

1 **State-dependent, visually-guided behaviors in the nudibranch *Berghia***  
2 ***stephanieae***

3

4 Running title: Visual behaviors in a nudibranch

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6 Phoenix D. Quinlan <sup>a,\*</sup>, Paul S. Katz <sup>a,b</sup>

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8 a. Neuroscience and Behavior Graduate Program, University of Massachusetts Amherst,  
9 611 North Pleasant Street, Amherst, MA 01003, United States

10

11 b. Department of Biology, University of Massachusetts Amherst, 611 North Pleasant  
12 Street, Amherst, MA 01003, United States

13

14 \*Corresponding author: Phoenix D. Quinlan

15

16 Email:

17 Phoenix D. Quinlan: [pquinlan@umass.edu](mailto:pquinlan@umass.edu)

18 Paul S. Katz: [pkatz@umass.edu](mailto:pkatz@umass.edu)

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20 Key words: Gastropod mollusc, visual navigation, internal state, thigmotaxis

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22 **Summary statement:** Behavioral analyses demonstrate that the nudibranch *Berghia*  
23 *stephanieae* is capable of spatial vision and has visually-guided behaviors that are  
24 influenced by olfactory information and hunger state.

## 25 **Abstract**

26 Nudibranch molluscs have structurally simple eyes whose behavioral roles have  
27 not been established. We tested the effects of visual stimuli on the behavior of the  
28 nudibranch *Berghia stephanieae* under different food and hunger conditions. In an arena  
29 that was half shaded, animals spent most of their time in the dark, where they also  
30 decreased their speed and made more changes in heading. These behavioral differences  
31 between the light and dark were less evident in uniformly illuminated or darkened arenas,  
32 suggesting that they were not caused by the level of illumination. *Berghia* responded to  
33 distant visual targets; animals approached a stripe that was at least 15° wide and 50%  
34 darker than the background. They did not approach a stripe that was lighter than the  
35 background but approached a stripe that was isoluminant with the background,  
36 suggesting the detection of spatial information. Animals travelled in convoluted paths in  
37 a featureless arena but straightened their paths when a visual target was present even if  
38 they did not approach it, suggesting that visual cues were used for navigation. Individuals  
39 were less responsive to visual stimuli when food-deprived or in the presence of a food  
40 odor. Furthermore, when given a food odor, they had a weaker preference for the dark  
41 and behaved similarly in the light and dark. Thus, *Berghia* exhibits visually-guided  
42 behaviors that are influenced by odors and hunger state.

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44

## 45 **Introduction**

46 Gastropod molluscs have been shown to have visual responses ranging from  
47 phototaxis (Matsuo et al., 2014; Zieger et al., 2009) to high-resolution spatial vision (Irwin  
48 et al., 2021; Land, 1982). Moreover, gastropod species display a wide diversity of eye  
49 types ranging from open pit eyes to simple and complex lens eyes (Serb & Eernisse,  
50 2008; Zieger & Meyer-Rochow, 2008). Nudibranchs have relatively simple lens eyes,  
51 whose behavioral functions are not known. Studying the role of visually-guided behaviors  
52 in nudibranchs has been challenging because animals are often wild-caught, limiting  
53 control over the animal's life history and internal state. To better understand the role of  
54 nudibranch eyes, we examined visually-guided behaviors of a laboratory-raised aeolid  
55 nudibranch, *Berghia stephanieae* (Valdés, 2005).

56 Nudibranch eyes are located beneath the integument near the brain. Many  
57 nudibranchs lack epithelial pigment over the eye, allowing it to be visible as a small black  
58 spot (Hughes, 1970) (Fig. 1). Each eye contains a spherical lens that is covered by a  
59 cellular cornea (Chase, 1974; Eakin et al., 1967; Hughes, 1970). Several pigment-  
60 producing cells shield light from entering the eye from behind. The eyes of adult  
61 nudibranchs possess only three to five photoreceptor cells (Chase, 1974; Eakin et al.,  
62 1967; Hughes, 1970), which is fewer than other gastropods that can have hundreds or  
63 thousands of photoreceptor cells forming an organized retina (Bobkova et al., 2004;  
64 Jacklet, 1969; Meyer-Rochow & Bobkova, 2001; Zhukov et al., 2002). Nonetheless, the  
65 positioning and neural connectivity of the photoreceptors in the aeolid nudibranch  
66 *Hermisenda crassicornis* suggest that they could support spatial vision (Stensaas et al.,  
67 1969; Tabata & Alkon, 1982).

68 Although spatial vision has not been demonstrated in nudibranchs, they have been  
69 shown to have phototactic responses to light. For example, the dorid *Onchidoris*  
70 *bilamellata* spends more time in the dark when given a choice between light and dark  
71 (Barbeau et al., 2004), whereas another dorid, *Chromodoris zebra*, and *Hermisenda*  
72 spend more time in illuminated areas and approach light sources (Crozier & Arey, 1919;  
73 Lederhendler et al., 1980). Furthermore, when *Hermisenda* encounters a shadow in an  
74 otherwise illuminated environment, it stops moving forward and returns to the light  
75 (Lederhendler et al., 1980).

76 Anecdotal observations of *Berghia* suggest that it spends most of its time in dark  
77 environments, such as underneath objects or in dark crevices. *Berghia*'s responses to  
78 visual stimuli have not been experimentally tested. Here, we tested the responses of  
79 *Berghia* to visual stimuli to gain insights into the visual behaviors and capabilities of these  
80 nudibranchs. We found that *Berghia* exhibits visually-guided behaviors and provide  
81 evidence of low-resolution spatial vision. Furthermore, we tested animals under different  
82 conditions and found that visually-guided behaviors are state- and context-dependent.

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## 86 **Materials and methods**

### 87 **Animal care and husbandry**

88           Specimens of *Berghia stephanieae* were initially obtained from Salty Underground  
89 (Crestwood, MO) and Reeftown (Boynton Beach, FL). They were propagated in the  
90 laboratory by placing an egg mass into a plastic petri dish and incubating at 30°C. Artificial  
91 seawater (ASW) (Instant Ocean, Blacksburg, VA, USA) was maintained at a specific  
92 gravity of 1.020-1.022, temperature of 22-26°C, and pH of 8.0-8.5. ASW was exchanged  
93 twice weekly through manual pipetting. Late stage juvenile *Berghia* were transferred in  
94 groups of ten to 1-gallon acrylic aquariums filled with ASW and kept on a 12:12 LD cycle.  
95 *Exaiptasia diaphana* (Carolina Biological Supply, Burlington NC) were added twice  
96 weekly as a food source. *Exaiptasia* were kept in glass aquariums filled with artificial  
97 seawater maintained at the above conditions. *Exaiptasia* were fed brine shrimp (*Artemia*  
98 *nauplii*, Carolina Biological Supply Co) twice per week.

