1   **Route following by one-eyed ants suggests a revised model of normal route following**

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9   **Summary**

10   The prevailing account of visually controlled routes is that an ant learns views as it follows a

11   route, while guided by other path-setting mechanisms. Once a set of route views is memorised,

12   the insect follows the route by turning and moving forwards when the view on the retina

13   matches a stored view. We have engineered a situation in which this account cannot suffice in

14   order to discover whether there may be additional components to the performance of routes.

15   One-eyed wood ants were trained to navigate a short route in the laboratory guided by a single

16   black, vertical bar placed in the blinded visual field. Ants thus had to turn away from the route

17   to see the bar. They often turned to look at or beyond the bar and then turned to face in the

18   direction of the goal. Tests in which the bar was shifted to be more peripheral or more frontal

19   than in training produced a corresponding change in the ants' paths, demonstrating that they

20   were guided by the bar, presumably obtaining information during scanning turns towards the

21   bar. Examination of the endpoints of turns away from the bar suggest that ants use the bar for

22   guidance by learning how large a turn-back is needed to face the goal. We suggest that the

23   ants' zigzag paths are an integral part of visually guided route following. In addition, on some

24   runs in which ants did not take a direct path to the goal, they still turned to face and sometimes

25   approach the goal for a short stretch. This off-route goal facing indicates that they store a

26   vector from start to goal and use path integration to track their position relative to the
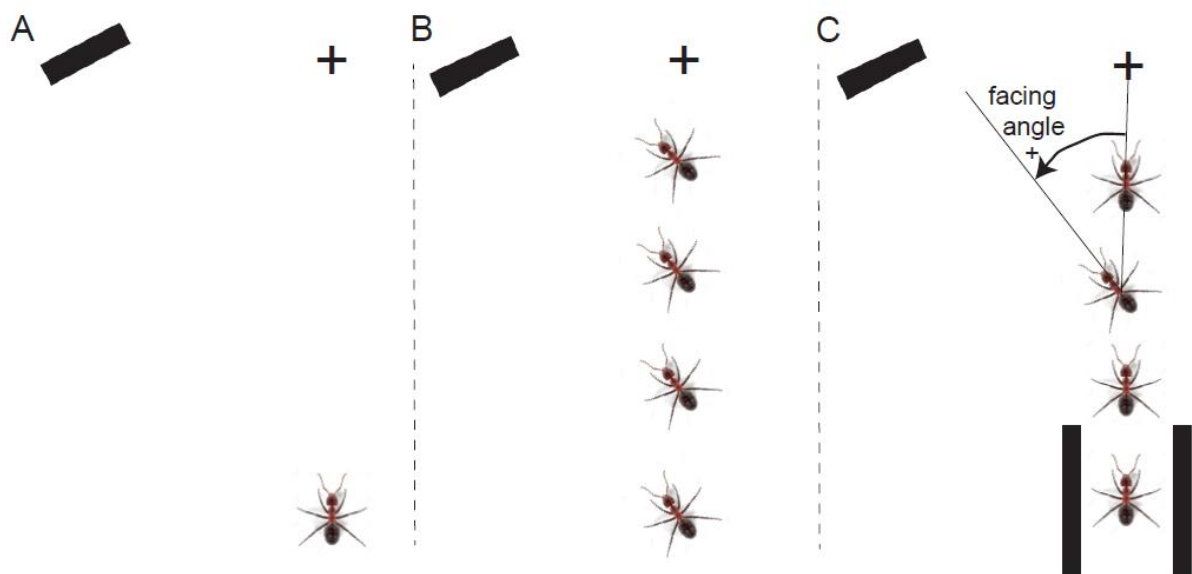
27   endpoint of the vector.

28 **Introduction**

29   Foraging ants, with their faculties intact, readily learn and follow visually guided routes

30 between their nest and a foraging site (Collett et al. 1992, Wehner et al. 1996, Collett, M., 2010,

31 Mangan and Webb 2012, Narendra et al. 2013). They can do so despite a large mismatch

32 between their position on the route and their path integration (PI) state (Kohler and Wehner

33 2005, Graham and Cheng 2009, Narendra et al., 2013), implying that visual guidance does not

34 require support from PI, even though the two guidance mechanisms are normally co-active

35 (Collett, M., 2012; Wehner et al., 2016; Hoinville and Wehner 2018). Such experiments and

36 modelling (Baddeley 2012) had suggested that visual route following involves 'alignment image

37 matching' (AIM) (review: Zeil, 2012, Collett et al., 2013). In brief, ants that are guided initially by

38 PI and by innate responses to obstacles and visual features that they encounter along the way,

39 memorise routes by recording retinotopic views when facing along the route. Thereafter, they

40 can follow the route by turning until they face in the direction that best matches a recorded

41 view from the set of recorded views and then walking forward.

42   Since wood ants often use the same foraging path when going to and from their nest and

43 on their outward route turn back to face along the path they have just followed, they can use

44 what they have learnt on outward routes to help guide their return (Graham and Collett, 2006;

45 Schwartz et al, 2020). A set of views may thus be associated with a particular motivational

46 context so that an ant interrogates a different set on its approach to food than it does when

47 homing (Harris et al., 2005; Wehner et al., 2005). A recently suggested alternative (Murray et al.,

48 2020) is that ants activate both sets for guidance with one as attractive, mediating approach,

49 and the other repulsive, causing avoidance, so improving the precision of route following. To

50 account for the behaviour of wood ants from this perspective (Harris et al. 2005), the

51 motivational context would determine which views are attractive and which repulsive.

52   Can one-eyed ants follow visually guided routes in the restricted surroundings of a

53 laboratory lacking celestial compass information? In earlier experiments (Buehlmann et al.

54 unpublished data), ants with one eye painted over were trained to find a drop of sucrose at the

55 edge of a circular arena. They were released at the centre of the arena and learnt a straight

56   route from there to a point on the circumference. This location was specified by a black vertical

57   bar fixed to the white inner wall of a rotatable cylinder surrounding the arena. When the ants

58   faced along the route, the bar was well outside the visual field of the ants' seeing eye (e.g.,

59   Zollikofer et al., 1995). The cylinder and the position of the food were rotated together from

60   trial to trial to ensure that the black bar was the principal visual cue and also to remove any

61   reliance on a magnetic compass (Çamlitepe and Stradling,1995). Ants with their left eye capped

62   were approximately normal in route learning when the bar was on the right side of the route

63   going from the centre to the periphery of the arena, but struggled when the shape was on the

64   left side (Buehlmann et al. unpublished data). From the perspective of image alignment, this

65   failure is not surprising. To the one-eyed ant, the view along the desired route is of an empty

66   cylinder (Fig 1A) and the ant can fulfil this condition by picking from a wide swath of possible

67   paths. One possible solution to the problem is to walk sideways while facing the cue (Fig 1B) – a

