

1 **The environmental geometry in spatial learning by zebrafish (*Danio rerio*)**

2

3

4

5 Greta Baratti¹, Davide Potrich¹, Valeria Anna Sovrano^{1,2}

6

7

8

9 ¹Center for Mind/Brain Sciences, University of Trento, Italy.

10 ²Department of Psychology and Cognitive Science, University of Trento, Italy.

11

12

13

14 Address for correspondence:

15 Valeria Anna Sovrano and Greta Baratti, Center for Mind/Brain Sciences (CIMEC),

16 University of Trento, Piazza Manifattura 1, Borgo Sacco, I-38068 Rovereto (Trento), Italy;

17 valeriaanna.sovrano@unitn.it; greta.baratti@unitn.it

18

19 **Abstract**

20 During navigation, disoriented animals learn to use the spatial geometry of rectangular
21 environments in order to gain rewards. The length of macroscopic surfaces (metric:
22 short/long) and their spatial arrangement (sense: left/right) are powerful cues that animals
23 prove to encode for reorientation. The aim of this study was to investigate if zebrafish
24 (*Danio rerio*) could take advantage of such geometric properties in a rewarded exit task,
25 hence by applying a reference memory procedure. The experiment was performed in a
26 rectangular arena having four white walls, where fish were required to choose the two
27 geometrically equivalent exit-corners lying on the reinforced diagonal. Results showed
28 that zebrafish encoded the geometry of the arena during reorientation, solving the spatial
29 task within the first five days of training. With the aim to avoid the possible influence of
30 extra-visual cues on the zebrafish' success, we performed a geometric test in extinction
31 of response after the learning day. At test, fish persisted in choosing the two correct
32 corners, thus confirming that the navigation strategy used at training was based on
33 geometric cues. This study adds evidence about the role of geometric frameworks in fish
34 species, and it further validates an effective spatial learning paradigm for zebrafish.

35

36 **Keywords:** spatial cognition, navigation, spatial reorientation, geometry, zebrafish

37 **Introduction**

38 Habitat and ecosystems share some characteristics that allow animals to find
39 resources, as the position of something worthwhile, ensuring survival. The ability to take
40 advantage of such features falls within “spatial navigation”, a series of orientation
41 strategies used by organisms to better adapt in the life space. This set includes widely
42 known mechanisms like dead reckoning, celestial and magnetic compass, landmarks,
43 cognitive maps, and environmental geometry (see broad-ranging reviews^{1,2,3,4}).

44 Geometric orientation has been studied in depth along three decades of research and,
45 in those fruitful years, several core approaches have been proposed (see review⁵). The
46 theoretical model that better contextualizes the present study is the “boundary-based”
47 view, stating that boundary structures, both manmade (e.g. walls, ledges) and naturalistic
48 (e.g. cliffs, hills), are crucial for navigation (see review⁶). Given the relevance of these
49 like-terrain structures, it shall be assumed that animals can spontaneously take
50 advantage of them to find food sources, companions, and shelters scattered in their
51 habitat. Conventionally, such cues refer to large-scale properties of three-dimensional
52 surfaces, as their length (‘metric’: short/long) and their arrangement in relation to a
53 specific reference point (‘directional sense’: left/right). In lay words, the position of an
54 object can be characterized on the basis of the shape of the surrounding environment,
55 thus, on its geometric boundaries (see for instance⁷).

56 The use of geometry has been widely studied in vertebrates (see reviews⁸⁻¹⁰) and
57 invertebrates¹¹⁻¹⁴, highlighting the critical role of the environmental boundaries in
58 reorientation behavior. A geometry-based reorientation task is usually performed in
59 rectangular arenas with visible walls. The spatial problem, as proposed for the first time

60 by Cheng¹⁵⁻¹⁶, consists in placing an object in correspondence of one corner and then
61 requesting animals, after an inertial disorientation, to approach the corner where that
62 object was previously present (see also¹⁷). Note that in rectangular arenas two out of four
63 corners (those placed on one of the two diagonals) have the same geometric properties
64 (e.g. a short wall on the left and a long wall on the right), being indistinguishable. This
65 spatial ambiguity leads animals to systematically confound the two geometric-twin
66 corners.

67 Fish species have been used for long as model in reorientation tasks¹⁸⁻²⁸, showing that
68 they were able to encode the geometry of rectangular-shaped arenas. It has been also
69 proven that boundaries can be detected through extra-visual stimulation in blind fishes²⁸.
70 Generally, in these studies authors corroborated two behavioral techniques: a working
71 memory procedure in a social cuing task (spontaneous choice without experience), where
72 animals approached the location of a social object no longer present; a reference memory
73 procedure in a rewarded exit task (training over time with experience), where animals
74 learned to navigate to a given position (two geometrically equivalent exit-corners within a
75 rectangular arena) in order to gain a reward (to leave such arena for getting food,
76 companions, and a comfortable environment). Focusing on zebrafish (*D. rerio*), three
77 study by Lee and colleagues²⁴⁻²⁶ investigated the spontaneous use of geometric cues
78 during navigation in an opaque arena composed of four white walls, thus under the
79 working memory procedure. In such visual conditions, fish encoded the spatial geometry
80 in relation to a specific goal (i.e. a conspecific acting as social stimulus), in order to
81 reorient.

82 Gaining popularity with Streisinger²⁹, the use of zebrafish in behavioral neuroscience
83 has spread increasingly in the course of the last twenty years³⁰. Despite this, there is a
84 lack of paradigms assessing their cognitive abilities through operant conditioning
85 protocols (see reviews³¹⁻³³). To our knowledge, although more and more researchers
86 investigating zebrafish' behavioral patterns agree about a clear difficulty in applying
87 training procedures, a dearth of outspoken null evidence complicates matters. One crucial
88 issue would be to determine what kind of stimuli this species is susceptible to,
89 dependently on skills it has developed down its natural history. Until now, the most of
90 studies has examined simple behavioral patterns (see review³⁴); notwithstanding, some
91 authors have started employing operant conditioning with the aim to probe zebrafish'
92 visual (see for instance³⁵⁻³⁸), spatial (see for instance^{35,38-40}), and numerical abilities⁴¹.

