Discrimination of edge orientation by bumblebees

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12   Simple feature detectors in the visual system, such as edge-detectors, are likely to underlie

13   even the most complex visual processing, so understanding the limits of these systems is

14   crucial for a fuller understanding of visual processing. We investigated the ability of

15   bumblebees (*Bombus terrestris*) to discriminate between differently angled edges. In a

16   multiple-choice, "meadow-like" scenario, bumblebees successfully discriminated between

17   angled bars with 7° differences, significantly exceeding the previously reported performance

18   of eastern honeybees (*Apis cerana*, limit: 15°). Neither the number of choices required to

19   learn, nor the bees' final discrimination performance were affected by the angular orientation

20   of the training bars, indicating a uniform performance across the visual field. Previous work

21   has found that, in dual-choice tests, eastern honeybees cannot reliably discriminate between

22   angles with less than 25° difference, suggesting that performance in discrimination tasks is

23   affected by the training regime, and doesn't simply reflect the perceptual limitations of the

24   visual system. We used high resolution LCD monitors to investigate the limits of bumblebees'

25   angular resolution in dual-choice experiments. Bumblebee could still discriminate 7° angle

26   differences under such conditions (exceeding the previously reported limit for *Apis mellifera*,

27   of 10°, as well as that of *A. cerana*), eventually reaching similar levels of accuracy, but required

28   longer learning periods than under multiple-choice conditions. Bumblebees show impressive

29   abilities to discriminate between angled edges, performing better than two previously tested

30   species of honeybee. This high performance may, in turn, support complex visual processing

31   in the bumblebee brain.

Introduction

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33 Low-level feature detectors [1–5] such as edge orientation detector neurons [6] underlie

34 visual object recognition, even in complex cognitive tasks [2,6–10]. Roper *et al.* [11]

35 demonstrated that it is possible to identify and discriminate a wide variety of complex visual

36 patterns, using a low number of edge orientation detectors, without any need for storing

37 "snapshot" visual memories. Differences in edge detection performance are thus likely to

38 underpin interspecies differences in many visual discrimination tasks, so a detailed

39 understanding of visual learning by bees and other insects will require an understanding of

40 the limits of edge orientation detection. Bumblebees are a popular model for studies of insect

41 visual learning [12,13], providing significant advantages in that they can be can be bred and

42 kept in indoor settings, which allows researchers to test them year-round in precisely defined

43 laboratory conditions. There is currently no behavioural data available on how well

44 bumblebees can discriminate between angled edges.

45 Wehner and Lindauer [14,15] trained European honeybee workers (*Apis mellifera*) to collect

46 food from feeders bearing either vertical, horizontal or 45° black bars. In tests, bees could

47 discriminate the training pattern from one with only a 10° difference in orientation; at 8°,

48 bees' performance deteriorated; and they failed completely at 5°. Chandra *et al.* [6] trained

49 eastern honeybee workers (*Apis cerana*) to feeders with black stripes of various angular

50 orientations, presented on a white disk. Two tests were used: in one, the bees chose between

51 two cues, one with stripes matching the trained orientation and one in a different orientation;

52 the other test employed a multiple-choice paradigm, in which bees chose between the trained

53 orientation and 11 deviations from it. Honeybees performed better in the multiple-choice

54 situation, successfully choosing the trained orientation over alternatives rotated by 15°,

55 whereas under the dual-choice conditions they only succeeded in differentiating between

56 stripes that differed by 25°. This result gives a clear demonstration of the risks of inferring the

57   limits of discrimination from behavioural performance, since such performance depends not

58   just on what the bee sees, but also on the training procedure. There is mounting evidence

59   that the conditioning procedure plays an important role in how animals perform in visual

60   discrimination tasks [16–19]. Comparative methodological approaches allow us to understand

61   the advantages and limits of each type of protocol [9,20], and provide insight into the ways

62   performance depends not just on sensory limitations but on how information is processed

63   and used in different contexts.

64   We investigated the ability of bumblebee workers (*Bombus terrestris audax*) to discriminate

65   between cues with differently oriented edges, in both a multiple-choice, meadow-like

66   environment and in a dual-choice set-up, to determine whether their performance differs

67   from that of honeybees and how it is affected by the behavioural context.

Methods

Experiments 1 and 2: Multiple-choice (meadow paradigm)

Setup and pre-training

71   We tested bumblebee workers from two commercially bred colonies (Biobest, Belgium). Each

72   colony was kept in a custom-built wooden nest box (280 × 160 × 110 mm high) and connected

73   via a Perspex tunnel to a foraging arena (700 × 700 × 400 mm high) with white painted walls

74   and green laminated paper floor. High frequency fluorescent lighting (TMS 24F lamps with HF-

75   B 236 TLD ballasts, Phillips, Netherland; fitted with Activa daylight fluorescent tubes, Osram,

76   Germany) were used to illuminate the foraging arena; the flicker frequency of the lights was

77   ~42kHz which is well above the flicker fusion frequency for bees [21,22].

78   During a pre-training phase, bees were allowed free access to the arena where they could

79   forage on *ad libitum* sucrose solution (20% w/w) from ten feeders placed on the arena floor.

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80   Each feeder consisted of a sucrose-solution-filled feeding tube (Ø 7mm) placed horizontally at

81   the centre of a vertically aligned, circular disk (Ø 110mm), made of laminated white paper.

82   Each feeder was supported by an attached block weight (15 × 25 × 45 mm high) that allowed

83   the feeder disk to be aligned perpendicular to the floor arena (Figure 1b). Bees foraging from

84   the feeders were individually marked with number tags (Opalithplättchen, Warnholz &

85   Bienenvoigt, Germany) for identification. Numbered bees that were observed to forage in the

86   arena frequently were allowed to advance to the individual training phase. Pollen was

87   provided *ad libitum* into the colony.

