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1 **TITLE:**

2 Assessing the Influence of Personality on Sensitivity to Magnetic Fields in Zebrafish

3

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21 **KEYWORDS:**

22 Zebrafish, personality, fish behavior, orientation, magnetic field, rheotaxis

23

24 **SUMMARY:**

25 We describe a behavioral protocol designed to assess how zebrafish's personalities influence
26 their response to water currents and weak magnetic fields. Fishes with the same personalities
27 are separated based on their explorative behavior. Then, their rheotactic orientation behavior in
28 a swimming tunnel with a low flow rate and under different magnetic conditions is observed.

29

30 **ABSTRACT:**

31 To orient themselves in their environment, animals integrate a wide array of external cues, which
32 interact with several internal factors, such as personality. Here, we describe a behavioral protocol
33 designed for the study of the influence of zebrafish personality on their orientation response to
34 multiple external environmental cues, specifically water currents and magnetic fields. This
35 protocol aims to understand whether proactive or reactive zebrafish display different rheotactic
36 thresholds (i.e., the flow speed at which the fish start swimming upstream) when the surrounding
37 magnetic field changes its direction. To identify zebrafish with the same personality, fish are
38 introduced in the dark half of a tank connected with a narrow opening to a bright half. Only
39 proactive fish explore the novel, bright environment. Reactive fish do not exit the dark half of the
40 tank. A swimming tunnel with low flow rates is used to determine the rheotactic threshold. We
41 describe two setups to control the magnetic field in the tunnel, in the range of the earth's
42 magnetic field intensity: one that controls the magnetic field along the flow direction (one
43 dimension) and one that allows a three-axial control of the magnetic field. Fish are filmed while
44 experiencing a stepwise increase of the flow speed in the tunnel under different magnetic fields.

45   Data on the orientation behavior are collected through a video-tracking procedure and applied
46   to a logistic model to allow the determination of the rheotactic threshold. We report
47   representative results collected from shoaling zebrafish. Specifically, these demonstrate that
48   only reactive, prudent fish show variations of the rheotactic threshold when the magnetic field
49   varies in its direction, while proactive fish do not respond to magnetic field changes. This
50   methodology can be applied to the study of magnetic sensitivity and rheotactic behavior of many
51   aquatic species, both displaying solitary or shoaling swimming strategies.
52
53   **INTRODUCTION:**
54   In the present study, we describe a lab-based behavioral protocol which has the scope of
55   investigating the role of fish personality on the orientation response of shoaling fish to external
56   orientation cues, such as water currents and magnetic fields.
57
58   The orienting decisions of animals result from weighing various sensory information. The decision
59   process is influenced by the ability of the animal to navigate (e.g., the capacity to select and keep
60   a direction), its internal state (e.g., feeding or reproductive needs), its ability to move (e.g.,
61   locomotion biomechanics), and several additional external factors (e.g., time of day, interaction
62   with conspecifics)[1].
63
64   The role of the internal state or animal personality in the orientation behavior is often poorly
65   understood or not explored[2]. Additional challenges arise in the study of the orientation of social
66   aquatic species, which often perform coordinated and polarized group movement behavior[3].
67
68   Water currents play a key role in the orientation process of fish. Fish orient to water currents
69   through an unconditioned response called rheotaxis[4], which can be positive (i.e., upstream
70   oriented) or negative (i.e. downstream oriented) and is used for several activities, ranging from
71   foraging to the minimization of energetic expenditure[5,6]. Moreover, a growing body of literature
72   reports that many fish species use the geomagnetic field for orientation and navigation[7–9].
73
74   The study of rheotaxis and swimming performance in the fish is usually conducted in flow
75   chambers (flume), where fish are exposed to the stepwise increase of the flow speed, from low
76   to high speeds, often until exhaustion (called critical speed)[10,11]. On the other hand, previous
77   studies investigated the role of the magnetic field in the orientation through the observation of
78   the swimming behavior of the animals in arenas with still water[12,13]. Here, we describe a
79   laboratory technique that allows researchers to study the behavior of fish while manipulating
80   both the water currents and the magnetic field. This method was utilized for the first time on
81   shoaling zebrafish (*Danio rerio*) in our previous study, leading to the conclusion that the
82   manipulation of the surrounding magnetic field determines the rheotactic threshold (i.e., the
83   minimal water speed at which shoaling fish orient upstream)[14]. This method is based on the use
84   of a flume chamber with slow flows combined with a setup designed to control the magnetic field
85   in the flume, within the range of the earth's magnetic field intensity.
86
87   The swimming tunnel utilized to observe the behavior of zebrafish is outlined in **Figure 1**. The
88   tunnel (made of a nonreflecting acrylic cylinder with a 7 cm diameter and 15 cm in length) is