99

### 100 **Behavioral assays**

101           Individual *Berghia* were video recorded while freely moving inside a circular arena,  
102 which consisted of a 9.5-cm diameter glass dish filled with 240 mL ASW. The dish was  
103 placed in the center of a piece of 11.5-cm diameter white PVC pipe with a height of 9.5  
104 cm. White cardstock paper was inserted between the pipe and the glass dish. Visual  
105 stimuli were printed onto the paper using a Color Laser Jet Pro M454dw (HP, Palo Alto,  
106 CA).

107           An LED tracing board (tiktecklab) was fixed 15.25 cm above the testing apparatus  
108 to illuminate the arena. To shade half of the arena, black cardstock paper was placed on  
109 top and on one side of the arena. The hemisphere that was shaded was rotated between  
110 trials. For tests in illuminated and darkened arenas without a visual target, an 850 nm  
111 infrared light (CMVision) illuminated the arena from 30 cm above at a 60° angle. A USB  
112 infrared sensing camera with OV2710 CMOS sensor (webcamera\_usb) was fixed 16 cm  
113 below the dish, recording at 30 frames per second (for trials in a half-shaded arena and  
114 uniformly illuminated/darkened arenas) or 2 frames per second (for trials with a visual  
115 target).

116 All animals used were reproductive adults (1-2 cm length) and were tested at least  
117 12 weeks post-hatching. Like other nudibranchs, *Berghia* is hermaphroditic. Each animal  
118 was used only once, except when paired testing was performed as indicated. Animals  
119 were tested 24-48 hours after being fed. For experiments on food-deprived animals,  
120 testing was performed 5-6 days after their last feeding. To create conditioned ASW for  
121 food odor, six *Exaiptasia* were kept in 200 mL ASW for 24 hours. 10 mL of conditioned  
122 ASW was diluted with 230 mL ASW to provide food odor.

123 For each trial, a single *Berghia* was gently pipetted to the center of the glass dish.  
124 Animals were given 5 minutes to acclimate to the arena, after which they were recorded  
125 for 10 minutes. Sample size was chosen using the resource equation approach, which  
126 suggested 11-21 animals for within-subjects repeated measures. In the half-shaded  
127 arena, 15 animals were tested for each feeding and odor condition (60 animals total).  
128 When animals were tested in arenas that were completely illuminated or darkened, the  
129 order of the light and dark trial were counterbalanced, and 15 animals were tested.

130 When testing animals with a visual target, 18 animals were tested for each stimulus  
131 type, but individuals were excluded if they did not right themselves immediately upon  
132 being pipetted into the arena. No acclimation period was used, and animals were  
133 recorded until they reached the edge of the arena or until 6 minutes elapsed. Animals that  
134 did not reach the edge were tracked and plotted but excluded from further analysis.

135

## 136 **Analyses and statistics**

137 The location of each individual within the arena was tracked for the duration of the  
138 trial using the markerless pose estimation software DeepLabCut (Nath et al., 2019).  
139 Networks were trained to detect *Berghia* using training datasets in which animals were  
140 manually marked posterior to the first ceratal row. A different network and training dataset  
141 were used for each behavioral assay. The trajectories of each animal were exported into  
142 CSV files, after which they were analyzed using custom MATLAB scripts. Incorrectly  
143 labeled points were removed using criteria such as a likelihood score and the maximum  
144 possible distance to travel between frames. For trials with a visual target recorded at 2  
145 frames/second, only *Berghia* that could be tracked from the center to the wall of the arena  
146 were included. For trials in a half-shaded arena and uniformly illuminated/darkened

147 arenas, trials were only used if at least 50% of frames (a total of 15000 frames were  
148 recorded at 30 frames per second) were labelled correctly. Tracking accuracy was around  
149 98% for fed animals and 88% for food-deprived animals (Fig. S1). Tracking accuracy was  
150 higher for fed animals presumably because *Berghia* becomes darker in color after eating,  
151 making it more visible against the illuminated background of the video. Arena boundaries  
152 were determined by manual segmentation using Make Sense (Skalski, 2019).

153 To determine whether *Berghia* approached visual targets, the distribution of the  
154 locations where *Berghia* touched the wall of the dish were analyzed. Videos were trimmed  
155 from when the animal righted itself to when it came in contact with the wall. The  
156 trajectories of animals were adjusted so that the first coordinate of each trace was located  
157 at the origin, and the location where each animal travelled 95% of the distance to the  
158 edge was identified. The R package CircMLE (Fitak & Johnsen, 2017) was used to rank  
159 how well 10 models of animal orientation (Schnute & Groot, 1992) describe the  
160 distribution of these locations (Table S2). The AICc criterion (Hurvich & Tsai, 1991) was  
161 used to compare models, and the model with the highest AICc value was reported as the  
162 best fit for the data. Further models were also reported if the relative differences to the  
163 best model ( $\Delta\text{AICc}$ ) were less than 2, as these models were also strongly supported  
164 (Burnham et al., 2011). A visual target was considered to be approached by *Berghia* if  
165 the best fitting model was of unimodal distribution directed toward the target.

166 Behavioral measures such as mean speed, straightness, and mean change in  
167 heading were calculated. Mean speed was calculated by dividing total distance travelled  
168 by time elapsed. Straightness (straightness index) was calculated by dividing the distance  
169 from the center of the arena to the wall by the total distance travelled from the center to  
170 the wall. Mean change in heading was calculated as the mean change in direction of 2  
171 vectors defined by the animal's location across 3 subsequent points in time. Statistical  
172 significance was assessed using the Student's one-sample, two-sample, and paired  
173 sample *t*-tests as indicated, with  $\alpha = .05$  (function 'ttest' and 'ttest2' in MATLAB). A one-  
174 way ANOVA was used to assess whether means from multiple groups of animals were  
175 significantly different, with  $\alpha = .05$  (function 'anova1' in MATLAB). A two-way ANOVA  
176 (function 'anovan' in MATLAB) was performed to determine the main effect of food-  
177 deprivation, the presence of a food odor, and the interaction effect of these two conditions

178 on behavioral measures, including mean speed and mean change in heading. Following  
179 the one- or two-way ANOVA, pairwise comparison was performed using Fisher's Least  
180 Significant Difference Test (function 'multcompare' in MATLAB) and statistically  
181 significant differences were reported. For comparisons of non-parametric data, the  
182 Kruskal-Wallis test (function 'kruskalwallis' in MATLAB) was used. For detailed results  
183 from statistical testing, see supplementary information (Tables S1-S8).

184

## 185 **Results**

### 186 ***Berghia* preferred dark environments**

187 When placed in an environment that was half-shaded, animals spent most of their  
188 time in the dark. The movements of fifteen animals were tracked for ten minutes in an  
189 arena that was half-illuminated and half-shaded (Fig. 2A,B). Following a 5-minute  
190 acclimation period, 13 of 15 animals (86.7%) were located in the dark side of the arena.  
191 During the 10-minute trial, all animals spent a majority of their time in the dark (Fig. 2C).  
192 On average, animals spent  $83.6 \pm 14.2\%$  of their time in the dark half of the arena ( $n=15$ ,  
193 one-sample  $t$ -test:  $P<.001$ ).

