foraging to feed the brood, which places demands on learning and memory (Klein
70 et al., 2017) and has been linked to foraging success (Raine & Chittka, 2008; Pull et al., 2022).
71 Accordingly, bumblebee queens have been shown to not only successfully complete associative
72 learning tasks quickly, but to perform them better than workers (Muth, 2021; Evans & Raine, 2014),
73 suggesting they invest relatively heavily in learning and memory processes.

74

75 Here we ask whether the demands of learning to identify rewarding flower species come at a cost to
76 colony founding success in bumblebee queens, using a laboratory protocol that allows isolation of
77 learning from movement between flowers. We train queens on a visual associative learning task over
78 a six-day period, in which they must repeatedly learn to associate a colour (blue/yellow) with a sugar
79 reward, over multiple reversals. In honeybees, there is evidence to suggest that learning even a single
80 association leads to synaptic reorganisation in the brain. For example, Hourcade et al. (2010) trained
81 individuals to a single odour association and found an increase in synaptic densities in the mushroom
82 body lip, compared with bees in the control groups, suggesting that neural changes can be elicited by
83 learning a single stimulus association.

84

85 We compare colony initiation success, brood production, and offspring size in queens exposed to the
86 learning protocol with control groups of queens either (i) exposed to the same stimuli and rewards, in
87 the absence of pairing (to preclude learning), and (ii) not exposed to the task at all. Since the effects
88 of any stressor may not become apparent unless energy is restricted, we expose half of our queens in
89 all groups to a low-quality diet, by providing them with a lower concentration of *ad libitum* sucrose
90 solution throughout the experiment, following a fully crossed design. We track colony formation all
91 the way from initial egg-laying to emergence of the first brood. Thus, our study represents a unique
92 direct test of the impact of repeated exposure to a learning task on reproductive success.

93 **Methods**

94

95 *Queen production and diapause*

96

97 Bumblebee queens (*Bombus terrestris audax*; N = 210) were hatched and mated at Koppert Biological
98 Systems, Slovakia and placed into diapause at 2-4°C and 90-95% RH in three experimental blocks, each
99 staggered by one week. Queens were free from parasites for the duration of the experiment. At weeks
100 8 – 10 of diapause, queens were shipped (at 4°C, without breaking their diapause) to Royal Holloway
101 University of London, UK, where they completed the remaining period of diapause (total diapause
102 time = 12 weeks). Queens were then weighed and placed into individual Perspex nest boxes
103 (67×127×50 mm), and maintained in a temperature-controlled room at 27-29°C and 55-60% RH. Ten
104 queens did not awake from diapause and thus did not contribute further to the experiment. Egg-laying
105 was stimulated through introduction of an 8:16 hour light:dark cycle for the first 14 days post-
106 diapause, and queens were then housed under continuous darkness/red light for the remainder of
107 the experiment. Queens were assigned to diet and learning treatment groups (see below) based on
108 their mass, such that there was an equal distribution of mass in each treatment group (linear model,
109 post-diapause mass does not significantly improve the model fit, Δ AIC between full model and null
110 model = 9.35; mean \pm standard error (SE) mass = 814.61 \pm 4.79 mg).

111

112

113 *Diet treatment*

114

115 Queens were allocated to either a high-quality (40% w/w sucrose solution) or low-quality (20% w/w
116 sucrose solution) diet. When carbohydrates are restricted in this way, consumption may be slightly
117 increased, but bees are unable to compensate fully for the lower sugar concentration by consuming a
118 proportionately higher volume of liquid (Brown & Brown, 2020; see also our Results). Fresh sucrose
119 of the assigned concentration was provided *ad libitum* for the duration of the experiment. We also
120 provided queens from both diet treatments with the same *ad libitum* pollen (polyfloral fresh-frozen
121 honeybee-collected corbicular pollen, Agralan, UK; pollen patty 4:1 pollen:water). Fresh sucrose and
122 pollen were replaced every three days, and we measured the consumption of sucrose and pollen when
123 replacing food (Advanced Portable Balance Scout STX123 120g, OHAUS Corporation \pm 1 mg). Nest
124 boxes were also cleaned on these days.

125

126

127 *Learning trials*

128

129 Following Muth et al. (2018) and Muth (2021), we adapted a visual associative learning task in which
130 queens learnt to associate a colour with a sucrose reward in an unrestrained set-up (Fig. 1). Two hours
131 before learning trials commenced, queens were removed from their boxes and placed into individual
132 tubes (clear Acrylic tubes, 24 mm internal diameter, 150 mm length), which were sealed at one end
133 and contained a Perspex disc with openings for reward delivery at the other. Tubes were covered with
134 black material such that only the first 3 cm and entrance disc were exposed to light. Queens were
135 initially kept in the tubes for two hours in the dark, to allow time to acclimatise. Trials were performed
136 under natural daylight at approximately 24°C. Each day we began by giving queens two motivation
137 trials, during which they were presented with a clear pipette tip filled with 5µL 50% w/w sucrose
138 solution and allowed to drink this fully.

139

140 Queens were then presented with the first of three blocks of ten learning trials over two days (five
141 trials per day), in which we simultaneously presented a blue and a yellow painted pipette tip (Beautiful
142 Blue Gloss Finish, Paint Factory; Sunshine Yellow Gloss Finish, Paint Factory) filled with either 5µL 50%
143 w/w sucrose solution (reward) or 5µL distilled water (no reward). We adapted the original protocol to
144 use this novel method of reward delivery so that all queens received equal volumes of sucrose across
145 the experiment. Once a queen had made a choice (approach, antennation and extension of the
146 proboscis to the colour, or prolonged biting of the coloured tip) the liquid was dispensed from the
147 pipette tip so that she could drink it, and the pipette tip was removed from the set-up. To ensure
148 exposure to both the positive and negative associations, in each trial, the queen was additionally
149 allowed to drink from the colour she had not initially selected, prior to it being removed. Trials during
150 which a queen inspected both colours but did not make a choice for over five minutes were marked
151 as unsuccessful. The inter-trial interval was 12 minutes, which is known to induce long-term memory
152 formation (Menzel et al., 2001; Menzel, 2012). On the final trial of each block (i.e., trial ten) we tested
153 queens with both colours unrewarded (both filled with distilled water). Starting colours were
154 randomised between groups. For each queen, we then repeated the entire process twice, reversing
155 the colour association in each case (but see small adjustment for block 1 in Supplementary
156 Information; Fig. S1). Thus, each queen participated in learning trials for six consecutive days, with an
157 unrewarded test trial at the end of every two-day learning bout followed by reversal of the rewarding
158 colour.

159

160 We included two control groups, (i) an exposure control, and (ii) a full control. Queens in the exposure
161 control group underwent the same protocol as queens in the learning treatment, but coloured pipette
162 tips were presented empty (we allowed queens to interact with the pipette tips in each trial). Queens
163 were then fed the total volume of 50% sucrose solution that they would have received across the
164 learning trials at the end of training each day. Queens in the full control group were not removed from
165 their nest boxes and received an equivalent volume of 50% sucrose solution delivered directly into
166 their boxes for each day of training. The volume of sucrose was adjusted based on the number of
167 rewarding trials experienced by queens in the learning treatment that day (accounting for the
168 unrewarded test trial every 10th trial).

169

170 We noticed that some queens became inactive in between trials, and they appeared to be resting. To
171 allow queens to participate in trials voluntarily, we left inactive queens undisturbed and
172 recommenced from the last completed trial when the queen became active again. We measured the
173 additional time spent by queens in the tube for each day of learning (see Supplementary Results).

174

175

176 *Colony monitoring*

177



178 Following testing, we carried out daily inspections and noted the presence of eggs, callows and their
179 sex, and any larvae or pupae that had been discarded, to monitor colony founding. Callows were
180 removed from the colony within 24 hours of hatching, and their thorax width measured (mm;
181 Axminster Digital Electronic Callipers, ± 0.01 mm). After 55 days, queens were euthanised, and their
182 thorax width was also measured. We dissected the remaining brood and counted the numbers of eggs,
183 larvae and pupae remaining.