Training and testing

89   During the training phase, bees were allowed to forage individually in the arena. Ten feeders

90   (Figure 1B) were arranged in a circle, such that a bee could see all ten stimuli from the centre

91   of the arena (Figure 1A). These feeders were identical to those used in pre-training, except

92   that each white circle contained a single black bar (75 x 5 mm). Use of a 360° protractor

93   attached to each feeding station, and spirit level on the arena base, allowed the experimenter

94   to align the bar to precise angular orientations (Figure 1). Five feeders, randomly assigned,

95   had the bar oriented at one angle, *A*, and provided 10µl sucrose solution (CS+: 30% w/w); the

96   remaining five feeders had the bar oriented to a different angle, *B*, and were non-rewarding,

97   filled with a 10µl water droplet (CS-). Feeding tubes were refilled from the rear, preventing

98   sucrose or water from being deposited at the entrance of the tube.

99   **Figure 1. Experimental set-up**

100   **A:** Multiple-choice arena. A bumblebee colony was housed in a wooden box, connected to

101   the experimental arena by a transparent tunnel. Ten feeders were presented in a circle

102   configuration so that all were visible from the centre of the arena. **B:** Feeder. Each feeder

103   consisted of a white paper disc with a printed black bar, and a feeding tube in the centre. A

104   heavy block weight at the rear of the feeder supported a rotatable 360° protractor, allowing

105   the bar to be rotated to the required angle. Five feeders, allocated at random, had the bar

106   oriented to angle *A* and contained a drop of sucrose solution; the others were oriented to

107   angle *B* and contained water only. **C:** Dual-choice arena. Two computer monitors were
108   arranged at a 60° angle at the rear of the arena. Each presented a black bar in a white circle.
109   A feeding tube was placed at the centre of each bar. One bar, allocated at random was
110   oriented to angle *A* and the feeding tube contained a drop of sucrose solution; the other was
111   oriented to angle *B* and contained water. **D:** Example stimuli used in dual-choice
112   experiments. Top row, two stimuli used during pre-training, one on each screen. Bottom row,
113   example bar stimuli used for training and testing: left, 0° bar; right, 7° bar. All stimuli were
114   displayed in magenta (RGB: 255,0, 255) on a white background (RGB: 255, 255, 255).

115   This training paradigm was used for two experiments: in the first, three bees were trained to

116   rewarding and unrewarding feeders with bars differing in orientation by 15°. Each bee

117   experienced one angle *A* (CS+) and one angle *B* (CS-), but three different sets of angles were

118   used, with one set assigned to each bee at random. Angle *A* was either 30°, -60° or -75°

119   (clockwise from vertical); angle *B* was 45°, -75°, or -60° respectively.

120   In the second experiment, 25 bees were trained to bar orientations differing by 7°. A greater

121   range of bar orientations was used in order to examine discrimination performance across the

122   full range of possible orientations. We started with four base angles at -60°, 0°, 45° and 90°.

123   These were paired with an angle that differed by 7°, either clockwise or anticlockwise (e.g. 45°

124   could be paired with 38° or 52°), giving 8 pairs of angles. We assigned pair of angles to each

125   bee at random and further randomised which of the two angles in the pair was associated

126   with the sucrose reward and which with water. There were thus 16 possible combinations of

127   angles *A* and *B*, although only 12 were assigned to bees in practice. Table 1 gives details of

128   how many bees were trained with each pair of angles.

129 **Table 1. Number of bees tested with each pair of bar angles in experiment 2**

Base angle (°)	Direction of difference	Base angle assignment	Angle *A* (°)	Angle *B* (°)	No bees
-60	+	A	-60	-53	1
-60	+	B	-53	-60	2
-60	-	A	-60	-67	4
-60	-	B	-67	-60	2
0	+	A	0	7	1
0	+	B	7	0	0
0	-	A	0	-7	0
0	-	B	-7	0	0
45	+	A	45	52	4
45	+	B	52	45	3
45	-	A	45	38	2
45	-	B	38	45	0
90	+	A	90	97	1
90	+	B	97	90	1
90	-	A	90	83	2
90	-	B	83	90	2

130

131 Before training, all bees were removed from the arena and the setup was cleaned with 70%

132 ethanol. The focal bee was allowed repeated access to the foraging arena until a total of 100

133 consecutive feeder choices were recorded. A choice was defined as a bee landing on a feeding

134 tube, and for each choice we recorded whether the feeder was rewarding or unrewarding.

135 After each foraging bout (consisting of multiple feeder choices, typically 4-10, continuing until

136 the bee filled its crop, and returned to the nest box), all feeders were cleaned and the

137 positions of rewarding and unrewarding were randomized before the bee was allowed to re-

138 enter the arena. Rewarding feeders were refilled with 10µl sucrose solution once the bee each

139 time a bee had drunk and departed from the feeder. Bees did not consume water from the

140 unrewarding feeders, so they did not require refilling after each choice, but unrewarding

141 feeders were cleaned and refilled after every foraging bout.

Experiments 3 and 4: Dual-choice

Set-up

We tested bumblebee workers from a third colony (Biobest, Belgium) on dual-choice tests, including even smaller angular deviations. To test the bees' ability to discriminate below 7°, it is important to ensure that the angular alignment is more precisely controlled than we could guarantee using physical stimuli. Instead, we presented stimuli on two high-resolution, high-refresh-rate LCD computer monitors (Acer Predator GN246HLB, with 144Hz refresh rate, which is above the flicker fusion frequency of bees [22,23]). These monitors were aligned and fixed in position, and software-generated oriented bars were used to ensure uniform angles.