93 In this study we investigated the reorientation behavior of zebrafish in an opaque
94 rectangular arena composed of four white walls, carrying out rewarded exit task
95 (reference memory procedure). Fish were trained to choose the two corners lying on the
96 same diagonal, by a trial-and-error learning procedure, allowing them prolonged
97 exploration times. After the training, fish underwent a geometric test in extinction of
98 response, in order to verify whether the learning had actually been achieved on the basis
99 of geometric cues, rather than by taking advantage of other environmental cues (e.g. the
100 difference in the amount of water filtered through the open corridors with respect to the
101 closed ones). Our hypothesis was that zebrafish could learn to choose the correct-
102 geometry diagonal *versus* the incorrect one, as they are able to spontaneously use a
103 rectangular spatial framework during reorientation²⁴⁻²⁶. In case of learning, in the
104 geometric test (after blocking all the exits), we expected that zebrafish would have

105 persisted in choosing the two correct corners more than chance level, showing the mere
106 use of geometric cues for solving the spatial task during training.

107

108 **Materials and Methods**

109

110 *Subjects and housing*

111 Subjects were 12 mature males zebrafish (*D. rerio*), ranging from 4-5 cm in body length
112 and coming from breeding stocks in our laboratory. In order to attract the experimental
113 fish, three females conspecifics were used as sexual and social stimuli³⁷. Fish were
114 maintained under a 10:14-h LD cycle and raised in glass-made home tanks (25 l
115 capacity). Tanks were enriched with gravel and plants, thus cleaned with suitable filters
116 (Aquarium Systems Duetto, Newa, I), with the aim to ensure comfortable habitats. The
117 water temperature was maintained at 26° C through heaters (Newa Therm), and fish were
118 fed twice a day with dry food (GVG-Mix, Sera® GmbH, D).

119

120 *Apparatus*

121 The apparatus was the same used in Sovrano and colleagues^{18,19,22,23}, consisting of a
122 white polypropylene (Poliplak®) rectangular arena (length: 31 cm; width: 14 cm; height:
123 16 cm), inserted in a larger rectangular tank (length: 60 cm; width: 36 cm; height: 25 cm).
124 Embedded in correspondence of each corner, there was a rectangular small corridor (2 x
125 3 cm; 2.5 cm in length; 4.5 cm from the floor), allowing fish to leave the arena and access
126 an external surrounding region (Fig. 1a). At the end of each corridor there was a flexible
127 door (2.5 x 3.5 cm) that could be easily pushed and bent by the fish with its snout: the

128 upper part of such door (2.5 x 2.5 cm) was an opaque plastic sheet, while the lower part
129 (2.5 x 1 cm) was a transparent acetate sheet. The four doors were visually identical, but
130 only two (i.e. those on the correct-geometry diagonal) could be opened; contrariwise, the
131 two other doors (i.e. those on the incorrect-geometry diagonal) could not be opened
132 because glued on the final part of the corridors with a minimum amount of transparent
133 silicone (see details in Fig. 1b). Although the corridors were blocked, a regular water
134 passage was guarantee through three small holes (diameter: 0.5 cm) cut out in the lower
135 transparent part, avoiding any nonvisual cues eventually detected by extra-visual sensory
136 modalities²⁸.

137

138 *Procedure*

139 The experiment was divided in two parts: a “training-phase”, by using an operant
140 conditioning procedure (reference memory) and a “geometric test”, by using an extinction
141 procedure (hence, in absence of differential reinforcements). Both procedures are
142 schematized in Fig. 2.

143 The training-phase consisted of daily sessions of 8 trials until reaching the learning
144 criterion (a number of correct choices greater or equal to 70% for C1 + C2 – the
145 geometrically correct corners – in two consecutive daily sessions), or at least for a
146 minimum of 5 consecutive daily sessions, in order to describe a learning curve for all
147 animals.

148 Before starting each trial, the fish was gently transferred from the region surrounding
149 the arena into an opaque plastic jar (diameter: 13 cm; height: 7.5 cm) and passively
150 disoriented (i.e. slowly rotated 360° clockwise and counterclockwise) on a rotating device

151 with the aim to reduce the use of compass and inertial information. Then, the fish was
152 moved into a glass-made cylinder (diameter: 6 cm; height: 8 cm), placed in the center of
153 the arena, leaving it there for 30 seconds. After this period, the cylinder was carefully lifted
154 up and the fish was free to swim in the environment for a 10 minutes time-limit. At the
155 end, the apparatus was rotated 90° right. In each trial, the choices (or attempts) for the
156 four corridors were sequentially noted down until the fish exit the arena. Such approaches
157 were thus codified as follows: letter “C1” and letter “C2”, for the correct corners on the
158 reinforced diagonal; letter “X1” and letter “X2” for the incorrect corners on the
159 nonreinforced diagonal.

160 A correction method was used⁴²: the fish was allowed to change one or more wrong
161 attempts, until it was able to choose one out of two right corridors (leaving the arena), or
162 until the time-limit was elapsed. Intervals among trials, where the fish could remain in the
163 apparatus’ external region, were 6 minutes (complete reinforcement, with administration
164 of a small amount of food and presence of conspecifics) if the fish identified the correct
165 corner (C1 or C2) as single attempt, and 2 minutes (partial reinforcement, without
166 administration of food but in presence of conspecifics) if the fish identified the correct
167 corner after two or more attempts. In case of the fish did not respond within the 10 minutes
168 time-limit, it was given a 5 minutes time-break in the outer region, not providing any
169 reward. Multiple choices for the correct corridors could occur, for instance when the fish
170 explored them without leaving the arena. An attempt to the corner was considered as an
171 effective choice if the fish entered the corridor with more than half of its body. Exit attempts
172 were clearly visible in video-recordings, through characteristic tail and body movements.

