1 **Earth-strength Magnetic Field Affects the Rheotactic Threshold of Zebrafish**

2 **Swimming in Shoals**

3

4 **Alessandro Cresci[1], Rosario De Rosa[2,3], Nathan F. Putman[4,5], Claudio Agnisola[6*]**

5 *[1] Rosenstiel School of Marine & Atmospheric Science, 4600 Rickenbacker Causeway, Miami, FL 33149-1098, USA*

6 *[2] Department of Physics, University of Naples Federico II, MSA Campus, Via Cinthia 21, 80126 Napoli, ITALY*

7 *[3] INFN, section of Naples, MSA Campus, Via Cinthia 21, 80126 Napoli, ITALY*

8 *[4] Cooperative Institute for Marine & Atmospheric Studies, Rosenstiel School of Marine & Atmospheric Science, 4600*

9 *Rickenbacker Causeway, Miami, FL 33149-1098, USA.*

10 *[5]Atlantic Oceanographic and Meteorological Laboratory, National Oceanic and Atmospheric Administration, Miami,*

11 *FL 33149, USA.*

12 *[6] Department of Biology, University of Naples Federico II, MSA Campus, Via Cinthia 21, 80126 Napoli, ITALY*

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18 [*]Corresponding author:

19 C. Agnisola

20 Department of Biology, University of Naples Federico II

21 V. Cinthia 21, 80126 Napoli, ITALY

22 Tel. Office: +390812535144; Tel. Lab: +390812534128

23 agnisola@unina.it

24

25

Abstract

Rheotaxis, the unconditioned orienting response to water currents, is a main component of fish behaviour. Rheotaxis is achieved using multiple sensory systems, including visual and tactile cues. Rheotactic orientation in open or low-visibility waters might also benefit from the stable frame of reference provided by the geomagnetic field, but this possibility has not been explored before. Zebrafish (*Danio rerio*) form shoals living in freshwater systems with low visibility, show a robust positive rheotaxis, and respond to geomagnetic fields. Here, we investigated whether a static magnetic field in the Earth-strength range influenced the rheotactic threshold of zebrafish in a swimming tunnel. The direction of the horizontal component of the magnetic field relative to water flow influenced the rheotactic threshold of fish as part of a shoal, but not of fish tested alone. Results obtained after disabling the lateral line of shoaling individuals with Co^{2+} suggest that this organ system is involved in the observed magneto-rheotactic response. These findings constitute preliminary evidence that magnetic fields influence rheotaxis and suggest new avenues for further research.

1. Introduction

Spatial orientation is essential for many of the activities animals undertake, including habitat selection, foraging, and migration (Aidley, 1981; Jander, 1975). Numerous environmental cues are available to animals for orientation and identifying the sensory thresholds by which animals perceive each cue can provide valuable insight to their behavioral ecology (Phillips et al., 2002). Yet, how animals weight different sensory information or how changes in one sensory cue modulates the response to other cues (as likely occurs in nature) remains challenging to determine (Lohmann et al., 2008a; Jorge et al., 2009; Putman et al., 2014a; Beason and Wiltschko, 2015). For instance, in aquatic environments, rheotaxis (i.e., the unconditioned orienting response to water currents) is especially important for facilitating movement decisions and is observed across diverse taxa (Chapman et al., 2011). In fish, positive rheotactic behavior is widespread (Montgomery et al., 1997; Kanter and Coombs, 2003; Suli et al., 2012) allowing fish to either maintain their upstream-oriented position or move against the current (Baker and Montgomery, 1999). The rheotactic process, necessarily, depends on information coming from the surrounding environment (Bak-Coleman et al., 2013). In zebrafish, rheotaxis is mediated by several sensory modalities including visual, vestibular, and tactile (Bak-Coleman et al., 2013; Montgomery et al., 2014), each of which

60 requires some frame of reference for fish to gauge the direction of water flow. Given that zebrafish

61 occupy variable freshwater environments with highly turbid waters, such as seasonal floodplains,

62 rice paddies, and slow streams (Engeszer et al., 2007; Spence et al., 2008), multimodal rheotaxis

63 may have an important function for their ecology.

64   There has been long-standing speculation that the Earth's magnetic field could play a role in

65 rheotaxis (Arnold, 1974). A benefit of using this cue is that the geomagnetic field is ubiquitous, and

66 could provide a stable frame of reference by which current-induced displacement, water direction,

67 or both could be detected. Linking magnetic orientation and rheotactic responses could greatly

68 increase the efficiency of navigation, particularly in dynamic environments (Wyeth, 2010; Endres et

69 al., 2016). Previous studies indicate that diverse animals can detect and use the magnetic field as a

70 stationary cue for positional and compass information (e.g., Walker et al. 1997; Walker et al., 2002;

71 Gould, 2010; Putman et al., 2013; Putman et al., 2014c; Putman et al., 2015). Likewise, zebrafish

72 are known to detect Earth-strength static magnetic fields (Shcherbakov et al., 2005; Takebe et al.,

73 2012; Osipova et al., 2016) and biogenic magnetite, which is associated with magnetoreception

74 (Kirschvink et al., 2001), has been found in the region of the lateral line (Dixson, 2011). Neuromast

75 mechanosensors (canal and superficial) are involved in the fish orientation to water flows

76 (Montgomery et al., 1997; McHenry and van Netten, 2007; Suli et al., 2012) and play an important

77 role in the cohesive swimming of fish shoals (Faucher et al., 2010). However, whether

78 magnetoreception and mechanoreception are behaviorally interrelated or whether the magnetic field

79 influences rheotaxis, in general, has yet to be addressed experimentally.

80   Here we explore the hypothesis that rheotaxis of zebrafish is influenced by the presence and

81 direction of an Earth-strength magnetic field. We performed a series of laboratory-based

82 experiments, using a swimming tunnel that allowed us to quantify the rheotactic threshold of

83 animals while controlling the intensity and the direction of the magnetic field relative to the

84 direction of water flow. Specifically, we evaluated the rheotactic response by a stepwise increase in

85 the velocity of water current and measuring at each step the time spent by the animals oriented

86 upstream. As zebrafish naturally aggregate in shoals and schools (Pitcher and Parrish, 1993), we

87 compared the behavior of individuals swimming alone or as part of a shoal. Furthermore, we

88 examined whether the response of shoaling fish to rheotactic and magnetic stimuli involved the

89 lateral line by also performing experiments with animals pre-treated with cobalt, to inhibit the

90 lateral line functioning (Karlsen and Sand, 1987). We hypothesized that if the geomagnetic field

91 plays a role in rheotaxis, upstream orientation of zebrafish might be influenced by the direction of

92 the magnetic field with respect to water current. Our findings suggest that the rheotactic threshold is

93 affected by the magnetic field in the geomagnetic range when animals swim in a shoal and

94 implicate a possible role of the lateral line in this effect.

95

96 **2. Materials and methods**

97

98 *2.1 Ethic statement*

99 All animal procedures were approved by the Institutional Animal Care and Use Committee (CESA)

100 of the University of Naples Federico II, Naples, Italy.

101

102 *2.2 Animals*

103 Adult zebrafish of the short-fin wild-type were provided by a local supply store (CARMAR SAS, S.

104 Giorgio (NA), Italy). Animals were maintained in 200 l tanks, at a density of 1 animal per 2 l. Main

105 water parameters in the maintaining tanks were monitored daily: temperature 27°-28°C;

106 conductivity <500 µS; pH 6.5-7.5; NO_3 < 0.25 mg/l. Animals were fed once a day with commercial

107 pelleted fish food (Tetramin, Tetra, Germany; 47% crude protein content, 6% humidity, 20.1 kJ/g

108 dry mass) and displayed a normal shoaling behavior. Experimental individuals were selected

109 randomly from the maintenance tank. All experiments were performed at the same time of the day

110 (9.30-13.00), during March and October 2014. The average weight of selected animals was

111 0.76±0.1 g (Mean ± s.d., N= 45).