89   connected to a setup for the control of the flow rate[14]. With this setup, the range of flow rates in
90   the tunnel varies between 0 and 9 cm/s.
91
92   To manipulate the magnetic field in the swimming tunnel, we use two methodological
93   approaches: the first is one-dimensional and the second is three-dimensional. For any
94   application, these methods manipulate the geomagnetic field to obtain specific magnetic
95   conditions in a defined volume of water—thus, all the values of magnetic field intensity reported
96   in this study include the geomagnetic field.
97
98   Concerning the one-dimensional approach[15], the magnetic field is manipulated along the water
99   flow direction (defined as the x-axis) using a solenoid wrapped around the swimming tunnel. This
100 is connected to a power unit, and it generates uniform static magnetic fields (**Figure 2A**).
101 Similarly, in the case of the three-dimensional approach, the geomagnetic field in the volume
102 containing the swimming tunnel is modified using coils of electric wires. However, to control the
103 magnetic field in three dimensions, the coils have the design of three orthogonal Helmholtz pairs
104 (**Figure 2B**). Each Helmholtz pair is composed of two circular coils oriented along the three
105 orthogonal space directions (*x*, *y*, and *z*) and equipped with a three-axial magnetometer working
106 in closed-loop conditions. The magnetometer works with field intensities comparable with the
107 earth's natural field, and it is located close to the geometrical center of the coils set (where the
108 swimming tunnel is located).
109
110 We implement the techniques described above to test the hypothesis that the personality traits
111 of the fish composing a shoal influence the way they respond to magnetic fields[16]. We test the
112 hypothesis that individuals with proactive and reactive personality[17,18] respond differently when
113 exposed to water flows and magnetic fields. To test this, we first sort zebrafish using an
114 established methodology to assign and group individuals that are proactive or reactive[17,19–21].
115 Then, we evaluate the rheotactic behavior of zebrafish swimming in shoals composed of only
116 reactive individuals or composed of only proactive individuals in the magnetic flume tank, which
117 we present as sample data.
118
119 The sorting method is based on the different tendency of the proactive and reactive individuals
120 to explore novel environments[21]. Specifically, we use a tank divided into a bright and a dark
121 side[17,19–21] (**Figure 3**). Animals are acclimated to the dark side. When access to the bright side is
122 open, proactive individuals tend to quickly exit the dark half of the tank to explore the new
123 environment, while the reactive fish do not leave the dark tank.
124
125 **PROTOCOL:**
126 The following protocol has been approved by the Institutional Animal Care and Use Committee
127 of the University of Naples Federico II, Naples, Italy (2015).
128
129 **1. Animal maintenance**
130
131 1.1. Use tanks of at least 200 L to host a shoal of at least 50 individuals of both sexes in each tank.
132

133 NOTE: The density of the fish in the tank has to be one animal per 2 L or lower. Under these
134 conditions, zebrafish will display normal shoaling behavior.

135

136 1.2. Set the maintenance conditions as follow: temperature at 27–28 °C; conductivity at <500 μS;
137 pH 6.5–7.5; NO_3 at <0.25 mg/L; and a light:dark photoperiod at 10 h:14 h.

138

139 NOTE: Identical holding conditions must be used for both the mixed population and the
140 separated proactive and reactive populations.

141

142 **2. Personality selection in zebrafish**

143

144 2.1. Prepare and place the personality selection tank in a quiet room (**Figure 3**) with the same
145 water as used in the maintenance tanks.

146

147 2.2. Place a video camera above or at the side of the tank. Connect the camera to a computer
148 with a monitor located in an area where there is no visual contact with the tank.