68   strategy that we have not seen the ants adopt

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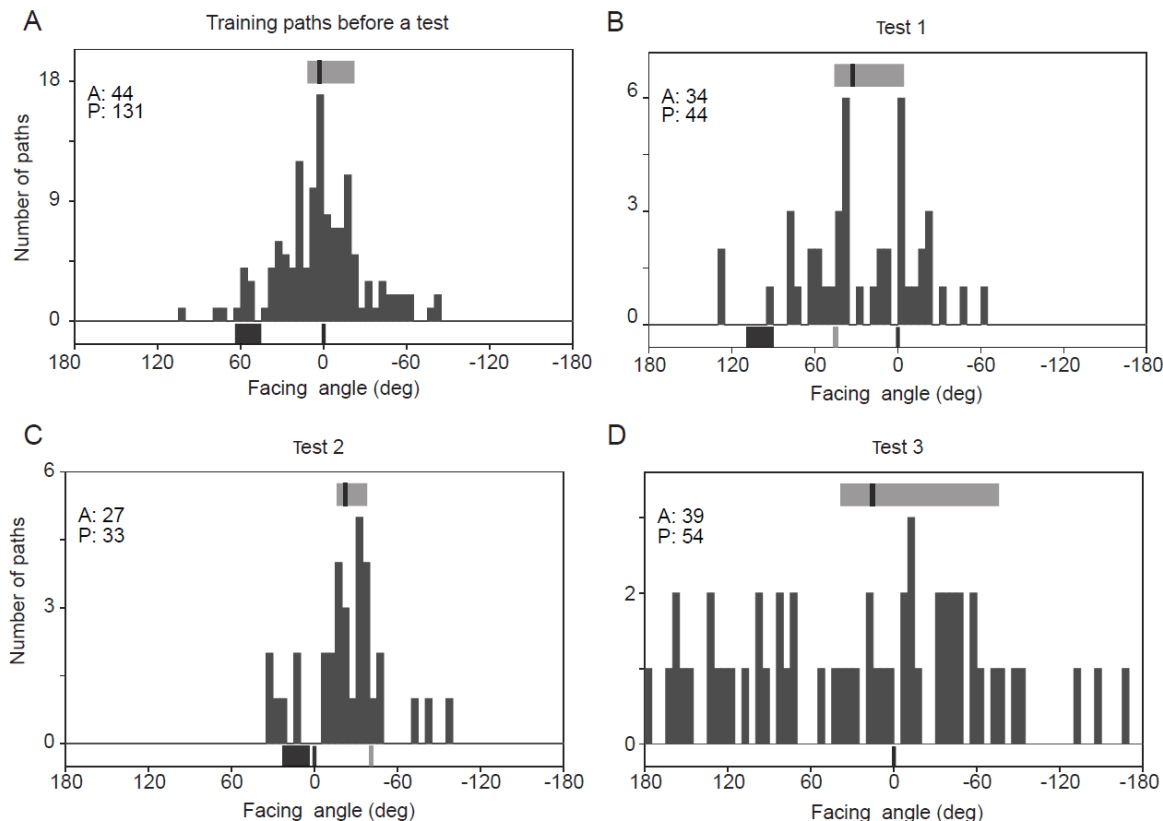**Figure 1. Schematic of problem and solution**

A: Ant with left eye covered has difficulties in reaching its goal when the only directional aid is a black, vertical stripe outside the right eye's visual field. **B**: Ant could, but does not approach, the goal when moving sideways. **C**: Ant is aided by mechanical guides forming a channel at the start of the route. It then turns to face towards and then away from the stripe and can reach the food (+).

75   In the present study, performed in 2017, we followed the same procedure except that we

76   added extra information that seemed to help the ants learn a route. Ants were pointed in the

77   right direction at the start of the route by two short parallel strips of wood aligned along the

78   route (Fig. 1C). We examine the possibility that these guides help ants to develop a food vector

79   (Collett et al., 1999; Bolek et al 2012) (i.e. a memory of the distance and direction from the start

80   to the food); and that the ants' routes are to some degree guided by PI, as they follow this

81   vector. Tests with the vertical bar shifted further or closer to the route direction show that the

82   ants also made use of the visual cue.

83   **Results**

84   ***Routes of monocular ants***

85   Although ants were able to learn the route, the paths are much more variable than those of

86   ants with normal vision guided by a similar cue (c.f. Woodgate et al. 2016). The directions of

87   individual paths from the end of the channel to 15 cm from the food are shown plotted relative

88   to the position of the food during training. If we select all the paths in the training trials before

89   a test (Figure 2A, S1A), the bulk of paths are in a direction that lies within the expected

90   quadrant (circular median: 2.85^{o}; IQR: -22.35^{o} to 11.69^{o}). This value does not differ significantly

91   from the direction of the food (binomial test, N: 131, P: 0.16; Zar, 1999). The details of the

92   distribution are complex with a peak just to the right of the goal and another ca 20° to the left

93   of the goal and subsidiary peaks close to the direction of the bar.

94   *Figure 2. Path histograms*

95   *Path directions during training and three tests (see Methods). **A**: histogram showing the overall path*
96   *directions taken by ants in training trials immediately preceding a test. Bin width is 5° in all histograms.*
97   *Zero is the direction of the starting channel points from the centre of the arena (i.e., direction of food*
98   *during training). In each histogram in this and other figures, A gives the number of ants and P the*
99   *number of paths; the black line and grey rectangle above the histogram show the median and IQR of the*
100 *distribution. The black rectangle below the histogram shows the width and position of the visual cue; the*
101 *black line at 0° shows the food direction. **B**: path directions in tests with visual cue shifted left, relative to*
102 *the starting channel. Black line at 0° shows the direction in which the starting channel was pointing; the*
103 *grey line is the predicted goal direction if ants use the visual cue for guidance. **C**: path directions in tests*
104 *with the visual cue shifted right, relative to the starting channel. **D**: path directions in the absence of the*
105 *visual cue.*

106   Tests in which trained ants were tested with the bar displaced to the left or to the right of
107 its training position suggest that the ants' direction of travel shifts with the displacement. In
108 test 1, the right edge of the bar is about 90° from the direction in which the starting channel
109 points (Figure 2 B, Figure S1 B). The number of paths is small (n= 44). One of two 'major' peaks
110 (n=6) of the path directions (Figure 3 B) is 10° to the right of the expected goal relative to the

111 shifted bar (bar-defined predicted heading: 45°). Another peak (n=6) is in the channel direction

112 (channel-defined predicted heading: 0°). The circular median path direction (plotted relative to

113 the channel-defined prediction; median 32.41°; IQR -4.55° to 45.92°) differs significantly from

114 the bar-defined goal (a), but does not differ from the direction of the channel (b) (binomial test,

115 a: N=44, P: 0.010; b: N=44, P: 0.096). A comparison of path directions shows that the test 1

116 circular path median differed significantly from the median of training paths both when

117 measured relative to the bar-defined goal direction (a) and relative to the channel direction (b)

118 (Common median test, a: N=44,131, test statistic=10.11, P=0.001; b: N=44,131, test

119 statistic=4.19, P=0.041).

120   In a second test, the bar was moved to the right with its right edge almost in line with the

121 channel. In this case, the paths shifted to the right of the training path (Figure 2 C, Figure S1 C).

122 The peak of the distribution of path directions relative to the food lay between the training

123 direction and the goal relative to the shifted bar (circular median -22.41°; IQR = -38.08° to -

124 15.98°, N:33). This median differs from both the bar-defined goal (a, bar-defined predicted

125 heading: -41.2°) and from the channel direction (b, channel-defined predicted heading: 0°)

126 (binomial test, a: N: 33, P: 0.0003 ; b: N: 33, P: 0.0003) The circular median of test 2 differs from

127 the training path median, whether it is measured relative to the bar-defined goal direction (a)

128 or the channel direction (b) (Common median test, a: N=33,131, test statistic=10.96, P=0.0009;

129 b: N=33,131, test statistic=16.73, P<0.0001).

130   In the last test the bar was removed (Figure 2 D, Figure S1 D). The median direction is

131 15.54° (IQR = -75.87° to 38.74°, N 54) and does not differ significantly from the channel

132 direction (binomial test, N: 54, P: 0.497). The dispersion of paths around the median was

133 broader than in training (circular Watson U^2 test, U 1.133; P<0.01; N: 54, 131). Nonetheless, the