112

113 *2.3 Swimming tunnel and magnetic field control*

114 The swimming apparatus for zebrafish was from M2M Engineering Custom Scientific Equipment

115 (Naples, Italy). As schematically shown in Fig. 1A, water flow was continuously measured by a

116 SMC Flow switch flowmeter and controlled by a digital feedback system. Water in the swimming

117 apparatus was identical to the maintenance water, was continuously aerated (oxygen content ~7.9

118 mg/l) and its temperature was maintained at 27°C via a TECO 278 thermo-cryostat. The tunnel was

119 a non-reflecting Plexiglas cylinder (7 cm diameter, 15 cm length) (Fig. 1B). Micro-turbulence in the

120 tunnel was avoided by two perforated Plexiglas plates.

121   The intensity and direction of the horizontal component of magnetic field in the lab,

122 measured along the major axis of the tunnel in the upstream direction (x axis, +70° of geographic

123 north, Fig. 1C and E), was 11µT; the field along y axis was -25µT and that of z axis was 55µT

124 (F=62 µT; I=64°; D=44°). These magnetic conditions were similar to those in the aquarium room

125 for maintenance of animals, measured along the same axes (x= 22 µT; y= -27 µT; z= 43 µT). No

126 variation in the lab magnetic field was observed during the period of experimentation, being likely

127 below the measurement equipment resolution (92nT). For geographic context, the International

128 Geomagnetic Reference Field (IGRF-12) predicted the geomagnetic field at site of the lab (40°N,

129 14°E) to be F = 45.8 µT; I = 56.1 degrees; H = 25.6 µT; D = 2.91 degrees at the time of testing

130 (www.geomag.nrcan.gc.ca).

131   We manipulated the magnetic field along one dimension, the same axis as the water flow

132 (Fig. 1C, i.e., the x-axis) (one-dimensional magnetic field manipulation, according to Tesch, 1974).

133 The magnetic field intensity and direction along the tunnel major axis were modified by wrapping

134 the swimming tunnel with a solenoid (0.83 turns cm^{-1}) connected with a power unit to generate

135 static magnetic fields (DC power supply ALR3003D, Elc, France). Fields of -50 µT, 0 µT and +50

136 µT were utilized in order to observe the effect of field direction with respect to the water flow (y

137 and z components were unchanged, see Fig. 1E). Positive induced magnetic field along the x axis

138 was oriented opposite to the water current direction (upstream). The manipulated magnetic field did

139 not vary along the swimming tunnel. The magnetic field was measured with a Gauss/Teslameter

140 (9500 Gauss meter, DC 10 kHz, 0.092 µT resolution, FW Bell, Orlando, USA).

141

142 *2.4 Experimental protocol*

143 We used a mixed design with one within-subject factor (flow speed) and three between-subject

144 factors (solitary *vs* in-shoal swimming, magnetic field and cobalt pre-treatment). A repeated-

145 measure design was used to manipulate flow within each animal group – magnetic field

146 combination.

147   Animals were tested inside the magnetic swimming tunnel solitary or in a shoal of 5

148 individuals. Animals were acclimated for 1 h (Fig. 1D). During acclimation, the water flow was

149 1.73 cm sec^{-1}. This flow rate was sufficient, according to preliminary experiments, to maintain the

150 oxygen supply for the animals. After acclimation, we started the test with the induced magnetic

151 field. At first, animals stayed for 10 minutes with no water current. Successively, we exposed the

152 fish to a stepwise increase in the velocity of water current, from 1.95 cm sec^{-1} to 8.45 cm sec^{-1}

153 (about 0.7-2.9 BL sec^{-1}). The flow rate increased by 1.3 cm sec^{-1} (about 0.4 BL sec^{-1}) every 10

154 minutes for six times (60 minutes in total). The range of water speed was in the lower range of flow

155 rates that induce continuous oriented swimming in zebrafish (3-15% of U$_{crit}$) (Plaut, 2000).

156

157 *2.5 Experimental groups*

158 Only naïve fish were used, and each fish experienced only one magnetic field condition. A first

159 group was made of 15 animals that swam alone in the tunnel (mean body weight: 0.78 ± 0.05 g,

160 standard length, 3.10 ± 0.05 cm); this group was divided in 3 sub-groups of 5 animals. Each sub-

161 group was tested with a different magnetic field induced along the axis of water flow: -50 µT, +50

162 µT and a null magnetic field, 0 µT (see above). A second group was made of 15 animals which

163 were let to swim in groups of 5 animals (mean body weight: 0.80 ± 0.05 g; standard length, $3.00 \pm$

164 0.05 cm); each of the three groups was tested with a different induced magnetic field: -50 µT, +50

165 µT and a null magnetic field, 0 µT (see above). These animals were tested in order to observe how

166 the magnetic field affects the rheotactic orientation of individual zebrafish in the shoal, which is the

167 natural state of aggregation for this species. Finally, as the integrity of lateral line may be important

168 for the interaction of individuals in a shoal (Faucher et al., 2010) and for magnetoreception (Dixson,

169 2011), we tested a third group of 15 animals, which were tested in groups of 5. These fish were

170 previously treated with cobalt (24h exposure to Ca^{2+} free, 0.1 mmol l^{-1} $CoCl_2$ solution), which

171 reversibly inhibits the lateral line (Karlsen and Sand, 1987) (mean body weight: 0.69 ± 0.03 g,

172 standard length, 2.81 ± 0.04 cm). Again, each of the three groups of 5 animals were tested with a

173 different induced magnetic field as above. The efficacy of the cobalt (Co^{2+}) treatment on

174 neuromasts was confirmed by treating animals from the same batch with 0.1 mM DASPEI (2-[4-

175 (di-methylamino) styryl-N-ethylpyridinium iodide, Sigma) in fish water for 1 hr, followed by deep

176 anaesthesia in ice-cold 50 mg/ml MS222 (Sigma). Neuromasts were observed with a binocular

177 fluorescent microscope (Leica AF6000) (Yoshizawa et al., 2010). Examples of observations are

178 reported in Fig. 2, which show the virtual absence of DASPEI staining of both canal and superficial

179 neuromasts in Co^{2+}-treated animals. The animals used to check the effect of Co^{2+} were not used for

180 the swimming test.

181

182 *2.6 Observational analysis and data collection*

183 The whole run in the tunnel was video recorded with the Sony DCR-TRV18E video camera and

184 saved as mpg files (25 frames/sec). Zebrafish behavior in the tunnel was studied from videos of the

185 trials in all the experimental conditions. Behavior was blindly recorded and analyzed by trained

186 observers (intra-rater reliability > 0.90). Only the second half (5 min) of each of the 10 min

187 intervals corresponding to each experimental condition was analyzed, to allow for recovery from a

188 possible anxiety response to the change in environment/flow rate (Wong et al., 2010). Fish behavior

189 was analyzed by a video-tracking software (Tracker 4.84 Video Analysis and Modeling Tool).

190 Tracking was performed manually at eight-frame intervals (0.32 sec). The reference system used for

191 the analysis had the x axis along the tunnel with the origin fixed in the lowest point of the ending

192 wall from which the current came. The eye of each fish was used as reference for the fish tracking,

193 allowing us to distinguish individuals in the shoal. Any video distortion was corrected using the

194 perspective and radial distortion filters.

195

2.7 Fish behavior in the tunnel and parameters evaluated

In the tunnel, both in the absence and the presence of water flow, animals displayed two types of

behavioral patterns: oriented swimming and maneuvering. Most of the time (total oriented time,

usually higher than 95% of total recording time) they swam along the major axis of the tunnel (i.e.

oriented along the axis with an angle lower than 45°), both in the upstream and downstream

directions, turning when they came close to the end walls; often displaying thigmotaxis, i.e. they

swam close to the tunnel wall (Kalueff et al., 2013). In absence of water flow and at the lowest flow

rates, animals displayed a burst and coasting type of swim (Kalueff et al., 2013) but moved to

continuous swimming with minimal turns at the highest water speed used in the tests. The rest of

the time, they showed a maneuvering or non-oriented activity, i.e. they stayed almost in place,

usually close to an end wall, quickly moving caudal and lateral fins, or displaying two or more

successive turns, with intervals between turns less than 1.0 sec. Four animals of the solitary group

displayed a third type of behavior, i.e. they stayed still at the bottom of the tunnel (freezing, Kalueff

et al., 2013). Freezing in these animals was always upstream oriented. It is worth noting that this

response was observed only after acclimation. So, it was not likely related to a fear response to new

environment, but rather to the isolated condition, and could depend on other inter-individual

differences, e.g. "proactive" vs "reactive" behavior (Moretz et al., 2007).