149

150 NOTE: Try to limit the interactions with the tanks and fish to the least amount of time possible.
151 Avoid noise and fast movements.

152

153 2.3. Select nine fish at random from the maintenance tank and transfer them to the dark side of
154 the personality selection tank, using a knotless net.

155

156 NOTE: If necessary, transfer the animals in a small-volume transporting tank (about 2 L) with
157 water from the holding tank. The fish must be fed ad libitum prior to the transfer to the
158 experimental tank. This limits the possibility that different tendencies of food-seeking behavior
159 would affect the behavior of the individuals during the following experiment[22]. Try to minimize
160 the capture time, avoid collecting multiple fish as it might cause physical damage to the animals,
161 and do not hold the fish for more than a few seconds in the net as these factors can increase
162 stress. Conduct replicate experiments at the same time of the day. This minimizes variability in
163 the behavior of the experimental groups caused by possible circadian rhythms[23].

164

165 2.4. After 1 h of acclimatization, open the sliding door.

166

167 NOTE: Individuals who exit from the hole, exploring the bright side of the tank within 10 min, are
168 considered proactive[21].

169

170 2.5. After 10 min, gently remove the proactive individuals from the tank and transfer them to the
171 proactive maintenance tank.

172

173 2.6. After 15 min, collect the fish that remain in the dark box, which are considered reactive[21],
174 and transfer them to the reactive maintenance tank.

175

176 NOTE: Discard fish that move to the bright side of the tank after 10 min[21]. Perform the personality

test with nine fish at a time until the desired number of proactive and reactive fish necessary for the tests described in section 5 are collected. Consistency of the proactive and reactive personality can be checked regularly using the same approach.

3. Set up of the magnetic field with the one-dimensional magnetic field manipulation[27]

3.1. Switch on the Power unit (**Figure 2A**).

3.2. Place the coiled tunnel in the location where the rheotactic protocol will be performed (section 5) but keep it disconnected from the swimming apparatus (**Figure 2A**). Place a magnetic probe connected with a Gauss/Teslameter inside the tunnel and verify which voltage is necessary to obtain the chosen magnetic field value along the major axis of the tunnel.

NOTE: Because of the magnetic properties of a solenoid, the field is reasonably uniform inside the tunnel; this can be checked by slowly moving the probe both horizontally and vertically.

3.3. Disconnect the probe and connect the flow tunnel to the swimming apparatus.

3.4. Start with the rheotactic protocol (section 5).

4. Set up of the magnetic field with the three-dimensional magnetic field manipulation[27]

4.1. Switch on the CPU, DAC, and coil drivers (**Figure 2B**).

4.2. Set the chosen magnetic field on each one of the three axes (x, y, and z).

4.3. Place the tunnel in the center of the Helmholtz pairs set.

4.4. Start with the rheotactic protocol (section 5).

5. Test of the zebrafish rheotaxis in the flow chamber

5.1. Transfer one to five fish to the flow tunnel using a 2 L tank with the sides and the bottom obscured.

5.2. Turn on the pump and set the flow rate in the tunnel to 1.7 cm/s.

NOTE: This slow-moving water will keep the water in the tunnel oxygenated and it will facilitate animal recovery.

5.3. Let the animals acclimate to the swimming tunnel for 1 h.

5.4. Start the video recording of the behavior of the fish in the tunnel.

221 NOTE: We used a camera (e.g., Yi 4K Action) with remote control (e.g., Bluetooth) and saved the
222 video as .mpg (30 frames/s).

224 5.5. Start the stepwise increase of the flow rate according to the chosen experimental protocol
225 (1.3 cm/s in this study; **Figure 4**).

227 NOTE: For this protocol, we used low flow rates which, for zebrafish, range from 0 to 2.8 BL (body
228 lengths)/s. These flow speeds are in the lower range of flow rates that induce continuous
229 oriented swimming in zebrafish (3%–15% of critical swimming speed [U_{crit}])[24]. The use of low flow
230 rates (following Brett's protocol[25]) is linked to the specific behavioral characteristics of this
231 species in the presence of water currents. Zebrafish tend to swim along the major axis of the
232 chamber, turning frequently, even in the presence of water flow, and tend to swim both
233 upstream and downstream[24,26]. This behavior is affected by the water flow rate, disappearing at
234 relatively high speeds (>8 BL/s)[26], when the animals continuously swim facing upstream (full
235 positive rheotactic response). Vertical and transversal displacements are very rare.

