



Earth-strength magnetic field affects the rheotactic threshold of zebrafish swimming in shoals



Alessandro Cresci^a, Rosario De Rosa^{b,c}, Nathan F. Putman^{d,e}, Claudio Agnisola^{f,*}

^a Rosenstiel School of Marine & Atmospheric Science, 4600 Rickenbacker Causeway, Miami, FL 33149–1098, USA

^b Department of Physics, University of Naples Federico II, MSA Campus, Via Cinthia 21, 80126 Napoli, Italy

^c INFN, Section of Naples, MSA Campus, Via Cinthia 21, 80126 Napoli, Italy

^d Cooperative Institute for Marine & Atmospheric Studies, Rosenstiel School of Marine & Atmospheric Science, 4600 Rickenbacker Causeway, Miami, FL 33149–1098, USA

^e Atlantic Oceanographic and Meteorological Laboratory, National Oceanic and Atmospheric Administration, Miami, FL 33149, USA

^f Department of Biology, University of Naples Federico II, MSA Campus, Via Cinthia 21, 80126 Napoli, Italy

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ABSTRACT

Rheotaxis, the unconditioned orienting response to water currents, is a main component of fish behavior. 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.

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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 requires some frame of reference for fish to gauge the direction of water flow. Given that zebrafish occupy variable freshwater environments with highly turbid waters, such as seasonal floodplains, rice paddies, and slow streams (Engeszer et al., 2007; Spence et al., 2008), multimodal rheotaxis may have an important function for their ecology.

There has been long-standing speculation that the Earth's magnetic field could play a role in rheotaxis (Arnold, 1974). A benefit of using this cue is that the geomagnetic field is ubiquitous, and could provide a stable frame of reference by which current-induced displacement, water direction, or both could be detected. Linking magnetic orientation and rheotactic responses could greatly increase the efficiency of navigation, particularly in dynamic environments (Wyeth, 2010; Endres et al., 2016). Previous studies indicate that diverse animals can detect and use the magnetic field as a stationary cue for positional and compass information (e.g., Walker et al., 1997; Walker et al., 2002; Gould, 2010; Putman et al., 2013; Putman et al., 2014c; Putman et al., 2015). Likewise, zebrafish are known to detect Earth-strength static magnetic fields (Shcherbakov et al., 2005; Takebe et al., 2012; Osipova et al., 2016) and biogenic magnetite, which is associated with magnetoreception

* Corresponding author.

E-mail address: agnisola@unina.it (C. Agnisola).

(Kirschvink et al., 2001), has been found in the region of the lateral line (Dixon, 2011). Neuromast mechanosensors (canal and superficial) are involved in the fish orientation to water flows (Montgomery et al., 1997; McHenry and van Netten, 2007; Suli et al., 2012) and play an important role in the cohesive swimming of fish shoals (Faucher et al., 2010). However, whether magnetoreception and mechanoreception are behaviorally interrelated or whether the magnetic field influences rheotaxis, in general, has yet to be addressed experimentally.

Here we explore the hypothesis that rheotaxis of zebrafish is influenced by the presence and direction of an Earth-strength magnetic field. We performed a series of laboratory-based experiments, using a swimming tunnel that allowed us to quantify the rheotactic threshold of animals while controlling the intensity and the direction of the magnetic field relative to the direction of water flow. Specifically, we evaluated the rheotactic response by a stepwise increase in the velocity of water current and measuring at each step the time spent by the animals oriented upstream. As zebrafish naturally aggregate in shoals and schools (Pitcher and Parrish, 1993), we compared the behavior of individuals swimming alone or as part of a shoal. Furthermore, we examined whether the response of shoaling fish to rheotactic and magnetic stimuli involved the lateral line by also performing experiments with animals pre-treated with cobalt, to inhibit the lateral line functioning (Karlsen and Sand, 1987). We hypothesized that if the geomagnetic field plays a role in rheotaxis, upstream orientation of zebrafish might be influenced by the direction of the magnetic field with respect to water current. Our findings suggest that the rheotactic threshold is affected by the magnetic field in the geomagnetic range when animals swim in a shoal and implicate a possible role of the lateral line in this effect.

2. Materials and methods

2.1. Ethic statement

All animal procedures were approved by the Institutional Animal Care and Use Committee (CESA) of the University of Naples Federico II, Naples, Italy.

2.2. Animals

Adult zebrafish of the short-fin wild-type were provided by a local supply store (CARMAR SAS, S. Giorgio (NA), Italy). Animals were maintained in 200 l tanks, at a density of 1 animal per 2 l. Main water parameters in the maintaining tanks were monitored daily: temperature 27°–28 °C; conductivity < 500 μ S; pH 6.5–7.5; $\text{NO}_3^- < 0.25$ mg/l. Animals were fed once a day with commercial pelleted fish food (Tetramin, Tetra, Germany; 47% crude protein content, 6% humidity, 20.1 kJ/g dry mass) and displayed a normal shoaling behavior. Experimental individuals were selected randomly from the maintenance tank. All experiments were performed at the same time of the day (9.30–13.00), during March and October 2014. The average weight of selected animals was 0.76 ± 0.1 g (Mean \pm s.d., $N = 45$).