184

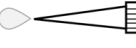


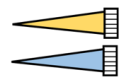

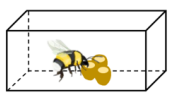

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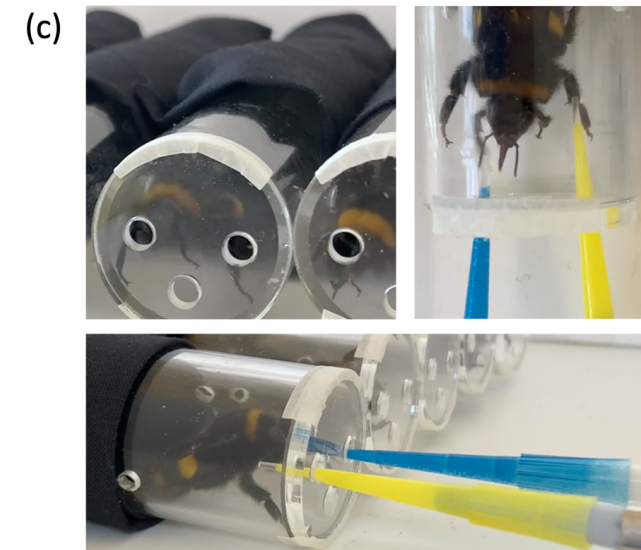
186 *Ethical note*

187 No licenses were required for these experiments. However, we ensured high welfare of our
188 bumblebees through regular feeding and cleaning of housing conditions as described above, and use
189 of red light to minimise disturbance. All queens were euthanised using liquid nitrogen. Our
190 experiments meet the approval of our Institutional ethics board.

	Day 1					Day 2					Day 3					Day 4					Day 5					Day 6				
Trial	1	2	3	4	5	6	7	8	9	10 (T)	1	2	3	4	5	6	7	8	9	10 (T)	1	2	3	4	5	6	7	8	9	10 (T)
CS+ (sucrose )	Colour A					Colour B					Colour A																			
CS- (water )	Colour B					Colour A					Colour B																			

T = unrewarded test trial

	Motivation trials x2	Colour trials x5	Reward equivalent
Learning group			
Exposure control			
Full control			



191

192 **Figure 1.** Bumblebee queens were trained on an associative learning protocol adapted from Muth *et al.* (2018) and Muth (2021). (a) Timeline of learning trials
 193 for queens in the learning group. Queens were presented with a yellow and a blue pipette tip, one filled with 5µL 50% w/w sucrose (rewarded) and the other
 194 filled with 5µL water (unrewarded), for five trials per day over a six-day period. The rewarding colour was reversed every 10 trials. On trial 10 (the final trial
 195 prior to colour reversal, indicated by grey shading), both pipette tips were presented unrewarded. (b) Queens in the learning and exposure control groups

196 were both presented with two motivation trials at the start of each training day (clear pipette tip baited with 5 μ L sucrose, indicated by a grey droplet). Queens
197 in the learning group were then simultaneously presented with one rewarded (grey droplet) and one unrewarded (5 μ L water, white droplet) pipette tip,
198 whereas queens in the exposure control group were presented with both pipette tips unfilled and allowed to interact with them, but were not rewarded.
199 Queens in the full control group did not leave their nest boxes. Queens in both control groups received the equivalent volume of sucrose as queens in the
200 learning group at the end of trials for that day, and the volume was adjusted each day based on how many rewarding trials queens in the learning treatment
201 had experienced. (c) Example of queens in the set-up. Queens in the learning and exposure control groups were transferred to cylindrical tubes for learning
202 trials. Tubes had a Perspex disc with opening for reward delivery at one end and were covered with black material to reduce stress. Coloured pipette tips
203 were presented through openings in the Perspex discs.

204 *Data analysis*

205

206 We used (generalised) linear models and mixed effects models ((G)LM / (G)LMM) for data analysis.
207 For each model set, we created a full model, the null model, and all subsets of fixed factors, while
208 retaining any random factors. We selected the best model from this set based on Akaike information
209 criterion (AIC; where two nested competing best models were within $\Delta\text{AIC} \leq 2.00$ of each other, we
210 selected the simplest; Burnham & Anderson, 2002). We then estimated each parameter estimate and
211 its 95% confidence interval (CI) from the final model.

212

213 To confirm that queens in the learning treatment learnt the association, we modelled the proportion
214 of correct choices (response variable) against trial number, including diet treatment and rewarding
215 colour as additional fixed effects, and individual as a random effect (binomial error structure; link
216 function = “logit”). Trial numbers reflected the trial (1 – 10) for that colour prior to a reversal. To
217 analyse unrewarded test trials (the last trial for each colour in that set of 10 trials), we followed a
218 similar approach but did not include trial number. To check that additional time spent in tubes by
219 queens was not an effect of diet treatment, and did not affect learning, we tested for differences
220 between means of groups (t-test) and correlated additional time and learning success, respectively.

221

222 For all models of colony founding measures (oviposition probability, oviposition timing, callow
223 production, callow thorax width, total brood production and discarded brood), we used diet
224 treatment, learning treatment and their interaction as fixed predictors. In addition, we included queen
225 thorax width as a covariate in models of oviposition probability, and callow type (worker or male) as
226 a covariate in models of callow thorax width. In models of callow thorax width, we accounted for
227 multiple callows per colony by including colony as a random factor. Total brood production was
228 calculated by adding together the total numbers of callows, larvae and pupae in the brood at the end
229 of the experiment, and any larvae, pupae or callows found dead during the experiment. Discarded
230 brood was the measure of any larvae, pupae or callows found dead during or at the end of the
231 experiment. The response variable error structures were: binomial (link function = “logit”) for
232 oviposition probability (yes/no), Poisson (link function = “log”) for total brood production, negative
233 binomial (link function = “logit”) for discarded brood, and normal for callow thorax width. To account
234 for a number of queens producing no callows, callow production was modelled using a zero-inflated
235 model with a binomial (probability of callow production) and negative binomial (callow count) error
236 structure. To determine whether oviposition timing differed between treatments, we used a Cox
237 proportional hazards model with diet treatment, learning treatment and their interaction as

238 predictors, and day of oviposition as the response. Additionally, we used only queens in the learning
239 treatment to ask whether oviposition probability, and separately the total number of callows
240 produced, were predicted by individual learning performance (i.e., the total number of correct choices
241 made across all learning trials for that individual). We used a binomial and negative binomial error
242 structure for these models, respectively.

243

244 Finally, we modelled whether the consumption of sucrose and pollen differed between treatments.
245 We used diet treatment, learning treatment and their interaction as covariates, and queen as a
246 random effect. For measures of sucrose consumption, we adjusted values for evaporation, as low diet
247 sucrose had a 1.25× higher evaporation rate. Pollen measures were not adjusted for evaporation, as
248 the pollen provided was the same across both diet treatments, and measuring pollen evaporation is
249 inaccurate as queens will often add sucrose to the pollen. For pollen consumption, we performed a
250 square root transformation and included day after oviposition for each measure of pollen
251 consumption as a covariate.

252

253 All statistical analysis was carried out in R version 4.2.1 (R Core Team, 2022) using the packages lme4
254 (Bates *et al.*, 2015), RVAideMemoire (Hervé, 2022), car (Fox & Weisberg, 2019), survival (Therneau,
255 2022a; Therneau & Grambsch, 2000), survminer (Kassambara, Kosinski & Biecek, 2021), coxme
256 (Therneau, 2022b), dplyr (Wickham *et al.*, 2022), DHARMA (Hartig, 2022), performance (Lüdecke *et*
257 *al.*, 2021), pscl (Zeileis, Kleiber & Jackman, 2008), MASS (Venables & Ripley, 2002), ggplot2 (Wickham,
258 2016) and patchwork (Pedersen, 2024).