237 5.6. Perform morphometry of the animals (sex and total length [TL], fork length [FL], or BL) on
238 screenshots from the video recordings of the fish in the swimming tunnel.

240 5.6.1. Select the appropriate screenshot.

242 5.6.2. Open the screenshot in ImageJ.

244 5.6.3. Take note of the sex of the animal (male zebrafish are slender and tend to be yellowish,
245 while females are more rounded and tend to have blue and white colorings).

247 5.6.4. Click **Analyze** > **Set Scale** and set the scale of the image in centimeters, using the whole
248 horizontal length of the tunnel as reference.

250 5.6.5. Click **Analyze** > **Measure** and record the linear length of the animal.

252 5.6.6. Calculate its body weight (BW).

254 NOTE: BW is calculated from sex-FL-BW relationships previously built in the lab or from metadata.
255 The whole procedure avoids manipulation stress on the animals.

257 **6. Video tracking**

259 6.1. Open the video file with Tracker 4.84 Video Analysis and Modeling Tool.

261 NOTE: If necessary, correct any video distortion using perspective and radial distortion filters.

263 6.2. Click on **Coordinate system** in the upper menu and set the length units to centimeters and
264 the time units to seconds.

265

266 6.3. Click on **File** > **Import** > **Video** and open one of the videos in Tracker 4.84.

267

268 6.4. Click on **Coordinate axes** and set the reference system to track the position of the fish over
269 time with the x-axis along the tunnel. Set the origin at the low corner of the wall upstream (at
270 the water inlet).

271

272 6.5. Click on **Track** > **New** > **Point of mass** and start tracking one fish at a time. Track the last 5
273 min of each step that the fish spent at each flow rate.

274

275 6.6. Advance the video manually at five-frame intervals (0.5 s) and mark the time and position of
276 the animal at each upstream-downstream turn (UDt; red dots in **Figure 5**) and at each
277 downstream-upstream turn (DUt; blue dots in **Figure 5**).

278

279 NOTE: Use the fish eye position as a reference for the fish's position. Track the animal's position
280 using a Point mass. Exclude from the tracking any period of non-oriented swimming (i.e.,
281 maneuvering time).

282

283 6.7. At the end of each tracking session, select the x-values and time values from the table at the
284 bottom-right corner of the software window. Right-click on the data and click **Copy data** > **Full**
285 **precision**.

286

287 6.8. Save the time values and x-values of all turning positions on a template spreadsheet file to
288 calculate the total upstream time (sum of all the intervals between UDts and DUts) and the total
289 downstream time (sum of the intervals between DUts and UDt), as well as the values of the
290 rheotactic index in percentages (RI%) for each flow step (see **Figure 5**).

291

292 NOTE: The rheotactic behavior is quantified by the proportion of the total oriented time that the
293 fish spend facing upstream (swimming or rarely freezing [i.e., they stay still at the bottom of the
294 tunnel][27]). This proportion is defined as the RI% (**Figure 5**).

295

296
$$RI\ (\%) = \frac{upstream\ time}{(upstream\ time + downstream\ time)} \times 100$$

297

298 **REPRESENTATIVE RESULTS:**
299 As sample data we present results obtained controlling the magnetic field along the water flow
300 direction on proactive and reactive shoaling zebrafish[16] using the setup shown in **Figure 2A** (see
301 section 3 of the protocol). These results show how the described protocol can highlight
302 differences in responses to the magnetic field in fish with different personalities. The overall
303 concept of these trials relies on the finding that the direction of the magnetic field relative to the
304 water flow affects the rheotactic threshold in shoaling zebrafish[14]. Thus, as changes in the
305 magnetic field modulate the rheotaxis, this protocol can be used to assess if the response of
306 zebrafish to magnetic fields differ according to their proactive or reactive personality[28].