2.3. Swimming tunnel and magnetic field control

The swimming apparatus for zebrafish was from M2M Engineering Custom Scientific Equipment (Naples, Italy). As schematically shown in Fig. 1A, water flow was continuously measured by a SMC Flow switch flowmeter and controlled by a digital feedback system. Water in the swimming apparatus was identical to the maintenance water, was continuously aerated (oxygen content ~ 7.9 mg/l) and its temperature was maintained at 27 °C via a TECO 278 thermo-cryostat. The tunnel was a non-reflecting Plexiglas cylinder (7 cm diameter, 15 cm length) (Fig. 1B). Micro-turbulence in the tunnel was avoided by two perforated Plexiglas plates.

The intensity and direction of the horizontal component of magnetic field in the lab, measured along the major axis of the tunnel in the upstream direction (x axis, +70° of geographic north, Fig. 1C and E), was 11 μ T; the field along y axis was -25 μ T and that of z axis was 55 μ T ($F = 62$ μ T; $I = 64^\circ$; $D = 44^\circ$). These magnetic conditions were similar to those in the aquarium room for maintenance of animals, measured along the same axes ($x = 22$ μ T; $y = -27$ μ T; $z = 43$ μ T). No variation in the lab magnetic field was observed during the period of experimentation, being likely below the measurement equipment resolution (92 nT). For geographic context, the International Geomagnetic Reference Field (IGRF-12) predicted the geomagnetic field at site of the lab (40°N, 14°E) to be $F = 45.8$ μ T; $I = 56.1^\circ$; $H = 25.6$ μ T; $D = 2.91^\circ$ at the time of testing (www.geomag.nrcan.gc.ca).

We manipulated the magnetic field along one dimension, the same axis as the water flow (Fig. 1C, i.e., the x-axis) (one-dimensional magnetic field manipulation, according to Tesch, 1974). The magnetic field intensity and direction along the tunnel major axis were modified by wrapping the swimming tunnel with a solenoid (0.83 turns cm^{-1}) connected with a power unit to generate static magnetic fields (DC power supply ALR3003D, Elc, France). Fields of -50 μ T, 0 μ T and $+50$ μ T were utilized in order to observe the effect of field direction with respect to the water flow (y and z components were unchanged, see Fig. 1E). Positive induced magnetic field along the x axis was oriented opposite to the water current direction (upstream). The manipulated magnetic field did not vary along the swimming tunnel. The magnetic field was measured with a Gauss/Teslameter (9500 Gauss meter, DC 10 kHz, 0.092 μ T resolution, FW Bell, Orlando, USA).

2.4. Experimental protocol

We used a mixed design with one within-subject factor (flow speed) and three between-subject factors (solitary vs in-shoal swimming, magnetic field and cobalt pre-treatment). A repeated-measure design was used to manipulate flow within each animal group – magnetic field combination.

Animals were tested inside the magnetic swimming tunnel solitary or in a shoal of 5 individuals. Animals were acclimated for 1 h (Fig. 1D). During acclimation, the water flow was 1.73 cm s^{-1} . This flow rate was sufficient, according to preliminary experiments, to maintain the oxygen supply for the animals. After acclimation, we started the test with the induced magnetic field. At first, animals stayed for 10 min with no water current. Successively, we exposed the fish to a stepwise increase in the velocity of water current, from 1.95 cm s^{-1} to 8.45 cm s^{-1} (about 0.7–2.9 BL s^{-1}). The flow rate increased by 1.3 cm s^{-1} (about 0.4 BL s^{-1}) every 10 min for six times (60 min in total). The range of water speed was in the lower range of flow rates that induce continuous oriented swimming in zebrafish (3–15% of U_{crit}) (Plaut, 2000).

2.5. Experimental groups

Only naïve fish were used, and each fish experienced only one magnetic field condition. A first group was made of 15 animals that swam alone in the tunnel (mean body weight: 0.78 ± 0.05 g, standard length, 3.10 ± 0.05 cm); this group was divided in 3 sub-groups of 5 animals. Each sub-group was tested with a different magnetic field induced along the axis of water flow: -50 μ T, $+50$ μ T and a null magnetic field, 0 μ T (see above). A second group was made of 15 animals which were let to swim in groups of 5 animals (mean body weight: 0.80 ± 0.05 g; standard length, 3.00 ± 0.05 cm); each of the three groups was tested with a different induced magnetic field: -50 μ T, $+50$ μ T and a null magnetic field, 0 μ T (see above). These animals were tested in order to observe how the magnetic field affects the rheotactic orientation of individual zebrafish in the shoal, which is the natural state of aggregation for this species. Finally, as the integrity of lateral line may be important for the interaction of individuals in a shoal (Faucher et