Animals usually stayed in the lower half of the tunnel. Vertical displacements were rare. The

vertical position was relatively stable between 2 and 4 cm from the bottom and was not related with

any of the experimental conditions used (Supplementary Fig. 1). Transversal displacements, likely

constrained by the tunnel shape, were also very rare.

Under these conditions, the rheotactic response could be evaluated by the amount of time

that the animals spent oriented upstream (swimming or, in the few animals that displayed it,

freezing), as a proportion of the total oriented time. We define this proportion as the Rheotactic

Index (RI). In preliminary experiments, RI increased sigmoidally with water speed, thus

representing a simple way to quantify the rheotaxis of the animals tested. Under manipulated

magnetic fields, changes in the relationship between RI and water speed between the groups would

reveal whether magnetic fields influence the rheotaxis of fish. We also estimated the amount of time

of Polarized Directional Swimming (PDS) of shoals. We counted, in the 5 min interval, all frames

(0.32 sec apart) in which at least 4 fish in the group were synchronically upstream oriented and

express PDS as % of total time analyzed (5 min). This parameter measures the probability of

finding at least 4 animals simultaneously upstream oriented, which is expected to increase with flow

rate when a rheotactic response occurs. In contrast to the RI, the PDS allowed us to assess the level

229 of schooling behavior, by evaluating the degree of synchronization and polarization (swimming

230 towards a common direction) of the fish composing the shoal during the upstream orientation.

231 Effects of magnetic field manipulation on the values of this parameter would indicate whether

232 magnetic information influences the degree of synchronized and polarized swimming during

233 rheotaxis and thus identify a possible role of magnetic fields in group-swimming behavior. PDS

234 was also evaluated for the group of solitary swimmers. Solitary swimmers were, by definition, run

235 separately in different trials; thus, the PDS of these animals measured the probability that at least 4

236 individuals would display the same upstream orientation in the same moment during the runs, in

237 absence of any possible inter-individual interaction. Any difference from the pattern showed by the

238 solitary animals would indicate an among individual interaction effect in a group. We associated the

239 PDS measure in shoals with that of the average inter-individual distance (AvD) (Miller and Gerlai,

240 2012).

241 Other parameters evaluated were the frequency of turns (min^{-1}) and the swimming rate (cm

242 sec^{-1}).

243

244 *2.8 Statistical analysis*

245 As the major aim of the study was to check the effects on individual's rheotactic behavior while

246 swimming solitary or in shoal, individuals were considered as the statistical unit. The rheotactic

247 index from the 5 individuals of each experimental sub-group is shown as mean ± s.e.m.. AvD, also

248 expressed as mean ± s.e.m., was calculated as the mean of the 5 average distances of each animal

249 from the other 4 animals in a shoal (Miller and Gerlai, 2012). All statistics were performed on

250 arcsine transformed data.

251 The relationship between RI or PDS and water flow rate was fitted by the following logistic-

252 sigmoidal model:

253
$$y = min + \frac{(max - min)}{1 + 10^{(F50-x)slope}} \qquad (1)$$

254 For RI and PDS, constraints were posed on both the min value (>0) and the max value (<100), as

255 they can only vary between 0 and 100.

256 The threshold for the rheotactic response, i.e. the minimal flow at which a significant

257 rheotactic response can be elicited (Baker and Montgomery, 1999), was extrapolated from the

258 sigmoid fitting curves as the flow rate at which the curve displayed the highest rate of slope

259 variation, individuated as the positive peak of the second derivative of the curve.

260 Within each animal group (solitary, shoal, and shoal treated with Co^{2+}), we tested whether

263 one statistical sample). To this aim, within each group, extra sum-of-square F test ($p<0.05$) was

264 used to test if the model could be applied to the three curves (the three induced magnetic fields)

265 separately (significant effect of magnetic field), or if a single curve for all pooled data must be

266 drawn (no significant effect of magnetic field). In the first case, we repeated the test for all pairwise

267 comparisons of the three magnetic field curves, checking for significant differences between each

268 pair of magnetic field conditions (-50 vs 0; -50 vs + 50 and 0 vs +50). P values were corrected for

269 multiplicity. When a significant effect of magnetic field was found, a comparison among threshold

270 values was performed with a pairwise t-test comparison and a Bonferroni multiplicity correction of

271 probabilities. A similar approach was used for the comparison of PDS-flow rate curves. However,

272 as PDS is a parameter concerning the shoal behaviour, the comparison was among animal groups at

273 each magnetic field condition.

274   All the statistical tests were "two-tailed". Statistics was performed using GraphPad Prism

275 version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

276

277 **3. Results**

278

279 The Rheotactic Index (RI) of solitary swimming animals at increasing water flow under different

280 magnetic fields cannot be represented by different curves (p for comparison of fits >0.05),

281 suggesting independence of RI-flow rate relationship from the magnetic field (Fig. 3 left lower

282 panel). In contrast, the RI-water flow rate curves significantly differed depending on the magnetic

283 field for shoaling zebrafish with and without pre-treatment of cobalt (p for comparison of fits

284 <0.05) (Fig. 3, middle and right lower panels). Thus, the RI of zebrafish swimming in shoal is

285 influenced by the magnetic field.

286   The relationship between the Polarized Direction of Swimming (PDS) and water flow rate

287 also appears to be influenced by the magnetic field (Fig. 4). For instance, while at 0 µT there was

288 no significant difference among solitary, shoaling and Co^{2+}-treated shoaling animals (p for

289 comparison of fits >0.05), the three curves are significantly different at both -50µT and +50µT (p

290 for comparison of fits <0.05). Moreover, the field effect is clearly different if the field direction

291 changes with respect to the water current direction. At -50µT (i.e., when the field is downstream

292 oriented), at any given flow rate, there was a higher probability to find all the animals upstream

293 oriented when swimming in a shoal compared to the solitary swimming, possibly suggesting that

294 rheotaxis and schooling reinforce each other. At +50µT (i.e., when the field is upstream oriented)

295 the opposite occurs, suggesting a competition between rheotaxis and schooling. Cobalt treatment

296 affects this magnetic field dependent schooling effect. At -50 µT the probability to find the Co^{2+}-

297 treated animals, swimming in a shoal, upstream oriented at a given flow is even lower than the
298 solitary animals, while at +50 µT, the Co^{2+}-treated and solitary curves are not significantly
299 different.

300   We also detected an influence of the magnetic field on the inter-individual distance (AvD), a
301 parameter that may be linked to the degree of animal interaction in the shoal (Miller and Gerlai,
302 2012). When the magnetic field along the tunnel axis was set to zero the AvD was significantly
303 lower in Co^{2+}-treated (2.18±0.04 cm) than in the untreated (3.69±0.03 cm) fish. However, this
304 difference disappeared when a horizontal magnetic field is induced, whether with or against water
305 flow (see Supplementary Table 4 for the complete set of data).

306   During the trials, the swimming rate of animals tended to coincide, in its absolute value with
307 the water flow rate, particularly at the highest rates. Accordingly, the turn frequency tended to
308 reduce towards zero. Neither parameter was affected by the magnetic field and did not differ among
309 the three experimental groups (Supplementary Figs. 2 and 3).

310

311 **4. Discussion**

312

313 This study is consistent with others that show an influence of Earth-strength magnetic fields on
314 zebrafish behavior (Takebe et al., 2012; Osipova et al., 2016). Additionally, we present preliminary
315 experimental evidence that magnetic fields can affect the rheotactic threshold of aquatic animals.
316 Zebrafish swimming in a shoal displayed a rheotactic response (RI) that was influenced by the
317 induced magnetic field (Fig. 3). Likewise, we observed an influence of the magnetic field on
318 behavioral indices associated with schooling (PDS and AvD).