307

308   At first, using the dark/bright tank as shown in **Figure 3**, zebrafish were split into different groups
309   according to their proactive/reactive personality. Following such a test, shoals of five fish with
310   the same personality were then tested in the solenoid swimming tunnel (**Figure 1** and **Figure 2A**).
311   A total of 20 fish were tested: two shoals composed of five reactive fish each (10 reactive fish)
312   and two shoals composed of five proactive fish each (10 proactive fish).

313

314   One shoal at a time was video recorded while swimming in the tunnel and the water current was
315   accelerated with a stepwise increase of the flow rate as schematically shown in **Figure 4**. The fish
316   were allowed to acclimate for 1 h in the tunnel. After that, we applied the protocol for the
317   quantification of the rheotactic behavior, using a stepwise increase of the flow rate according to
318   the classic Brett protocol[25]. Specifically, the flow rate increased by 0.4 BL/s every 10 min for a
319   total of seven consecutive steps (**Figure 4**). The behavior of the zebrafish was recorded
320   throughout the whole duration of the run in the tunnel (70 min), and the RI value at each step
321   was calculated (see protocol step 6.8).

322

323   During the runs in the swimming tunnel, the magnetic field was set at one of the two following
324   conditions: 50 µT downstream (i.e., the horizontal component [along the x-axis] of the magnetic
325   field had the same direction of water flow) and 50 µT upstream (i.e., the horizontal component
326   of the magnetic field had an opposite direction with respect to the water flow)[16]. The intensity
327   along axes y and z were not affected, as well as the total intensity and inclination of the magnetic
328   field vector. Each shoal of five fish was exposed to only one of the two magnetic conditions. For
329   example, considering proactive fish, one proactive shoal had the magnetic field directed
330   downstream and the other proactive shoal had the magnetic field directed upstream.

331

332   The videos were then analyzed with the video-tracking software (section 6 of the protocol). The
333   fish were video recorded for the whole duration of the run in the swimming tunnel. However,
334   only the last 5 min of each 10 min-long stepwise increase of flow rate (**Figure 4**) were tracked.
335   During the tracked time, the turns of each fish at each flow rate were highlighted (**Figure 5**, red
336   and blue data points). These were then used as references to calculate the RI of each fish and
337   each flow speed (**Figure 5**). The RI index ranges between 0% and 100%. When below 50%, the RI
338   index indicates that the fish displayed negative rheotaxis (prevalence of downstream swimming);
339   when the RI is higher than 50%, it shows that the animal had a positive rheotactic response
340   (prevalence of upstream swimming). An RI not significantly different from 50% would indicate an
341   absence of rheotactic response. The values of RI% of all the five fish in a shoal were then averaged
342   at each flow rate. These averaged data were arcsine transformed and used to fit the curves
343   displayed in **Figure 6A**. Thus, the rheotactic index increases sigmoidally when the water speed
344   increases, allowing the quantification of the rheotaxis with a simple mathematical method. The
345   relationship between the RI and the flow rate can be fitted to the following logistic-sigmoidal
346   model.

347

348 $$RI = RI_{bottom} + \frac{(RI_{plateau} - RI_{bottom})}{(1 + 10^{(R_{tr} - flow\ rate)})}$$

349

350   Three parameters and their variability can be derived from the fit curve. The $RI_{plateau}$ measures
351   the maximal tendency of the animals to orient upstream in the range of flow rates used in the
352   experiment. RI_{bottom} is the RI value in the absence of water flow and, hypothetically, should not
353   differ from 50%. R_{tr} is the is the flow rate at which the maximum slope of the curve occurs, and
354   it can be used as a measure of the rheotactic threshold[6].

355

356   The results indicate that the rheotactic threshold (R_{tr}) of zebrafish is very low, in the range of a
357   few centimeters per second. Variations of the magnetic field do not affect the R_{tr} of proactive
358   fish (no effect of the magnetic field, t-test, $P > 0.05$). Oppositely, magnetic field changes have a
359   pronounced effect on the rheotactic behavior of reactive zebrafish. When the magnetic field
360   component along the swimming tunnel was directed downstream, the R_{tr} is very low and similar
361   to that of proactive fish. The threshold was significantly higher when the magnetic field was
362   directed upstream (t-test, $P < 0.01$).