173 After the training-phase, hence when the fish reached the learning criterion, there was
174 the geometric test. It was performed in two consecutive daily sessions of 4 trials each, in
175 extinction of response (the two open corridors were closed with the aid of a thin green
176 metal wire). Such test was useful to verify whether the learning had actually been
177 achieved on the basis of geometric cues, rather than by taking advantage of other
178 potential environmental cues (e.g. a different water passage between open and closed
179 corridors). Each test trial lasted 2 minutes. If the fish did not make any attempt in that
180 period, the time available was prolonged, with the aim to collect at least one valid choice.
181 In any case, the maximum time for the test trial was not longer than 10 minutes. In
182 absence of any choice within the maximum time supplied, the trial was considered as null
183 and therefore repeated. The inter-trial interval was 5 minutes, during which the fish was
184 free to rest in the surrounding comfortable environment. To avoid a loss of motivation and
185 null trials (since all the corridors were not traversable), the test trials were interspersed
186 with recall trials, carrying out the usual training procedure, with both C1 and C2 corridors
187 open.

188 The first choices and the total choices made by fish in correspondence of the four
189 corridors, during the training-phase (summing the first five sessions), in the learning day
190 (the single session where fish reached the criterion), and in the geometric test were used
191 as individual data per each session, combining the attempts for the two diagonals
192 (correct: C1 + C2; incorrect: X1 + X2). The inter-observer reliability criterion⁴² was applied
193 in the recoding of a subset of 10% of different videos ($p < 0.001$, Pearson's correlation
194 between the ratio calculated on the original coding and on the *de novo* coding performed
195 by an experimenter blind on the test condition of the fish).

196

197 *Statistical analyzes*

198 The dependent variables measured were the mean number of trials (with 95% CI) to
199 reach the learning criterion (greater or equal to 70% for C1 + C2), the first choices and
200 the total choices made by fish for the two diagonals (C1 + C2 *versus* X1 + X2) in the
201 summed first five training sessions (of 8 trials each), in the learning day (the single session
202 where fish reached the criterion), and in the geometric test.

203 In order to assess the homoscedasticity, the tests applied were the Levene Test and
204 the Mauchly's Sphericity test. A repeated measures ANOVA was performed in order to
205 estimate the use of geometry over time (i.e. among the first five training sessions). With
206 the aim to compare the two corners on the same diagonal (C1 *versus* C2; X1 *versus* X2),
207 the nonparametric Wilcoxon test was applied on the first choices, while the parametric
208 Student's t-test was applied on the total choices. Moreover, with respect to the first
209 choices collected during the training-phase, the Wilcoxon test was performed on the five
210 training sessions one by one. To estimate the effect size, we reported partial eta-squared
211 (η_p^2) as index for the ANOVA, and 95% Confidence Intervals for the Student's t-test. Data
212 were analyzed with the IBM SPSS Statistics 20 software package.

213

214 **Results**

215 For this experiment, the reorientation behavior of 12 zebrafish (*D. rerio*) was observed
216 in an opaque all-white rectangular arena^{18,19,22,23}, carrying out a rewarded exit task.
217 During the training-phase (reference memory procedure), fish were allowed free
218 prolonged exploration times in each trial, until reaching a learning criterion greater or

219 equal to 70% for C1 + C2 (the correct-geometry diagonal). After the training-phase, fish
220 underwent a geometric test (extinction procedure), to assure that the learning was
221 occurred on geometric basis.

222 The number of trials needed to reach the learning criterion was 27 ± 3.087 (mean \pm
223 SEM).

224 Results of the learning day (the single session where zebrafish finalized the training)
225 are shown in Fig. 3 (left). The Wilcoxon test applied on the first choices revealed a
226 significant effect of Geometry (diagonals C1 + C2 *versus* X1 + X2: $Z = -3.078$, $p = 0.002$),
227 while did not reveal any differences between corners C1 and C2 ($Z = -0.240$, $p = 0.810$),
228 and between corners X1 and X2 ($Z = -0.921$, $p = 0.357$). The two-tailed t-test applied on
229 the total choices revealed a strongly significant effect of Geometry (diagonals C1 + C2
230 *versus* X1 + X2: $t(11) = 17.728$, $p \leq 0.0001$), while did not reveal any differences between
231 corners C1 and C2 ($t(11) \leq 0.0001$, $p = 1$), and between corners X1 and X2 ($t(11) = 1.254$,
232 $p = 0.236$). Results provided by the training-phase showed that zebrafish successfully
233 solved the rewarded exit task set up in the opaque all-white rectangular arena.

234 Results of the geometric test are shown in Fig. 3 (right). The Wilcoxon test applied on
235 the first choices revealed a significant effect of Geometry (diagonals C1 + C2 *versus* X1
236 + X2 = $Z = -3.070$, $p = 0.002$), while did not reveal any differences between corners C1
237 and C2 ($Z = -1.500$, $p = 0.134$), and between corners X1 and X2 ($Z = -0.061$, $p = 0.951$).
238 The two-tailed t-test applied on the total choices revealed a strongly significant effect of
239 Geometry (diagonals C1 + C2 *versus* X1 + X2: $t(11) = 7.140$, $p \leq 0.0001$), while did not
240 reveal any differences between corners C1 and C2 ($t(11) = -1.945$, $p = 0.078$), and
241 between corners X1 and X2 ($t(11) = 1.302$, $p = 0.220$). Results provided by the geometric

242 test bore out that the learning was actually occurred on geometric cues during the training-
243 phase.

244 Performance over time in the first 5 days of training is shown in Fig. 4. Considering the
245 data collected per fish for the two diagonals (C1 + C2 *versus* X1 + X2) in the summed
246 first five training sessions of 8 trials each, the repeated measures ANOVA with Time (days
247 1-5) and Geometry (diagonals C1 + C2 *versus* X1 + X2) as within-subject factors, applied
248 on the first choices, revealed a significant effect of Geometry ($F(1,11) = 23.866$, $p \leq$
249 0.0001 , $\eta_p^2 = 0.685$) and of Time x Geometry ($F(4,44) = 3.744$, $p = 0.010$, $\eta_p^2 = 0.254$),
250 while Time was not significant ($F(4,44) = 1.296$, $p = 0.286$). The Wilcoxon test applied on
251 the first choices specified the parametric statistics' outcome (day 1: $Z = -0.258$, $p = 0.796$;
252 day 2: $Z = -1.513$, $p = 0.130$; day 3: $Z = -2.971$, $p = 0.003$; day 4: $Z = -1.344$, $p = 0.179$;
253 day 5: $Z = -3.276$, $p = 0.001$). Results about the use of geometry over time, when
254 considering the first choices, showed that on day 3 the effect of the diagonals on the
255 learning performance of zebrafish began to consolidate.