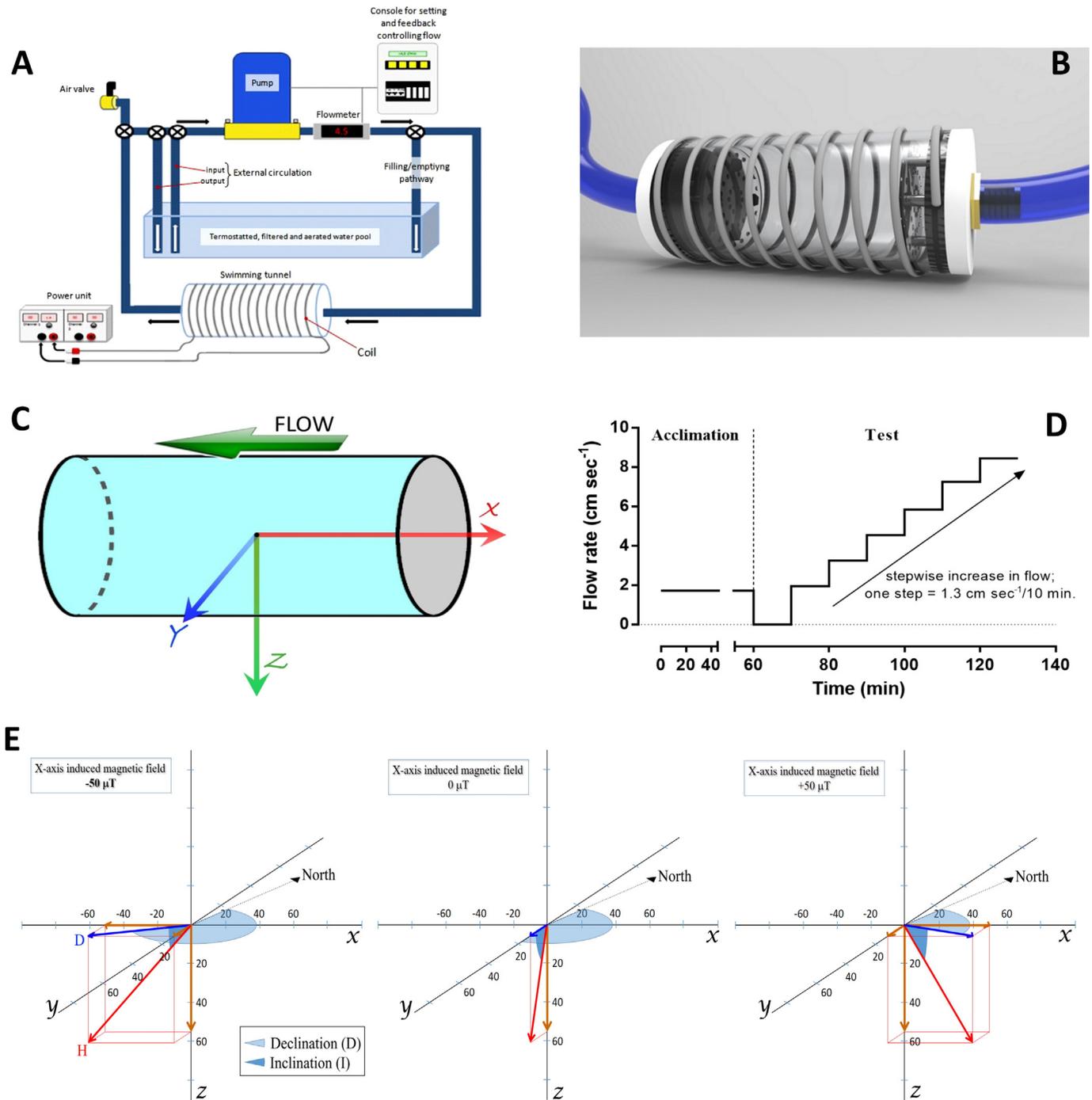


Fig. 1. Experimental apparatus and design. **A.** Simplified scheme of the swimming tunnel apparatus utilized in the present study. The coil around the tunnel allowed us to induce a static magnetic field inside the tunnel. Magnetic field lines were parallel to the water flow, and a positive induced magnetic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

al., 2010) and for magnetoreception (Dixson, 2011), we tested a third group of 15 animals, which were tested in groups of 5. These fish were previously treated with cobalt (24 h exposure to Ca^{2+} free, $0.1 \text{ mmol l}^{-1} \text{ CoCl}_2$ solution), which reversibly inhibits the lateral line (Karlsen and Sand, 1987) (mean body weight: $0.69 \pm 0.03 \text{ g}$, standard length, $2.81 \pm 0.04 \text{ cm}$). Again, each of the three groups of 5 animals were tested with a different induced magnetic field as above. The

efficacy of the cobalt (Co^{2+}) treatment on neuromasts was confirmed by treating animals from the same batch with 0.1 mM DASPEI (2-[4-(Dimethylamino) styryl]-1-ethylpyridinium iodide, Sigma) in fish water for 1 h, followed by deep anaesthesia in ice-cold 50 mg/ml MS222 (Sigma). Neuromasts were observed with a binocular fluorescent microscope (Leica AF6000) (Yoshizawa et al., 2010). Examples of observations are reported in Fig. 2, which show the virtual absence of