134 data are not uniformly distributed around the entire circle (Rayleigh test for uniformity: Z: 3.16,
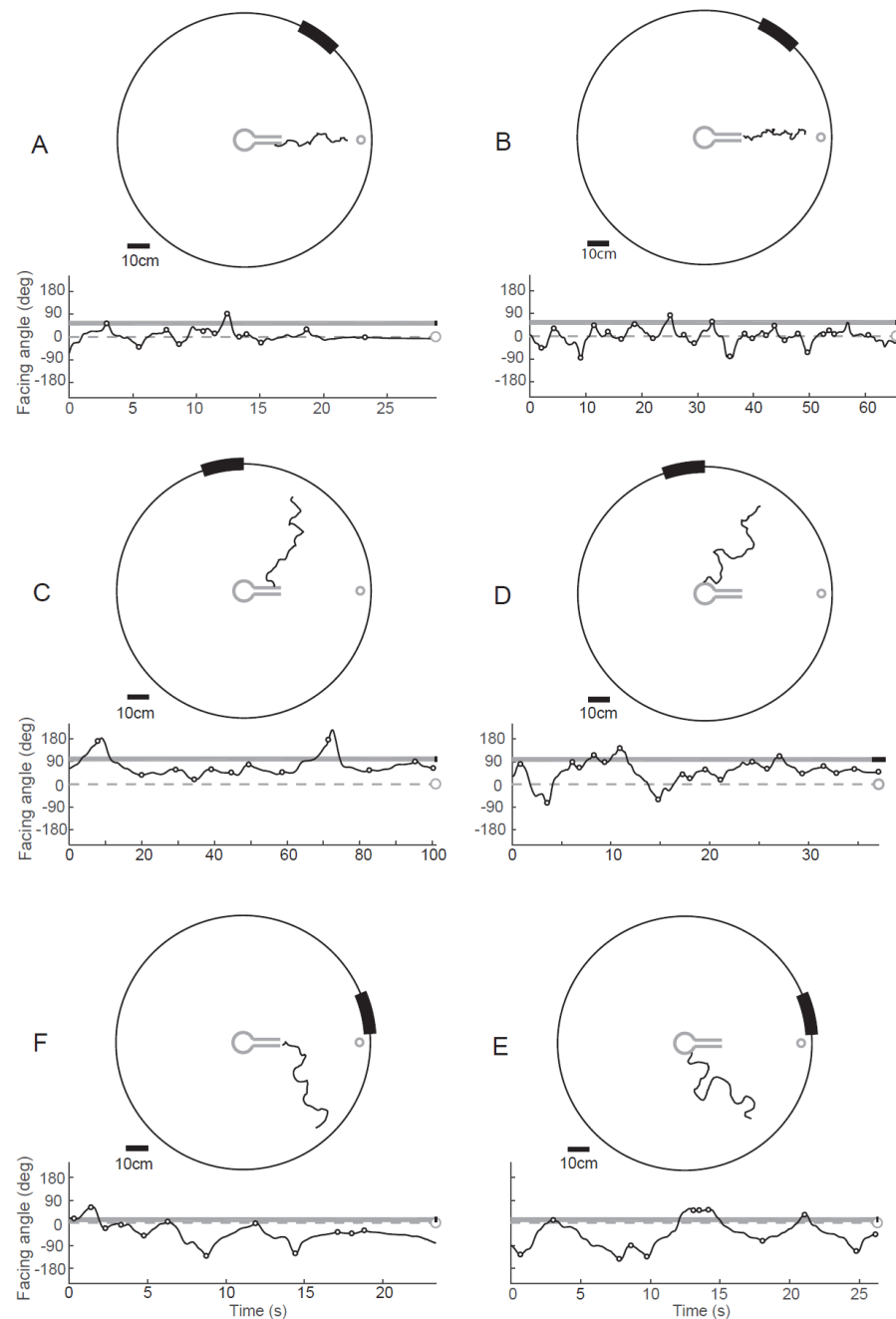
135 P: 0.042).

136   We conclude from these results that the ants' path directions are influenced by the

137 position of the bar, but that there are additional guidance mechanisms at play, such as a food

138 vector.

Individual paths

140   That the multiple peaks in the path direction data may be the consequences of following

141   different navigational mechanisms is supported by the patterns seen in individual paths.

142   Examples of test paths (Figure 3 C-F) indicate that ants can be guided by the bar, but also that

143   guidance along the bar defined route can be accomplished with very few frontal views of the

144   bar (Figure 3C). Training routes with few frontal views of the bar (Figure 3A) could mean that

145   ants, guided idiothetically, without compass information, follow their starting directions to the

146   food (e.g. Lent et al., 2009). Ants may also be guided idiothetically by PI, as in *Drosophila*

147   returning to a drop of sucrose that they have sampled (Kim and Dickinson, 2017).

148   Suggestive evidence for a food vector monitored through PI comes from examples of ants

149   that leave the direct route to the goal and turn and travel towards the food when they are

150   some distance from the trained path. Deviations from the trained path happens when ants

151   head towards the bar (Figure 4 A-C, G-J) or travel well to the right of the direct route from the

152   centre to the food (Figure 4 D-F). These deviant paths can be interrupted by the ant turning

153   towards the goal and moving a short (e.g. Figure 4, F, G) or a longer distance (e.g. Figure 4 E, I)

154   towards it. When the distance towards the goal is longer, and is not just a brief interruption to a

155   path elsewhere (Fig. 4 D, E, H), facing the goal tends to occur at the peaks and troughs of a

156   zigzag approach, as it does in binocular ants (Lent et al., 2013).

157   The examples mentioned so far come from training trials with a drop of sucrose at the goal.

158   Could the ants obtain guidance cues from the sucrose, itself? During experiments over many

159   years in this set up, ants never seem to notice the food until they have almost reached it. These

160   concerns do not arise during tests in which food is always absent. Ants continue to face the

161   channel-defined goal when approaching the bar in test 1 (Figure 4 J-L).
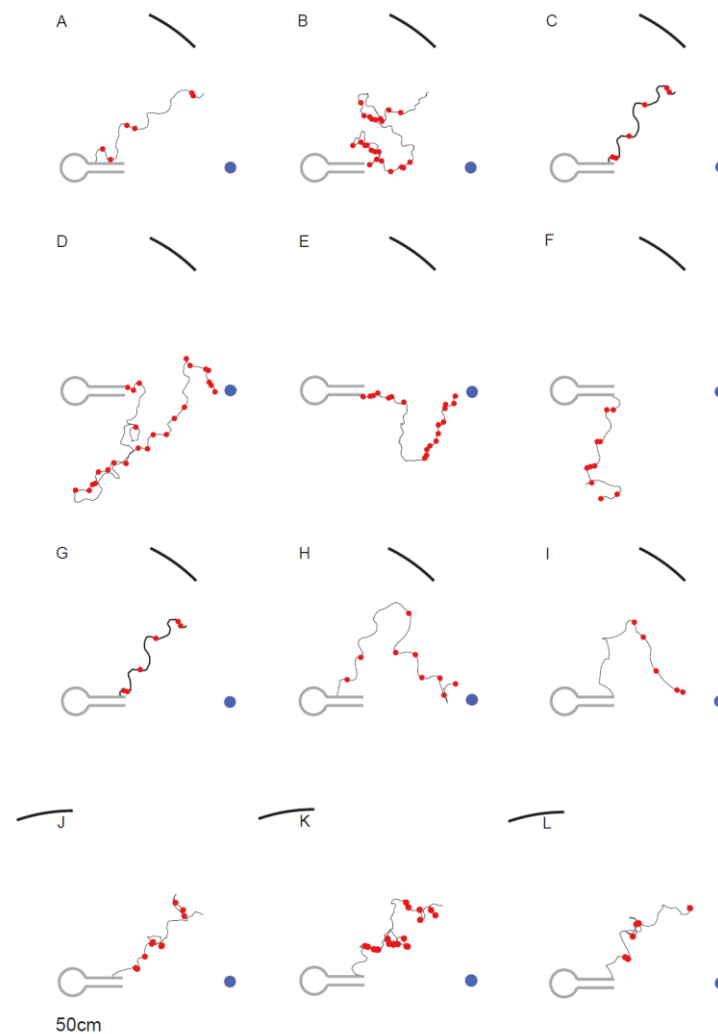
Figure 3. Individual examples of ants' trajectories during training and tests

A, B: Training paths. Each panel shows the path of a different ant. Top: path of each ant, seen from above, as it moves from the enclosure and guiding channel in the centre of the arena towards the food position (0°) 55 cm away. Black bar on circumference of arena is the projection of the vertical bar that is fixed to the surrounding 180 cm diameter cylinder. Bottom: Ants' facing angle relative to the food position (+ve angles counterclockwise) are plotted against time from start of the illustrated trajectory. Gray bar shows angular position of bar as measured from arena centre. Circles mark extracted turn extrema. **C, D**: Test with bar shifted more peripherally relative to the direction of the starting channel (no food present). Other details as in A. **E, F**: Test with bar shifted centrally relative to starting channel.

171   Facing and moving towards the goal can most easily be explained by supposing that ants

172   learn a vector that goes from the centre of the arena to the goal and rely on PI to monitor their

173   progress along the vector and to generate a home vector, whatever path they take (cf.