256 The same repeated measures ANOVA applied on the total choices revealed two
257 significant main effects: Geometry ($F(1,11) = 34.951$, $p \leq 0.0001$, $\eta_p^2 = 0.761$) and Time
258 ($F(4,44) = 3.990$, $p = 0.008$, $\eta_p^2 = 0.266$), while the interaction Time x Geometry was not
259 significant ($F(4,44) = 1.894$, $p = 0.128$). By analysing the total choices, the learning
260 performance of zebrafish appeared strongly progressive over time: the ability to solve the
261 rewarded exit task, disambiguating the correct-geometry diagonal with respect to the
262 incorrect one emerged soon, already in the first five training sessions.

263

264 **Discussion**

265 The crucial issue of this study was to investigate whether zebrafish (*D. rerio*), as well
266 as other fish species^{18-20,28}, learned to use the geometric properties (i.e. short/long,
267 left/right) of a rectangular arena composed of four opaque walls. For the purpose, a
268 reference memory procedure was applied to the observation of zebrafish' reorientation
269 behavior, by using an operant conditioning with prolonged exploration times. Such
270 technique is well-standardized even in redbtail splitfin fish (*Xenotoca eiseni*)^{18,19,22,23,27},
271 goldfish (*Carassius auratus*)²⁰, and cavefish (*Astyanax mexicanus*, *Phreatichthys*
272 *andruzzii*)²⁸, but it has never been employed with zebrafish. Specifically, we performed a
273 rewarded exit task where fish were required to choose two open corridors towards an
274 enriched and comfortable outer zone (with gravel, plants, and two conspecifics), in order
275 to gain additional reinforcement (a small amount of food associated with a correct choice).
276 Our experiment was divided in two parts: a training-phase and a geometric test, both
277 carried out in visual conditions, that are, within an environment with well-defined
278 boundaries.

279 About the training-phase, zebrafish had to learn to identify two rewarding corridors
280 taking advantage of their position in relation to the geometric context. Hence, basing their
281 reorientation behavior on spatial geometry, they should have focused their choices on the
282 correct-geometry diagonal (thus, on the two corners having the same metric-plus-sense
283 properties). Results showed that fish easily solved the spatial task, choosing the correct-
284 geometry diagonal more than the incorrect one. Moreover, they proved to have learned
285 the task within the first five days of training, in particular when considering the total
286 choices. Indeed, it seems that the total choices were a stronger indicator of learning than

287 the first choices, probably because the latter were affected by emotional responses⁴³ due
288 to experimental handling.

289 The spatial behavior of zebrafish perfectly matched with learning performance
290 obtained in the same geometric task by two other eyed fishes (*X. eiseni* and *C. auratus*),
291 within arenas characterized by a marked geometry (i.e. opaque rectangular enclosures¹⁸⁻
292 ²⁰).

293 Although a gradual improvement in performance generally emerged right away, a
294 component of behavioral variability seemed to arise during the spatial performance over
295 time, especially in relation to the first choices: on day 3, the correct-geometry diagonal
296 was chosen more than the incorrect one, but not in all fish. Indeed, 7 out of 12 zebrafish
297 achieved earlier the learning criterion (i.e. within the first three training sessions), while
298 the other 5 by the fifth day. The nature of this difference is unclear but, just speculating,
299 it could be due to individual levels in emotional response⁴³, boldness⁴⁴ and/or swim-
300 activity patterns⁴⁵.

301 With regards to the geometric test, it was essential to verify the actual use of geometric
302 cues during the training-phase. The reason underlying such test was the possibility of
303 other cues to be present in the whole apparatus. In particular, it could be a difference in
304 the amount of water filtered through the open corridors with respect to the closed ones.
305 According to a study by Sovrano and colleagues²⁸, extra-visual systems of fish detecting
306 slight hydrodynamic perturbations are involved in geometry-based reorientation. In order
307 to exclude that zebrafish could have solved the rewarded exit task by using such cues,
308 the geometric test with all exits closed (extinction procedure) was performed. Results
309 showed that, also with the four corridors blocked, fish continued to choose the correct-

310 geometry diagonal, demonstrating the mere encoding of metric-plus-sense information
311 during the training-phase.

312 Even though zebrafish is commonly considered an excellent model to study cognitive
313 phenotypes³³, some authors have argued that existing behavioral paradigms are not
314 optimal enough for investigating mental processes (e.g. learning and memory) in such
315 fish^{31,32}. Nevertheless, studies about spatial learning have picked out that zebrafish could
316 be trained through aversive or appetitive stimuli to choose a maze's compartment on the
317 basis of its position (thus, detecting intra-maze cues)^{35,38-40}. In our study we suggest an
318 effective training paradigm for assessing relational learning in zebrafish, that is, by
319 providing a geometric context where fish can take advantage of metric and sense
320 contingencies in order to get a reinforcement. This protocol is easily replicable, and it
321 could be further used to set up associative learning experiments, for instance, by adding
322 visual cues with specific color and/or pattern with the aim to deepen understand zebrafish'
323 discrimination capacities. In fact, prior investigation regarding visual discrimination in this
324 species has led to inconsistent findings (see for instance^{46,47}).

325 Even if zebrafish can spontaneously reorient by using the geometry of rectangular
326 enclosures, the social cuing task by Lee and colleagues²⁴⁻²⁶ highly differs from the
327 rewarded exit task here performed. Our protocol could gain value depending on further
328 studies assessing the encoding of geometric cues, both visual and nonvisual, with a
329 comparison targeted to highlight similarities and dissimilarity between such paradigms.

330 In conclusion, results obtained by zebrafish, a species never studied in visual
331 geometric navigation under training, also support the great ecological value of the
332 encoding of geometry per se, as a useful tool for processing macrostructural

333 environmental information (i.e. boundaries). In fact, it is likely that such cues, as for
334 instance, the presence of a mountain, a river, and their spatial relationships, keeping
335 unchanged over animals' lifespan, have become crucial for survival. Lastly, the possibility
336 to apply a combinational approach⁴⁸ makes zebrafish a prominent animal model for
337 looking into development and neural basis of this essential cognitive skill.