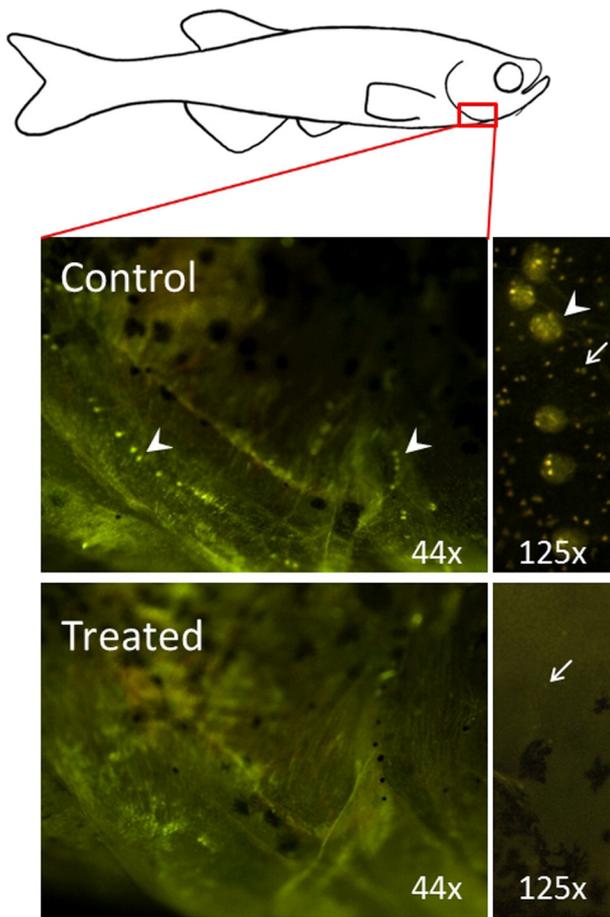


Fig. 2. Effect of cobalt treatment on zebrafish neuromasts, as checked by vital staining. The exposure of animals to 0.1 mM DASPEI (see [Materials and methods](#)) allowed visualization of both canal (arrowheads) and superficial (arrows) neuromasts in control zebrafish (upper pictures). Neuromasts appeared strongly reduced or absent in the animals treated for 24 h with 0.1 mmol l⁻¹ CoCl₂ (lower pictures).

DASPEI staining of both canal and superficial neuromasts in Co²⁺-treated animals. The animals used to check the effect of Co²⁺ were not used for the swimming test.

2.6. Observational analysis and data collection

The whole run in the tunnel was video recorded with the Sony DCR-TRV18E video camera and saved as mpg files (25 frames/s). Zebrafish behavior in the tunnel was studied from videos of the trials in all the experimental conditions. Behavior was blindly recorded and analyzed by trained observers (intra-rater reliability > 0.90). Only the second half (5 min) of each of the 10 min intervals corresponding to each experimental condition was analyzed, to allow for recovery from a possible anxiety response to the change in environment/flow rate (Wong et al., 2010). Fish behavior was analyzed by a video-tracking software (Tracker 4.84 Video Analysis and Modeling Tool). Tracking was performed manually at eight-frame intervals (0.32 s). The reference system used for the analysis had the x axis along the tunnel with the origin fixed in the lowest point of the ending wall from which the current came. The eye of each fish was used as reference for the fish tracking, allowing us to distinguish individuals in the shoal. Any video distortion was corrected using the perspective and radial distortion filters.

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 < 1.0 s. 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 s 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 of schooling behavior, by evaluating the degree of synchronization and polarization (swimming towards a common direction) of the fish composing the shoal during the upstream orientation. Effects of magnetic field manipulation on the values of this parameter would indicate whether magnetic information influences the degree of synchronized and polarized swimming during rheotaxis and thus identify a possible role of magnetic fields in group-swimming behavior. PDS was also evaluated for the group of solitary swimmers. Solitary swimmers were, by definition, run separately in different trials; thus, the PDS of these animals measured the probability that at least 4 individuals would display the same upstream orientation in the same moment during the runs, in absence of any possible inter-individual interaction. Any difference from the pattern showed by the solitary animals would indicate an among individual interaction effect in a group. We associated the PDS measure in shoals with that of the average inter-individual distance (AvD) (Miller and Gerlai, 2012).