363

364   The $RI_{plateau}$ value of reactive animals was significantly lower when the magnetic field was directed
365   upstream (t-test, $P < 0.01$). This result indicates that with these conditions, reactive fish would
366   reach the full positive rheotactic response (RI = 100%) only at very high flow rates. Thus, this
367   result highlights that, compared to R_{tr}, $RI_{plateau}$ provides less information about the swimming
368   behavior of the fish. In fact, based on the strong difference in the reactive $RI_{plateau}$ between the
369   two magnetic conditions, we can state that, under the upstream-oriented magnetic field, the
370   reactive animals will probably display a full rheotactic response at a higher water flow.

371

372   RI_{bottom} values tend to be higher (although not significantly) than 50% in the proactive animals
373   and in the reactive animals exposed to a downstream-oriented magnetic field. This may indicate
374   a bias in the protocol since the animals characterized by a very low threshold may remember the
375   flow direction experienced during acclimation. A proper protocol could be devised to test this
376   possibility.

377

378   **FIGURE AND TABLE LEGENDS:**

379

380   **Figure 1: Simplified representation of the swimming tunnel apparatus utilized in the present**
381   **study.**

382

383   **Figure 2: Setup for magnetic field control.** (**A**) Rendering of the swimming tunnel with a solenoid
384   for the induction of a static, horizontal magnetic field within the tunnel. The solenoid (0.83
385   turns/cm) is connected to a power unit and it generates fields in the range of ±250 µT (intensity
386   range that includes the earth's magnetic field range). On the right-hand side, a photo of the
387   solenoid tunnel connected to the swimming apparatus is shown. The tunnel is made of acrylic
388   and it has two perforated acrylic plates placed at the water inlet, which guarantee the flow to be
389   close to laminar. (**B**) Diagram and photo of the three orthogonal Helmholtz pairs set for the
390   control of the magnetic field in the geomagnetic range of intensities. The magnetic field probe,
391   the CPU, the digital-to-analog converter, and the coil drivers used to close the loop are also
392   shown. Each pair of coils is composed of two circular coils with a radius (r) of 30 cm and N = 50
393   turns of AWG-14 copper wires. A three-axes magnetometer (sensor) with selectable scale (± 88

394 μT to ± 810 μT) is placed close to the center of the coil set. The sensor range is set to values
395 ranging to ±130 μT. These values were also used for the measurements described in the
396 representative results (in these conditions, the nominal sensor resolution is about 0.1 μT). The
397 intensity and the direction of the magnetic field are controlled with a digital feedback system.
398 The sensor measures the three components of the magnetic field vector (the three axes), and
399 the corresponding error signals are extracted. Then, the correction signals are generated by a
400 simple integrator filter. The digital correction signals are converted to voltage by a digital-to-
401 analog converter and amplified by a suitable coil driver. These last signals are used to drive the
402 Helmholtz pairs. The sampling frequency is fixed to 5 Hz and the unity gain frequency of the loops
403 is about 0.16 Hz. Once the currents in the Helmholtz pairs of the coils are set, the total magnetic
404 field varies less than 2% from its mean intensity value in the central cubic volume (with edge [L]
405 = 10 cm) of the coils. During the measurements, the magnetic field rms is less than 0.2 μT. In both
406 the setups (panels **A** and **B**) a static electric field is generated by the current in the coils producing
407 the magnetic field[16]. The intensity of the electric field is about 0.4 V/m when the maximum
408 current is applied; this value is negligible compared to natural or artificial static fields present in
409 the environment whose intensity is of the order of 1 kV/m[17].

411 **Figure 3: Schematic representation of the tank (40 cm x 40 cm x 40 cm) used to separate**
412 **proactive from reactive zebrafish individuals (not in scale), according to Rey et al.[21].** The volume
413 of the personality selection tank is 50 L. Half of the tank was occupied by a dark box with a hole
414 of 5 cm in diameter on the side of the box facing the bright half of the tank. The hole was covered
415 by a sliding door (not shown), whose opening signed the start of a selecting trial. The dark side
416 of the tank needs a removable cover to allow access of hand nets. This facilitates placing or
417 catching fish before and after the behavioral trials.