319

320 *4.1 Implications for understanding fish magnetoreception*

321   The lateral line appears to be involved in mediating the effect of magnetic fields on
322 rheotaxis, given that impairing the lateral line alters the rheotactic threshold under different
323 magnetic fields. This finding could be linked with the observation that the lateral line system is
324 involved in rheotaxis at low rates of flow (Montgomery et al., 1997; Montgomery et al., 2000). It
325 should be noted that following Co^{2+} treatment, rheotactic threshold is still affected by the magnetic
326 field, but the sensitivity to the magnetic field direction is altered. Walker and coworkers (1997)
327 found that the ros V nerve in the anterior skull of rainbow trout (*Oncorhynchus mykiss*) responded
328 to changes in the intensity but not the direction of imposed magnetic stimuli of +/- 50µT. This nerve
329 links the brain to the lamina propria cells, in the olfactory lamellae, where magnetite crystals were
330 detected (Walker et al. 1997). These findings indicated that there are magnetoreceptive cells located

331 in the olfactory system of trout. In this species, the ros V nerve is closely linked to the dorsal

332 anterior lateral-line nerve (rosl) (Walker et al. 1997). In this context, it is worth noting that in

333 addition to manipulating the component of the magnetic field along the axis of the swimming

334 tunnel, the magnetic treatments altered the total field intensity and inclination angle (Fig. 1 and

335 Supplemental Table 1), two field parameters that are perceptible to diverse taxa (Phillips et al.

336 2002; Boles and Lohmann 2003; Putman et al. 2011; Kishkinev et al. 2015), including fishes

337 (Putman et al. 2014b, c). However, the change in total field intensity and inclination angle was

338 identical for two of the magnetic treatments (-50 µT and +50 µT). Thus, our results imply a

339 direction-dependent sensitivity of zebrafish to an imposed magnetic field that can be altered by

340 inhibiting the lateral line.

341   The ability to sense magnetic field direction may be related to the integration between

342 magnetosensation and the rheotactic response, which depends from several sensorial pathways,

343 tactile, visual and vestibular (Bak-Coleman et al., 2013; Montgomery et al., 2014), and that thus it is

344 only partially altered after Co^{2+} treatment. Two explanations could be hypothesized. One possibility

345 is that the magnetoreceptors are located in the olfactory cells only, so that rheotactic and magnetic

346 stimuli are post-processed and integrated in common neural pathways, allowing fish to elicit a

347 consequent behavioral response. A second explanation is that magnetite crystals are located also in

348 the lateral line, participating in magneto-sensation. In this last case, magnetoreception in fish would

349 be complex, with magneto-sensitive cells located in different parts of the body surface, and only in

350 part affected by Co^{2+} treatment. In this scenario, our results of cobalt treated zebrafish would

351 suggest that the lateral-line cells participate to the detection of magnetic field direction (while the

352 olfactory cells might detect magnetic intensity also in this species, as in trout). There is evidence

353 that magnetite is located in the lateral line region of zebrafish (Dixson, 2011), but further studies are

354 necessary in order to validate the hypothesis of a magnetosensitive-lateral line in fish. Although the

355 behavioral responses of cobalt treated zebrafish reported in this study suggest the lateral line is

356 involved in the "magneto-rheotactic" behavior, the mechanism of the interaction between magnetic

357 field detection and the neuromasts in rheotaxis appears to be complex and requires further research.

358

359 *4.2 Behavioral and ecological implications*

360   The activation of the rheotactic response in zebrafish is pointed out by two main evidences:

361 the decrease of the turning frequency (Plaut, 2000) and the increase of the time spent by the animals

362 upstream oriented when flow rate increases. The last parameter, when measured as the proportion of

363 time spent by the animals swimming upstream (RI), displays a sigmoidal relationship with flow

364 rate, and it is used in the present study to quantify the rheotactic threshold. This choice was linked

365 to the specific behavioral characteristics of this species. Zebrafish, is a very active fish with high

366 swimming performance (Palstra et al., 2010), that swims most of the time (Fuiman and Webb,

367 1988), usually in the speed range at which the oxygen consumption is scarcely affected by

368 swimming speed (< 50 U_{crit}, Palstra et al., 2010). This occurs also in respirometers or swimming

369 tunnels, where they tend to swim along the major axis of the chamber, turning frequently, even in

370 the presence of water flow, and tending to swim both upstream and downstream (Plaut and Gordon,

371 1994; Plaut, 2000). This behavior in the tunnel is affected by the water flow rate, disappearing at

372 very high speeds (>8 BL s^{-1}, Plaut and Gordon, 1994), when the animals swim continuously with

373 their heads directed upstream (full rheotactic response).

374   Our results show that the magnetic field significantly affects the rheotactic threshold when

375 fish swim in shoal. This could be of ecological significance, as the threshold values here reported

376 are in the range of zebrafish routine swimming speed in nature (Fuiman and Webb, 1988). As

377 shown in Fig. 3, we see that: (i.) the rheotactic response elicited in our studies reaches its maximum

378 at relatively low water speed (2-3 BL s^{-1}); (ii.) when swimming in shoals, small variations in the

379 water speed, or small variations in magnetic field at constant water flow significantly affect the

380 tendency of individuals to orient upstream in slowing moving waters; (iii) no significant effect of

381 magnetic field was observed in solitary animals.

382   In their natural environment, zebrafish inhabit narrow rivers and areas with slow water

383 currents (floodplain, rice-fields and blind canals connected to the main rivers), characterized by

384 considerable seasonal variability owing to monsoon winds (Spence et al., 2008). In these

385 environments, turbidity is typically high (Spence et al., 2008) and visual cues might often be

386 unreliable for detecting the drifting direction induced by the water flow. The relationship between

387 the rheotactic threshold and the magnetic field could be consistent with the hypothesis that zebrafish

388 may exploit magnetic cues for the seasonal migration to floodplain and back to streams (Spence et

389 al., 2008; Takebe et al., 2012).

390   The activation of a rheotactic response induces a polarization of shoals. Polarization could

391 be also the result of schooling, an effective behavior displayed by zebrafish that could be helpful for

392 predator avoidance (Bode et al., 2010) and for information exchanging through the group

393 (particularly important for migratory species) (Couzin et al., 2005). Schooling could reinforce the

394 rheotactic response compared to the isolated animals. Indeed, in our experimental conditions, the

395 polarization of a group of fish could be simply the result of the simultaneous rheotactic orientation

396 of the animals, without any contribution of inter-individual interactions. Interestingly, this appears

397 to be true only in absence of the magnetic field along the water flow axis. In presence of a magnetic

398 field, individuals in shoals clearly behave differently from individual tested alone (see Fig. 4). The

399 reduced inter-individual distance observed at 0 µT in Co^{2+}-treated, highlights the putative role of

400 lateral line in determining animal interaction in zebrafish shoals (Partridge and Pitcher, 1980).

401 Interestingly, the presence of the magnetic field seems to compensate for the lateral line deficiency

402 in defining the inter-individual distances in the shoal and again this effect is most effective when the

403 magnetic field has the same direction as the water current. Moreover, zebrafish naturally aggregate

404 in shoals (Spence et al., 2008). The negative result of solitary zebrafish might be due to the non-

405 natural state of isolation, as suggested by the fact that almost 30% of solitary fish displayed the

406 stress-related "freezing" behavior (Kalueff et al., 2013). It is conceivable that increasing the sample

407 size in individuals tested could result in detecting a statistical relationship for zebrafish in the

408 solitary condition. Indeed, previous studies on species such as Pacific salmon (*Oncorhynchus* spp.),

409 reported an effect of magnetic fields on the orientation of fish tested alone utilizing larger sample

410 sizes (>100 fish per treatment) (e.g., Quinn 1980; Putman et al., 2014c). Our results, show that with

411 a much smaller sample size, an influence of the magnetic field can be detected in fish when they are

412 in shoaling condition. For zebrafish, this may suggest that testing animals in groups might be a

413 more powerful way to study the sensory ecology of orientation.

414

415 *4.3 Conclusions*

416   The results here reported strongly suggest that the ability of zebrafish to sense the

417 geomagnetic field could affect their ability to react to water current when swimming in shoals. In

418 particular, results imply that fish display the highest rheotactic response (i.e., the lowest threshold)

419 when fish swim in shoal, have a functioning lateral line and the horizontal magnetic field has the

420 same direction as the water current. These results suggest that the geomagnetic field, which is

421 already known to provide numerous animals with stationary reference information (Walker et al.,

422 2002; Lohmann et al., 2008b; Putman et al., 2014c), might also provide a reference point for fish

423 rheotaxis. However, these results should not be considered definitive, but as an initial, exploratory

424 investigation in the topic. Additional studies are required to understand the mechanisms of the

425 integration of magnetic signal detection with the other sensorial systems involved in the multimodal

426 rheotaxis.