338

339 **Acknowledgments**

340 We wish to thank Tommaso Pecchia for his technical support with the experimental
341 setting, Ciro Petrone, Cristina Pascu, and Giampaolo Morbioli for the animal care and
342 welfare.

343

344 **Author Contributions**

345 V.A.S. conceived the study, V.A.S. and D.P. designed it; G.B. performed the
346 experiments; V.A.S. analyzed the results; all authors interpreted them and wrote the
347 paper.

348

349 **Conflict of Interests**

350 The authors declare no competing financial and nonfinancial interests.

351

352 **Ethical Regulations**

353 This research was carried out in the Animal Cognition and Neuroscience Laboratory
354 (A.C.N. Lab.) of CIMeC (Center for Mind/Brain Sciences) at the University of Trento (Italy).
355 All husbandry and experimental procedures complied with European Legislation for the

356 Protection of Animals used for Scientific Purposes (Directive 2010/63/EU) and were
357 previously authorized by the University of Trento's Ethic Committee for the Experiments
358 on Living Organisms, and by the Italian Ministry of Health (auth. num. 1111-2015-PR).

359

360 **Data Availability**

361 Data are available in a submitted supplementary Excel file.

362

363 **References**

- 364 1. Wang RF, Spelke ES. Comparative approaches to human navigation. In The
365 neurobiology of spatial behaviour. KJ Jeffery, (ed), pp. 119-143, Oxford University
366 Press, Oxford, OXF, 2003.
- 367 2. Collett TS, Graham P. Animal navigation: path integration, visual landmarks and
368 cognitive maps. *Curr Biol* 2004;14:R475-R477.
- 369 3. Jeffery HAM, Menzel R, Newcombe NS. Animal Navigation – A Synthesis. 2010.
370 Unpublished manuscript.
- 371 4. Mouritsen H. Long-distance navigation and magnetoreception in migratory animals.
372 *Nature* 2018;558:50-59.
- 373 5. Cheng K, Huttenlocher J, Newcombe NS. 25 years of research on the use of
374 geometry in spatial reorientation: a current theoretical perspective. *Psychon Bull*
375 *Rev* 2013;20:1033-1054.
- 376 6. Lee SA. The boundary-based view of spatial cognition: a synthesis. *Curr Opin*
377 *Behav Sci* 2017;16:58-65.

- 378 7. Spelke ES, Lee SA. Core systems of geometry in animal minds. *Philos Trans R Soc*
379 *B* 2012;367:2784-2793.
- 380 8. Cheng K, Newcombe NS. Is there a geometric module for spatial orientation?
381 Squaring theory and evidence. *Psychon B Rev* 2005;12:1-23.
- 382 9. Cheng K. Whither geometry? Troubles of the geometric module. *Trends Cogn Sci*
383 2008;12:355-361
- 384 10. Tommasi L, Chiandetti C, Pecchia T, Sovrano VA, Vallortigara G. From natural
385 geometry to spatial cognition. *Neurosci Biobehav R* 2012;36:799-824.
- 386 11. Wystrach A, Beugnon G. Ants learn geometry and features. *Curr Biol* 2009;19:61-
387 66.
- 388 12. Wystrach A, Cheng K, Sosa S, Beugnon G. Geometry, features, and panoramic
389 views: ants in rectangular arenas. *J Exp Psychol Anim B* 2011;37:420-435.
- 390 13. Sovrano VA, Rigosi E, Vallortigara G. Spatial reorientation by geometry in
391 bumblebees. *Plos ONE* 2012;7:e37449.
- 392 14. Sovrano VA, Potrich D, Vallortigara G. Learning of geometry and features in
393 bumblebees (*Bombus terrestris*). *J Comp Psychol* 2013;127:312-318.
- 394 15. Cheng K. A purely geometric module in the rat's spatial representation. *Cognition*
395 1986;23:149-178.
- 396 16. Cheng, K. Rats use the geometry of surfaces for navigation. In *Cognitive processes*
397 *and spatial orientation in animal and man*. Ellen P, Thinus-Blanc C, (eds), pp. 153-
398 159. Springer, Dordrecht, ZH, 1987.
- 399 17. Gallistel CR. *The organization of learning*. MIT Press, Cambridge, United States,
400 1990.

- 401 18. Sovrano VA, Bisazza A, Vallortigara G. Modularity and spatial reorientation in a
402 simple mind: Encoding of geometric and nongeometric properties of a spatial
403 environment by fish. *Cognition* 2002;85:B51-B59.
- 404 19. Sovrano VA, Bisazza A, Vallortigara G. Modularity as a fish views it: Conjoining
405 geometric and nongeometric information for spatial reorientation. *J Exp Psychol*
406 *Anim B* 2003;29:199-210.
- 407 20. Vargas JP, Lopez JC, Salas C, Thinus-Blanc C. Encoding of geometric and featural
408 spatial information by Goldfish (*Carassius auratus*). *J Comp Psychol* 2004;118:206-
409 216.
- 410 21. Sovrano VA, Dadda, M, Bisazza, A. Lateralized fish perform better than
411 nonlateralized fish in spatial reorientation tasks. *Behav Brain Res* 2005;163:122-
412 127.
- 413 22. Sovrano VA, Bisazza A, Vallortigara G. Animals' use of landmarks and metric
414 information to reorient: effects of the side of the experimental space. *Cognition*
415 2005;97:121-133.
- 416 23. Sovrano VA, Bisazza A, Vallortigara G. How fish do geometry in large and in small
417 spaces. *Anim Cogn* 2007;10:47-54.
- 418 24. Lee SA, Vallortigara G, Ruga V, Sovrano VA. Independent effects of geometry and
419 landmark in a spontaneous reorientation task: a study of two species of fish. *Anim*
420 *Cogn* 2012;15:861-870.
- 421 25. Lee SA, Vallortigara G, Flore M, Spelke ES, Sovrano VA. Navigation by
422 environmental geometry: the use of zebrafish as a model. *J Exp Biol* 2013;216:3693-
423 3699.