A second flight arena (1150 × 1300 × 500 mm high) was used for experiments 3 and 4. The flight arena was covered with a red Gaussian random dot pattern (generated with custom MATLAB code), printed onto white laser copy paper and laminated. At the top of the arena a fine fabric net was attached, this extended to the laboratory ceiling. High frequency fluorescent lighting (TMS 24F lamps with HF-B 236 TLD ballasts, Phillips, Netherland; fitted with Activa daylight fluorescent tubes, Osram, Germany) were used to illuminate the apparatus.

Two monitor screens were positioned at the rear wall of the flight arena and aligned at 60° angle from each other, allowing the bee to see both screens from the entrance of the arena (980 mm in front of the monitors). A transparent Plexiglas sheet was placed 15 mm from each monitor screen with a small hole (Ø 10mm) at the centre, leading to a feeding tube (Ø 8mm, 15mm long). As the tube was behind the Plexiglas from a bee's point of view, it did not block any of her flight movements in front of presented stimuli and allowed us an unobstructed view of the bee's movements.

Stimuli

Stimuli were created and displayed on the monitors using custom MATLAB (Mathworks) code and the PsychToolbox [24]. Each monitor displayed a single circle open circle (Ø 260 mm) on a white background, with one or more shapes inside. All stimuli were magenta (RGB: 255,0, 255), allowing observers to easily see the dark body of a bee in front of the monitor while still providing high levels of green-photoreceptor-contrast for the bee, which is required for edge detection and angle discrimination tasks [4,25,26]. During pre-training, one monitor showed a Ø 20 mm filled circle surrounded by four concentric circles (Ø 95, 160, 210 and 260 mm), while the other showed the Ø 260 mm circle containing an arrangement of 17 filled circles (Ø 20 mm; Fig 1D).

During training and tests, each monitor showed a circle containing a single bar (180 mm x 20 mm, with rounded ends; Fig 1D). Each rewarding bar orientation was paired with an identical, unrewarding bar, at a different angle. Two experiments were carried out: in experiment 3, rewarding stimuli were assigned to 6 bees at random (angle *A*: 0°, 38°, 83°, 90°, 90° and 97° clockwise from vertical). The bar for the unrewarding feeder was rotated either +7° or -7°, relative to the rewarding stimulus (angle *B*: 7°, 45°, 90°, 83°, 97° and 90°, respectively). In experiment 4, three bees were trained to stimuli with angle *A* set to 85°, 90° and 90° clockwise from vertical. The bar for the unrewarding feeder was rotated by +5° or -5° relative to the rewarding stimulus (angle *B*: 90°, 85° and 85°, respectively).

Pre-training

During the pre-training phase, bees were individually trained to feed from the feeding platforms and to learn that certain visual stimuli were associated with a sucrose reward. During this phase, no oriented bars were presented, but bees learned to discriminate between filled or open circles placed within the 260 mm outer circles (Fig 1D). One stimulus, assigned

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189   at random, provided 20µL of 50% (w/w) sucrose solution in the feeding tube (CS+). The other

190   contained 20µL of water (CS-).

191   A choice was recorded whenever a bee landed on one of the two feeding tubes. If the bee

192   chose the tube in front of the unrewarding stimulus it was allowed to continue making choices

193   it landed on the rewarding feeder. Once the bee had located and consumed the 20µL sucrose

194   solution from the rewarding feeder, it was captured in a transparent ventilated transfer tube

195   (Ø 30mm; 70mm long) and released again from the entrance of the arena to make another

196   choice. Each foraging bout consisted of approximately four feeder visits, and lasted until the

197   bee's crop was full and it returned to the nest (mean crop capacity: 80 to 120µL, [27]). The

198   positions of the rewarding and unrewarding stimuli were pseudo-randomized between bouts

199   (with no more than two consecutive visits permitted with the same positions). Once a bee

200   chose the rewarding pattern eight times out of the last ten feeder visits, it progressed to the

201   experimental training phase.

202   **Training and testing**

203   The experimental training phase followed the same procedure as above. During each foraging

204   bout, each of the two monitors showed an oriented bar (one feeder providing reward of 20µL

205   of 50% sucrose solution w/w, and the other providing 20µL water). The location of the

206   rewarding stimulus was pseudo-randomized for each foraging bout. Each bee was trained for

207   approximately 150 choices, continuing until the bee achieved at least 80% correct choices

208   over two consecutive 10-choice batches. If the bee did not achieve this 80% accuracy after

209   250 choices, the training was abandoned.

210   If the bee achieved ≥80% correct choices in two consecutive batches of 10 choices, it was

211   subjected to a test bout. During tests, both feeding tubes had a 20µL drop of water within

212   them regardless of the stimulus. After each batch of 10 choices between unrewarding stimuli,

213   the bee was given 10 choices under training conditions, with the trained stimulus again

214   rewarded with sucrose, to maintain its motivation to visit the feeders. The proportion of

215   choices for the trained stimulus was recorded for each batch of 10 choices for unrewarded

216   stimuli. The test bout ended when the bee no longer attempted to visit either feeding tube.

217   **Analysis**

218   Learning performance was evaluated using the percentage of correct choices for every block

219   of 10 consecutive feeder visits. Individual bees were categorised as having learned the task if

220   they made more than 80% correct choices over two consecutive 10-choice blocks at any point

221   during the learning phase. Experiments 1 and 4 had small sample sizes (of only 3 bees per

222   experiment), so we evaluated bees' performance based solely on how many had reached this

223   learning criterion.

224   For experiments 2 and 3, we further tested whether the number of correct choices over the

225   last 20 feeder visits by each bee, during training, was significantly greater than chance

226   performance, using one-sample Wilcoxon signed-rank tests to compare the median number

227   of correct choices to a chance level of 10 correct choices out of 20. The bees in experiment 3

228   were also tested on their performance in unrewarding probe s. We tested whether their

229   performance exceeded chance in these tests using a paired Wilcoxon signed-rank test, in

230   which the number of correct choices made was compared to an expected value of half the

231   total number of feeder visits during the test.