Other parameters evaluated were the frequency of turns (min⁻¹) and the swimming rate (cm s⁻¹).

2.8. Statistical analysis

As the major aim of the study was to check the effects on individual's rheotactic behavior while swimming solitary or in shoal, individuals were considered as the statistical unit. The rheotactic index from the 5

individuals of each experimental sub-group is shown as mean \pm s.e.m. AvD, also expressed as mean \pm s.e.m., was calculated as the mean of the 5 average distances of each animal from the other 4 animals in a shoal (Miller and Gerlai, 2012). All statistics were performed on arcsine transformed data.

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

$$y = \min + \frac{(\max - \min)}{1 + 10^{(F50 - x)/\text{slope}}} \quad (1)$$

For RI and PDS, constraints were posed on both the min value (>0) and the max value (<100), as they can only vary between 0 and 100.

The threshold for the rheotactic response, i.e. the minimal flow at which a significant rheotactic response can be elicited (Baker and Montgomery, 1999), was extrapolated from the sigmoid fitting curves as the flow rate at which the curve displayed the highest rate of slope variation, individuated as the positive peak of the second derivative of the curve.

Within each animal group (solitary, shoal, and shoal treated with Co^{2+}), we tested whether the magnetic field affects the RI by checking the probability that the data fit three different curves (3 fields) rather than one (null hypothesis: no effect of field, all data from an animal group represent one statistical sample). To this aim, within each group, extra sum-of-square F test ($p < 0.05$) was used to test if the model could be applied to the three curves (the three induced magnetic fields) separately (significant effect of magnetic field), or if a single curve for all pooled data must be drawn (no significant effect of magnetic field). In the first case, we repeated the test for all pairwise comparisons of the three magnetic field curves, checking for significant differences between each pair of magnetic field conditions (-50 vs 0 ; -50 vs $+50$ and 0 vs $+50$). p values were corrected for multiplicity. When a significant effect of magnetic field was found, a comparison among threshold values was performed with a pairwise t -test comparison and a Bonferroni multiplicity correction of probabilities. A similar approach was used for the comparison of PDS–flow rate curves. However, as PDS is a parameter concerning the shoal behavior, the comparison was among animal groups at each magnetic field condition.

All the statistical tests were “two-tailed”. Statistics was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

3. Results

The Rheotactic Index (RI) of solitary swimming animals at increasing water flow under different magnetic fields cannot be represented by different curves (p for comparison of fits > 0.05), suggesting independence of RI–flow rate relationship from the magnetic field (Fig. 3 left lower panel). In contrast, the RI–water flow rate curves significantly differed depending on the magnetic field for shoaling zebrafish with and without pre-treatment of cobalt (p for comparison of fits < 0.05) (Fig. 3, middle and right lower panels). Thus, the RI of zebrafish swimming in shoal is influenced by the magnetic field.

The relationship between the Polarized Direction of Swimming (PDS) and water flow rate also appears to be influenced by the magnetic field (Fig. 4). For instance, while at $0 \mu\text{T}$ there was no significant difference among solitary, shoaling and Co^{2+} -treated shoaling animals (p for comparison of fits > 0.05), the three curves are significantly different at both $-50 \mu\text{T}$ and $+50 \mu\text{T}$ (p for comparison of fits < 0.05). Moreover, the field effect is clearly different if the field direction changes with respect to the water current direction. At $-50 \mu\text{T}$ (i.e., when the field is downstream oriented), at any given flow rate, there was a higher probability to find all the animals upstream oriented when swimming in a shoal compared to the solitary swimming, possibly suggesting that rheotaxis and schooling reinforce each other. At $+50 \mu\text{T}$ (i.e., when the field is upstream oriented) the opposite occurs, suggesting a

competition between rheotaxis and schooling. Cobalt treatment affects this magnetic field dependent schooling effect. At $-50 \mu\text{T}$ the probability to find the Co^{2+} -treated animals, swimming in a shoal, upstream oriented at a given flow is even lower than the solitary animals, while at $+50 \mu\text{T}$, the Co^{2+} -treated and solitary curves are not significantly different.