- 424 26. Lee SA, Ferrari A, Vallortigara G, Sovrano VA. Boudary primacy in spatial mapping:
425 Evidence from zebrafish (*Danio rerio*). Behav Process 2015;119:116-122.
- 426 27. Sovrano VA, Chiandetti C. Reorientation ability in redbtail splitfin (*Xenotoca eiseni*):
427 Role of environmental shape, rearing in group and exposure time. Biol Comm
428 2017;62:48-56.
- 429 28. Sovrano VA, Potrich D, Foà A, Bertolucci C. Extra-visual systems in the spatial
430 reorientation of cavefish. Sci Rep 2018;8:17698.
- 431 29. Streisinger G, Walker C, Dower N, Knauber D, Singer F. Production of clones of
432 homozygous diploid zebra fish (*Brachydanio rerio*). Nature 1981;291:293-296.
- 433 30. Levin ED, Cerutti DT. Behavioral Neuroscience of Zebrafish. In Methods of Behavior
434 Analysis in Neuroscience. 2nd edition. Buccafusco JJ, (ed), CRC Press/Taylor &
435 Francis, Boca Raton, FL, 2009.
- 436 31. Gerlai R. Associative learning in zebrafish (*Danio rerio*). In Methods in cell biology.
437 HW Detrich, M Westerfield, L. Zon (eds.), pp. 249-270, Academic Press,
438 Cambridge, MA, 2011.
- 439 32. Bailey JM, Oliveri AN, Levin ED. Pharmacological analyses of learning and memory
440 in zebrafish (*Danio rerio*). Pharmacol Biochem Be 2015;139:103-111.
- 441 33. Meshalkina DA, Kizlyk MN, Kysil EV, Collier AD, Echevarria DJ, Abreu MS, et al.
442 Understanding zebrafish cognition. Behav Process 2017;141:229-241.
- 443 34. Orger MB, de Polavieja GG. Zebrafish behavior: opportunities and challenges. Annu
444 Rev Neurosci 2017;40:125-147.
- 445 35. Arthur D, Levin ED. Spatial and non-spatial visual discrimination learning in
446 zebrafish (*Danio rerio*). Anim Cogn 2001;4:125-131.

- 447 36. Colwill RM, Raymond MP, Ferreira L, Escudero H. Visual discrimination learning in
448 zebrafish (*Danio rerio*). Behav Process 2005;70:19-31.
- 449 37. Al-Imari L, & Gerlai R. Sight of conspecifics as reward in associative learning in
450 zebrafish (*Danio rerio*). Behav Brain Res 2008;189:216-219.
- 451 38. Sison M, Gerlai R. Associative learning in zebrafish (*Danio rerio*) in the plus maze.
452 Behav Brain Res 2010;207:99-104.
- 453 39. Darland T, Dowling JE. Behavioral screening for cocaine sensitivity in mutagenized
454 zebrafish. PNAS 2001;98:11691-11696.
- 455 40. Williams FE, White D, Messer Jr WS. A simple spatial alternation task for assessing
456 memory function in zebrafish. Behav Process 2002;58:125-132.
- 457 41. Potrich D, Rugani R, Sovrano VA, Regolin L, Vallortigara G. Use of numerical and
458 spatial information in ordinal counting by zebrafish. Sci Rep 2019;9:18323.
- 459 42. Caro TM, Roper R, Young M, Dank R. Inter-observer reliability. Behaviour
460 1979;69:303-315.
- 461 43. Kalueff AV, Stewart AM, Kyzar EJ, Cachat J, Gebhardt M, Landsman S, et al. Time
462 to recognize zebrafish 'affective' behavior. *Behaviour*, 2012;149:1019-1036.
- 463 44. Kalueff AV, Gebhardt M, Stewart AM, Cachat JM, Brimmer M, Chawla JS, et al.
464 Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. Zebrafish
465 2013;10:70-86.
- 466 45. Tran S, Gerlai R. Individual differences in activity levels in zebrafish (*Danio rerio*).
467 Behav Brain Res 2013;257:224-229.
- 468 46. Agrillo C, Miletto Petrazzini ME, Tagliapietra C, Bisazza A. Inter-specific differences
469 in numerical abilities among teleost fish. Front Psychol 2012;3:483.

- 470 47. Oliveira J, Silveira M, Chacon D, Luchiari A. The zebrafish world of colors and
471 shapes: preference and discrimination. *Zebrafish*, 2015;12:166-173.
- 472 48. Grunwald DJ, Eisen JS. Headwaters of the zebrafish – emergence of a new model
473 vertebrate. *Nat Rev Genet* 2002;3:717-724.
- 474
- 475
- 476

477 **FIGURE LEGENDS**

478

479 **FIG. 1.** Photographs of the experimental apparatus used for investigating the reorientation
480 behavior of zebrafish (*D. rerio*) in visual conditions. The all-white rectangular arena is surrounded
481 by a comfortable environment for fish (with gravel, plants, and two conspecifics), becoming a
482 positive reinforcement to exit the arena through the small embedded corridors. Animals were
483 allowed prolonged exploration times to solve the spatial task. **(A)** Top view of the experimental
484 apparatus. **(B)** Detail of an open door.

485

486 **FIG. 2** Schematic representation of the experimental procedures used for the training-phase and
487 the geometric test. The correct corners laying on the reinforced diagonal are labeled “C1” and
488 “C2”, while the incorrect ones “X1” and “X2”. Different animals were reinforced on different
489 diagonals. The experiment consisted of two phases: training, where fish was trained to choose
490 two geometrically equivalent exit-corners (here indicated with “+”) to get a reward, and geometric
491 test, in extinction of response (where all corridors were closed, here indicated with “-“), aimed to
492 bear out that the learning had been achieved on environmental geometric information. Each trial
493 of training and geometric test started with a “disorientation” period (30 seconds), where fish was
494 put into a plastic cylinder and taken out the arena, hence slowly rotated 360°
495 clockwise/counterclockwise on a rotating device. Such period was followed by an “acclimation”
496 period (30 seconds), where fish was placed into a glass cylinder, in the center of the arena, and
497 it could accustom to the environment. The training-phase’s third period was “exploration” (600
498 seconds time-limit), where fish could experience the arena and choose the four corridors, until
499 exiting. Instead, the geometric test’s third period was “test” (120 seconds time-limit), where fish
500 was free to swim towards the corners, but in absence of differential reinforcement (extinction

501 procedure). If fish made no choice in 120 seconds, the time available was extended until at least
502 one choice occurred.