232   We investigated whether there was any effect of bar orientation on the final performance

233   reached by each bee in experiment 2. Bees were assigned to one of four orientation groups,

234   in which we tested their ability to differentiate between bars at one of four angles (-60°, 0°,

235   45°, 90°) and bars ±7° from each of those angles (Table 1). Only one bee was tested in the

236   0°±7° group, so it was excluded from this analysis. The other three groups (-60°, 45°, 90°)

237   contained 9, 9 and 6 bees, respectively. We tested for differences in performance between

238   these groups using a Kruskal-Wallis test, in which the dependent variable was the number of

239   correct choices over the last 20 feeder visits made by bees in each orientation group. Among

240   those bees that reached a performance of 80% correct choices over at least 20 visits, we

241   tested whether there was an effect of bar orientation on the speed of learning, using another

242   Kruskal-Wallis test in which the dependent variable was the number of choices required to

243   reach the 80% learning criterion by bees in each orientation group.

244   We investigated whether there were differences in final performance between bees trained

245   to discriminate 7° orientation differences under dual- and multiple-choice conditions, using a

246   pair of Kruskal-Wallis tests to compare the performance of bees from experiment 2 to those

247   from experiment 3. The dependent variables were the number of correct choices by each bee

248   during feeder visits 80-100 and the number of correct choices by each bee over its last 20

249   choices. We tested whether there was a difference in the speed of learning between

250   experiments 2 and 3, using another Kruskal-Wallis test in which the dependent variable was

251   the number of choices required to reach the 80% learning criterion by bees in each

252   experiment.

253   All statistical tests were 2-tailed. All statistics were calculated in Matlab (version R2018a;

254   Mathworks Inc., Natick, USA).

Results

256   **Experiment 1: Multiple-choice, 15° angular difference**

257   The first experiment tested bumblebees' ability to discriminate a 15° difference in edge

258   orientation. The first three bees tested all reached the criterion for success (mean ≥80%

259   correct choices over two consecutive blocks of 10 feeder visits) within 40-50 feeder choices

260   (proportion correct choices over final 20 choices, mean ± standard error of the mean: 0.917 ±

261   0.033; Fig 2). Due to this clear evidence that bees could discriminate an angular difference of

262   15°, we proceeded immediately to test a smaller difference of 7°.

263   **Figure 2. Learning curves for bees in multiple- and dual-choice experiments.**

264   Each marker shows the mean proportion of choices (±S.E.) for the trained angle, *A*, across a

265   block of 10 consecutive choices in multiple-choice experiments (red lines) and dual-choice

266   experiments (blue lines). Dark red triangles indicate experiment 1 (multiple-choice, angles *A*

267   and *B* differ by 15°, N=3); light red circles, experiment 2 (multiple-choice, angles *A* and *B*

268   differ by 7°, N=25); dark blue circles, experiment 3 (dual-choice, angles *A* and *B* differ by 7°,

269   N=6); light blue squares, experiment 4 (dual-choice, angles *A* and *B* differ by 5°, N=3). Dashed

270   grey horizontal lines indicate a chance level of performance (0.5) and the criterion for our

271   bees to be classified as having learned (0.8, but note that bees had to reach this proportion

272   of correct choices over 20 feeder visits).

Experiment 2: Multiple-choice, 7° angular difference

274   Out of 25 bees tested on angled bars with a 7° difference, just three failed to reach 80% correct

275   choices over at least two consecutive blocks of 10 feeder visits. Those bees that reached the

276   80% learning criterion, did so after a mean of 71.36 ± 3.37 choices (range: 40-100; Fig 2). The

277   mean proportion of correct choices by all 25 bees in this group over their last 20 feeder visits

278   was 0.870 ± 0.025, significantly greater than expected by chance (Wilcoxon signed-rank test:

279   V = 325, N = 25, P <0.0001), and similar to the performance seen in the 15° experiments (Fig

280   2).

281   The three bees that failed to reach 80% successful choices were each trained with a different

282   bar orientation, *A* (-60°, 52° and 45°) and each of these angles was also assigned to other bees

283   that did reach the criterion (5, 2 and 5 successful learners, respectively). There were no

284   significant differences between the groups of bees trained on each of three different base bar

285   orientations (-60°±7°, 45°±7°, 90°±7°) in the number of correct choices over their final 20

286   choices (Kruskal-Wallis test: χ^2_2 = 4.98, P = 0.083; Fig 3A-B). Among the 21 bees in these three

287   orientation groups that did reach the 80% learning criterion, there was no effect of bar

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288   orientation on the number of choices required to reach this level of performance (Kruskal-

289   Wallis test: $\chi^2_2 = 1.60$, P = 0.450; Fig 3C).

290   **Figure 3. Angular discrimiation is unaffected by training bar angles, in multiple-choice
291   tests**

292   **A:** Mean proportion of choices (±S.E.) for the trained angle, *A*, across a block of 10

293   consecutive choices for bees in experiment 2. Dark red triangles indicate bees that were

294   trained to discriminate bars of -60° ± 7° (N=9); blue circles, bees trained to discriminate bars

295   of 45° ± 7° (N=9); light green squares, bees trained discriminate bars of 90° ± 7° (N=6). Dashed

296   grey horizontal lines indicate a chance level of performance (0.5) and the criterion for our

297   bees to be classified as having learned (0.8 but note that bees had to reach this proportion

298   of correct choices over 20 feeder visits). **B:** Proportion of choices for angle *A* (correct choices)

299   across the last 20 feeder visits by bees trained to each group of angular orientations (-60° ±

300   7°, 45° ± 7°, 90° ± 7°). Red lines indicate group median, boxes indicate the interquartile range

301   and whiskers indicate the range. Filled circles show the proportion of choices for angle *A,*

302   made by each individual. There are no significant differences between groups. **C:** Number of

303   feeder visits required to reach a performance level of at least 80% correct choices across a

304   block of 20 consecutive feeder visits, by bees trained to each group of angular orientations

305   (-60° ± 7°, N=8; 45° ± 7°, N=7; 90° ± 7°, N=6). Three bees that never reached this criterion

306   have been excluded (one from the -60° ± 7° group and two from the 45° ± 7° group). There

307   are no significant differences between groups.