259 **Results**

260

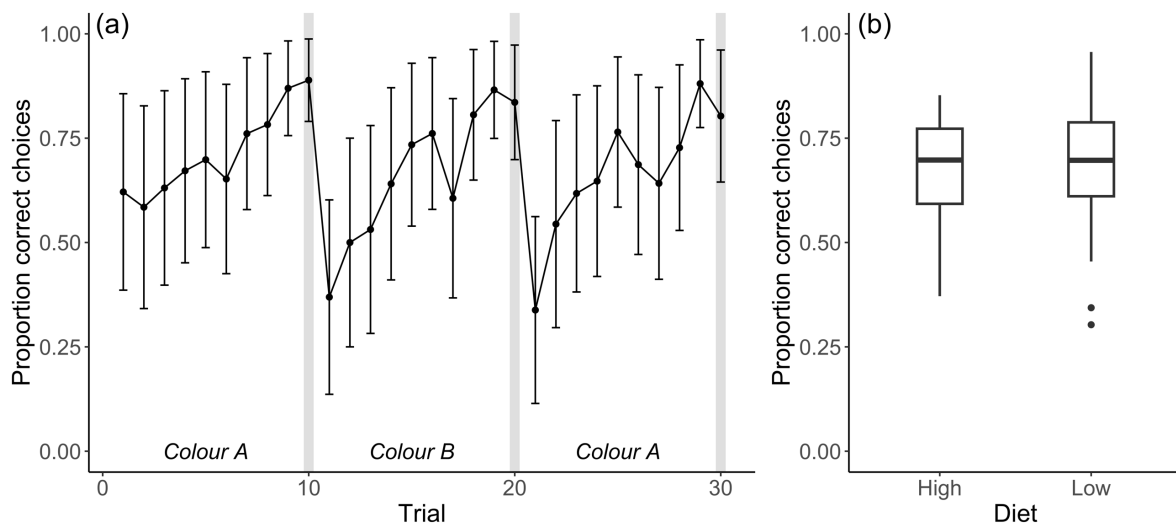
261 *Learning trials*

262

263 A total of 68 queens performed 2,040 individual learning trials across a six-day period. The probability
264 of making a correct choice significantly increased across each set of 10 trials (GLMM, trial parameter
265 estimate: 0.21, 95% confidence intervals (CI): 0.18 to 0.25, Fig. 2a), and was also significantly higher
266 when the rewarding colour was yellow compared with blue (colour parameter estimate: 0.99, 95% CI:
267 0.78 to 1.21; Fig. S2a). There was no effect of diet quality on learning (diet parameter was not included
268 in the best model; Fig. 2b). When looking only at the unrewarded test trials (the last trial for each
269 colour in that set of 10 trials), the probability of making a correct choice was 0.84 ± 0.14
270 (mean \pm variance). There was no effect of diet or colour type on correct choices made during test trials
271 (GLMM, neither diet nor colour parameters were included in the final model). Thus, overall, the
272 conditioning protocol was effective in eliciting learning, as intended.

273

274



275

276 **Figure 2.** (a) Learning curve showing the proportion mean \pm variance (calculated as variance = $p(1-p)$,
277 where p is the probability of making a correct choice across all bees for that trial) of correct choices
278 for each trial across the six-day learning period (five trials per day, total trials = 30, N queens per trial
279 = 63-68). Grey bars show the final trial of each colour, which was unrewarded. The rewarding colour
280 (blue/yellow) was reversed every 10 trials. (b) Proportion of correct choices made by queens fed a
281 high-quality and low-quality diet (40% and 20% w/w sucrose solution, respectively) across all learning
282 trials. Central line shows the median, box shows the interquartile range.

283 *Colony founding*

284

285 There was no effect of learning treatment on any colony founding measures (treatment was not
286 included in the final model). However, diet quality significantly decreased the probability of egg-laying,
287 with 49 vs 83% of queens laying eggs on a low-quality vs high-quality diet, respectively, over the 55-
288 day experimental period (GLM, diet parameter estimate = -1.63, 95% CI: -2.30 to -0.99; Fig. 3a).
289 Queens on the low-quality diet also appeared to lay eggs later than queens on a high-quality diet (Cox
290 proportional hazards model, diet coefficient: -1.04, 95% CI: -1.40 to -0.68, median (range) day low-
291 quality diet: 55 (10-55), high-quality diet: 24.5 (7 to 55); Fig. 3b). There was no interaction between
292 learning treatment and diet on oviposition probability or timing (interaction term not included in the
293 final model).

294

295 In total, queens produced 579 callows (offspring), of which 30 were males produced by eight queens.
296 Two out of 200 queens produced only male callows. Significantly fewer queens that had been fed a
297 low-quality diet produced callows (zero-inflated GLM binomial section, diet parameter estimate: 1.22,
298 95% CI: 0.44 to 2.00). Of queens that did produce callows, queens fed a low-quality diet produced
299 fewer callows in total, although this was not significant (zero-inflated GLM count section, diet
300 parameter estimate: -0.46, 95% CI: -0.93 to 0.02; mean±standard error (SE) number of callows
301 produced by queens on a low-quality diet: 1.92±0.59 vs high-quality diet: 5.83±0.78; Fig. 3c). Callow
302 size was not affected by diet or learning treatment (both parameters were not included in the final
303 model; Fig. 3d), but, as expected, workers were significantly smaller than males (mean±SE male thorax
304 width: 4.99±0.07 mm, worker thorax width: 4.27±0.02 mm; LMM, type parameter estimate: -0.81,
305 95% CI: -1.00 to -0.62).

306

307 When looking only at queens in the learning treatment, learning performance (i.e., the proportion of
308 correct choices made across all learning trials) did not predict probability of egg laying (GLM, trial
309 choice did not make the final model, Fig. S4a) or the number of offspring produced (GLM, callow
310 number did not make the final model, Fig. S4b).

311

312 Total brood production (all callows, plus larvae and pupae in the brood at the end of the experiment,
313 and any dead or discarded larvae, pupae and callows) was significantly lower for queens on a low-
314 quality diet (GLM, diet parameter estimate: -0.80, 95% CI: -1.24 to -0.34; Fig. S3). Discarded brood
315 (dead larvae, pupae and callows discarded during the experiment or found in the brood at the end of

316 the experiment) were found in 95 out of 200 colonies. Queens on a low-quality diet had a significantly
317 lower number of discarded brood (GLM, diet parameter estimate: -0.68, 95% CI: -1.19 to -0.16).

318

319 Queen mortality was low, with only eight queens dying over the 55-day experimental period (five in
320 the low-quality diet treatment and three in the high-quality diet treatment).