427

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554

555

556

557 **Legends**

558

559 **Figure 1. Experimental apparatus and design**. **A.** Simplified scheme of the swimming tunnel
560 apparatus utilized in the present study. The coil around the tunnel allowed us to induce a static
561 magnetic field inside the tunnel. Magnetic field lines were parallel to the water flow, and a positive
562 induced magnetic field was oriented opposite to the water current (downstream). **B.** The swimming
563 tunnel utilized in the present study with the solenoid for the induction of a static, horizontal
564 magnetic field within the experimental chamber. **C.** The set of axis used as reference for the
565 induced magnetic field. Only direction and intensity along the x axis was controlled. **D.** Diagram of
566 the experimental design used in this study. During the acclimation fish experienced a water flow
567 sufficient to guarantee adequate oxygen supply, but with a null horizontal magnetic field (0 µT).
568 This step allowed fish to acclimate to the novel environment and experience water flow direction in
569 the swimming tunnel. Fields of -50 µT, 0 µT and +50 µT were applied along the x axis during the
570 following test phase. Each fish, individually or in group, experienced only one field value. **E.**
571 Vectorial representation of magnetic field (units: µT) in the tunnel under the three experimental
572 conditions applied, using the reference axis shown in panel C. Declination and inclination are also
573 shown. Numeric values of the components of the induced magnetic fields are reported in
574 Supplementary Table 1.

575

576 **Figure 2**. **Effect of cobalt treatment on zebrafish neuromasts, as checked by vital staining**. The

577 exposure of animals to 0.1 mM DASPEI (see Materials and Methods) allowed visualization of both

578 canal (arrowheads) and superficial (arrows) neuromasts in control zebrafish (upper pictures).

579 Neuromasts appeared strongly reduced or absent in the animals treated for 24h with 0.1 mmol l^{-1}

580 $CoCl_2$ (lower pictures).

581

582 **Figure 3**. **Rheotactic response in zebrafish**. **Upper panels**: Rheotactic threshold, of solitary (one

583 combined value as the rheotactic response was not significantly affected by the magnetic field) and

584 of shoaling (untreated and Co^{2+}-treated) animals at the 3 induced magnetic field values. A pairwise

585 comparison of thresholds by t-test was performed. The Bonferroni method was used to correct p

586 values for multiplicity. **Lower panel**: RI (%) *vs* water flow rate in zebrafish of solitary and of

587 shoaling (untreated and Co^{2+}-treated) animals at the 3 induced magnetic field values (-50 µT, 0 µT

588 and +50 µT exposed animals). The curves were fitted to a logistic sigmoidal model (see Materials

589 and Methods). Within each group, the curves from the three different magnetic field conditions

590 were compared by the Extra sum-of-square F test to check if the points represent one single sample

591 (one curve) or separated sample (three curves). Where p< 0.05, as was the case of the solitary and

592 Co^{2+}-treated animals, the test was repeated for a pairwise comparison of the three curves. The p

593 values, which were corrected for multiplicity, are reported in Supplementary Table 2.

594

595 **Figure 4. PDS (%) *vs* water flow rate relationship of zebrafish at the 3 induced magnetic field**

596 **values (-50 µT, 0 µT and +50 µT exposed animals) of solitary and of shoaling (untreated and**

597 **Co^{2+}-treated) animals.** Data were statistically analyzed as described in Fig. 3. At 0µT the Extra

598 sum-of-square F test p value was not significant, whereas significant differences (p< 0.05) were

599 observed when at fields of -50 µT and +50 µT. For these conditions, the test was repeated for a

600 pairwise comparison of the three curves and p values were corrected for multiplicity (see

601 Supplementary Table 3).

602

603 **Legends to supplementary figures**

604

605 **Supplementary Figure 1**. Vertical displacement of animal of the 3 experimental groups (solitary;

606 shoaling; and shoal pre-treated with Cobalt) in the tunnel in absence of water flow and with

607 growing flow rate at the 3 induced magnetic fields. Tunnel diameter was 8 cm. Animals tended to

608 stay at 2-4 cm from the bottom. There was no significant effect of flow or magnetic field on the

609 vertical position in any of the animal groups (2-way ANOVA). Only in the Cobalt-treated animals

610 was a significant effect of magnetic field observed at some flow rates (asterisks, p<0.05). Indeed, in

611 the range 2-6 cm sec-1, these animals tended to stay closer to the bottom at 0 and +50 µT but not at

612 -50 µT.

613

614 **Supplementary Fig. 2.** Relationship between turning frequency and flow rate in the 3

615 experimental groups (solitary; shoaling; and shoaling pre-treated with Cobalt) at the 3 induced

616 magnetic fields. The number of turns decreased as flow increased (in all groups and magnetic field

617 conditions with an exponential relationship), becoming lower than 10 turns min^{-1} at the highest

618 flow. According to Plaut (2000), this behaviour is typical of zebrafish in the swimming tunnel at

619 low water flow rates. There was no significant effect of magnetic field on this parameter.

620

621 **Supplementary Fig. 3.** Relationship between swimming rate and water flow rate in the 3

622 experimental groups (solitary; shoaling; and shoaling pre-treated with Cobalt) at the 3 induced

623 magnetic fields. Data were fitted with a second order polynomial. Dashed lines represent water flow

624 rate. Downstream swimming rate in presence of water current was lower than water flow (animals

625 were dragged by water). Upstream swimming rate tended to coincide numerically to minus water

626 flow (represented by the dotted lines). No clear effect of magnetic field was observed, although

627 upstream curves from the S group tend to differentiate at low flow rate, possibly reflecting