194 In the dark half of the area, animals turned frequently and did not stay on the edge  
195 (Fig. 2B). In contrast, when animals were in the illuminated half of the arena, their paths  
196 were straighter, and they tended to stay near the edge (2B). Fig. 2Di shows an example  
197 of an individual that started in the lighted half of the arena, moving along the edge, but  
198 once it reached the darkened side, it moved away from the edge and increased the  
199 frequency of turns (Fig. 2Di). Fig. 2Dii shows a different individual that started on the dark  
200 side, but, after entering the light side, promptly turned around and re-entered the dark.  
201 Thus, *Berghia* had a strong preference for being in the dark and showed notable  
202 differences in behavior between the light and dark sides.

203

### 204 ***Berghia* behaved differently in uniformly and partially illuminated arenas**

205 To test whether differences in *Berghia*'s behavior could be attributed to the level  
206 of ambient lighting, the movements of fifteen animals were tracked in an arena that was  
207 either fully darkened or fully illuminated (Fig. 3A). The trajectories of individual animals  
208 were more consistent in the dark and light than when in a half-illuminated arena. For

209 example, an individual that circled the perimeter of the arena did so under both the dark  
210 and the light conditions (Fig. 3B*i*), and an individual that entered the interior of the arena  
211 did so in both conditions (Fig. 3B*ii*). However, in the completely darkened arena, the  
212 animals rarely came as close to the edge as they did under uniformly illuminated  
213 conditions as can be seen in the individual trajectories (Fig. 3B) as well as the density  
214 plots (Fig. 3C*i*).

215 Under uniform illumination, animals frequently made contact with the edge of the  
216 dish and spent most of their time within a body's length (about 1 cm) of the edge of the  
217 9.5 cm-diameter dish (Fig. 3C*i*). However, the proportion of time spent within a body's  
218 length of the edge in the lighted side of a partially illuminated arena was much higher than  
219 in a uniformly illuminated one (Fig. 3C*i,ii*).

220 There were other behavioral differences between animals in uniformly and  
221 partially-illuminated arenas. Although animals crawled about 75% faster in a fully  
222 darkened arena compared to one that was fully illuminated, the difference was more  
223 pronounced in a partially illuminated arena where animals crawled 300% faster in the  
224 dark side than the light side (Fig. 3D). Additionally, although animals did not exhibit  
225 significantly different turning behavior in uniformly darkened and uniformly illuminated  
226 arenas, there was a strong decrease in the mean change in heading when animals were  
227 on the light side of a partially illuminated arena, indicating that they turned less in the light  
228 (Fig. 3E). Thus, *Berghia* behaved differently in partially-illuminated versus uniformly-  
229 illuminated arenas, suggesting that they may be responding to visual features of the  
230 environment and not just ambient light levels.

231

### 232 ***Berghia* approached visual targets**

233 Animals were placed in a uniformly illuminated environment with or without a single  
234 vertical stripe on the wall outside of the arena (Fig. 4A). Animals placed in the center of  
235 an arena with no external markings typically changed directions several times before  
236 approaching the edge and 29.4% of them did not reach the wall at all (Fig. 4B). However,  
237 with a black stripe that extended 45° around the arena, every animal reached the wall and  
238 most animals approached the wall near the stripe, either moving directly toward it or  
239 making a large orienting turn before moving in a straight path toward the stripe (Fig. 4C).



240 When tested with a white stripe on a black background, all animals approached the black  
241 part of the wall rather than the stripe (Fig. 4D).

242 Animals were tested with stripes of various widths and contrast from the  
243 background (Fig. 4E). Animals approached a black stripe that was at least 15° of the  
244 circumference of the arena. While some animals approached a 10° stripe, most animals  
245 went to the opposite side, and the locations where animals approached the edge followed  
246 an axial distribution. Animals approached the widest stripe tested, which was 90°. The  
247 locations where animals reached the wall followed a unimodal distribution that was  
248 centered on the visual target (Fig. 4E). Animals did not approach a 25% gray stripe but  
249 approached a stripe that was 50% gray or darker (Fig. 4G). Overall, *Berghia* most reliably  
250 approached a 45° black stripe on a white background (Fig. 5).

251

### 252 ***Berghia* used spatial vision**

253 *Berghia* could be approaching visual targets through non-visual phototaxis or by  
254 using coarse spatial vision. Spatial vision is required for the detection of a visual target  
255 that is isoluminant with the background. An isoluminant visual target was created by  
256 surrounding a 25° black stripe with two 12.5° white stripes on a 50% gray background, so  
257 that the average luminance over the 50° is the same as the rest of the arena. Animals  
258 approached this isoluminant stripe near the target (Fig. 6B). A similar percentage of  
259 animals approached the 25° black stripe on an isoluminant background as approached a  
260 25° black stripe on a white background (Fig. 5). This suggests that *Berghia* has spatial  
261 vision rather than just sensing light and dark.

262

### 263 ***Berghia* used visual landmarks for navigation**

264 Animals travelled in straighter paths when a visual target was present even if they  
265 did not approach it. For example, although only about half of the animals approached a  
266 25° stripe, all of those animals travelled in a straight path to the edge of the arena (Fig.  
267 6A). Similarly, although a stripe that was 5° did not elicit approach, several individuals  
268 travelled in direct paths to the edge (Fig. 4E). Animals also travelled in straight paths  
269 when presented with a gray stripe that was only 25% darker than the white background,

270 which was below the contrast threshold for which animals began approaching a visual  
271 target (Fig. 4G).

272 The paths were straighter when a visual target of at least 15° was present (Fig.  
273 6C). Straightness was significantly higher both for animals that approached the target and  
274 for animals that did not approach the target compared to animals tested without a visual  
275 target. Straightness was also significantly higher in animals tested with an isoluminant  
276 visual target than with no target, suggesting that animals are responding to contrast rather  
277 than luminance (Fig. 6C). Thus, *Berghia* seems to use contrasting visual landmarks as a  
278 navigational aid, even when they do not approach it.

279

### 280 **Visually-guided behaviors were state- and context-dependent**

281 *Berghia's* preference for being in the dark changed with food-deprivation and the  
282 presence of a food odor. Animals were either fed or food-deprived for 5 days and tested  
283 in normal seawater or water that was conditioned with a food odor (Fig. 7). Animals spent  
284 most of their time in the dark with food-deprivation, the presence of food odor, or both  
285 (Fig. 7C). Food-deprivation alone also seemed to strengthen the preference for dark, as  
286 8 of 15 (53.3%) food-deprived animals spent the entire 10-minute period in the dark,  
287 whereas this occurred in only 3 of 15 (20%) fed animals. However, 4 of 15 (26.7%)  
288 animals that were food-deprived and given a food odor spent a majority of time in the  
289 illuminated side, whereas this did not occur with any of the fed animals and was observed  
290 in only 2 of 15 (13.3%) food-deprived animals and 1 of 15 (6.7%) animals tested with a  
291 food odor. This suggests that food-deprivation strengthens the preference for dark  
292 whereas the combination of being food-deprived and sensing a food odor reduces it.