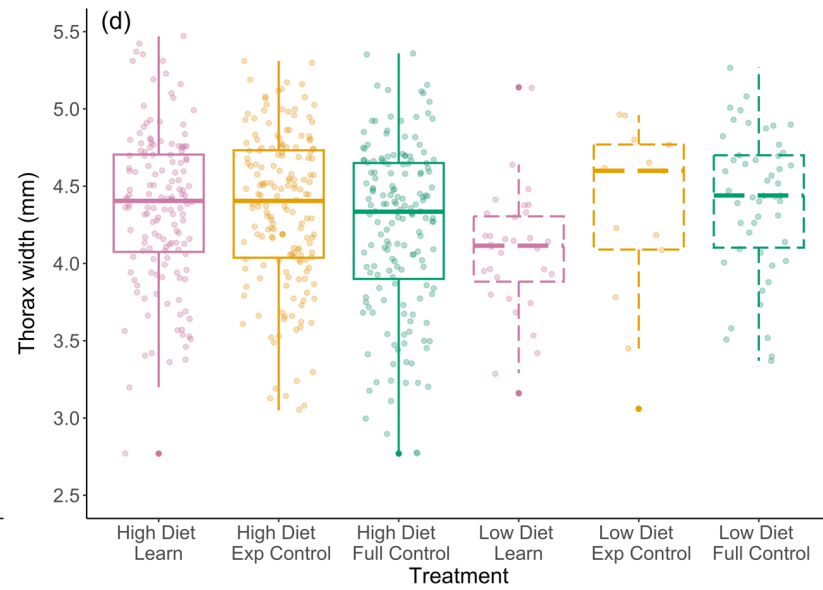
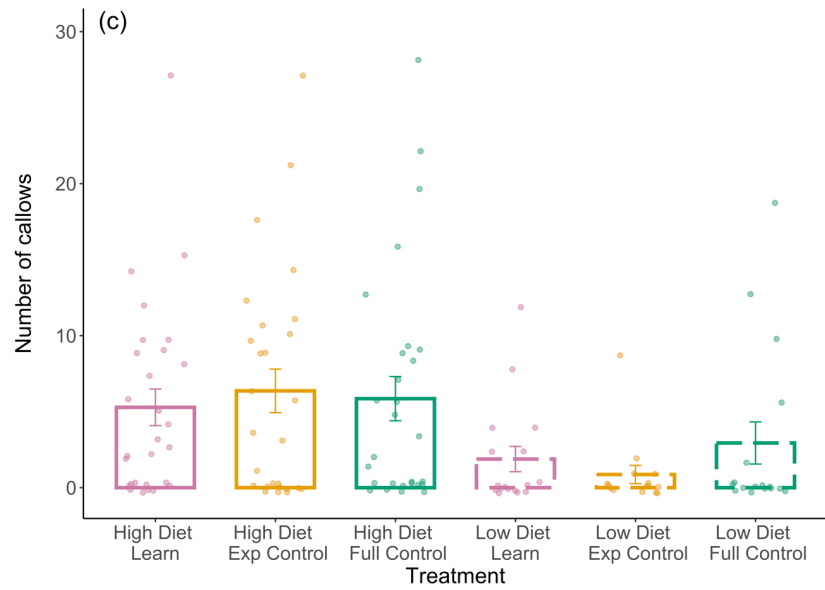
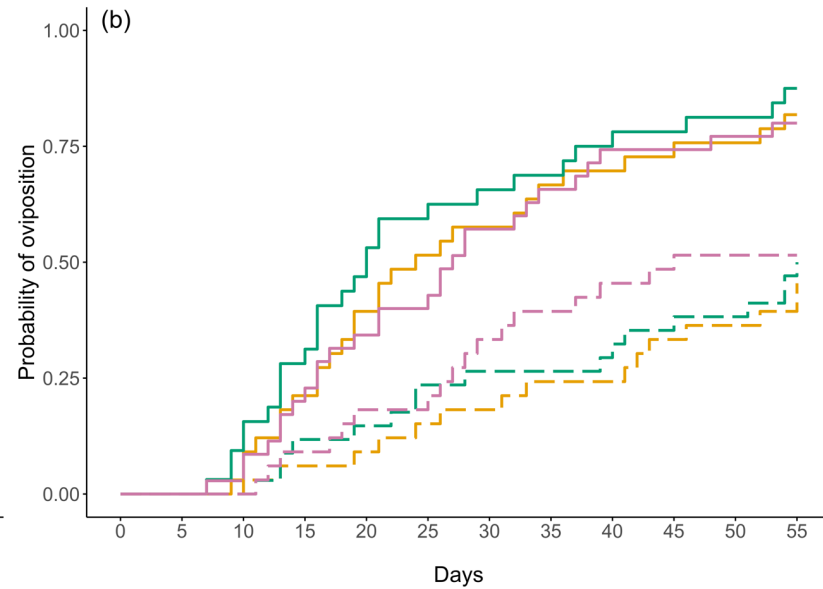
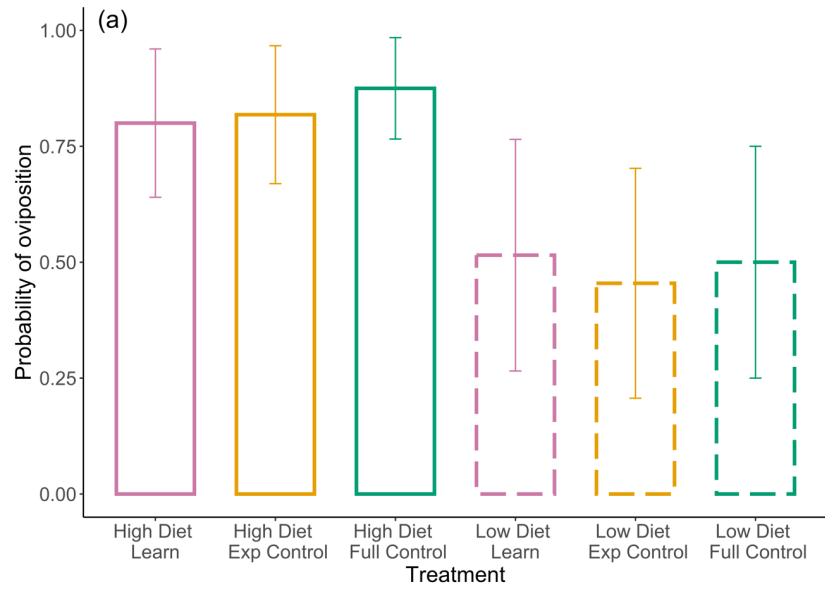
321

322

323 *Consumption*

324

325 Queens on the low-quality diet consumed 1.25× more sucrose than queens on the high-quality diet
326 (mean±SE consumption over a three-day period: low diet = 3452±60 mg, high diet = 2771±35 mg, diet
327 parameter estimate: 646.80, 95% CI: 213.71 to 1079.97; Fig. S5a). Pollen consumption was
328 significantly lower for queens on the low-quality diet (diet parameter estimate: -2.72, 95% CI: -3.58 to
329 -1.85; Fig. S5b) and significantly increased with days after oviposition across all treatments (day post
330 oviposition parameter estimate: 0.34, 95% CI: 0.32 to 0.36). There was no difference in sucrose or
331 pollen consumption between learning treatments (learning treatment was not retained in the final
332 model).



334 **Figure 3.** Colony founding measures taken over the 55-day experimental period in bumblebee queens. Treatments: high-quality diet (solid lines), low-quality
335 diet (dashed lines); learning group (pink), exposure control (yellow) and full control (green). (a) Mean \pm variance probability of oviposition (N=200 queens
336 across all treatments); (b) timing of oviposition; (c) mean \pm standard error number of offspring (workers and males combined) produced by queens in each
337 treatment (points show the raw data); (d) thorax widths of offspring (workers and males combined) that hatched during the experiment (box shows the
338 median and quartiles, points show the raw data).

339 **Discussion**

340

341 Aspects of learning and memory are predicted to bring about induced energetic costs (Burns, Foucaud
342 & Mery, 2010), yet we have limited evidence of the potential proximate impacts on other energy-
343 demanding processes that result when an individual invests in the learning process. We predicted a
344 trade-off between investment in learning and reproductive output based on previous studies in which
345 individuals that learn show lower survival, egg-laying and immune function compared with non-
346 learning individuals (e.g., Mery & Kawecki, 2004; Jaumann et al., 2013; Mallon, Brockmann & Schmid-
347 Hempel, 2003). Our bumblebee queens successfully learnt and reversed the association between a
348 colour and a sucrose reward, with, on average, a >80% chance of making a correct choice during
349 unrewarded test trials. However, we found no evidence for an impact of learning on reproductive
350 success.