503

504 **FIG. 3.** Bar graph shows the frequency of choice (mean \pm SEM) obtained by zebrafish, in terms
505 of number of attempts in correspondence of the two corridors placed on the correct-geometry
506 diagonal (C1 + C2) *versus* the incorrect-geometry diagonal (X1 + X2), in the Learning day (left)
507 and in the Geometric test (right). The geometrically correct corners are indicated as C1 and C2
508 (both open during the training-phase), while the incorrect ones are indicated as X1 (near C1) and
509 X2 (near C2). Grey bars refer to first choices, black bars to total choices. Statistically significant
510 differences between diagonals C1 + C2 and X1 + X2 are indicated with an asterisk.

511

512 **FIG. 4.** Line graph shows the frequency of choice (mean \pm SEM) obtained by zebrafish, in terms
513 of number of attempts in correspondence of the two corridors placed on the correct-geometry
514 diagonal (C1 + C2) *versus* the incorrect-geometry diagonal (X1 + X2) over time (days 1-5 of
515 training). Grey lines refer to first choices, black lines to total choices. Zebrafish (*D. rerio*) learned
516 to solve the rewarded exit task in visual conditions, achieving the learning criterion in the training-
517 phase and maintaining a performance higher than chance in the geometric test (see Fig. 3). Note
518 that the improvement of performance becomes apparent from the first three days of training.