293 There were additional changes in the behavior of animals following food-  
294 deprivation and/or exposure to a food odor. All animals crawled faster in the dark than the  
295 light, regardless of feeding state or whether a food odor was present. However, this  
296 increase was significantly lower in animals that were both food-deprived and given a food  
297 odor (Fig. 7D). Additionally, whereas all animals had an increased mean change in  
298 heading angle in the dark compared to the light, this difference was smaller for food-  
299 deprived animals that were given a food odor in comparison to animals tested without a  
300 food odor (Fig. 7E). There was a significant main effect of food odor on this heading

301 increase (Table S7). In particular, animals that were given a food odor had a larger mean  
302 change in heading in the light side of the arena compared to animals that were tested  
303 without a food odor (Fig. 7F). Thus, in addition to having a weaker preference for the dark,  
304 food-deprived animals that were given a food odor behaved more similarly in the light and  
305 dark.

306 Food-deprivation and sensing a food odor also reduced *Berghia*'s propensity to  
307 approach a stripe. Animals that were food-deprived approached the edge randomly, with  
308 or without a food odor (Fig. 8A). Fed animals that were given a food odor approached a  
309 45° stripe (Fig. 8A). Just over half of the animals approached the quadrant with the stripe,  
310 while 22.2% did not approach the edge at all, suggesting that there was a reduction in  
311 the propensity to approach the stripe when animals were given a food odor (Fig. 8B).  
312 Additionally, fed animals that were given a food odor made notably sharper turns and  
313 sometimes reversed directions completely rather than travelling directly to the edge,  
314 however this was not observed in food-deprived animals that were given a food odor (Fig.  
315 8A). Fed animals travelled in more direct paths with a stripe than without a stripe, both  
316 with and without a food odor (Fig. 8C). Food-deprived animals travelled in straighter paths  
317 than fed animals (two-sample two-tailed *t*-test,  $P = .04$ ), but travelled with a similar  
318 straightness regardless of whether a visual target was present (Fig. 8C). Finally, fed  
319 animals crawled significantly faster with a stripe than without a stripe, however this was  
320 not true for food-deprived animals, animals given a food odor, or animals that were both  
321 food-deprived and given a food odor (Fig. 8D). Overall, animals that were food-deprived  
322 or given a food odor were less responsive to visual cues.

323

## 324 **Discussion**

325 We found that *Berghia* exhibits visually-guided behaviors. Animals spent more time  
326 in dark environments and approached a contrasting visual target. When a visual target  
327 was present, animals crawled in straight paths even when they did not approach it,  
328 suggesting that visual cues are important for navigation. Animals that were food-deprived  
329 or given a food odor had a reduction in behavioral responses to visual stimuli,  
330 demonstrating that visual responses are state- and context-dependent. Additionally, there  
331 was an even stronger reduction in behavioral responses when animals were both hungry

332 and encountered a food odor, indicating an interaction between visual information,  
333 olfactory information, and hunger state.

334

### 335 **Visual navigation**

336 When given a choice between light and dark areas, *Berghia* spent most of its time  
337 in the dark and had distinctive behaviors in the light versus the dark. For example, animals  
338 followed along the edge of the arena when exposed to ambient light, which was rarely  
339 observed in the dark. Commonly referred to as thigmotaxis, this behavior is a spatial  
340 navigation strategy that has been observed in other animals, including insects (Jin et al.,  
341 2020), fish (Champagne et al., 2010; Sharma et al., 2009), rodents (Simon et al., 1994;  
342 Treit & Fundytus, 1988), and humans (Kallai et al., 2005, 2007). Thigmotaxis is thought  
343 to be performed when animals are trying to avoid or escape an environment. In *Berghia*,  
344 thigmotaxis appeared to be involved in helping animals leave illuminated environments;  
345 it was more prevalent in a partially illuminated arena, than one that was uniformly lit (Fig  
346 3C).

347 *Berghia* also approached a dark vertical stripe on a light background. Although  
348 other gastropods have similarly been shown to approach a dark vertical stripe (Chiussi &  
349 Díaz, 2002; Hamilton & Winter, 1982; Hamilton & Winter, 1984; Shepeleva, 2013), this is  
350 the first demonstration of this behavior in a species of nudibranch. Similar responses in  
351 other gastropods have been suggested to be related to seeking shelter (Chiussi & Díaz,  
352 2002) or habitat selection (Hamilton & Winter, 1982; Shepeleva, 2013). Anecdotal  
353 observations in the laboratory suggest that *Berghia* prefers to spend most of its time dark  
354 areas, such as in dark crevices and underneath objects. Additionally, *Berghia* feeds on  
355 anemones that are found in shaded areas on the roots of mangrove trees (Bedgood et  
356 al., 2020; Bellis et al., 2018). Thus, it is likely that *Berghia* approaches visual targets to  
357 seek out dark habitats that provide food and shelter.

358 Animals travelled in a straight path when a visual target was present even if they  
359 did not approach it, suggesting that *Berghia* uses visual landmarks to navigate its  
360 environment. External cues are indispensable in allowing animals to navigate in a straight  
361 line (Cheung et al., 2007, 2008). In addition to approaching objects, moving in a straight  
362 path allows animals to navigate to new locations, whereas convoluted paths may lead

363 animals to re-enter previously explored areas. In the absence of directional sensory  
364 information, even humans fail to navigate in a straight path (Dacke & el Jundi, 2018).  
365 When *Berghia* was placed into an illuminated arena without any visual targets, animals  
366 changed direction several times before reaching the edge. The tortuosity of *Berghia*'s  
367 path could be a result of the arena being void of directional olfactory or visual information.

368 In addition to approaching objects, moving in a straight path allows animals to  
369 navigate to new locations, whereas convoluted paths may lead animals to re-enter  
370 previously explored areas. After we deprived *Berghia* of food for 5 days, animals crawled  
371 with straighter paths than animals that were regularly and recently fed. Moving in a  
372 straight path may facilitate animals travelling to new locations when food is scarce and  
373 could thus be beneficial for animals that are hungry.

374

### 375 **Visual capabilities of nudibranchs**

376 In this study, we provide evidence that *Berghia* is capable of low-resolution spatial  
377 vision. Differences in *Berghia*'s behavior in the light and dark were stronger when light in  
378 the environment varied spatially than when it was uniformly illuminated. *Berghia* most  
379 effectively approached a black stripe subtending an arc of 45° around the arena while  
380 thinner or wider stripes were approached less, suggesting that *Berghia* is not simply  
381 moving toward darkness. Additionally, animals approached a 25° stripe that was  
382 isoluminant with the background, which suggests the detection of contrast rather than  
383 light intensity. It is therefore likely that *Berghia* uses spatial vision to detect objects in the  
384 environment.