351

352 Energetic investment in learning is likely to be dependent on the difficulty of the learning task. We
353 designed our learning task to maximise complexity by (i) using an inter-trial interval of 12 minutes to
354 induce long-term memory formation, which is likely to carry a relatively higher cost compared with
355 other memory phases as it requires *de novo* protein synthesis (Menzel, 2012); (ii) adding a reversal
356 learning element, during which interference from previously rewarding memories may increase the
357 cost in coding/over-writing with new ones (Tello-Ramos et al., 2019); and (iii) performing trials over
358 six consecutive days, mimicking the potential foraging patterns of new queens after emergence from
359 hibernation (Goulson, 2010). Given that foraging individuals often focus on a single flower species
360 until it becomes unrewarding (known as floral constancy; Chittka, Thomson & Waser, 1999), we expect
361 our task to be ecologically relevant. Nonetheless, foraging bees likely employ multiple modes of
362 learning, including using visual and olfactory cues to identify flowers (Menzel, 1993), thus performing
363 only one learning assay may be an over-simplification of the investment that occurs when foraging in
364 the wild. Furthermore, individuals may use both short- and long-term memory to make within- and
365 between-patch decisions when foraging (Pull et al., 2022). There is evidence to suggest potential
366 trade-offs between memory phases (Lagasse et al., 2012), thus underlying costs may not be revealed
367 when measuring a single memory type under laboratory conditions.

368

369 Individuals may compensate for the expression of costly traits by increasing their energetic intake
370 (Plaçais et al., 2017), and memory formation may be restricted under energy-limited scenarios (Plaçais
371 & Preat, 2013). To limit potential compensation for energy invested into memory formation in our
372 queens, and because costs may not be revealed unless other stressors are present, we added a

373 nutritional limitation by feeding half of our queens on a low-quality diet with a 50% lower
374 carbohydrate concentration. Queens in this group were exposed to the low-quality diet from
375 emergence and for 48 hours prior to starting learning trials, and then for the duration of the
376 experiment. We found queens fed a low-quality diet consumed 1.25× more artificial nectar compared
377 with queens fed a high-quality diet, however this did not vary between learning treatment groups. We
378 therefore found no evidence for compensatory energy intake in learners on a low-quality diet, and
379 feeding on a low-quality diet did not appear to affect learning performance.

380

381 One potential explanation for the lack of a detected cost is that our study precluded stressors other
382 than nutrition, such that queens did not incur the costs of flight, thermoregulation or infection that
383 they would in the wild (Moret & Schmid-Hempel, 2000 *Science*; Silvola, 1984). Uniquely, our queens
384 were laboratory bred, and therefore aseasonal, free from parasites, with no previous exposure to the
385 external environment and a standardised diapause time. Whilst this allowed us to standardise for
386 potential confounds (e.g., parasites may negatively affect learning; Gegear, Otterstater & Thomson,
387 2006; previous experiences affect learning, Cheng & Wignall, 2006), in the wild stressors may act
388 synergistically (Goulson et al., 2015; Siviter et al., 2021), meaning a potential trade-off may not be
389 large enough to detect in such a controlled set-up. Furthermore, the ecological costs incurred during
390 the learning process may be greater than potential physiological costs, and these are difficult to
391 recreate in laboratory studies (Liefting, 2022). These include making unfavourable decisions during
392 the learning process (Dunlap & Stephens, 2016; Lavery & Plowright, 1988), memory interference of
393 previous memories leading to mistakes in new learning tasks (Cheng & Wignall, 2006), and certain
394 memory types being maladaptive in different environments (Pull et al., 2022). The consequences of
395 such mistakes are likely to be more severe in the wild than in the laboratory (e.g., there is no cost of
396 predation or extreme weather in the laboratory), meaning a potential cost is not detected in such
397 controlled studies. For example, bumblebee workers that showed relatively better learning abilities in
398 a laboratory assay had shorter foraging careers when released into the wild, suggesting a potential
399 cost to learning proficiency was revealed when foraging in a natural setting (Evans, Smith & Raine,
400 2017). However, within the confines of our protocol, we can suggest that the potential costs of a
401 simple associative learning task do not appear to negatively affect reproductive success in laboratory-
402 bred bumblebee queens.

403

404 Whilst we did not detect a cost of learning that impacted life-history traits, we found that diet
405 treatment was a strong predictor of colony founding success. Queens fed a relatively low-quality diet,
406 manipulated by providing artificial nectar with a 50% lower sucrose concentration compared to the

407 high-quality diet, had a >50% lower probability of egg-laying, delayed egg-laying, and were ~50% less
408 likely to rear offspring. Nectar is the main source of carbohydrates for bees (Brodschneider &
409 Crailsheim, 2010), and we predict that the low-quality diet restricted energy available for ovary
410 maturation and brood incubation (Cartar & Dill, 1991; Vogt, Heinrich & Plowright, 1998), resulting in
411 reduced and/or delayed egg-laying, and fewer offspring. However, nectar quality did not appear to
412 affect offspring size, suggesting a potential number versus size trade-off in offspring production, with
413 all queens investing similar energy into each of their offspring, but energy-deprived queens being
414 limited in the number of offspring they can invest in. Fewer and later production of workers is likely
415 to negatively impact colony growth and the future production of sexuals (Pomeroy & Plowright, 1982).
416 Another factor that affects brood production and offspring size is pollen quality (Brodschneider &
417 Crailsheim, 2010), which we did not vary between diet treatments in our experiment. We chose not
418 to manipulate pollen diets, as the brain uses glucose as its primary energy source (Sokoloff, 1999),
419 thus nectar quality is likely to be more relevant for learning and memory traits. Furthermore, the ratio
420 of protein to carbohydrate (P:C) could be important to consider. P:C affects survival, growth, and ovary
421 activation in honeybees (Helm et al., 2017; Pirk et al., 2010) and may have impacted the number of
422 offspring produced by queens on our low-quality diet. Our results add to a growing body of evidence
423 suggesting that both nectar availability, and pollen quality and/or diversity, are important for queen-
424 right colony growth and development (Leza et al., 2018; Rotheray, Osbourne & Goulson, 2017;
425 Watrobska et al., 2021; Watrous, Duennes & Woodard, 2019; Woodard et al., 2019). Given ongoing
426 bumblebee population declines that have been linked in part to land-use change and floral resource
427 availability (Goulson et al., 2015; Woodard & Jha, 2017), further work is needed to identify the
428 nutritional needs of bumblebee queens at the colony-founding life cycle stage.

429

430 In conclusion, we found that any potential energetic costs of visual associative learning do not appear
431 to impact reproductive success in bumblebee queens when they emerge from diapause in a controlled
432 laboratory set-up, suggesting that detecting a potential cost is dependent on environmental variables
433 and the interaction with other stressors. Nectar quality did not affect learning performance, but was
434 an important predictor of colony success across all treatment groups, with queens fed an artificial
435 nectar of higher sucrose concentration showing relatively higher reproductive success compared with
436 queens fed a diet with a lower sucrose concentration. Our results draw into question the widespread
437 assumption that the active learning process trades-off against other energetically costly traits.

438

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448

449 **Author contributions**

450 C.M.W.: conceptualisation, data curation, formal analysis, investigation, writing – original draft,
451 writing – review & editing.