174   Fernandes et al., 2015). The examples in Figure 4 also show how readily ants switch between

175   following PI and controlling their path with a visual cue.

176   **Figure 4. Suggestive evidence that ants can be guided by path integration to the food position**
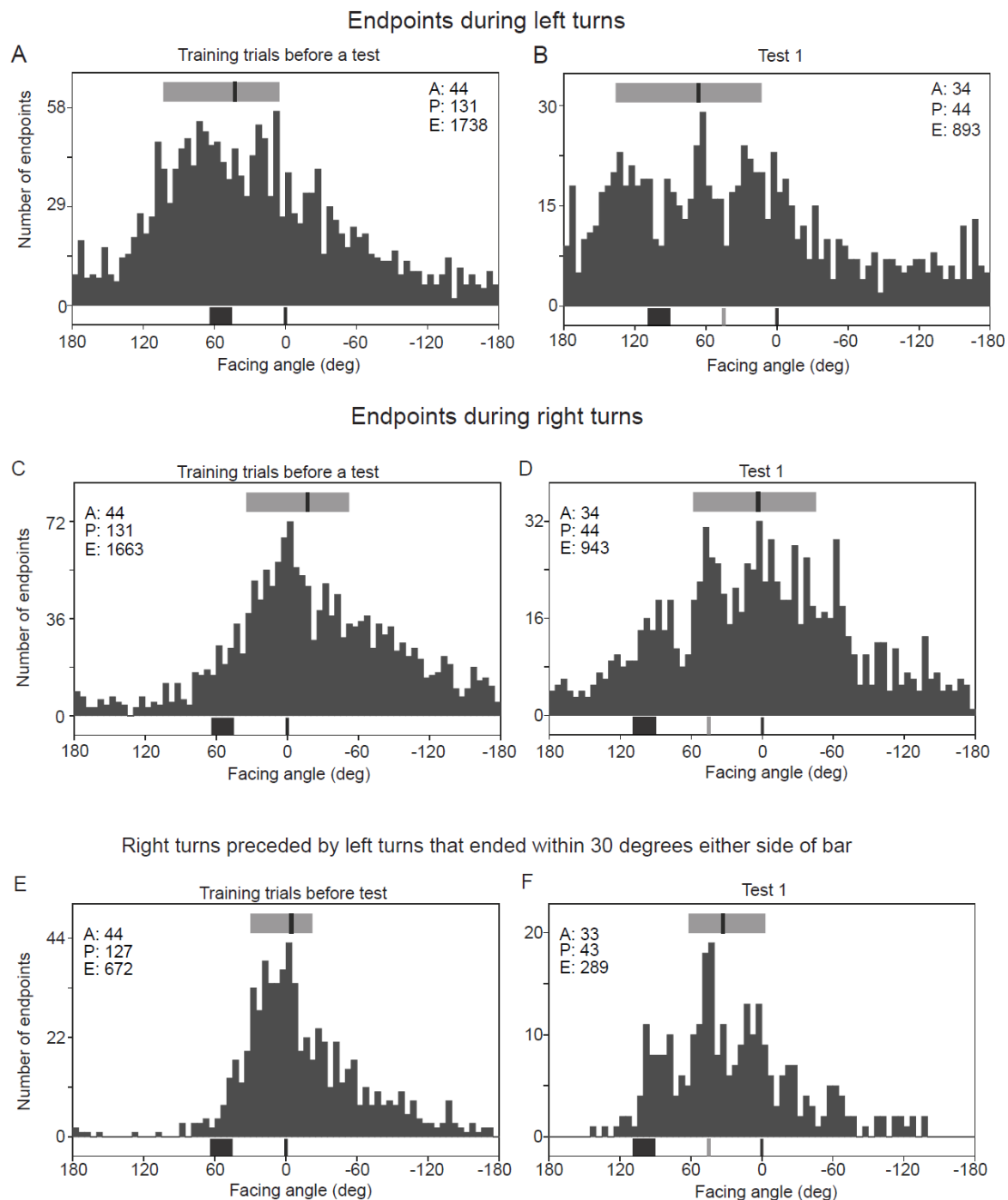
177   *Example paths of ants that deviated far from the direct path to the bar defined goal. Points or path*

178   *segments in which the ants face the goal within ±10° are marked with red dots. Panels D, E, H and I show*

179   *ants that stray far from the direct route between the start and the food, but later make move directly*

180   *toward the goal, suggestive of the action of a food vector monitored by PI. Other panels show shorter*

181   *periods of movement in the goal direction and illustrate the range of circumstances under which goal*

182   *facing occurs. Panels J, K and L show similar goal-facing in tests in which the position of the visual cue*

183   *has changed, demonstrating that this behaviour cannot be entirely visually mediated.*

184   *Facing directions at the end points of turns*

185   The distributions of path directions (Figure 2) indicate that the bar has a major influence on

186   the ants' direction of travel. More detail comes from examining separately the endpoints of the

187   left and right turns that ants make during their paths. We extracted two kinds of turn endpoints

188   from the data: i) the maxima and minima in plots of the ants' facing angles (e.g. Figure 3B); ii)

189   turns that end in a plateau, where the ant keeps its facing direction steady for at least 0.2s (e.g.

190   Figure 3A) and combined the two kinds of endpoints in all the histograms below (Figure 5).

191   Despite the wide spread of the distributions, the pattern of peaks is informative. In the pre-

192   test training trials, the distributions of left turn endpoints have a clear peak associated with the

193   food which is likely to reflect turns generated through PI (Figure 5A). A second peak consists of

194   left turns that end close to the bar. The endpoints of right turns during training are in the food

195   direction and these end points could either be generated through PI or reflect a right turn of a

196   remembered size after facing the bar (Figure 5C).

197   In test 1, the endpoints of left turns are shifted to the left in comparison with training, but

198   the details of the pattern are too noisy to be informative (Figure 5B). The distribution of

199   endpoints of right turns has two peaks, similar to the pattern of path directions during test 1

200   (Figure 5D, c.f. Figure 2B). One peak is in the bar-specified goal direction and a second is in the

201   channel-specified direction. The peak in the bar-specified goal direction is enhanced, when we

202   select just those right turns that occur after viewing the bar (Figure 5F). To do so, we included

203   only endpoints that followed turns ending within 30° either side of the edges of the bar. The

204   comparison of this plot with the plot of all right turns during test 1 provides our best evidence

205   that ants do learn the amplitude of the turn that they must make to face in the goal direction