385 Studies of the anatomy and electrophysiology of nudibranch eyes provide potential  
386 neural mechanisms that could underlie spatial vision. Although nudibranchs lack an  
387 organized retina, the microvillous regions of photoreceptors in nudibranchs form distinct  
388 areas within the eye (Dennis, 1967; Hughes, 1970; Stensaas et al., 1969). Further,  
389 photoreceptor cells in *Hermisenda* have been shown to have distinct receptive fields  
390 (Dennis, 1967). Additionally, there are inhibitory connections between the five  
391 photoreceptor cells in each eye (Crow & Tian, 2003; Detwiler & Alkon, 1973).  
392 Photoreceptor cells also inhibit cells in the contralateral optic ganglion, demonstrating a  
393 convergence of visual information between the two eyes (Alkon, 1973). It was suggested

394 that inhibition between photoreceptors or contralateral optic ganglia may support the  
395 detection of contrast (Alkon, 1973). Thus, nudibranchs may have the necessary  
396 components for spatial vision, and the results from the current study provide behavioral  
397 evidence to support this.

398

### 399 **State and context dependence**

400 Visually-guided behaviors in *Berghia* are influenced by hunger state and the  
401 sensation of food odor. When water was conditioned with *Berghia*'s prey, a sea anemone,  
402 animals still preferred dark environments, but they had a slight reduction in their  
403 propensity to approach a black stripe. Additionally, they showed changes in the style of  
404 locomotion, with animals performing sharper rather than smooth turns following the  
405 addition of a food odor. Following food-deprivation, animals did not approach a black  
406 stripe. When both food-deprived and given a food odor, animals had a weaker preference  
407 for being in the dark and behaved more similarly in the light and dark. Additionally, fed  
408 animals that were tested with a food odor often sharply reversed directions, while this  
409 never occurred in food-deprived animals that were given a food odor. Together, these  
410 results indicate that there are interactions between hunger state, olfactory information,  
411 and visual information that lead to changes in *Berghia*'s behavior.

412 Visual responses in other gastropods are also dependent on internal state or the  
413 presence of olfactory information. Similar to *Berghia*, the sea snail *Nerita fulgarans*  
414 approaches dark visual targets. When presented with a predator odor, *Nerita* avoids  
415 rather than approaches visual stimuli (Chiussi & Díaz, 2002). The dorid nudibranch  
416 *Chromodoris zebra* ceased orienting to light when they were with conspecifics (Crozier &  
417 Arey, 1919). The sea slug *Pleurobranchaea californica* responds to food preferentially to  
418 light (Davis & Mpitsos, 1971). The aeolid nudibranch *Hermisenda crassicornis* changes  
419 its preference for light according to the time of day, with bright light being preferred during  
420 the day but not during the night (Lederhendler et al., 1980). When given a choice,  
421 *Hermisenda* approaches a light over a food source, except when hungry (Alkon et al.,  
422 1978). Furthermore, stimulation of tentacular chemoreceptors inhibits the responses of  
423 photoreceptor cells in *Hermisenda* (Alkon et al., 1978). In these gastropods, as well as

424 *Berghia*, visual information seems to be ignored during other behaviors such as foraging  
425 or seeking mates.

426

## 427 **Conclusion**

428 Although vision was not previously considered an important sensory modality for  
429 nudibranchs, the current study provides behavioral evidence that nudibranchs respond to  
430 visual features of their environment. Our findings demonstrate that *Berghia* has visually-  
431 guided behaviors that are influenced by hunger state and odors. It is likely that *Berghia*  
432 uses its eyes for low-resolution visual tasks such as seeking dark habitats, approaching  
433 objects, and navigating its environment.

434

435

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438 collecting pilot data. We also thank Niah Holtz and Jackson Southard for assistance  
439 with data collection. Additionally, we thank Thi Bui for determining *Berghia*'s sensitivity  
440 to long-wavelength light.

441

442

## 443 **Competing interests**

444 The authors declare no competing or financial interests.

445

446

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449

450

## 451 **Data availability**

452 Data and analysis code available upon request.

453 **Figure Legends**

454 **Figure 1.** Photograph of adult *Berghia* with eye visible. The eye of adult *Berghia*  
455 *stephanieae* is located dorsolaterally on the head in a non-pigmented zone.

456

457 **Figure 2.** Dark preference in a half-shaded arena. **A.** Diagram of half-shaded arena. The  
458 arena was illuminated overhead using white LEDs. A camera was mounted below to  
459 record each animal for 10 minutes. **B.** Trajectories of individual animals overlaid, with the  
460 darkened (left) and lighted (right) sides marked. **C.** Histogram of the percentage of time  
461 spent in dark over 10 minutes ( $n=15$ ). **D.** Example traces from two individuals, one that  
462 travelled along the edge of the arena in the light but moved away from the edge and  
463 increased turning in the dark (*i*) and one that entered the lighted side, promptly turned  
464 around, and re-entered the dark side (*ii*). The starting position (star) and ending position  
465 (circle) of each individual is marked.

466

467 **Figure 3.** Behavior in arenas with uniform vs partial illumination. **A.** Trajectories of  
468 animals ( $n=15$ ) crawling in a completely darkened arena (left) and in one that was  
469 uniformly illuminated (right). **B.** Examples of individuals that behaved consistently under  
470 both conditions. One individual moved around the edge of the arena in both the light (gray  
471 trace) and the dark (black trace), although it got closer to the wall in the light (*i*). A different  
472 individual explored the center of the arena under both conditions (*ii*). The start and end  
473 positions of each trace are indicated by the star and circle, respectively. **C.** Density plots  
474 showing the relative amount of time spent at different distances from the edge of the  
475 arena in the light (white) and dark (gray) in an arena that was uniformly illuminated (*i*) and  
476 one that was only partially illuminated (*ii*). **D-E.** Box and scatter plots of the mean crawling  
477 speed (**D**) and mean change in heading (**E**) of animals in the dark (gray box) and light  
478 (white box) in a uniformly versus partially illuminated arena. For all box plots, the median  
479 value (red line) is reported. Connected data points are from the same individual; line styles  
480 indicate whether the value was higher in the dark (solid gray line) or light (dotted teal line)  
481 for each individual. A paired *t*-test was used to test whether mean speed (**D**) or mean  
482 change in heading (**E**) was significantly different in the dark compared to the light;  
483 significant differences: \*\* $P<.01$ , \*\*\* $P<.001$ .



484

485 **Figure 4.** *Berghia* approached visual targets. **A.** Diagram of visual target assay. Animals  
486 were placed in the center of a brightly illuminated circular arena surrounded by a white  
487 wall with a single vertical stripe. A camera was mounted below to record animals until  
488 they reached the wall. **B-D.** Trajectories of animals crawling from the center to the edge  
489 of an arena with no visual target (**B**), a 45° black stripe on a white background (**C**), or a  
490 45° white stripe on a black background (**D**). The location where each animal approached  
491 the edge is marked with a circle. Animals that did not reach the edge within the allotted  
492 time were plotted (open circles) but excluded from analysis. **E.** Trajectories of animals  
493 crawling from the center to the edge of an arena with black stripes of different widths. **F.**  
494 Histogram of the locations where 97 animals approached the edge of an arena with a  
495 black stripe with a width of 15-90°. **G.** Trajectories of animals crawling in response to 45°  
496 stripes of different levels of gray on a white background. Maximum likelihood analysis of  
497 circular data was used (**B-D,F,G**; Table S2); black triangles mark the direction(s) of best-  
498 fitting model for unimodal and bimodal models. Sample sizes were  $n=17$  for none;  $n=17$   
499 for 45° black;  $n=18$  for white;  $n=17, 17, 15, 18,$  and  $14$  for 5°, 10°, 15°, 60°, and 90° black,  
500 respectively;  $n=12, 16, 17$  for 25%, 50%, and 75% gray, respectively.