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

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

4. Discussion

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

4.1. Implications for understanding fish magnetoreception

The lateral line appears to be involved in mediating the effect of magnetic fields on rheotaxis, given that impairing the lateral line alters the rheotactic threshold under different magnetic fields. This finding could be linked with the observation that the lateral line system is involved in rheotaxis at low rates of flow (Montgomery et al., 1997; Montgomery et al., 2000). It should be noted that following Co^{2+} treatment, rheotactic threshold is still affected by the magnetic field, but the sensitivity to the magnetic field direction is altered. Walker et al. (1997) found that the ros V nerve in the anterior skull of rainbow trout (*Oncorhynchus mykiss*) responded to changes in the intensity but not the direction of imposed magnetic stimuli of $\pm 50 \mu\text{T}$. This nerve links the brain to the lamina propria cells, in the olfactory lamellae, where magnetite crystals were detected (Walker et al., 1997). These findings indicated that there are magnetoreceptive cells located in the olfactory system of trout. In this species, the ros V nerve is closely linked to the dorsal anterior lateral-line nerve (ros1) (Walker et al., 1997). In this context, it is worth noting that in addition to manipulating the component of the magnetic field along the axis of the swimming tunnel, the magnetic treatments altered the total field intensity and inclination angle (Fig. 1 and Supplemental Table 1), two field parameters that are perceptible to diverse taxa (Phillips et al., 2002; Boles and Lohmann, 2003; Putman et al., 2011; Kishkinev et al., 2015), including fishes (Putman et al., 2014b, 2014c). However, the change in total field intensity and inclination angle was identical for two of the magnetic treatments ($-50 \mu\text{T}$ and $+50 \mu\text{T}$). Thus, our results imply a direction-dependent sensitivity of zebrafish to an imposed magnetic field that can be altered by inhibiting the lateral line.

The ability to sense magnetic field direction may be related to the integration between magnetosensation and the rheotactic response, which depends from several sensorial pathways, tactile, visual and vestibular (Bak-Coleman et al., 2013; Montgomery et al., 2014), and that thus it is only partially altered after Co^{2+} treatment. Two explanations could be hypothesized. One possibility is that the magnetoreceptors