206   after viewing the bar.
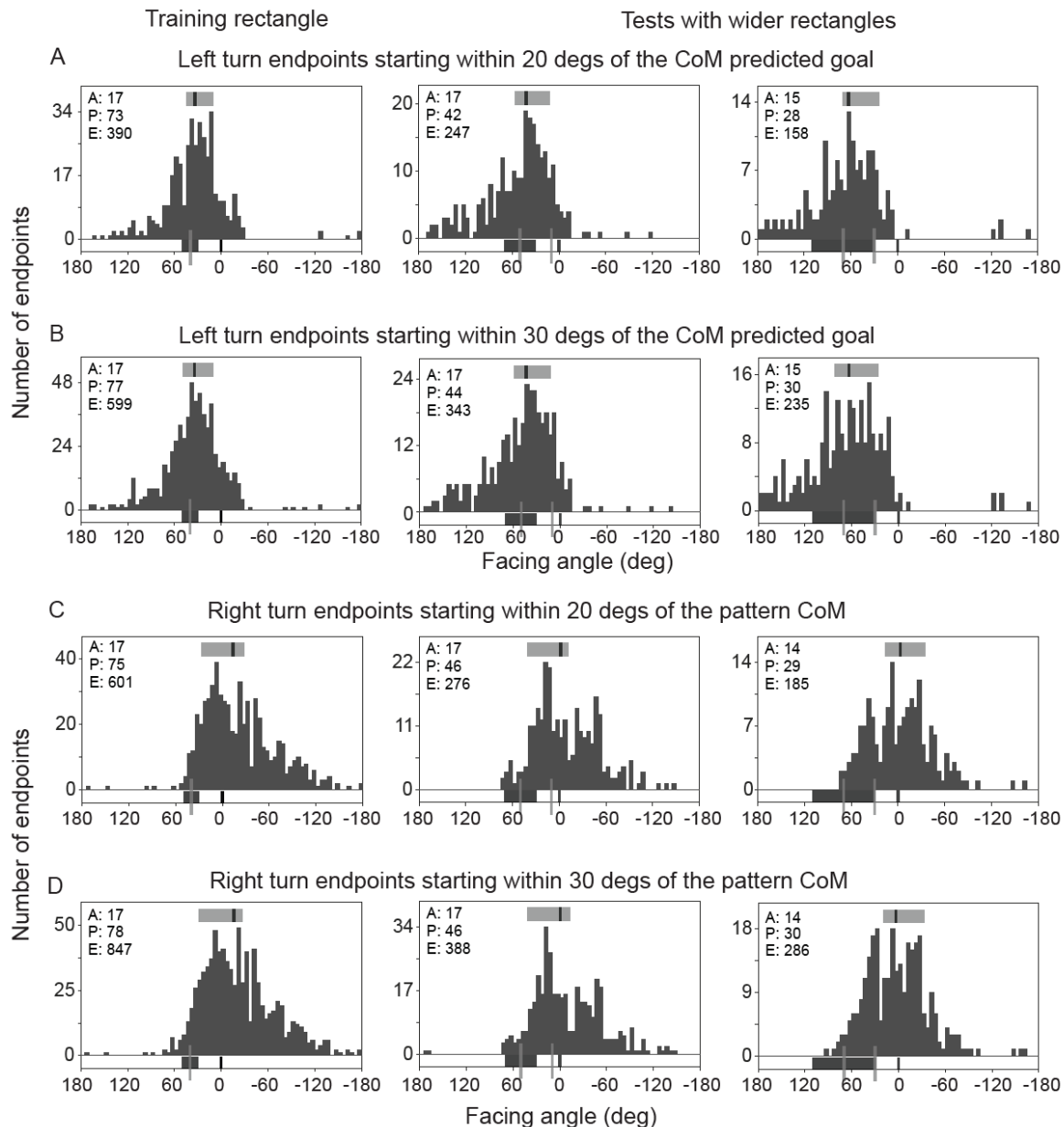
Figure 5. Histograms of endpoints of left and right turns.

A: All endpoints of left turns during pre-test training trials. B: Endpoints of left turns during Test 1 trials, with visual cue shifted left relative to the starting channel. C, D: Endpoints of right turns in pre-test training and Test 1, respectively. E, F: Endpoints of selected right turns that began ±30° from the bar edges. In all panels, E denotes number of endpoints. Other details as in Figure 2

212   Since we cannot suppose that turn endpoints are independent of each other, we have not

213   attempted statistical analysis. The same pattern is seen when we look at just the median

214   endpoint from each ant path (Figure S2). The small sample number and variability of the data

215   means that the only useful comparison to be made is whether the circular median directions of

216   test 1 (median, relative to the bar-defined predicted goal = -18.2^o IQR = -31.9^o to 14.2^o, 43

217   paths) differ from that of test 2 (median, relative to the bar-defined predicted goal = 0.8^o; IQR =

218   -13.7^o to 13.5^o; 32 paths). We selected all the median endpoints from ants that undertook both

219   tests and aligned the distributions on the bar-defined goals. A common median test

220   demonstrated that the two distributions differed significantly (Common median test, N: 19, 19,

221   test statistic: 5.157, P: 0.023, Fisher, 1995). We conclude that the endpoints of right turns

222   cannot be driven by the position of the visual cue alone but must also be influenced by the

223   mismatch between the bar and channel positions.

224   Right turn endpoints during test 2 peak at a position that falls between the bar-specified

225   and channel-specified directions. This compromise may be another example of ants averaging

226   directions of the outputs of independent navigational strategies (visual guidance and PI or

227   idiothetic guidance) when the directions of the two outputs are relatively close (Collett, 2012;

228   Wehner et al., 2016; Hoinville and Wehner, 2018).

229   *Binocular ants*

230   We have re-analysed an earlier study on binocular ants to see whether the findings on one-

231   eyed ants apply to normal route following. Wood ants (Woodgate et al 2016) were trained in

232   the same apparatus to approach a goal with the direction set by a single rectangle placed to the

233   left of the goal direction and tested with wider rectangles. The major result of various tests was

234   that ants seemed to learn their route relative to the centre of mass of the shape. We now ask

235   whether turn endpoints also suggest that ants learn the goal direction relative to the centre of

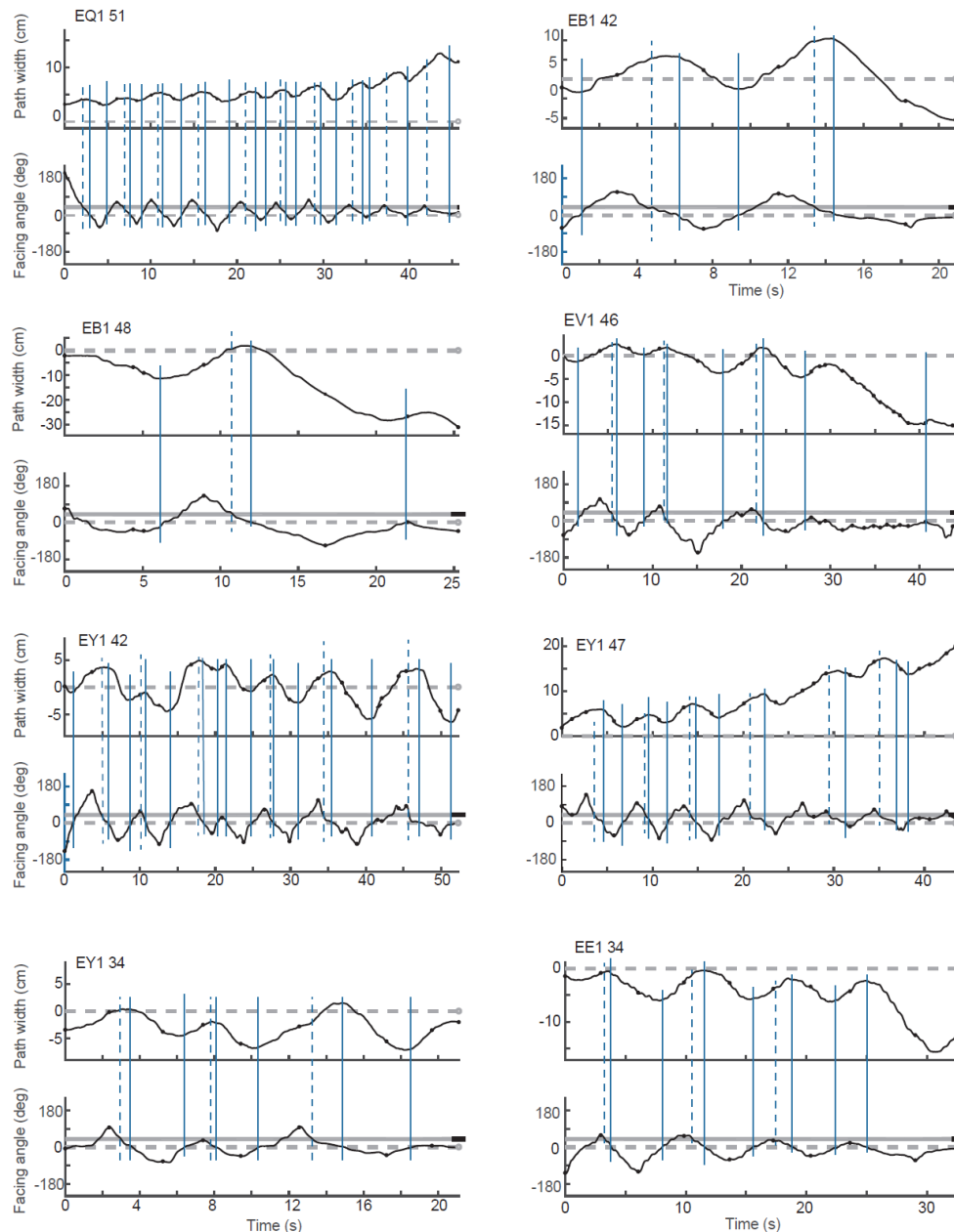236   mass of the training shape and wider test shapes.