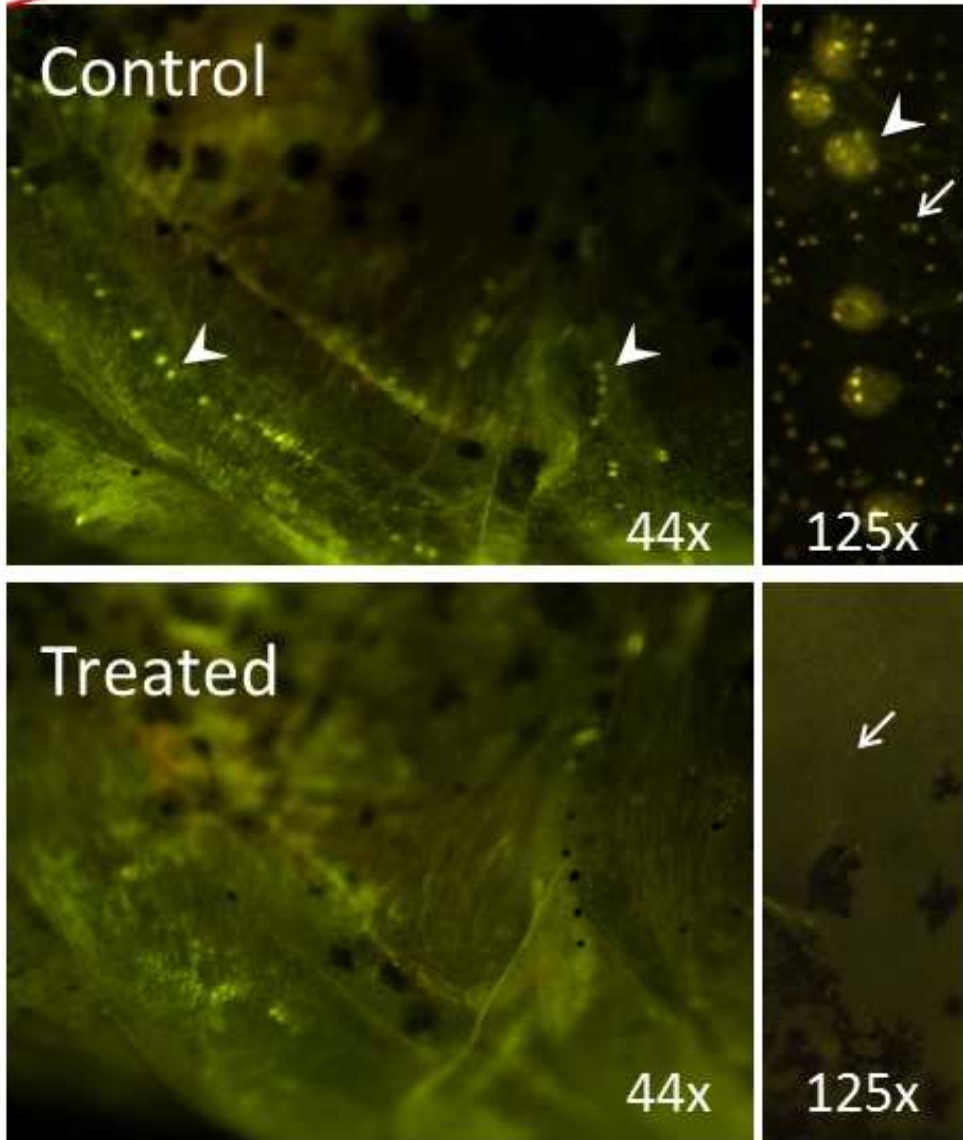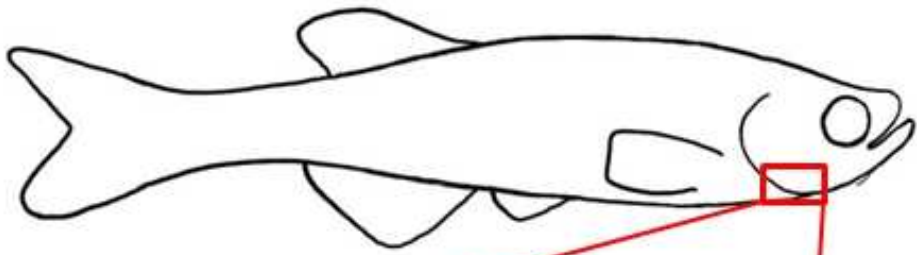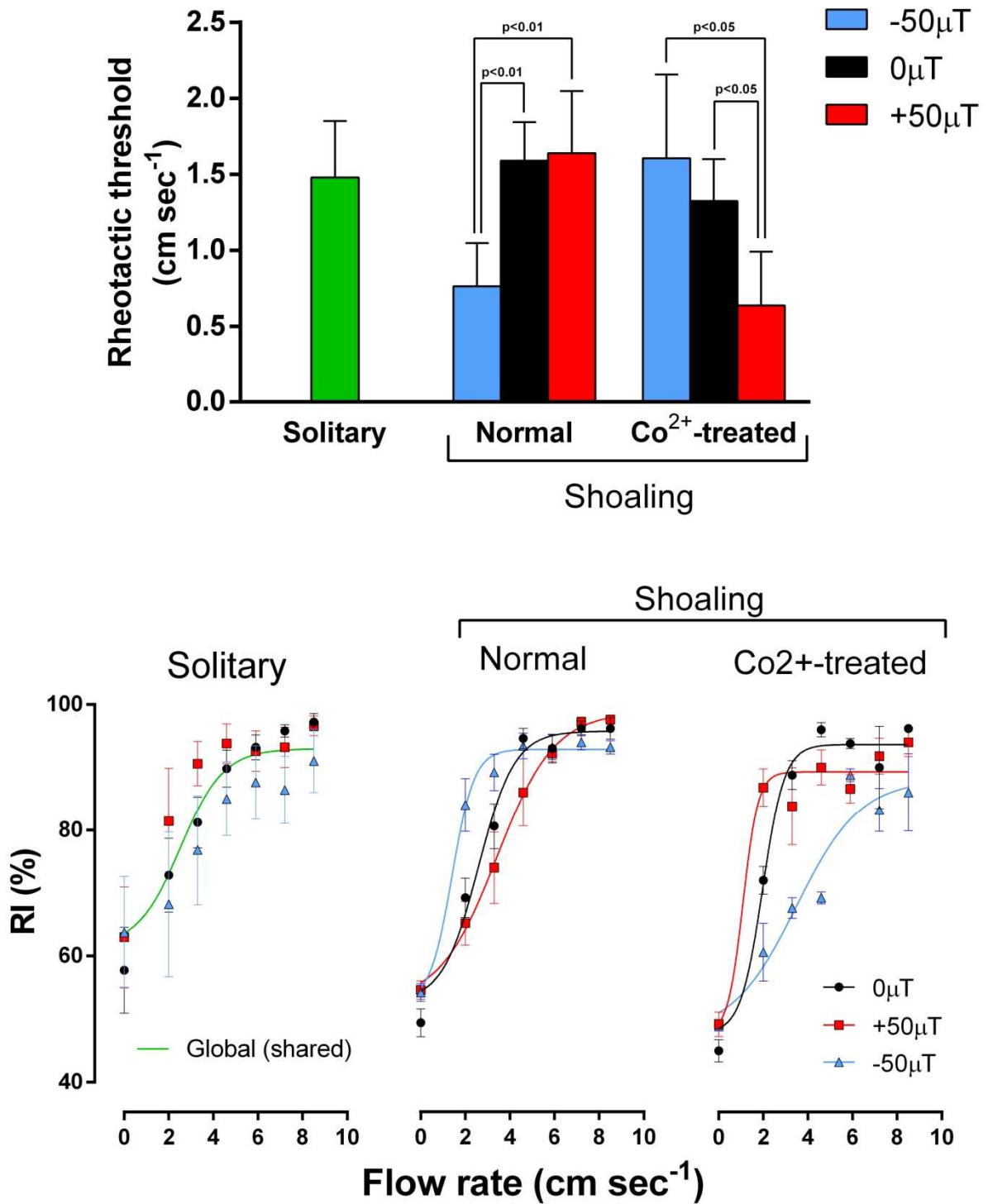628 differences in the rheotactic threshold.
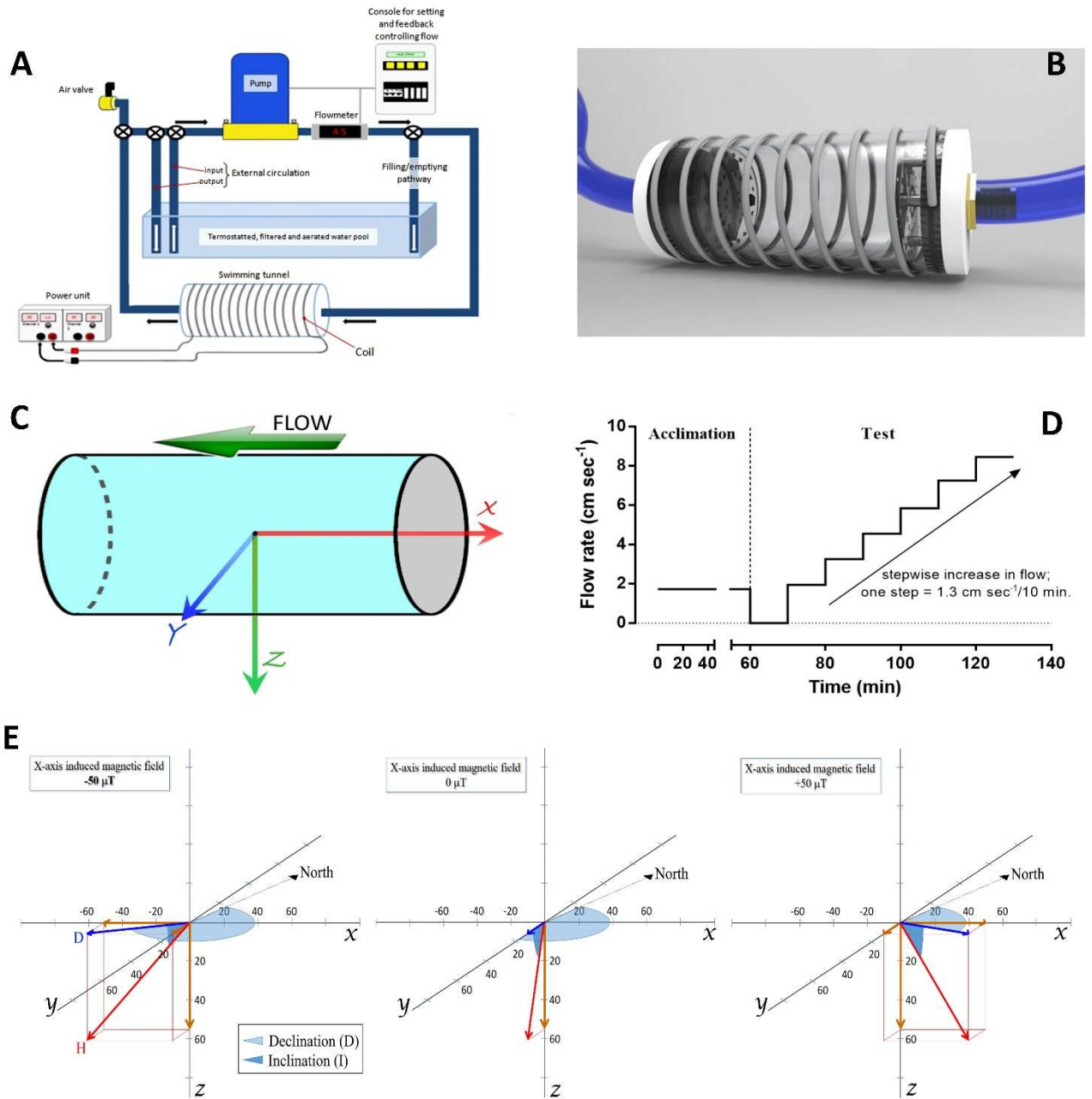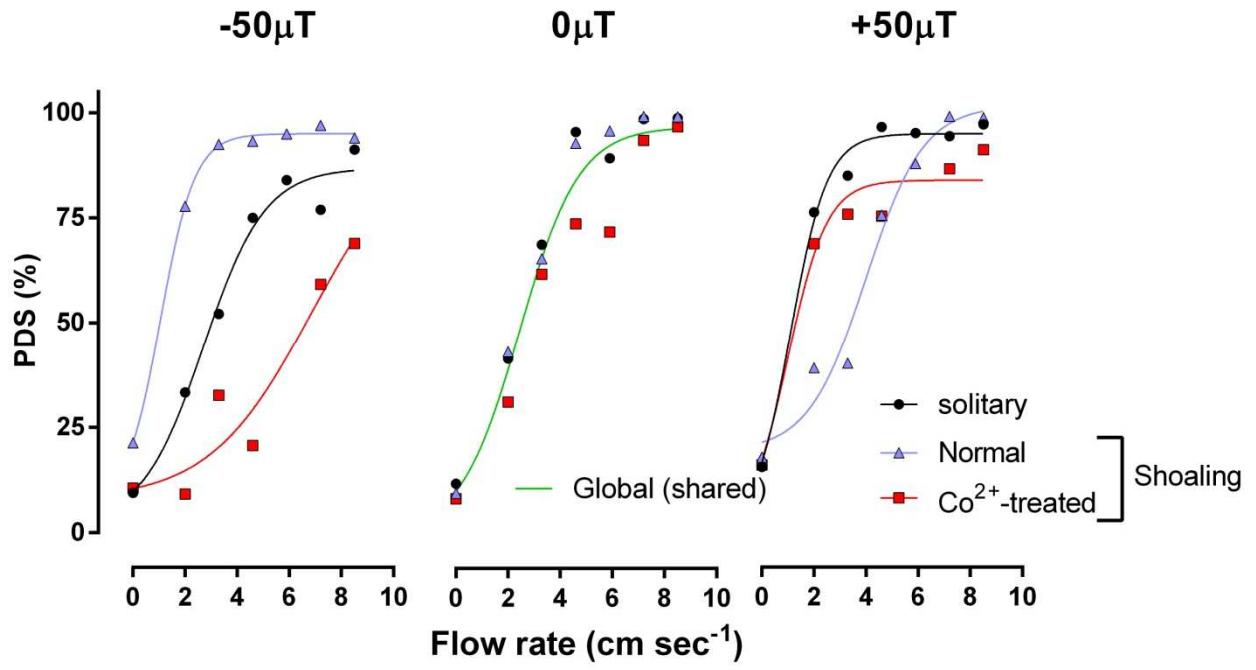
Figure 1