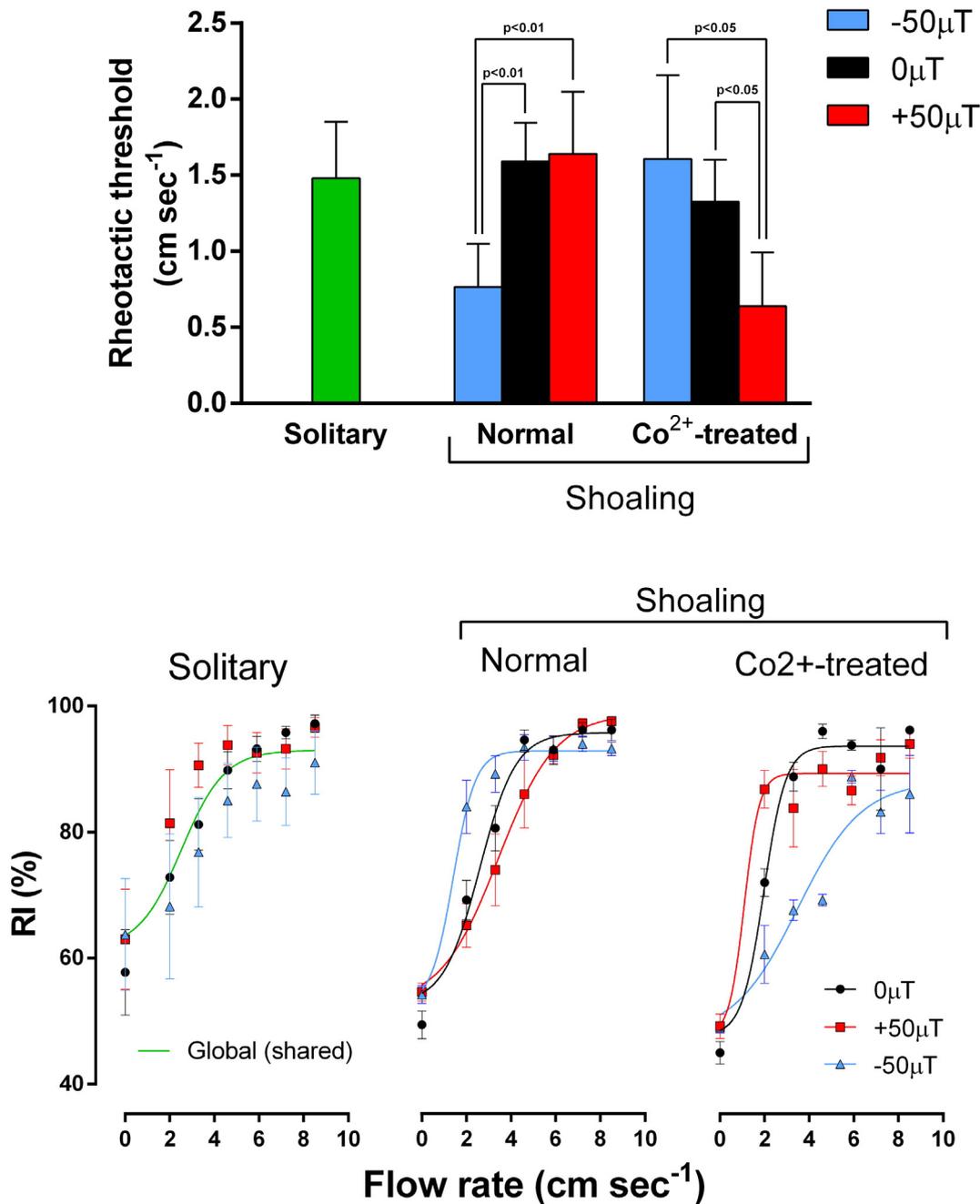


Fig. 3. Rheotactic response in zebrafish. Upper panels: Rheotactic threshold, of solitary (one combined value as the rheotactic response was not significantly affected by the magnetic field) and of shoaling (untreated and Co²⁺-treated) animals at the 3 induced magnetic field values. A pairwise comparison of thresholds by *t*-test was performed. The Bonferroni method was used to correct *p* values for multiplicity. Lower panel: RI (%) vs water flow rate in zebrafish of solitary and of shoaling (untreated and Co²⁺-treated) animals at the 3 induced magnetic field values (-50 μT, 0 μT and +50 μT exposed animals). The curves were fitted to a logistic sigmoidal model (see Materials and methods). Within each group, the curves from the three different magnetic field conditions were compared by the Extra sum-of-square *F* test to check if the points represent one single sample (one curve) or separated sample (three curves). Where *p* < 0.05, as was the case of the solitary and Co²⁺-treated animals, the test was repeated for a pairwise comparison of the three curves. The *p* values, which were corrected for multiplicity, are reported in Supplementary Table 2.

are located in the olfactory cells only, so that rheotactic and magnetic stimuli are post-processed and integrated in common neural pathways, allowing fish to elicit a consequent behavioral response. A second explanation is that magnetite crystals are located also in the lateral line, participating in magneto-sensation. In this last case, magneto-reception in fish would be complex, with magneto-sensitive cells located in different parts of the body surface, and only in part affected by Co²⁺ treatment. In this scenario, our results of cobalt treated zebrafish would suggest that the lateral-line cells participate to the detection of magnetic field

direction (while the olfactory cells might detect magnetic intensity also in this species, as in trout). There is evidence that magnetite is located in the lateral line region of zebrafish (Dixson, 2011), but further studies are necessary in order to validate the hypothesis of a magnetosensitive-lateral line in fish. Although the behavioral responses of cobalt treated zebrafish reported in this study suggest the lateral line is involved in the “magneto-rheotactic” behavior, the mechanism of the interaction between magnetic field detection and the neuromasts in rheotaxis appears to be complex and requires further research.

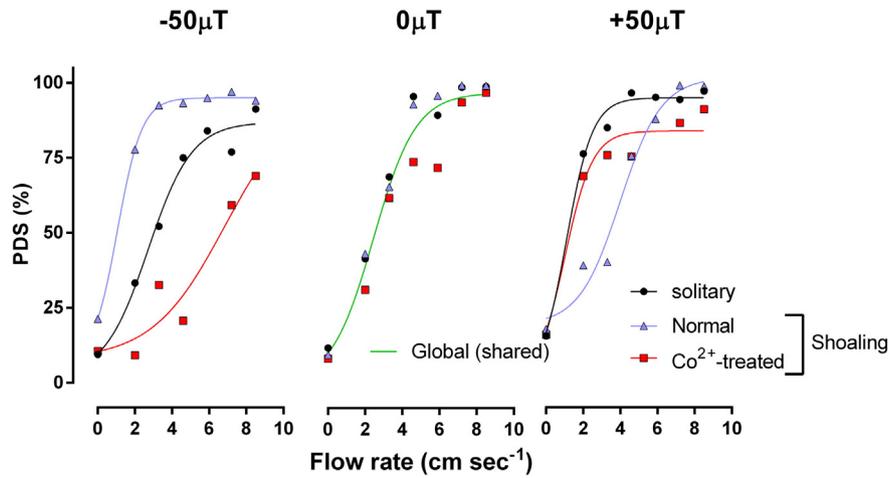


Fig. 4. PDS (%) vs water flow rate relationship of zebrafish at the 3 induced magnetic field values ($-50 \mu\text{T}$, $0 \mu\text{T}$ and $+50 \mu\text{T}$ exposed animals) of solitary and of shoaling (untreated and Co^{2+} -treated) animals. Data were statistically analyzed as described in Fig. 3. At $0 \mu\text{T}$ the Extra sum-of-square F test p value was not significant, whereas significant differences ($p < 0.05$) were observed when at fields of $-50 \mu\text{T}$ and $+50 \mu\text{T}$. For these conditions, the test was repeated for a pairwise comparison of the three curves and p values were corrected for multiplicity (see Supplementary Table 3).