Figure 6. Histograms of endpoints of binocular wood ants during selected left and right turns.

*Endpoints of turns in pre-test training trials, tests 1 and tests 2, in which rectangles were increasingly wide. **A**. Left turns with their starting points within ±20° of the predicted facing angle of the goal relative to the centre of mass (CoM) of each rectangle. Black lines below the histograms at 0° show the predicted goal direction during training. This direction is also the predicted direction were ants to match the right edge of the rectangle to its position in training. Grey lines below the histograms mark the CoM of the visual cue and the predicted goal direction, were ants to match the CoM of the rectangle to its position in training. Other details as in Figures 2 and 5. **B**. Left turns with starting points within ±30° of the predicted facing angle of the goal relative to the CoM of each rectangle. **C**. Right turns with starting points within ±20° of the CoM of each rectangle. **D**. Right turns with starting points within ±30° of the CoM of each rectangle.*

248   We extracted the endpoints of selected turns during training paths preceding a test and

249   during tests (Figure 6). The selected endpoints of left turns were those that began within ±20°

250   or ±30° of the predicted goal direction from the midpoint of the width of the training rectangle

251   and wider test rectangles. Selected right turns began within ±20° or ±30° of the midpoint of

252   these rectangles. As with monocular ants, the distribution of endpoints is complex and multi

253   peaked. Major peaks of left turns occur a little to the right of the midpoint of the training and

254   wider rectangles and peaks of right turns are a little to the left of the goal direction relative to

255   the midpoint of the rectangle. Given the complexity of the data, we cannot say more than the

256   data are consistent with the importance of the midpoint of the rectangles as one factor

257   influencing the pattern of endpoints and the turns that the ants make.

258   An earlier study of ants approaching visual features aligned with the route found that

259   facing along the route direction tended to occur at the apices of the ants' zigzag path (Lent et al.

260   2013). Examples of paths from (Woodgate et al 2016) show that moments when the ants face

261   the goal head on coincide closely with the peaks and troughs of the ants' paths (Figure 7). The

262   coincidences occur even when the zigzags are irregular.

Figure 7. Examples of zigzag paths in binocular wood ants.

Each panel gives one path. Top trace: ant's position along its path plotted against time. Horizontal grey dashed line shows the direct route from the starting point to the food. Bottom trace shows ants' facing angle relative to food. Horizontal dashed grey line shows when facing angle is directly towards the food; grey solid line represents the angular position of the bar as measured from the centre of the arena. Continuous vertical blue lines highlight points at which the ant is facing directly toward the food. These lines tend to intersect the peaks and troughs of the ant's Y position irrespective of the spatial or temporal regularity of the zigzag. Dotted blue vertical lines highlight points at which the ants faced toward the bar during turns in the direction of the food and show the variability of the interval between the ant facing the bar and facing the food.

Discussion

273

274   Alignment image matching, the prevailing account of visually guided navigation in insects,

275   suggests that an animal will turn until its current view matches a previously learned view and

276   then move in that direction (Baddeley et al., 2012). In our study, that process could not operate

277   because one eye was painted over and the only visual cue was on the blind side of the ant.

278   Consequently, when the ant faced in the correct direction, it saw no more than the white arena

279   wall. Ants still learnt routes to a feeder and, if the visual cue was shifted in tests, the ant's paths

280   were deflected in the appropriate direction, demonstrating that the ants' movements were

281   guided by the visual cue.

282   A wood ant's path tends to oscillate with the desired goal direction reached at the peaks

283   and troughs of the oscillations or zigzags (Lent et al. 2013). This is a common pattern in insect

284   locomotion. It is of clear benefit to insects that move in an oscillating path to follow an odour

285   trail (Hangartner, 1968; Cardé and Willis, 2008; Wllis et al, 2013; Namiki and Kanzaki, 2016).

286   When one-eyed ants zigzagged in the current experiments, they would see the bar during turns

287   to the left, giving them an opportunity to acquire information to guide their subsequent routes.

288   We suggest that, during route learning, the right turns after turning to face the visual cue were

289   guided by path integration so that turns ended with ants facing towards the goal. This turn size

290   can then be learned and later applied when following an acquired route. By this means, visual

291   scanning can support AIM and make it possible to navigate in surroundings with sparse visual

292   information.

293   The coincidence of facing the along the goal direction with the apices of the zigzag, even

294   when the zigzag is irregular suggests that turns in the goal direction help induce a reversal in

295   the ants' direction of rotation. Because the ants often sustain their direction towards the goal,

296   the influence of goal facing on controlling the direction of rotation seems to be more of a slowly

297   acting nudge than a kick.

298   One puzzle that the data present is that although ants seem able to reach the goal by

299   following a food vector without needing the bar, they mostly did not travel in the food direction

300   in tests with the bar absent (Figure 2D). A possible explanation comes from previous work on

301   the expression of home and goal vectors that suggests that the vectors are not fully expressed if

302   the surroundings in which they are tested differs markedly from the ant's accustomed

303   surroundings (Cheung et al., 2012). The bar may thus provide a contextual, scene-setting cue as

304   well as a cue for guidance. Another possibility is the lack of optic flow that the bar normally

305   provides.

306   While AIM as originally conceived is likely to be mediated by the mushroom body (Ardin et

307   al, 2016, Buehlmann et al., 2020; Kamhi et al., 2020) that signals an attractive or aversive

308   direction of travel (Aso et al., 2012), controlled turns to or from specific visual features are

309   probably mediated by visuomotor processing in the central complex (CX) (Collett and Collett,

310   2018). Visual learning of pattern orientation and elevation was some time ago shown to involve

311   ring cells in the ellipsoid body (EB) and cells in the fan-shaped body (Liu et al., 2006; Pan et al.,

312   2009). The experimental technique of these experiments prevented testing whether the

313   azimuth of a visual stimulus is also memorised within these structures.

314   Current research on the *Drosophila* CX has resolved this question in wonderful detail. First,

315   it was found that a fly's heading direction is correlated with the position of a single 'bump' of

316   electrical activity within the doughnut shaped EB, and that the position of the bump can be

317   locked to a compass direction or to a visual stimulus (Seelig and Jayaraman, 2015; Turner-Evans,

318   D. 2017; Green et al., 2017, Giraldo et al., 2018).

319   More recent imaging studies and recordings from ring and EP-G neurons in the EB have

320   uncovered the likely mechanism that underlies how the bump of activity within the EB becomes

321   linked to the direction of a visual cue (Fisher et al., 2019; Kim et al., 2019). Individual ring cells

322   in the EB respond to visual cues in a specific retinal position. When a ring cell is visually

323   activated, it inhibits E-PG cells at all positions within the EB, apart from where the ring cell is

324   itself visually activated. This system is plastic such that E-PG cells responsive at a specific

325   heading become linked to a visual cue in that direction after that cue has remained in a fixed

326   direction for a short time. This arrangement allows a facing direction within a visual scene to be

327   rapidly associated with a particular compass direction, just as is required for a visually

328   controlled route learnt through path integration.

329   The turn amplitudes that are likely to be mediated by known CX circuitry are of several

330   kinds. Turns that place a visual stimulus in its expected position, like turns towards and away

331   from the bar, can be understood within the framework of the previous paragraph (Fisher et al.,

332   2019; Kim et al., 2019). Turns in the direction of the home vector are also likely to involve the

333   CX, in this case, interactions between the fan-shaped body, the protocerebral bridge and the EB

334   (Neuser et al. 2008; Stone et al., 2017; Honkanen et al 2019, Le Moël et al., 2019). Lastly, the

335   lateral accessory lobes that contain neurons that mediate the turn of a zigzag path (Namiki et al.

336   2016, Steinbeck et al, 2020) may be responsible for the correlated phasing of goal facing and

337   the peaks and troughs of zigzags (Figure 7).