501

502 **Figure 5.** Approach rates for various visual targets. The percentage of animals that  
503 reached the wall in the quadrant centered by the stripe. When no stripe was present, the  
504 percentage of animals that reached a randomly chosen quadrant and semicircle was  
505 reported. Animals were tested with black stripes of various widths ranging from 5° to 90°  
506 (left), 45° stripes of various contrasts, and a 25° stripe that was isoluminant with the  
507 background (right). The probability of entering a random quadrant (25%) or semicircle  
508 (50%) are indicated (horizontal dashed lines). Sample sizes were  $n=17$  for none;  $n=17,$   
509  $17, 15, 16, 17, 18,$  and  $14$  for 5°, 10°, 15°, 25°, 45°, 60°, and 90°, respectively;  $n=12, 15,$   
510  $17$  for 25%, 50%, and 75% gray, respectively;  $n=18$  for white;  $n=17$  for isoluminant.

511

512 **Figure 6.** *Berghia* travelled in straighter paths with an isoluminant visual target. **A-B.**  
513 Trajectories of animals crawling from the center to the edge of an arena with a 25° black  
514 stripe on a white background (**A**) and isoluminant to the background (**B**). Maximum

515 likelihood analysis of circular data was used; black triangles mark the direction(s) of best-  
516 fitting model for unimodal and bimodal models. Sample sizes were  $n=16$  for a white  
517 background and  $n=17$  for an isoluminant background. **C.** The straightness index was  
518 calculated for each animal's path from the center to the edge of the arena. Higher values  
519 indicate a straighter path. Animals were tested without a visual target ( $n=12$ ) or with a  
520 dark stripe on a white background of at least  $15^\circ$ . Animals tested with a stripe were  
521 separated by whether they approached the edge of the arena within  $90^\circ$  of the center of  
522 the stripe ( $n=78$ ) or not ( $n=48$ ). Additionally, animals were tested with a  $25^\circ$  black stripe  
523 on a white background ( $n=16$ ), and a  $25^\circ$  stripe isoluminant to the background ( $n=17$ ).  
524 Animals tested with a  $25^\circ$  stripe were marked by whether they approached (gray triangle)  
525 or did not approach the stripe (red circle) but were plotted together due to the small  
526 sample size. Box plots report the median value (red line). A one-way ANOVA ( $F=3$ ,  $P=.02$ ;  
527 for detailed results see Table S3-S4) with Fisher's Least Significant Difference Test was  
528 used to compare straightness across groups; statistical differences: \*\*\* $P<.001$ .  
529

530 **Figure 7.** Effects of food-deprivation and food odor on behavior in a half-shaded arena.  
531 **A.** Trajectories of animals crawling in a half-shaded arena after food-deprivation ( $n=15$ ),  
532 in the presence of food odor ( $n=15$ ), and both of these conditions ( $n=15$ ). **B.** Trajectories  
533 from two animals that were food-deprived and presented with a food odor. The starting  
534 position (star) and ending position (circle) of each individual is marked. **C-E.** For animals  
535 that were fed or food-deprived and tested with or without a food odor, comparisons were  
536 made of the proportion of time spent in the dark (**C**), the percentage increase in the mean  
537 speed in the dark compared to the light (**D**), and the percentage increase in the mean  
538 change in heading angle in the dark compared to the light (**E**; for separate plots of the  
539 mean speed and mean change in heading in the light and dark side, see Fig. S2). **F.**  
540 Mean change in heading angle in the light for animals tested without ( $n=30$ ) or with a food  
541 odor present ( $n=30$ ). A Kruskal Wallis test (**C**), two-way ANOVA with a Fisher's Least  
542 Significant Difference Test (**D,E**; for detailed results see Table S5-S8), and two-sample  $t$ -  
543 test (**F**) were used; statistical differences: \* $P<.05$ , \*\*  $P<.01$ . Box plots report the median  
544 value (red line).  
545

546 **Figure 8.** Effects of food-deprivation and food odor on the approach of visual targets. **A.**  
547 Trajectories of animals crawling in the stripe assay following food-deprivation ( $n=14$ ), the  
548 addition of a food odor ( $n=14$ ), or both ( $n=15$ ). The location that each animal approached  
549 the edge of the arena is marked (colored circles). Animals that did not reach the edge  
550 within the allotted time (open circles) were plotted but excluded from analysis. Maximum  
551 likelihood analysis of circular data was used (Table S2); black triangles mark the  
552 direction(s) of best-fitting model for unimodal and bimodal models. **B.** The percentage of  
553 animals that entered the semi-circle and the quadrant containing the stripe were  
554 quantified for each combination of food-deprivation and food odor. For each group,  
555 percentages were quantified for animals tested with a visual target (diagonally lined bars)  
556 and without a visual target. When no stripe was present, the percentage of animals that  
557 entered a randomly chosen quadrant was reported. Results from fed animals with no  
558 stripe and a 45° black stripe are repeated from Figure 5. **C-D.** In addition to testing animals  
559 with a visual target, another set of animals were tested without a visual target following  
560 food-deprivation ( $n=17$ ), the addition of a food odor ( $n=13$ ), or both ( $n=14$ ). For each  
561 feeding or odor condition, straightness index (**C**) and mean speed (**D**) was compared  
562 between animals tested without a visual target (white boxes) and with a visual target (gray  
563 boxes). Box plots report the median value (red line). A two-sample  $t$ -test (**C,D**) was used;  
564 statistical differences:  $*P<.05$ ,  $**P<.01$ .  
565