Experiment 3: Dual-choice, 7° angular difference

309   We also tested bees' ability to discriminate a 7° angle variance in a dual-choice paradigm.

310   After 100 feeder visits (the total number experienced by bees in the multiple-choice

311   experiments), six bees had a mean success rate of just 0.600 ± 0.058 across choices 80-100.

312   This performance is significantly lower than in the multiple-choice setup (Kruskal-Wallis test:

313     $\chi^2_1 = 10.41$, P = 0.0013; Fig 2).

314   With continued training, all but two bees reached the 80% criterion within 130-170 choices

315   (mean ± S.E.: 147.50 ± 8.54), demonstrating that bumblebees are capable of learning to

316   discriminate angular deviations of 7° under these conditions. Bees in the dual-choice

317   experiment required significantly more training to reach this criterion than those in the

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318   multiple-choice experiment (Kruskal-Wallis test: χ^2_1 = 9.99, P = 0.0016), and examination of

319   the learning curves makes it clear that bees' performance in experiment 3 improved at a lower

320   rate than that of bees in experiment 2, throughout the entire training period (Fig 2).

321   Even the two bees that never reached the 80% threshold showed evidence of learning, with

322   final performances of 0.70 and 0.75 after 190 choices. The mean proportion of correct choices

323   by all 6 bees in this group over their last 20 feeder visits was 0.800 ± 0.034, significantly greater

324   than expected by chance (Wilcoxon signed-rank test: V = 21, N = 6, P = 0.031; Fig 2). There

325   was no significant difference between the final performance reached by these bees and those

326   discriminating 7° differences in experiment 2, under multiple-choice conditions (Kruskal-

327   Wallis test: χ^2_1 = 2.72, P = 0.099).

328   The bees in this test were subjected to a learning test after they had reached the 80% criterion,

329   or after 190 choices in the case of the two bees that never reached 80% performance. In this

330   test, all feeders were unrewarding. Under these conditions, bees were as successful in

331   discriminating angles as they were at the end of the training period, with a mean proportion

332   of choices for the previously rewarding stimulus of 0.764 ± 0.065, significantly greater than

333   expected by chance (Wilcoxon signed-rank test: V = 21, N = 6, P = 0.031).

Experiment 4: Dual-choice, 5° angular difference

335   We attempted to train a further three bees to discriminate oriented bars with only 5° angle

336   differences. This sample size is too low for formal analysis, but Fig 2 suggests that these bees

337   did show some improvement in performance with continued training. However, they

338   appeared to improve in performance at a far slower rate than those discriminating 7°

339   differences. Even after 250 feeder visits over the course of four to six full days, the bees did

340   not obviously choose the correct bars more often than expected by chance (mean proportion

341   correct choices ± S.E.: 0.600 ± 0.029; individual means: 0.500, 0.600, 0.650; Fig 2).

342   Performance in a subsequent learning test was similarly poor (mean proportion correct

343   choices ± S.E.: 0.636 ± 0.016; individual means: 0.625, 0.667, 0.615).

Discussion

345   In this study, we demonstrate that bumblebees (*Bombus terrestris*) are able to discriminate

346   between oriented bars with an angle difference of just 7°, in both a meadow-like multiple-

347   choice, and a dual-choice scenario, and regardless of bar orientation. Our data suggest that

348   bumblebees are unlikely to be able to discriminate angular differences of 5° or less. This

349   performance slightly exceeds that reported for European honeybees (*Apis mellifera*), which

350   discriminated 10° differences with certainty and still showed some evidence of discrimination

351   at 8°, in dual choice tests [14,15]. By contrast, previous work has found that eastern

352   honeybees (*Apis cerana*) could not be trained to discriminate angle differences below 15° in

353   a multiple-choice setup, and below 30° under dual-choice conditions [6].

354   While the final performance of bumblebees was similar in both experiments, it took far more

355   training for them to reach a high level of performance when choosing between two options

356   displayed on computer monitors than when 10 options were displayed in a physical array. A

357   number of studies have demonstrated an influence of conditioning paradigm on bees'

358   behavioural performance in a variety of tasks [28–30]. Our study, in concert with that of

359   Chandra *et al.* [6], demonstrates that the training procedure can affect the outcome of even

360   apparently simple perceptual tasks and highlights the dangers of attempting to derive

361   perceptual limits directly from behavioural studies.

362   Why might bees learn to discriminate angles more quickly in a multiple-choice experiment?

363   There were several methodological differences between the experiments, so it is not possible

364   to determine this conclusively, but one important factor may be the frequency with which

365   bees were able to get feedback on their choices. In the meadow-like, multiple-choice

366   experiment, bees visited several feeders during every foraging bout (round-trip from the nest

367   to the flight arena and back), but in the dual-choice experiment they could make only one

368   choice before being returned to the arena entrance. The comparative difference in how

369   rapidly they can sample different options and rewards may account for the difference in

370   learning speed.