Methods

Ants

340   Experiments were performed on wood ants from laboratory-maintained colonies collected

341   from Broadstone Warren, East Sussex, UK. The colonies were kept under a 12h:12h light-dark

342   cycle and the colony was sprayed with water daily. Water and sucrose dispensers were always

343   available except during experiments when the colony had limited access to sucrose to

344   encourage enthusiastic foraging. Frozen crickets were supplied several times a week.

345   Before ants were trained their left eye was painted with enamel paint and the integrity of

346   the paint cover checked daily under a binocular microscope. The cover appeared stable and it

347   was very rare to have to repaint.

Experimental set-up

349   The basic experimental procedures followed those described previously (Lent et al.,

350   2013). Individually marked wood ants were trained to go from the centre of a circular platform

351   (radius 60cm) towards a drop of sucrose on a microscope slide placed 55cm from its centre. The

352   slide was positioned relative to a single black vertical bar (50cm wide and 85cm high) that was

353   cut from black cotton sheeting and fixed to white netting on the white inner wall of a rotatable

354   cylinder (diameter 3m, height 1.8m). Seen from the centre of the arena, the right edge of the

355   bar was 45° to the left of the direction the feeder.

356   To provide idiothetic cues, a pair of guide sticks (3mm square cross-section) with a 24mm

357   path between them led from a ring (5cm radius and 25mm high) surrounding the centre of the

358   arena. In initial training trials, the sticks led most of the way to the food, their length was

359   gradually shortened to 13cm. 13cm and 10cm sticks were used for tests. See Fig. 3 for a top

360   view of the arena the arrangement of sticks, the sucrose and the radial position of the bar.

361   ***Experimental procedure***

362   Ants were given about 25 trials of group training over about 3 days before being trained

363   individually. On each trial, individually marked ants from a cohort of *ca.* 25, were taken from

364   the nest and separated into groups of between 6 and 8 individuals. One group at a time was

365   placed in the ring, sometimes overlapping with stragglers from a previous group. After the ants

366   had reached the feeder and had started to drink, they were placed in a box with sucrose and

367   drank to completion before being returned to the nest.

368   For individual training, ants were put singly into a 6.5cm diameter, cylindrical holding

369   chamber that lay within the ring. The wall of the holding chamber could be lowered remotely

370   from outside the cylinder to be flush with the arena floor. Once the wall was lowered the ant

371   was free to leave the ring. When the ant had reached the food reward and started to feed, or

372   reached the edge of the arena, the experimenter raised the wall of the holding chamber,

373   entered the cylinder, transferred the ant to a feeding box and placed the next ant in the holding

374   chamber. After the cohort of ants had completed a training trial, the guide sticks, the black bar

375   and the slide with food were rotated to a new position to avoid the ants learning other cues for

376   guidance.

377   Once individual training began, we recorded the ants' individual paths, starting just before

378   each ant was released from the holding chamber. Each ant's movements were recorded using a

379   tracking video camera (Trackit, SciTrackS GmbH), which gave as output the ant's position on the

380   arena and the orientation of its body axis every 20ms. Each ant was released from the holding

381   chamber and its path recorded until it reached the feeder or the edge of the platform. Once it

382   had started to feed, or if it was lost, it was placed in a feeding box.

383   After individuals had performed 30 training trials, three different tests were introduced

384   with a varying number of intervening training trials. In test 1, the right edge of the bar was

385   shifted to 90° to the left of the direction in which the starting channel was pointing. In a second

386   test the bar was shifted in the opposite direction, ca 5° to the left of the channel direction. In

387   the third test the bar was removed. No sucrose reward was present during tests. Ants were

388   removed from the arena once they reached the edge of the platform and allowed to feed to

389   satiety before being returned to the nest.

390   **_Data analysis_**

391   We examined two features of the ants' trajectories: 1. The direction of an ant's path; 2. The

392   ant's facing directions at the extrema of its turns to the left and the right, measured relative to

393   the position of the food (see Figure 1C).

394   *1. Path direction*

395   The direction of the ants' paths was analysed from when an ant left the guide channels,

396   beginning ca. 18cm from the centre of the arena, or sooner if ants climbed over the guide strips,

397   until they reached a radial distance of 45cm from the centre. The direction over this path-

398   segment was estimated in two stages. First, the path (consisting of x-y coordinates recorded at

399   50fps) was divided into successive 1.5cm segments, and orthogonal distance regression (Gene

400   et al., 1996) was used to calculate the angle of a best fit line through each segment, minimising

401   the error in both x and y dimensions (custom written MATLAB script based an algorithm

402   devised by Eitan, T. [https://stackoverflow.com/questions/12250422/orthogonal-distance-

403   regression-in-matlab]). The overall direction of the path was then taken to be the vector sum of

404   the directions of all path segments until the ant's path took it 45cm from the centre of the

405   arena.

406   *2. The amplitude of scans*

407   Plots of facing angles over time tend to have peaks and troughs. The facing angle at a peak

408   or a trough was defined as the most extreme angle to the left or right before a change in the

409   direction of rotation. These extrema were identified using the following procedure: first, to

410   reduce noise in the data, the ant's facing directions throughout each track were smoothed by

411   taking the circular mean facing direction across a 50-frame moving window. Points at which the

412   angular change between consecutive frames reversed direction gave the rough location of

413   inflection points in the track. To pick out the facing direction just prior to a reversal of direction,

414   we used the unsmoothed angle data to find the interval over which the facing angle was more

415   extreme than the facing angle at the inflection point. The mid-point of this interval was taken as

416   the facing angle at the peak or trough.

417   The algorithm used to extract extrema does not capture all the turn-endpoints that can be

418   seen by eye (e.g. Figure 3), because some turns were too small for the inflection point to show

419   up in the smoothed angle data. We saw no indication of false identification of extrema.

420   Some turns end in a clear plateau rather than a turn in the opposite direction. When this

421   was the case, the ant's facing angles at these plateaux were added to the extrema data to give

422   what we term turn endpoints. Plateaux were identified through the fulfilment of two criteria: (1)

423   an interval during a track that lasted at least 0.2s, in which the maximum change in facing

424   angles was less than 3°; (2) that the slope of the regression of facing angle against time of a

425   candidate plateau should be less than $1^{o}s^{-1}$. The facing angle of an identified plateau was then

426   defined as the circular mean of all facing angles in the interval.

427   ***Exclusion of inconsistent ants***

428   Some ants were very erratic. Paths of these ants are included in Figure S1 but excluded in

429   further analysis. To remove these ants, we calculated the overall direction of travel of each path

430   from every ant and calculated the resultant vector length of the path directions of each ant

431   across the entire training period. Ants whose resultant vector length >0.4 took similar

432   directions, relative to the visual and idiothetic cues, in each trial, despite the rotation of the

433   experimental arena. Those with a vector length <0.4 could not cope with rotation; 5 such ants

434   were removed from the training and test dataset. One of these 5 had taken part in tests 1 and

435   2, and none had participated in test 3.

436   **Descriptive statistics**

437   At the top of each histogram, we show the circular median of the distribution (black line;

438   Otieno and Anderson-Cook, 2003) and the interquartile range (IQR; grey bar; defined for

439   circular data, using the likelihood-based arc method, as the smallest arc of the circle containing

440   50% of all datapoints, Fisher and Hall, 1989). Rayleigh's tests for non-uniformity confirmed that

441   the ants' path directions and endpoints in training and tests 1 and 2 were not uniformly

442   distributed but showed a tendency to head in one direction (all $P<0.0001$).