452 P.S.: resources, writing – review & editing.

453 A.R.R.: investigation, writing – review & editing.

454 E.L.: conceptualisation, funding acquisition, supervision, writing – review & editing.

455

456 **Data Availability**

457 All data are available at <https://doi.org/10.6084/m9.figshare.c.6683525>.

458

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630

631 **Appendix**

632

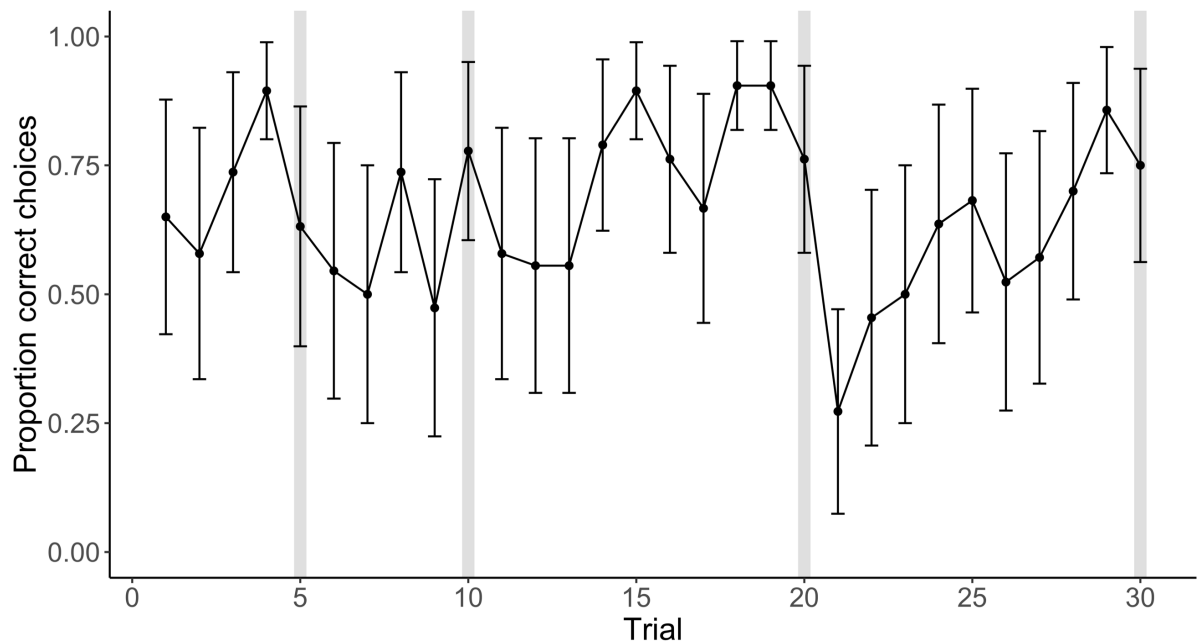
633 Supplementary Methods

634

635 Learning Trials

636 During the first block of the experiment, we began the first learning trials by switching the rewarding
637 colour after five, instead of 10, trials. However, we were not convinced that five trials allowed queens
638 enough time to sufficiently learn the association (Fig. S1). We therefore decided to extend each colour
639 learning time to ten trials, as per the protocol outlined in the methods of the main text. This allowed
640 queens to consolidate memories from the first day of trials during the second day, before having to
641 reverse the association. The switch in protocol occurred on day three of learning trials for the first
642 block (meaning only the first two days of trials for the first block of the experiment were affected). For
643 the main analysis of learning (probability of making a correct choice), we excluded trials 6-10 for
644 queens from block 1 (N=110 out of 2040 trials removed). However, including these data did not change
645 the overall result, and continued to show that the probability of making a correct choice significantly
646 increases with trial number (GLMM, trial parameter estimate: 0.20, 95% CI: 0.16 to 0.23) and was
647 significantly higher when the rewarding colour was yellow (colour parameter estimate: 1.03, 95% CI:
648 0.82 to 1.24).

649



651 **Figure S1.** Proportion of correct choices made during associative learning trials by queens in block 1
652 of the experiment (N.B.: we had three experimental blocks, each staggered by one week). Queens
653 completed five learning trials per day, on six consecutive days. Solid grey bars show the final test trial
654 (unrewarded) before a colour reversal. Initially, the rewarding colour was reversed after 5 trials (days
655 1 to 2), but we decided to extend the number of trials for each colour, so that reversals occurred every
656 ten trials. Queens in experimental blocks 2 and 3 (not shown) underwent colour reversals every ten
657 trials, as per the protocol in the main text.

658 Supplementary Results

659

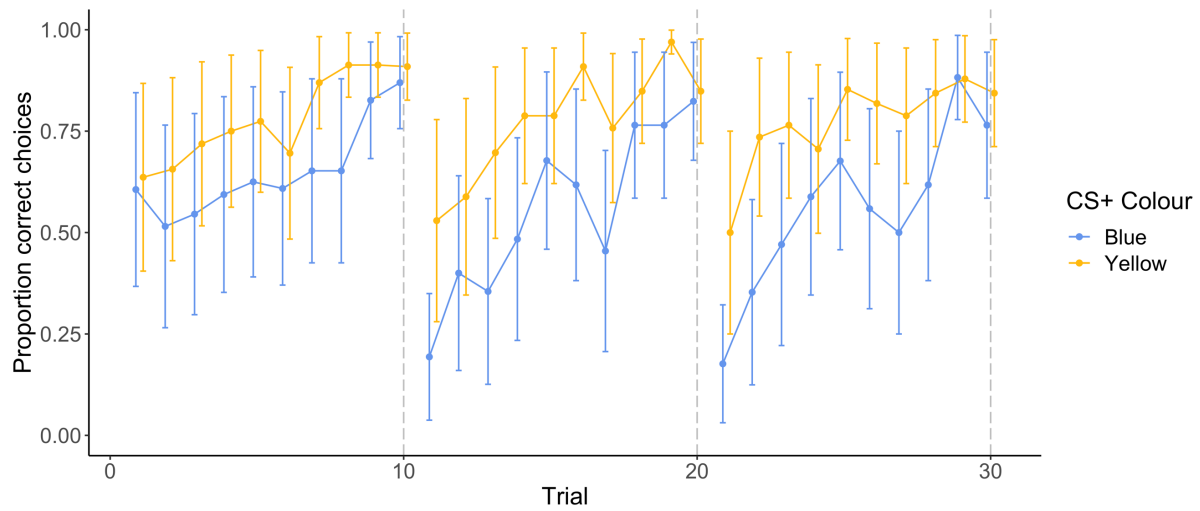
660 Additional time spent in tubes

661 Sixty-four out of 68 queens appeared inactive during one or more learning trials, and therefore spent
662 additional time in their learning tubes. The queens appeared to be resting, so we left them
663 undisturbed until they became active again, and then picked up from the last completed trial. Forty-
664 one trials (from eight different queens) were marked as unsuccessful as queens did not become active
665 again despite waiting until the end of the day. The total additional time spent in a tube by a queen
666 across the six days did not differ between diet treatments (mean additional time per queen each day
667 \pm SE = 56 ± 5 minutes; t-test, $t = 0.05$, $df = 62$, $P = 0.96$) and additional time in the tube was also not
668 correlated with learning score ($\chi^2 = 0.07$, $P = 0.16$).

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670 Supplementary Figures

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Figure S2. Proportion of correct choices made by queens presented with a yellow (yellow line) or blue (blue line) rewarding stimulus, across 30 trials. Every 10th trial was an unrewarded test trial (grey dashed line), after which the rewarding colour was reversed. Error bars show the variance around the proportion.