371   The stimuli for our multiple-choice test were printed on paper, while the dual-choice test was

372   presented on computer monitors, which have previously been found make fine

373   discriminations more difficult for bees [31]. However, Chandra et al. [6] reported differences

374   between dual- and multiple-choice setups for eastern honeybees, even though their stimuli

375   were printed on paper in both experiments, so the use of monitors is unlikely to account for

376   the differences we observe. Chandra et al. [6] suggested that the variation in performance

377   was due to their multiple-choice configuration providing only a one in twelve chance of the

378   bee selecting the correct stimulus. Under dual-choice conditions it may be efficient to sample

379   all stimuli at random, rather than investing time and computation in discriminating between

380   them, since there is a 0.5 chance of reward from each feeder sampled. By contrast, when the

381   odds of success fall to 0.0833, an investment in learning to discriminate between correct and

382   incorrect stimuli may lead to a greater medium- or long-term rate of energy gain. The odds of

383   success from random sampling were 0.5 in both of our experiments, however, so differences

384   in expected reward from random sampling cannot account for the difference between

385   paradigms.

386   While our results do show that aspects of the training procedure can affect bees' performance

387   in angular discrimination tasks, we can nonetheless draw some conclusions regarding the

388   sensory/perceptual limitations faced by bumblebees. At the end of training, when bees had

389   reached saturation-level performance, both groups of bees discriminated between edges

390   differing by 7° with a high level of accuracy, clearly demonstrating that they could perceive

391   differences of at least that magnitude. When trained to bars with a 5° difference under the

17

392   dual-choice paradigm, three bees did not perform above chance levels even after an extra 2-

393   3 days of training and 100 more choices than were required to learn a 7° difference. A greater

394   sample size would be required to confirm that bees are unable to learn a 5° difference, but

395   given the slow rate of improvement and the fact that bees typically invest relatively little time

396   in learning how to handle flowers [32–34], we suggest that the practical limit for bumblebee

397   angular perception during foraging tasks is likely to lie between 5°-7°. Note that it is difficult

398   to derive conclusions about perceptual limits from behavioural data: discriminating 5°

399   differences may not actually be beyond bees' perceptual ability, just sufficiently difficult or

400   costly that doing so was less efficient than random sampling in our experiment.

401   It is interesting to note that, even in a multiple-choice setup, the limit of angular discrimination

402   previously reported for eastern honeybees [6] is double what we found for bumblebees,

403   pointing to a significant variation in visual discrimination ability between the two species. One

404   explanation might be that visual-spatial resolution is constrained by eye optics: bumblebee

405   workers are generally larger than honeybees and their larger eyes have both larger

406   ommatidial facets and reduced interommatidial angles [35–39]. As a result, bumblebees have

407   been found to have higher visual acuity than European honeybees (*Apis mellifera*), in several

408   behavioural contexts [23,36,37]. Recent work on X-ray micro computed-tomography of the

409   honeybee and bumblebee eyes [37,38,40,41] may pave the way toward future experiments

410   in which we can relate the behavioural performance of individual insects directly to the acuity

411   of their eyes. Bumblebee workers exhibit a high degree of variation in body size, so a fruitful

412   avenue of investigation may be to ask whether larger individuals, with larger eyes, perform

413   better in angular discrimination tasks. In these experiments we did not take any measures of

414   body size, but we took care to use individuals that were within the normal size range for

415   bumblebee workers and the bees that failed to reach a high level of performance in our

416   experiments did not appear notably smaller than successful bees.

417 European honeybees have been reported to show levels of angular discrimination only a little

418 less than those we found in bumblebees [15]. The size and morphology of *A. mellifera* eyes

419 lies between that of *A. cerana* and *B. terrestris* in many aspects (e.g. ommatidial number: *A.*

420 *cerana* = ~4921, *A. mellifera* = ~5375, *B. terrestris* = ~5656; eye surface (mm2): *A. cerana* =

421 2.3, *A. mellifera* = 2.5, *B. terrestris* = 2.8; [39,42]), yet European honeybees performance was

422 closer to that of bumblebees than this might predict. This perhaps suggests that optical

423 resolution is not the limiting factor on honeybee performance; the characteristics of the

424 neurons mediating edge orientation discrimination are also likely to play an important role.

425 Our results and those of Chandra et al. [6], show that bees could discriminate angular

426 differences regardless of bar orientation. Chandra et al. [6] used a mathematical model to

427 suggest that a minimum of three orientation sensitive neurons would be required to account

428 for this orientation indifference. However, this model assumed neurons with horizontal and

429 vertical maximal sensitivities. Empirical work has subsequently identified two types of edge

430 orientation sensitive neurons in the lobula, the third visual ganglion of the honeybee, with

431 maximal sensitivity to edges angled at 115° and 220° from the vertical, respectively [43].

432 Roper *et al.* [11] presented computational-neuronal models based on the known properties

433 of honeybee [43] and dragonfly neurons [44], which predicted performances remarkably

434 similar to empirical honeybee results. Both models predicted that discrimination performance

435 should be independent of the orientation of the training bars, as found in all three species of

436 bees so far tested. These models support the hypothesis that angular discrimination

437 performance is largely determined by the very limited number of orientation detector

438 neurons identified in insects, and that these neurons' angular tuning is adaptive in allowing

439 uniform performance across a wide variety of orientations.