443

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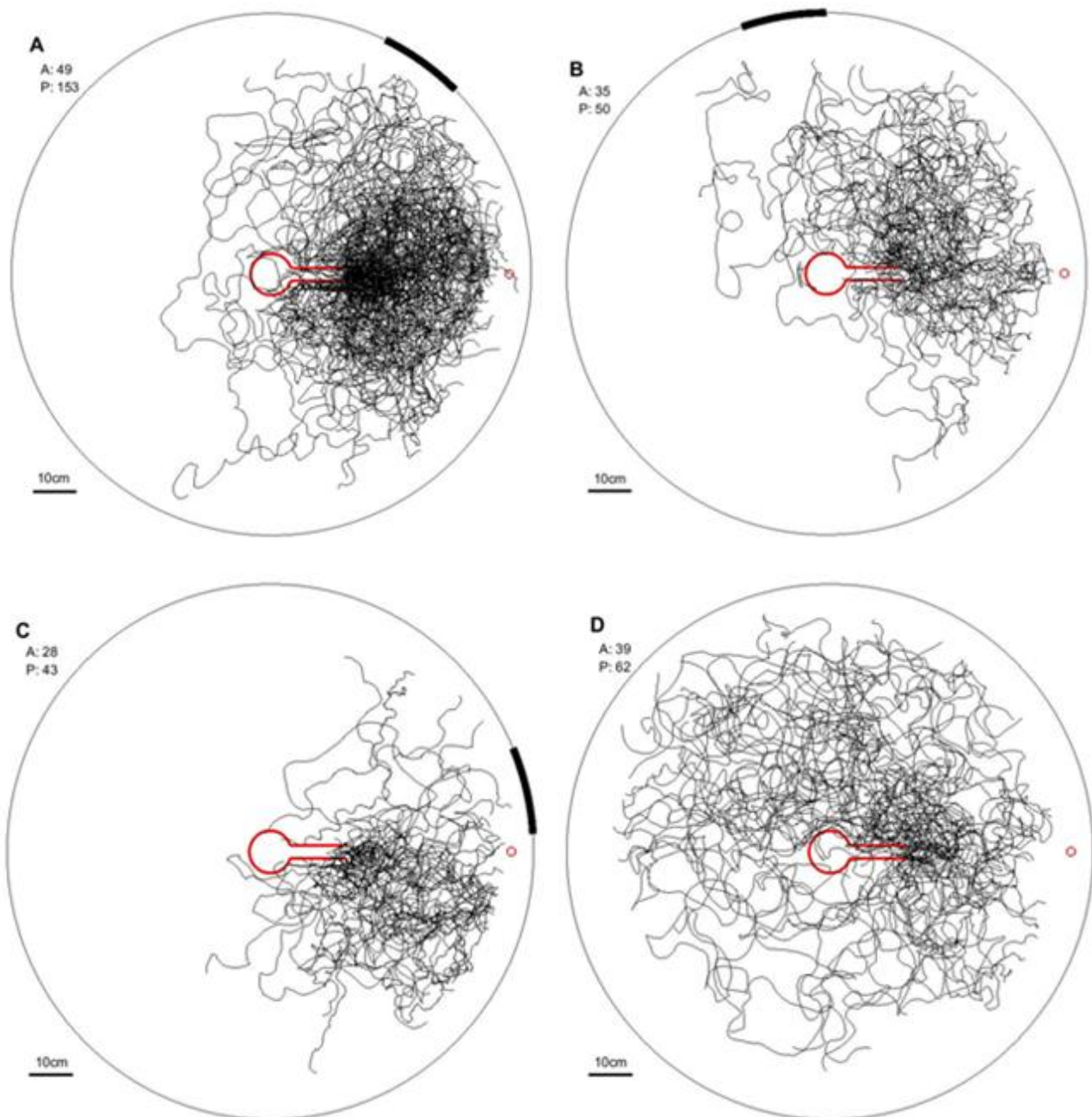560   *Biol. 219*, 1689-1696.

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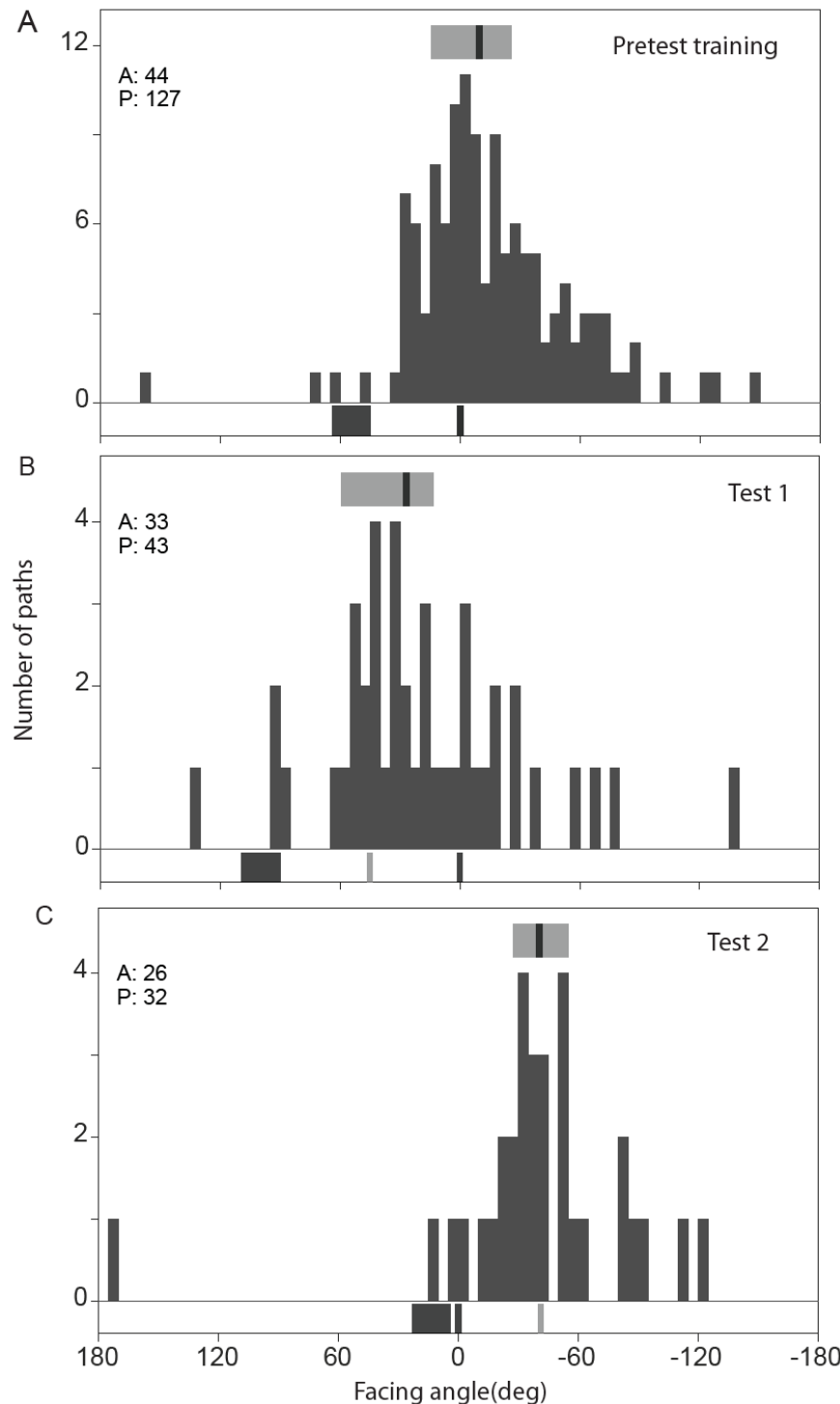
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566 **Supplementary figures**

567 *Figure S1. Superimposition of ants' paths during training conditions and tests.*

568 *All paths in training trials just before a test and in tests 1-3 are shown superimposed without any filtering*
569 *for consistency (See 'Exclusion of inconsistent ants' in Methods). **A.** paths recorded in training trials **B.***
570 *Paths in test 1 with bar shifted away from food. **C.** Paths in test 2 with bar shifted towards the food. **D.***
571 *Paths in test 3 with the bar removed. In each panel, A gives the number of ants and P the number of*
572 *paths.*
573

Figure S2. Replot of endpoints of selected right turns as in Figure 5.

*A: Histogram showing, for every pre-test training path, the median of the endpoints of selected right turns within the path. Within each path, selected turns began ±30⁰ from the bar edges. Each data point represents one path. The pattern of peaks resembles that of Figure 5 **B**: Median endpoints of selected turns in test 1 trials, with the visual cue shifted left, relative to the starting channel. **C**: Median endpoints of selected turns in test 2 trials, with the visual cue shifted right, relative to the starting channel.*