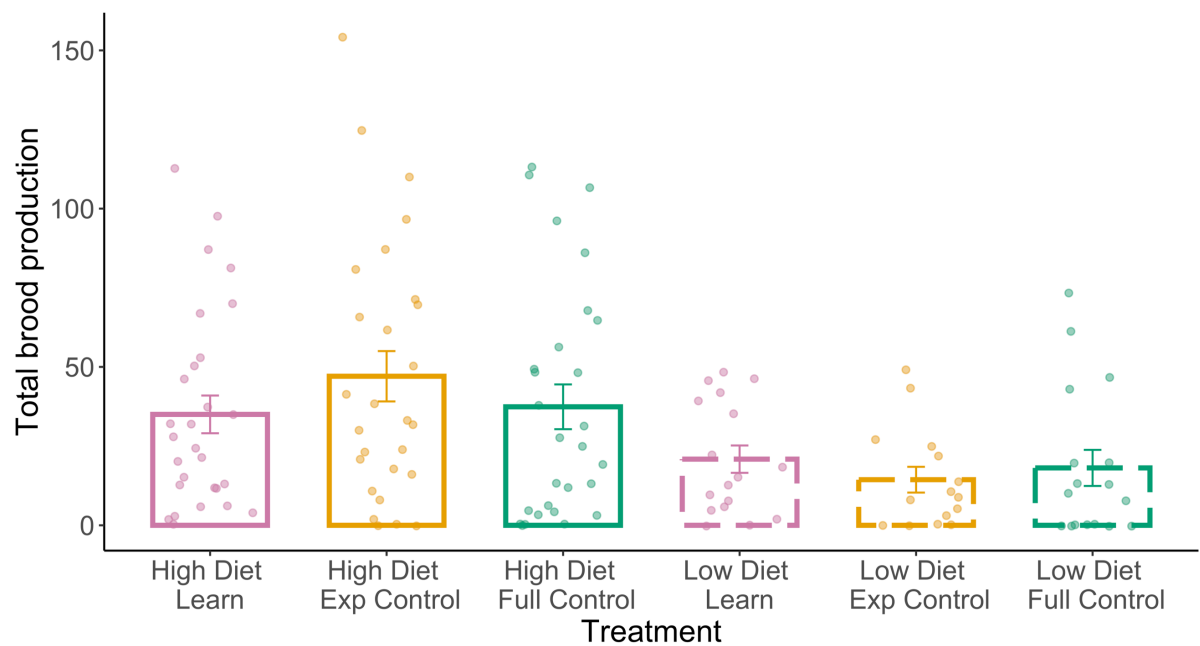
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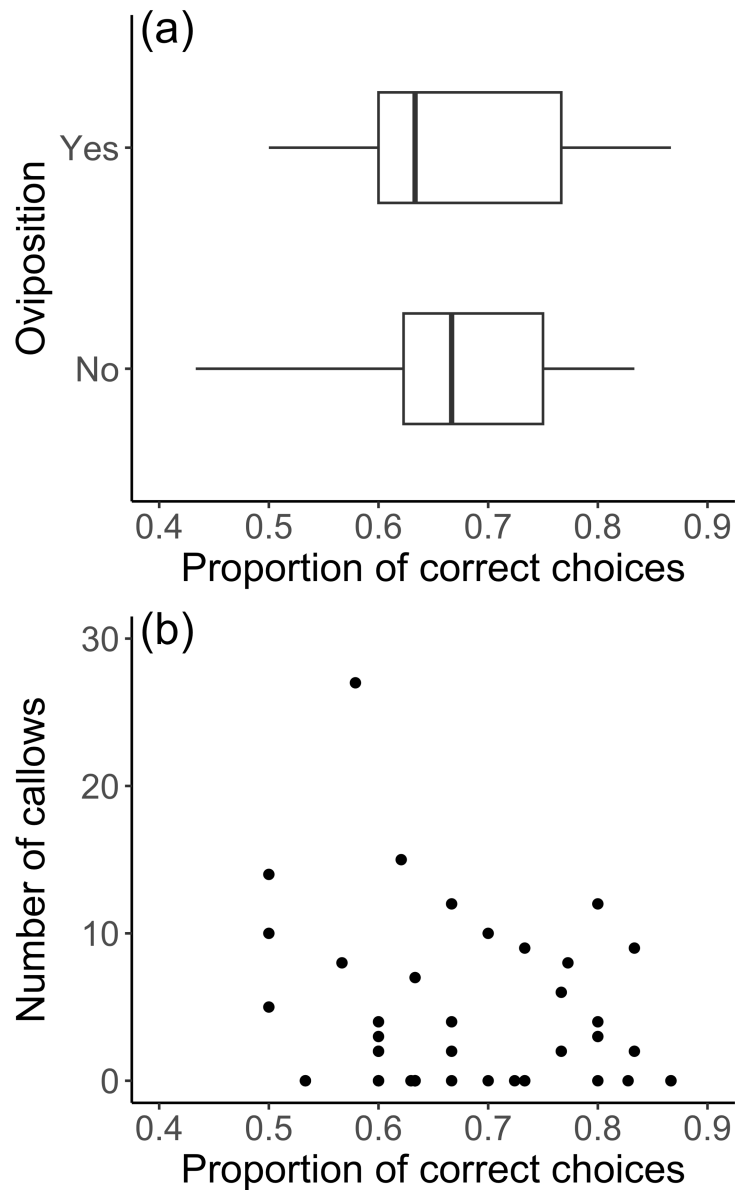
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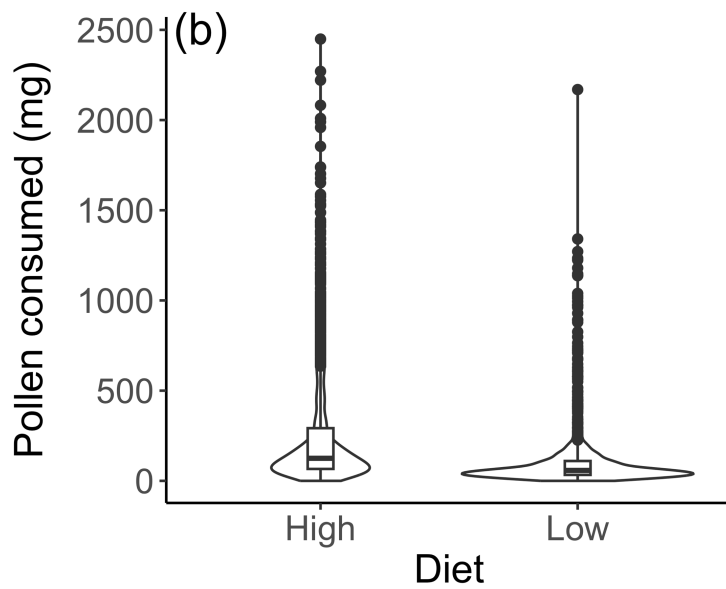
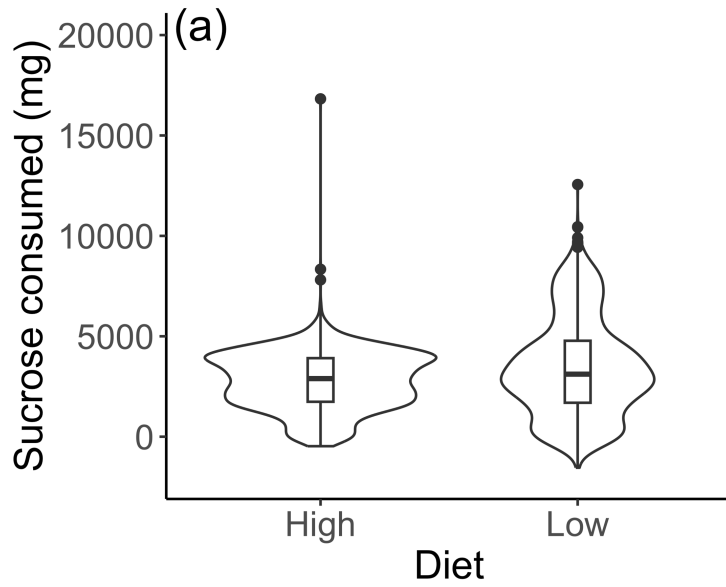
Figure S3. Total brood production (mean±standard error), which is the total number of callows that hatched over the 55-day experiment, plus any larvae and pupae dissected from colonies at the end of the experiment (including any dead larvae or pupae found during or at the end of the experiment, but

684 excluding eggs) across learning and diet treatments (learners = pink, exposure control = orange, full
685 control = green; high-quality diet = solid lines, low-quality diet = dashed lines).
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Figure S4. Colony founding measures against proportion of correct choices of queens in the learning treatment only for A) oviposition probability (N = 68 queens), and B) number of callows produced by queens across the 55 day experiment (N = 45 queens producing callows in the learning treatment).



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Figure S5. Consumption measured per queen every 3 days for A) sucrose and B) pollen on a high (40% w/w sucrose solution) or low (20% w/w sucrose solution) diet. Sucrose was corrected for evaporation by subtracting an average evaporation rate from each measure, with some measures resulting in negative values, which have been included to avoid bias against lower consumption measures.

699 **Model Tables**

700

701 **Table S1.** Nested candidate model set for a GLMM investigating the effect of trial number, diet
 702 treatment and rewarding colour (conditioned stimulus, blue/yellow) on the proportion of correct
 703 choices made during learning trials.