Figure 2
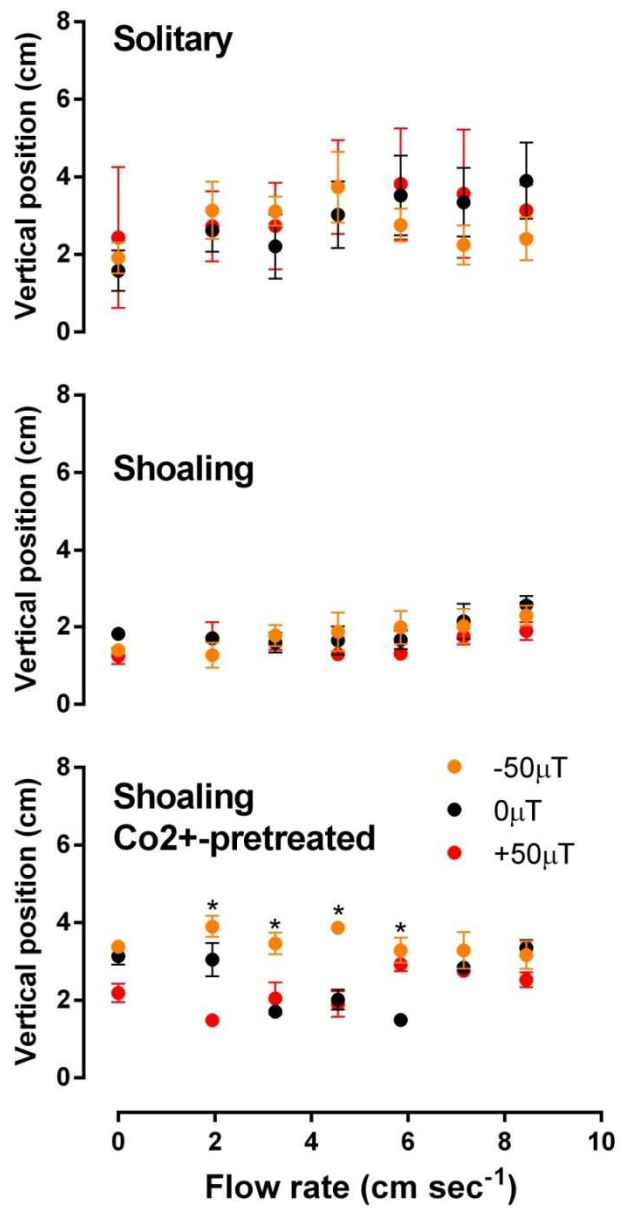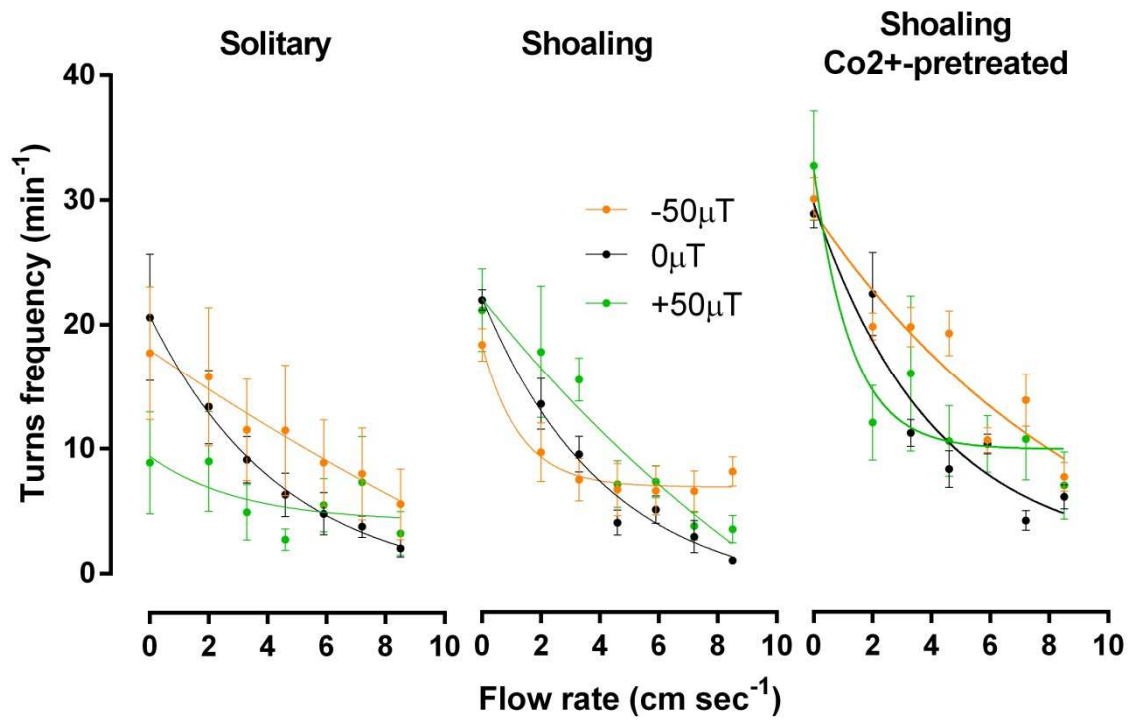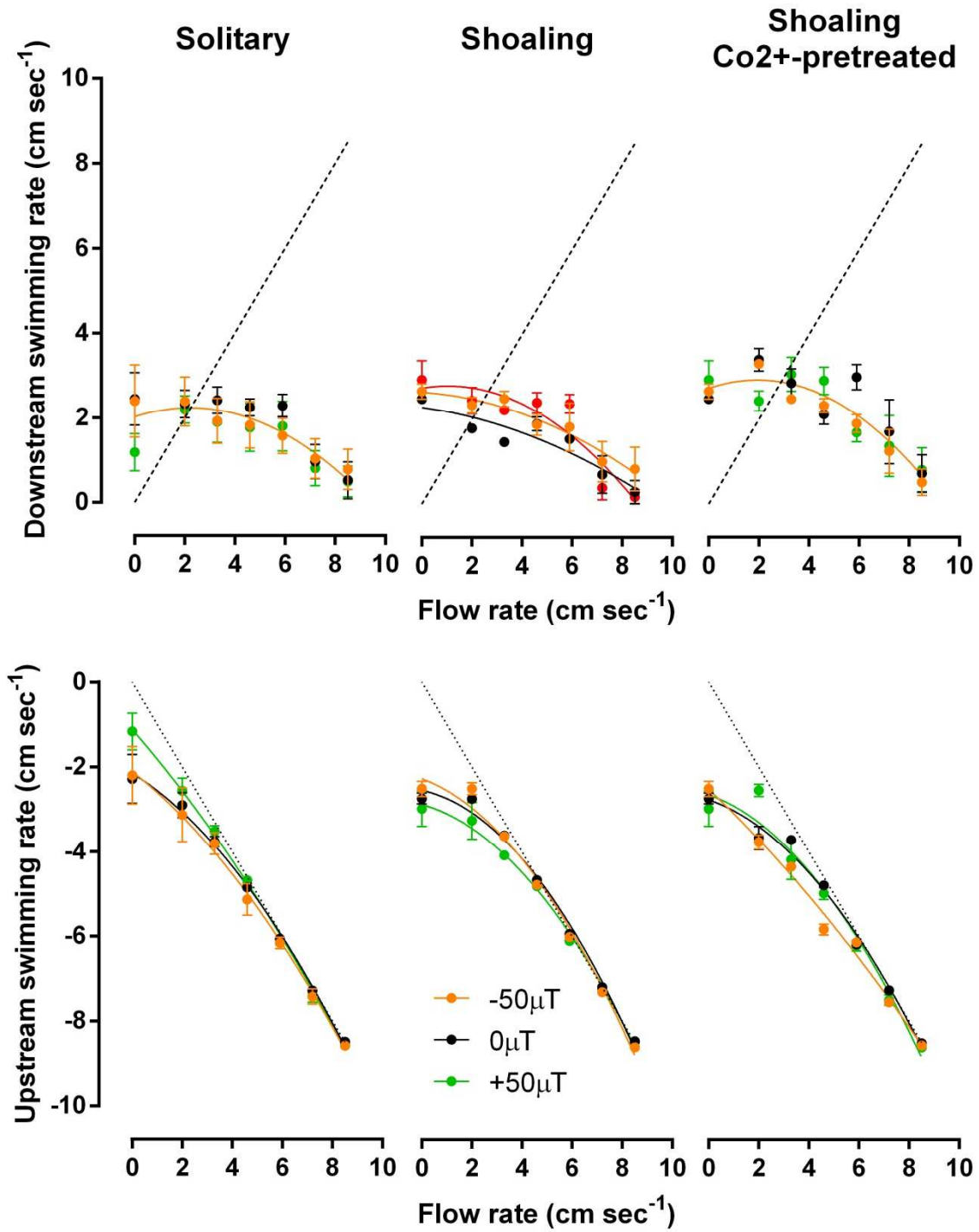
Figure 3