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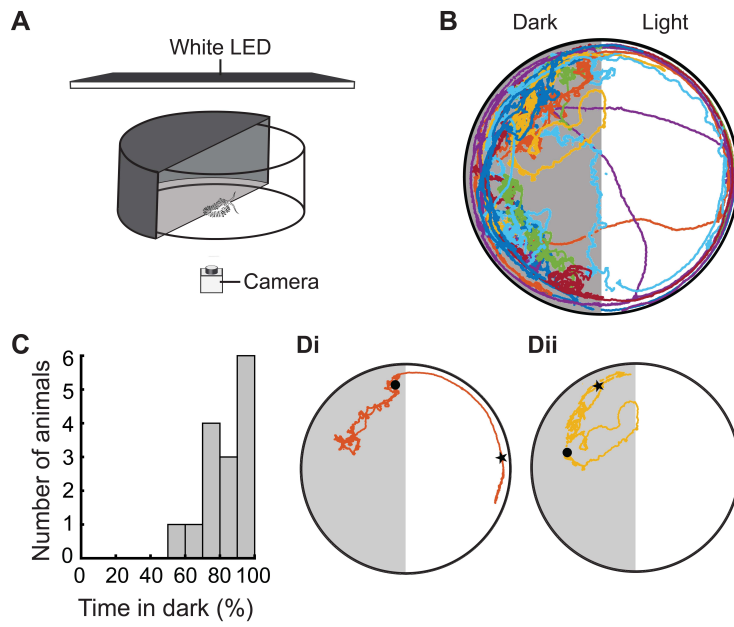
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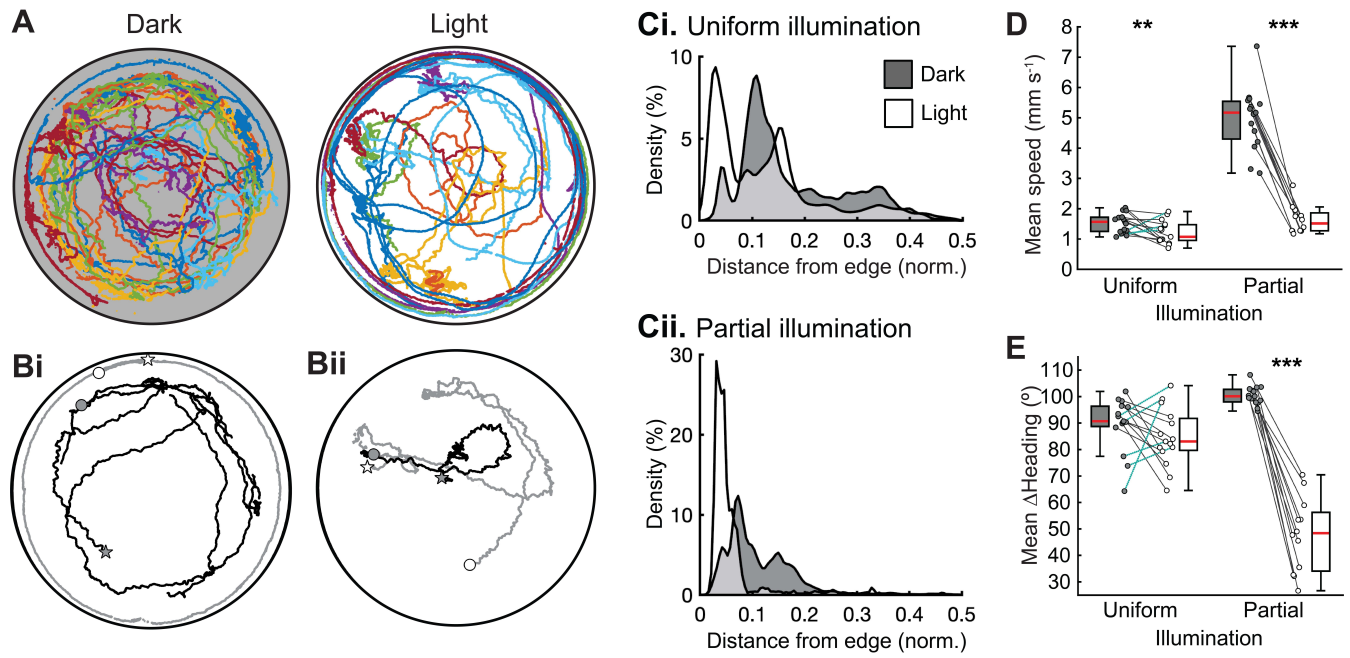
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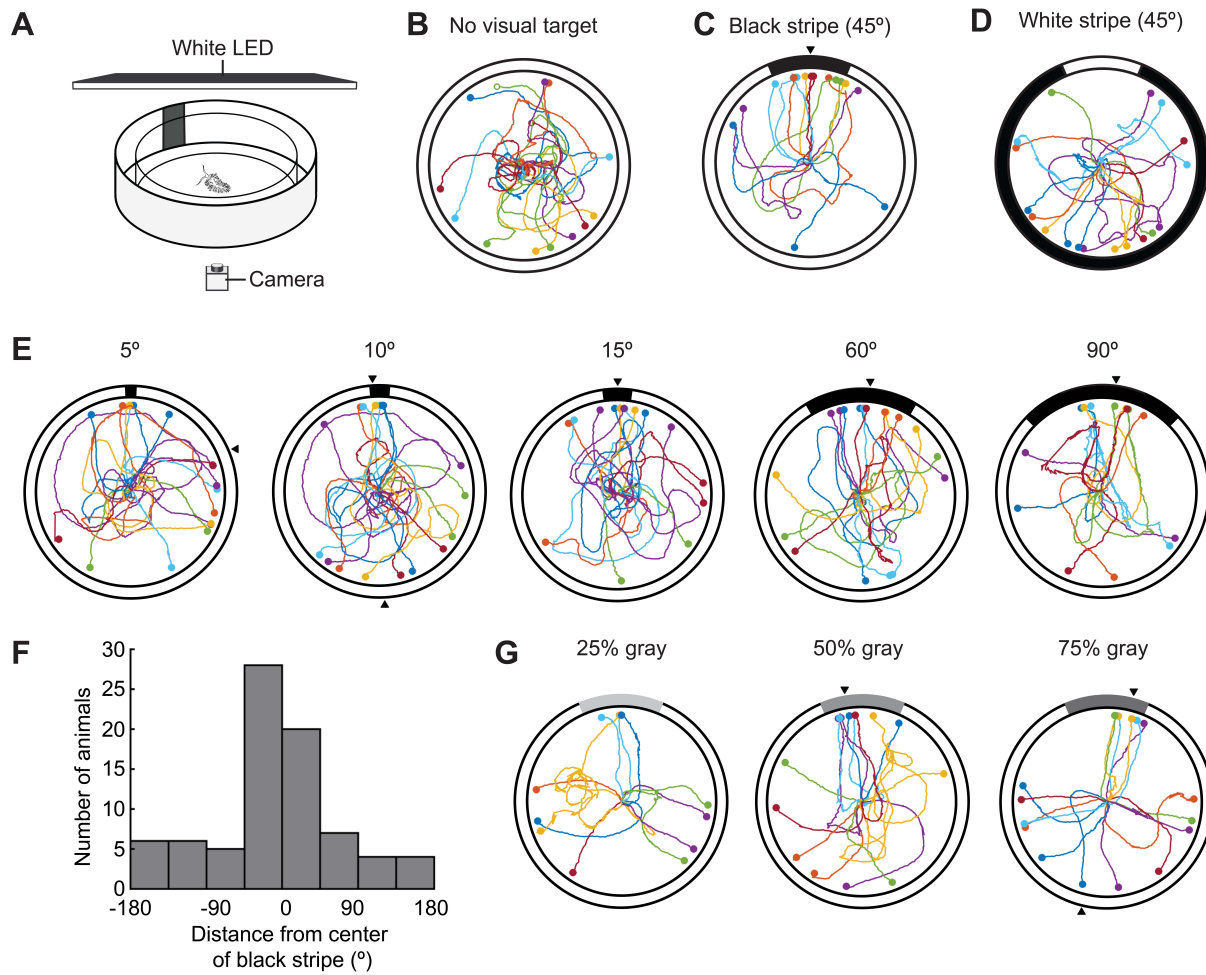