4.2. Behavioral and ecological implications

The activation of the rheotactic response in zebrafish is pointed out by two main evidences: the decrease of the turning frequency (Plaut, 2000) and the increase of the time spent by the animals upstream oriented when flow rate increases. The last parameter, when measured as the proportion of time spent by the animals swimming upstream (RI), displays a sigmoidal relationship with flow rate, and it is used in the present study to quantify the rheotactic threshold. This choice was linked to the specific behavioral characteristics of this species. Zebrafish, is a very active fish with high swimming performance (Palstra et al., 2010), that swims most of the time (Fuiman and Webb, 1988), usually in the speed range at which the oxygen consumption is scarcely affected by swimming speed ($< 5 U_{\text{crit}}$, Palstra et al., 2010). This occurs also in respirometers or swimming tunnels, where they tend to swim along the major axis of the chamber, turning frequently, even in the presence of water flow, and tending to swim both upstream and downstream (Plaut and Gordon, 1994; Plaut, 2000). This behavior in the tunnel is affected by the water flow rate, disappearing at very high speeds ($> 8 \text{ BL s}^{-1}$, Plaut and Gordon, 1994), when the animals swim continuously with their heads directed upstream (full rheotactic response).

Our results show that the magnetic field significantly affects the rheotactic threshold when fish swim in shoal. This could be of ecological significance, as the threshold values here reported are in the range of zebrafish routine swimming speed in nature (Fuiman and Webb, 1988). As shown in Fig. 3, we see that: (i.) the rheotactic response elicited in our studies reaches its maximum at relatively low water speed ($2\text{--}3 \text{ BL s}^{-1}$); (ii.) when swimming in shoals, small variations in the water speed, or small variations in magnetic field at constant water flow significantly affect the tendency of individuals to orient upstream in slowing moving waters; (iii) no significant effect of magnetic field was observed in solitary animals.

In their natural environment, zebrafish inhabit narrow rivers and areas with slow water currents (floodplain, rice-fields and blind canals connected to the main rivers), characterized by considerable seasonal variability owing to monsoon winds (Spence et al., 2008). In these environments, turbidity is typically high (Spence et al., 2008) and visual cues might often be unreliable for detecting the drifting direction induced by the water flow. The relationship between the rheotactic threshold and the magnetic field could be consistent with the hypothesis that zebrafish may exploit magnetic cues for the seasonal migration to floodplain and back to streams (Spence et al., 2008; Takebe et al., 2012).

The activation of a rheotactic response induces a polarization of shoals. Polarization could be also the result of schooling, an effective behavior displayed by zebrafish that could be helpful for predator avoidance (Bode et al., 2010) and for information exchanging through the group (particularly important for migratory species) (Couzin et al., 2005). Schooling could reinforce the rheotactic response compared to the isolated animals. Indeed, in our experimental conditions, the polarization of a group of fish could be simply the result of the simultaneous rheotactic orientation of the animals, without any contribution of inter-individual interactions. Interestingly, this appears to be true only in absence of the magnetic field along the water flow axis. In presence of a magnetic field, individuals in shoals clearly behave differently from individual tested alone (see Fig. 4). The reduced inter-individual distance observed at $0 \mu\text{T}$ in Co^{2+} -treated, highlights the putative role of lateral line in determining animal interaction in zebrafish shoals (Partridge and Pitcher, 1980). Interestingly, the presence of the magnetic field seems to compensate for the lateral line deficiency in defining the inter-individual distances in the shoal and again this effect is most effective when the magnetic field has the same direction as the water current. Moreover, zebrafish naturally aggregate in shoals (Spence et al., 2008). The negative result of solitary zebrafish might be due to the non-natural state of isolation, as suggested by the fact that almost 30% of solitary fish displayed the stress-related “freezing” behavior (Kalueff et al., 2013). It is conceivable that increasing the sample size in individuals tested could result in detecting a statistical relationship for zebrafish in the solitary condition. Indeed, previous studies on species such as Pacific salmon (*Oncorhynchus* spp.), reported an effect of magnetic fields on the orientation of fish tested alone utilizing larger sample sizes (> 100 fish per treatment) (e.g., Quinn, 1980; Putman et al., 2014c). Our results, show that with a much smaller sample size, an influence of the magnetic field can be detected in fish when they are in shoaling condition. For zebrafish, this may suggest that testing animals in groups might be a more powerful way to study the sensory ecology of orientation.

4.3. Conclusions

The results here reported strongly suggest that the ability of zebrafish to sense the geomagnetic field could affect their ability to react to water current when swimming in shoals. In particular, results imply that fish display the highest rheotactic response (i.e., the lowest threshold) when fish swim in shoal, have a functioning lateral line and the horizontal magnetic field has the same direction as the water

current. These results suggest that the geomagnetic field, which is already known to provide numerous animals with stationary reference information (Walker et al., 2002; Lohmann et al., 2008b; Putman et al., 2014c), might also provide a reference point for fish rheotaxis. However, these results should not be considered definitive, but as an initial, exploratory investigation in the topic. Additional studies are required to understand the mechanisms of the integration of magnetic signal detection with the other sensorial systems involved in the multimodal rheotaxis.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cbpa.2016.11.019>.

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