Figure 4

Supplementary Figure 1

Supplementary Figure 2

Supplementary Figure 3

Supplementary Table 1 – Components of the induced magnetic field under the three experimental conditions used in the present study. The X component was along the major axis of the tunnel (see Fig. S1) and was oriented +70° of geographic north. Magnetic declination is referred to the geographical north. The Y (-25μT) and Z (55μT) components were constant. The geomagnetic field at the lab location (GPS 40°N, 14°E) was: F = 45.8 μT; I = 56.1 degrees; H = 25.6 μT; D = 2.91 degrees (www.geomag.nrcan.gc.ca). No changes in the lab magnetic field were observed during the period of experimentation, being likely below the measurement equipment resolution (92nT).

X (μT)	F (μT)	I (degrees)	H (μT)	D (degrees)
-50	78,80	44,9	55,77	223,6
0	60,90	66,0	24,71	160,0
+50	78,80	44,9	55,77	96,3

F - Total Intensity; H - Horizontal Intensity; I - Magnetic Inclination; D - Magnetic Declination.

Supplementary Table 2. Curve fitting of data reported in Fig. 3. All p values were Bonferroni corrected for multiplicity. If the animals of the solitary group that displayed oriented freezing were excluded, the p value of the comparison of fits becomes = 0.4517. Where p≥0.05, only one shared curve drawn in the figure (null hypothesis true). n.d = not detectable as the p for the comparison of fits was >0.05.

Group	Comparison of fits	Pairwise comparison of fits		
			-50μT	0μT	+50μT
Solitary	p=0.1826	-50μT	-		
		0μT	n.d.	-	
		+50μT	n.d.	n.d.	-
			-50μT	0μT	+50μT
Shoaling	p<0.0001	-50μT	-		
		0μT	0.0210	-	
		+50μT	0.0003	0.1599	-
			-50μT	0μT	+50μT
Shoaling Co^{2+}-pretreated	p<0.0001	-50μT	-		
		0μT	0.0003	-	
		+50μT	0.0003	0.0291	-

Supplementary Table 3. Curve fitting of data reported in Fig. 4. All p values were Bonferroni corrected for multiplicity. Where p≥0.05, only one shared curve drawn in the figure (null hypothesis true). n.d = not detectable as the p for the comparison of fits was >0.05.

Group	Comparison of fits	Pairwise comparison of fits			
			Solitary	Shoaling	Shoaling Co^{2+}-pre-treated
-50µT	p<0.0002	Ind	-		
		S	0.0360	-	
		CoS	0.0006	0.0024	-
			Ind	S	CoS
0µT	p=0.1764	Ind	-		
		S	n.d	-	
		CoS	n.d.	n.d.	-
			Ind	S	CoS
+50µT	p<0.0001	Ind	-		
		S	0.0180	-	
		CoS	0.2208	0.0360	-

Supplementary Table 4. AvD values of Shoaling and Co^{2+}-pretreated animals, at the three induced magnetic field values (-50µT, 0µT and +50µT). Mean data are reported in absence and in the presence of water current. In all cases AvD was not affected by the water flow rate (tested by one-way ANOVA) so a mean value in the presence of water flow is reported. In Co^{2+}-pretreated at 0µT a significant difference was observed between absence/presence of water current. The asterisk indicates significant difference between absence and presence of current conditions (t-test, p<0.001, corrected for multiplicity).

Group	Flow rate	Magnetic field		
		-50µT	0µT	+50µT
Shoaling	O cm sec^{-1}	3.69±0.08	3.65±0.03	3.74±0.05
	3.3-8.5 cm sec^{-1}	3.74±0.08	3.67±0.05	3.51±0.06
Shoaling Co^{2+}-pretreated	O cm sec^{-1}	4.16±0.04	3.58±0.03	3.81±0.01
	3.3-8.5 cm sec^{-1}	3.76±0.07	2.18±0.04*	3.18±0.07