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Authors' contributions

448   Conceptualization, M.R., M.G., S.W. and L.C.

449   Methodology, M.R., M.G. and S.W.

450   Investigation, M.R., M.G. and S.W.

451   Data Curation, M.G.

452   Software, M.G.

453   Formal analysis, M.G. and J.L.W.

454   Visualization, M.G. and J.L.W.

455   Writing - Original draft, M.G.

456   Writing – Review & editing, M.G., M.R., J.L.W. and L.C.

457   Funding acquisition, L.C.

458   Supervision, L.C.

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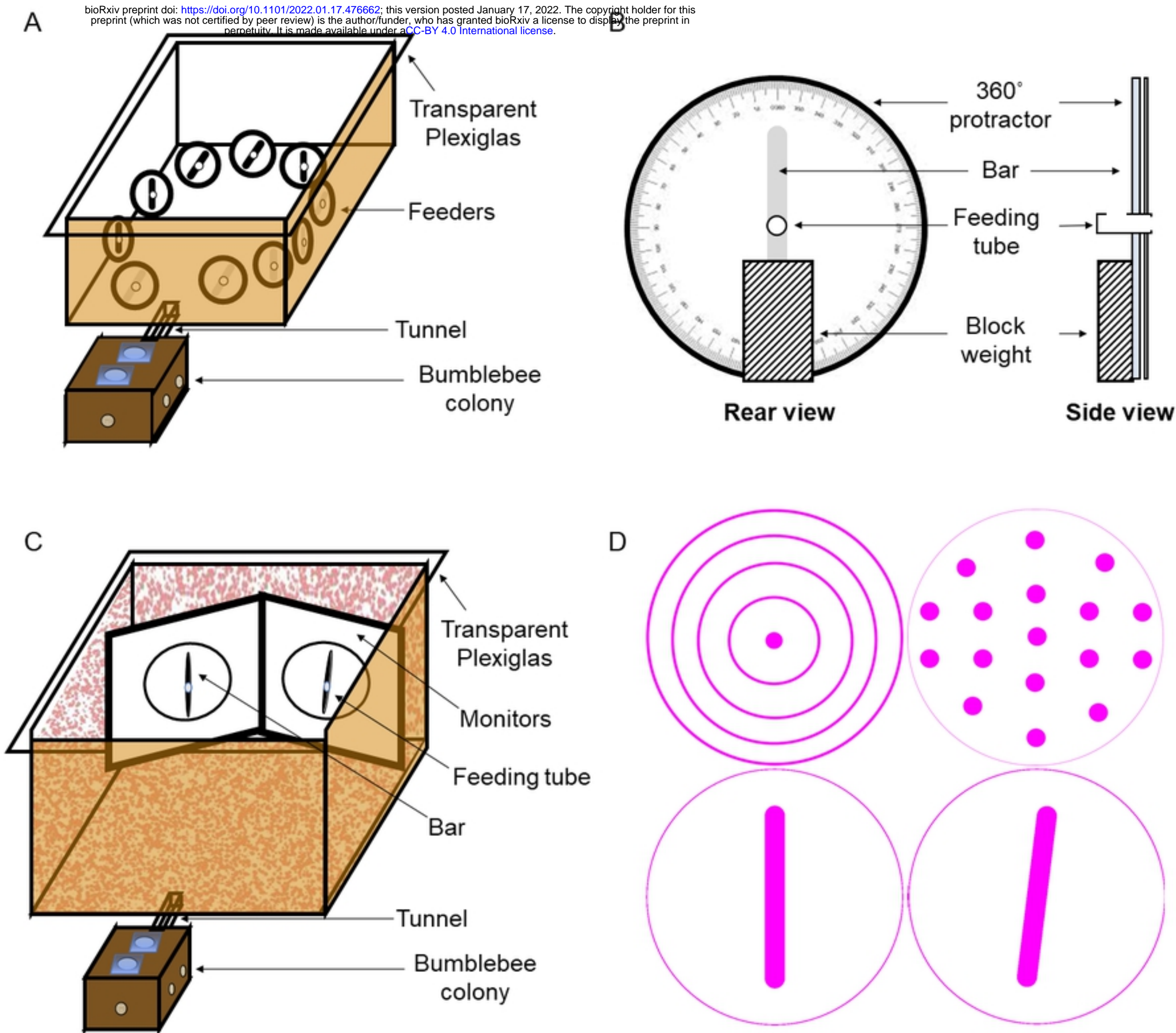571   44.   O'Carroll D. Feature-detecting neurons in dragonflies. Nature. 1993; 362(6420):541–3.
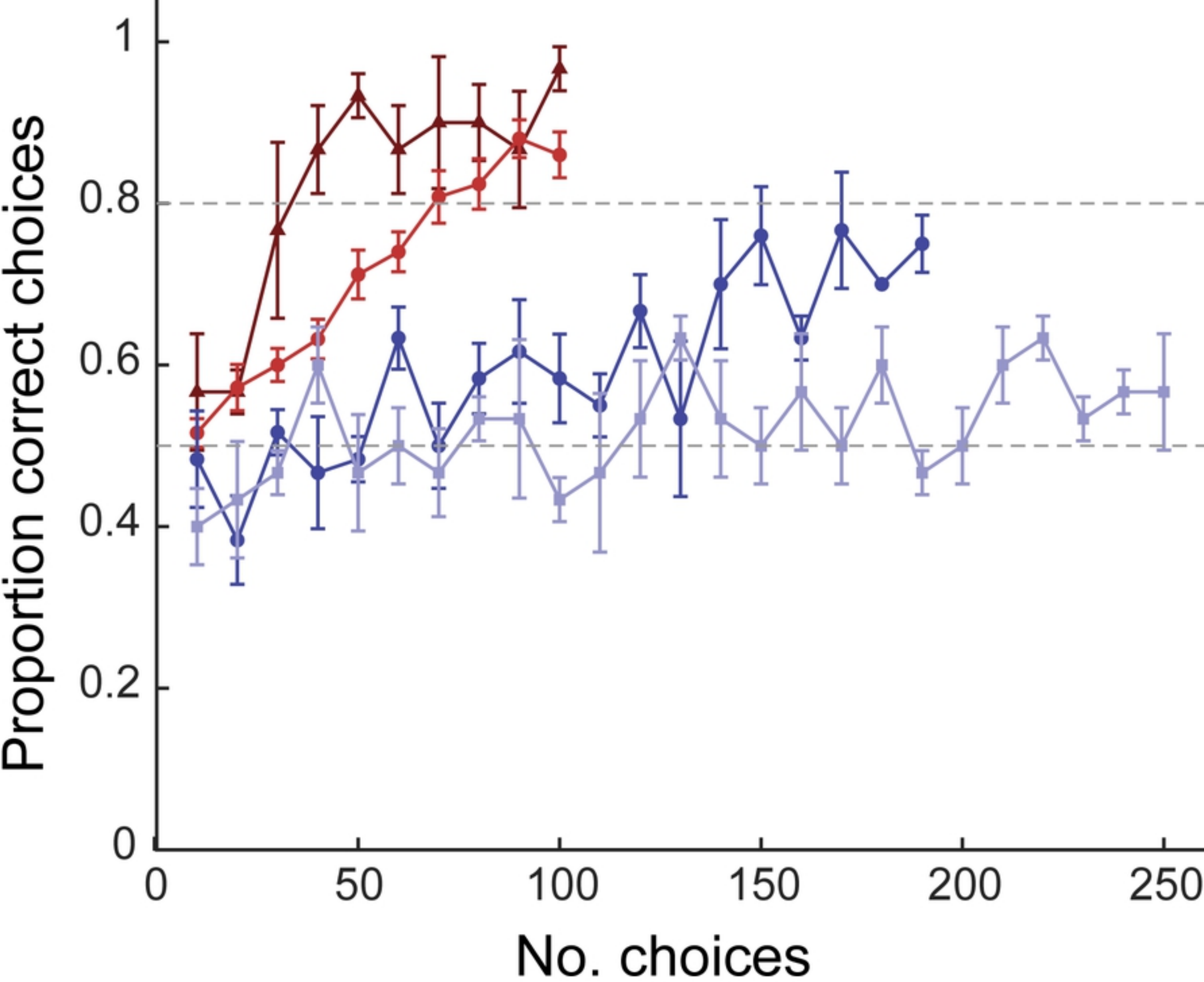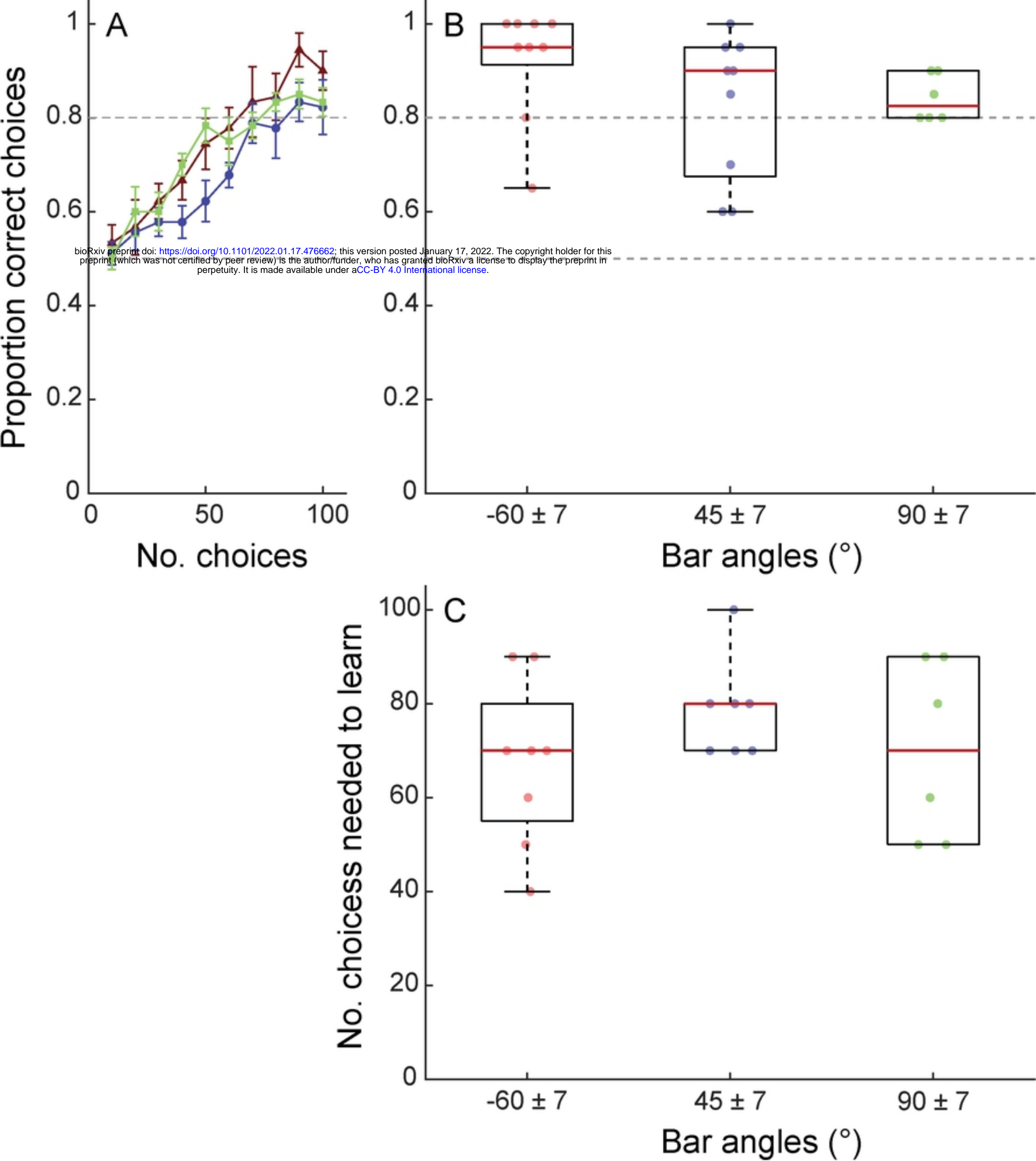
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Figure 1

Figure 2

Figure 3