Covariates: trial number + diet + colour				
Random effect: individual queen				
Model	DF	AIC	Δ AIC	
Trial + Colour	4	2159.91	0.00	
Trial + Colour + Diet	5	2161.41	1.50	
Trial	3	2243.88	83.87	
Trial + Diet	4	2245.45	85.54	
Colour	3	2290.52	130.61	
Colour + Diet	4	2291.99	132.08	
Null	2	2366.01	206.10	
Diet	3	2367.54	207.63	

704

705 The model in bold is the final model.

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707

708 **Table S2.** Nested candidate model set for a GLMM investigating the effect of diet treatment and
 709 rewarding colour (conditioned stimulus, blue/yellow) on the proportion of correct choices made
 710 during unrewarding memory trials.

Covariates: diet + colour				
Random effect structure: individual queen				
Model	DF	AIC	Δ AIC	
Diet	3	160.84	0.00	
Diet + Colour	4	162.08	1.24	
Null	2	162.11	1.27	
Colour	3	163.33	2.49	

711

712 The model in bold is the final model. Note where competing models were within Δ AIC \leq 2.00 of each
 713 other, the simplest model was selected.

714

715 **Table S3.** Nested candidate model set for a GLM investigating the effect of diet treatment, learning
 716 treatment, and their interaction, on the probability of oviposition (egg-laying).

Covariates: diet + learning treatment + diet:learning treatment + thorax width			
Model	DF	AIC	Δ AIC
Diet	2	233.77	0.00
Diet + Thorax width	3	235.46	1.69
Diet + Learning treatment	4	237.33	3.56
Diet + Learning treatment + Thorax width	5	239.03	5.26
Diet + Learning treatment + Diet:Learning treatment	6	240.76	6.99
Diet + Learning treatment + Diet:Learning treatment + Thorax width	7	242.56	8.79
Null	1	258.41	24.64
Thorax width	2	260.13	26.36
Learning treatment	3	262.11	28.34
Learning treatment + Thorax width	4	263.83	30.06

717
 718 Queen thorax width was included as an additional covariate in the model. The model in bold is the
 719 final model.

720
 721

722 **Table S4.** Nested candidate model set for a Cox proportional hazards model investigating the effect
 723 of diet treatment, learning treatment, and their interaction, on oviposition (egg-laying) timing.

Covariates: diet + learning treatment + diet:learning treatment			
Model	DF	AIC	Δ AIC
Diet	1	1250.70	0.00
Diet + Learning treatment	3	1253.82	3.12
Diet + Learning treatment + Diet:Learning treatment	5	1256.89	6.19
Null	0	1282.55	31.85
Learning treatment	2	1286.01	35.31

724
 725 The model in bold is the final model.
 726

727 **Table S5.** Nested candidate model set for a zero-inflated model investigating the effect of diet
 728 treatment, learning treatment, and their interaction, on the production of callows (offspring).

Covariates: diet + learning treatment + diet:learning treatment			
Model	DF	AIC	ΔAIC
Diet	5	581.17	0.00
Diet + Learning treatment	9	586.40	5.23
Diet + Learning treatment + Diet:Learning treatment	13	589.97	8.80
Null	3	591.54	10.37
Learning treatment	7	597.19	16.02

729

730 The model in bold is the final model.

731

732

733 **Table S6.** Nested candidate model set for a linear mixed-effects model (LMM) investigating the effects
 734 of diet treatment, learning treatment, and their interaction, on the size (thorax width) of callows
 735 (offspring) produced by bumblebee queens during the experiment.

Covariates: diet + learning treatment + diet:learning treatment + type			
Random effect structure: colony			
Model	DF	AIC	ΔAIC
Type	4	707.55	0.00
Diet + Type	5	708.62	1.07
Diet + Learning treatment + Diet:Learning treatment + Type	9	710.97	3.42
Learning treatment + Type	6	711.45	3.90
Diet + Learning treatment + Type	7	712.43	4.88
Null	3	774.26	66.71
Diet	4	775.74	68.19
Diet + Learning treatment + Diet:Learning treatment	8	777.26	69.71
Learning treatment	5	778.15	70.60
Diet + Learning treatment	6	779.64	72.09

736

737 Callow type (worker/male) was included as an additional covariate in the model. The model in bold is
 738 the final model.

739

740 **Table S7.** Nested candidate model set for a GLM investigating the effects of diet treatment, learning
 741 treatment, and their interaction, on total brood production.

Covariates: diet + learning treatment + diet:learning treatment			
Model	DF	AIC	Δ AIC
Diet	3	1155.68	0.00
Diet + Learning treatment	5	1159.58	3.90
Diet + Learning treatment + Diet:Learning treatment	7	1162.15	6.47
Null	2	1164.43	8.75
Learning treatment	4	1167.94	12.26

742
 743 Total brood production was calculated as the total number of callows produced, plus any larvae and
 744 pupae found at the end of the experiment, including ones that had died. The model in bold is the final
 745 model.

746
 747 **Table S8.** Nested candidate model set for a GLM investigating the effects of diet treatment, learning
 748 treatment, and their interaction, on discarded brood (dead larvae, pupae and callows found during or
 749 at the end of the experiment).

Covariates: diet + learning treatment + diet:learning treatment			
Model	DF	AIC	Δ AIC
Diet	3	791.67	0.00
Diet + Learning treatment	5	795.43	3.76
Null	2	795.90	4.23
Diet + Learning treatment + Diet:Learning treatment	7	798.54	6.87
Learning treatment	4	799.84	8.17

750
 751 The model in bold is the final model.

752
 753
 754 **Table S9.** Nested candidate model set for a GLM investigating the effects of proportion of correct
 755 choices across all learning trials on oviposition probability (yes/no), using only bees in the learning
 756 treatment.

Covariates: proportion of correct choices			
Model	DF	AIC	Δ AIC
Null	1	89.02	0.00
Choice	2	91.02	2.00

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 758
 759 The model in bold is the final model.

760 **Table S10.** Nested candidate model set for a GLM investigating the effects of proportion of correct
 761 choices across all learning trials on the total number of callows produced, using only bees in the
 762 learning treatment.

763

Covariates: proportion of correct choices			
Model	DF	AIC	ΔAIC
Null	2	214.20	0.00
Choice	3	215.58	1.38

764

765 The model in bold is the final model.

766

767

768 **Table S11.** Nested candidate model set for a LMM investigating the effects of diet treatment, learning
 769 treatment, and their interaction, on consumption of sucrose (measured as the total sucrose consumed
 770 per queen every three days, minus the evaporation rate).

771

Covariates: diet + learning treatment + diet:learning treatment			
Random effect structure: individual queen			
Model	DF	AIC	ΔAIC
Diet	4	57688.99	0.00
Diet + Learning treatment	6	57692.91	3.92
Diet + Learning treatment + Diet:Learning treatment	8	57694.66	5.67
Null	3	57695.46	6.47
Learning treatment	5	57699.37	10.38

772

773 The model in bold is the final model.

774 **Table S12.** Nested candidate model set for a LMM investigating the effects of diet treatment, learning
 775 treatment, and their interaction, on consumption of pollen.

Covariates: diet + learning treatment + diet:learning treatment + day post oviposition			
Random effect structure: individual queen			
Model	DF	AIC	Δ AIC
Diet + Day post oviposition	5	18849.60	0.00
Diet + Learning treatment	7	18850.21	0.61
+ Day post oviposition			
Diet + Learning treatment	9	18850.66	1.06
+ Diet:Learning treatment			
+ Day post oviposition			
Day post oviposition	4	18883.00	33.4
Learning treatment	6	18884.25	34.65
+ Day post oviposition			
Diet	4	20015.88	1166.28
Diet + Learning treatment	6	20019.10	1169.50
Diet + Learning treatment	8	20019.71	1170.11
+ Diet:Learning treatment			
Null	3	20068.77	1219.17
Learning treatment	5	20072.49	1222.89

776
 777 Pollen consumption was measured as the total pollen consumed per queen every three days with a
 778 square root transformation. The number of days post oviposition for each individual queen was
 779 included as a covariate in the model. The model in bold is the final model.