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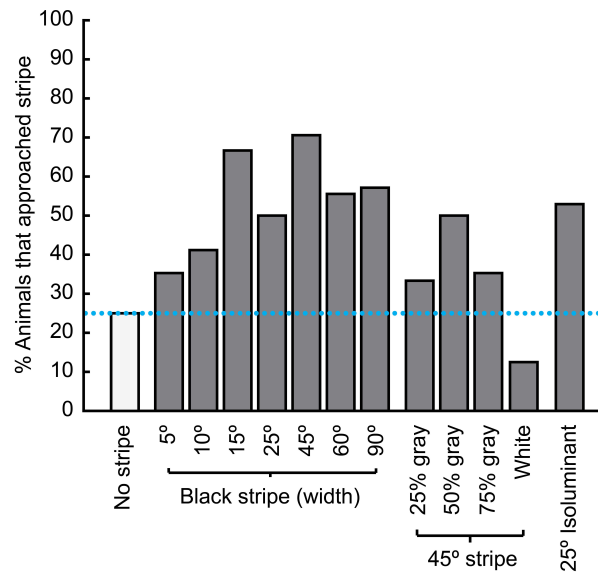
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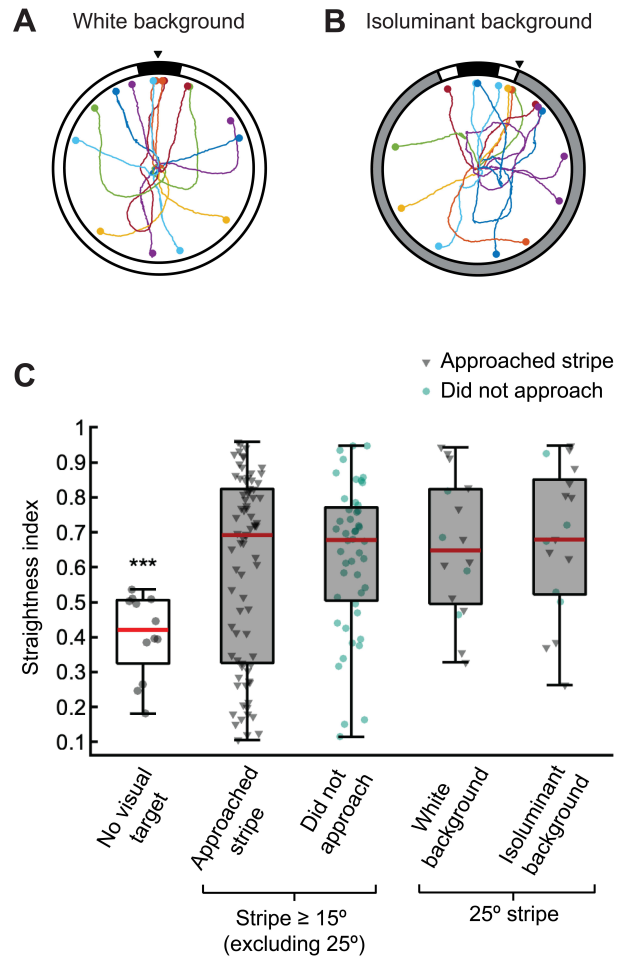
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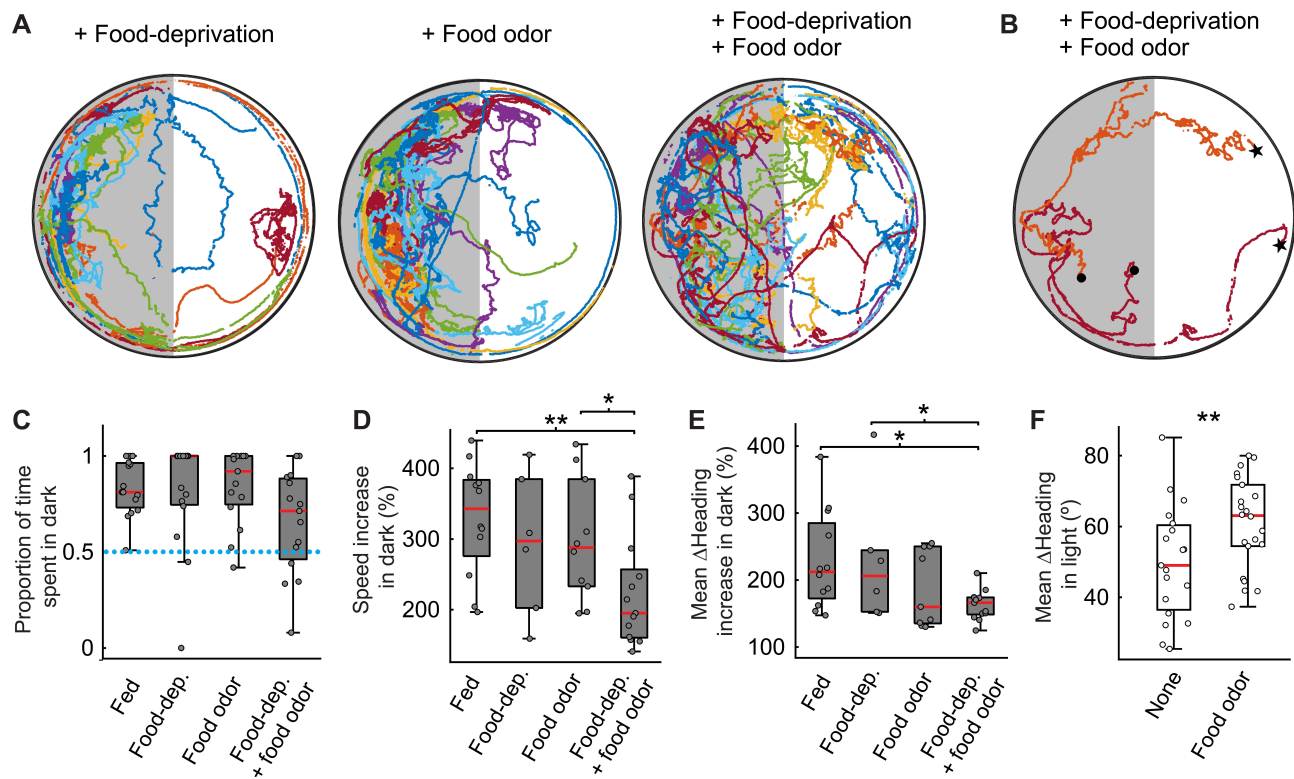
**Figure 5**



**Figure 6**



**Figure 7**



**Figure 8**