519

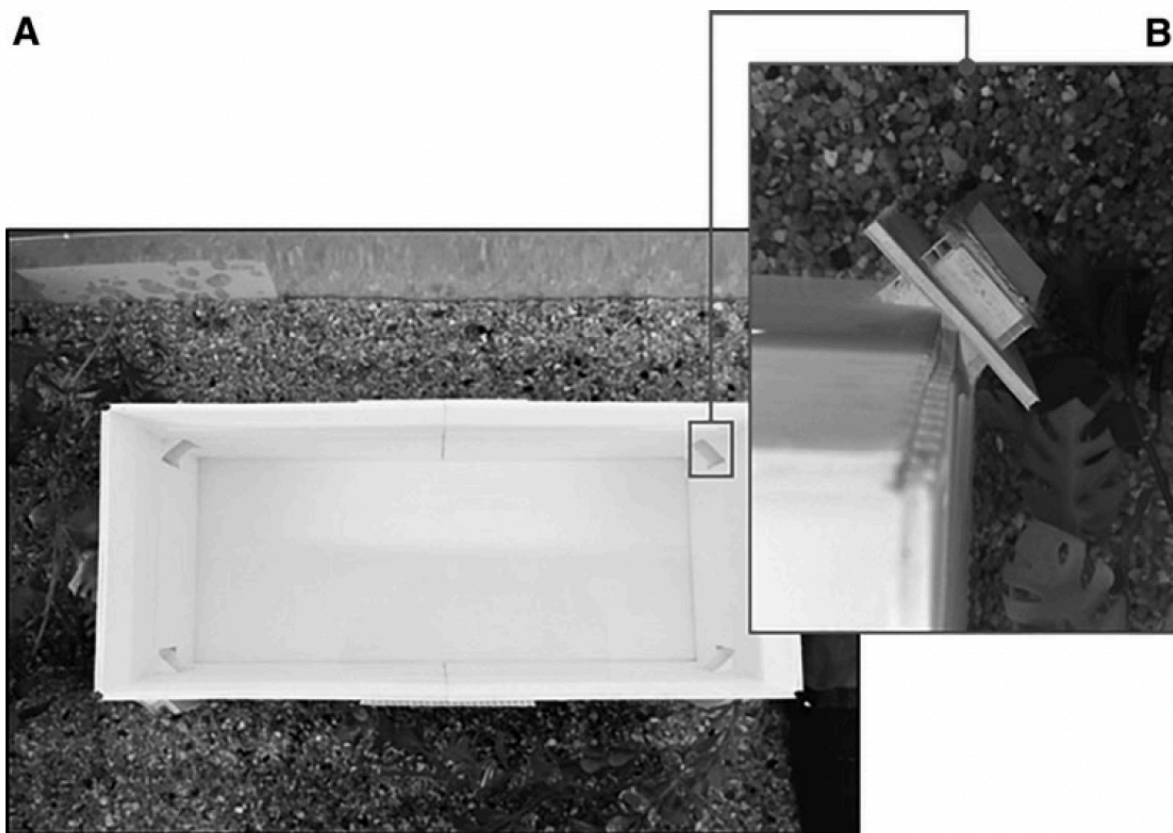
520

521

522

523 **FIGURE 1**

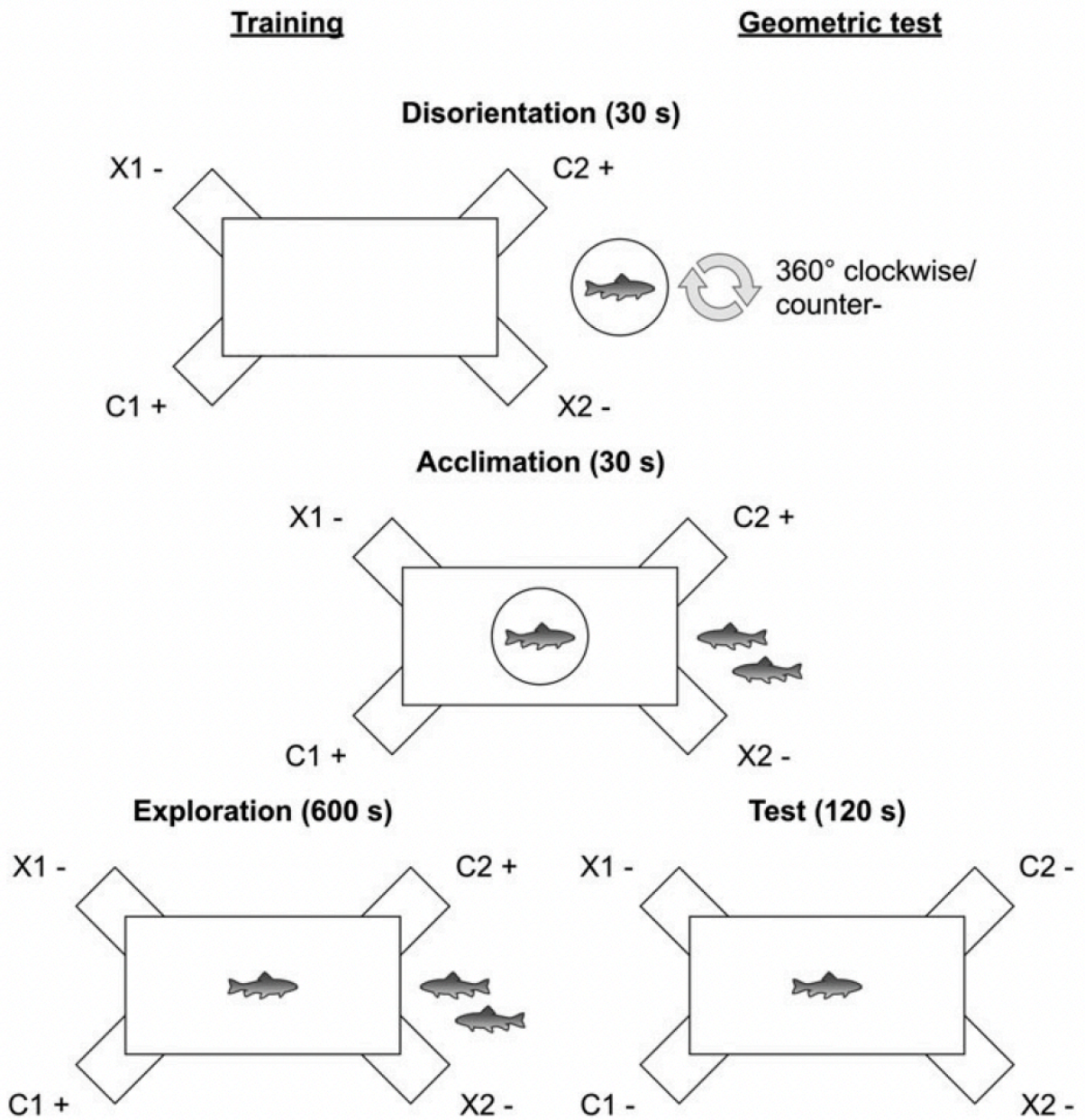
524



525

526

527

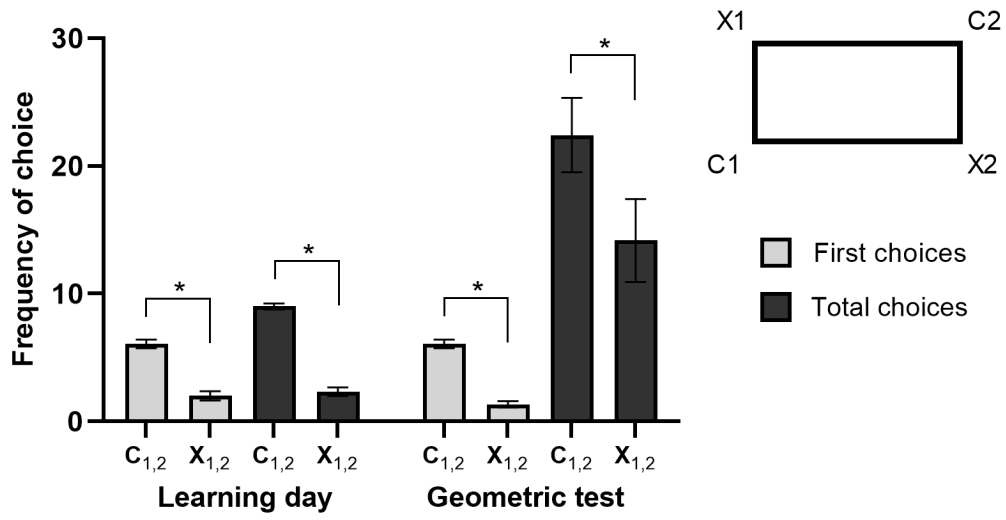


529

530

531 **FIGURE 3**

532



533

534

535

536

537

538

539

540

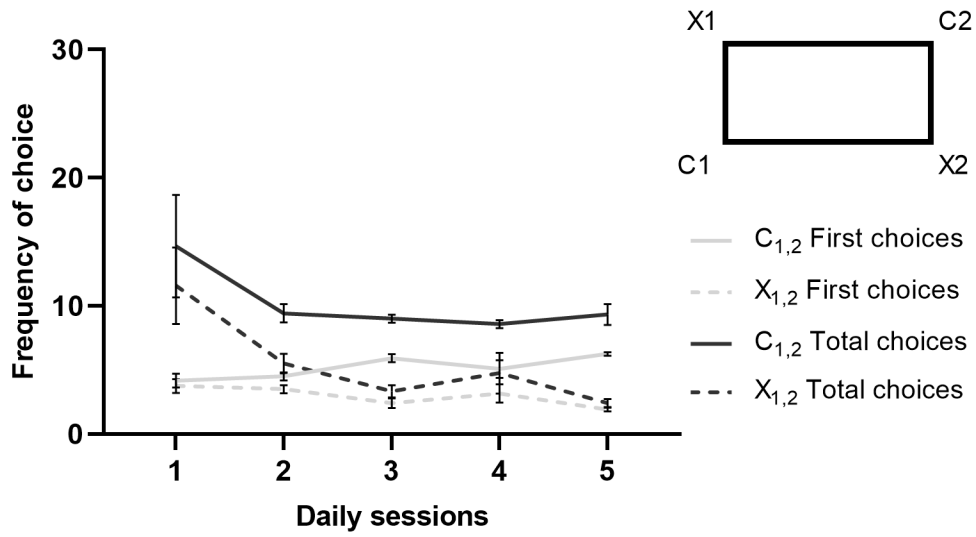
541

542

543

544 **FIGURE 4**

545



546

547

548

549

550

551