419 **Figure 4: Diagram of the flow rates used during the tests to determine the rheotactic threshold**
420 **of zebrafish.** The flow during the 1 h acclimation period was enough to guarantee an adequate
421 oxygen supply to the animals. It can be assumed that, with this design, oxygen supply is never a
422 limit, even in the first 10 min step with flow 0. Indeed, with an oxygen content of water at 27 °C
423 of about 7.9 mg/L and an animal oxygen consumption of 1 mg/h.g (an excess approximation for
424 zebrafish oxygen consumption both under routine conditions [Uliano et al.[29]] and at low-speed
425 swimming [Palstra et al.[30]]), it is possible to calculate that, in the absence of flow, the Po_2 in the
426 flume will not decrease more than 2% per animal, remaining well above the critical Po_2 (about
427 40 torr for zebrafish). This figure has been modified from Cresci et al.[14].

429 **Figure 5: Animal behavior in the tunnel and the calculation of RI.** The graphs present the
430 position of an individual animal along the x-axis during a 300 s record at three values of flow rate.
431 The red dots represent the downstream-to-upstream turns, the blue dots the upstream-to-
432 downstream turns. The corresponding time intervals spent by the animals downstream or
433 upstream are also reported, and the total upstream and downstream times are reported, from
434 which an RI value can be calculated. It can be observed that when increasing the flow rate, the
435 upstream time and the RI values increase.

437 **Figure 6: Representative results.** (**A**) Relationship between arcsine-transformed RI values (RI is

438   the percentage of the total oriented time that fish spend facing upstream) and the flow rate for
439   proactive and reactive shoaling zebrafish under two magnetic field conditions along the flow rate
440   direction (one-dimension control). Each data point is the average of the RI values of the five fish
441   composing the shoal, at each flow rate. Significant differences between curves were tested via a
442   sum-of-squares *F*-test (alpha = 0.05)[14]. (**B**) Magnetic field axes and direction of the water flow in
443   the tunnel. A three-dimensional representation of the magnetic vectors in the two magnetic field
444   conditions used in this study is also shown. The magnetic field in the lab (40°N, 14°E) was: F = 62
445   µT; I = 64°; D = 44°. This figure has been modified from Cresci et al.[16].
446

DISCUSSION:
447

448   The protocol described in this study allows scientists to quantify complex orientation responses
449   of aquatic species resulting from the integration between two external cues (water current and
450   geomagnetic field) and one internal factor of the animal, such as personality. The overall concept
451   is to create an experimental design that allows scientists to separate individuals of different
452   personality and investigate their orientation behavior while controlling separately or
453   simultaneously the external environmental cues.
454

455   The protocol described in this study, together with the mathematical definition of the rheotactic
456   index (RI), was designed following preliminary observations of the behavior of zebrafish in the
457   swimming tunnel. When placed in a tunnel, these animals display two types of behavioral
458   patterns, both in the absence or presence of water flow: oriented swimming and maneuvering.
459   They spend the majority of the oriented time (usually, the fish were video recorded for more
460   than 95% of the time) swimming along the tunnel (i.e., oriented along the long axis with an angle
461   lower than 45°), back and forth, turning in proximity of the end walls and often showing
462   thigmotaxis (i.e., swimming close to the walls of the tunnel)[27].
463

464   For this protocol to be successful, it is important that whoever performs the experiment pays
465   attention to the stress of the animals. The transportation of the fish between the experimental
466   setups must be executed with care. The usage of hand nets should be as quick as possible, and
467   some training before the actual experiment is highly recommended as zebrafish are fast
468   swimmers and hard to catch in a tank. Stress can dramatically affect the behavior of these
469   animals, and in the case of zebrafish, it can significantly change their swimming behavior[27]. This
470   would probably affect the results, as fish might display hyperactive behavior and be less sensitive
471   to water flows and magnetic field changes. Contact with the animals in the experimental setup
472   should be as quick and short as possible. The behavioral analysis requires remote observation,
473   which also requires practice. Moreover, it is important to analyze the videos blindly (i.e., without
474   knowing protocols and treatments).
475

476   The rheotactic behavior of many species of fish has been studied using swimming tunnels[5,10,11,31].
477   Numerous previous studies focused on the estimation of the swimming speed that fish can
478   sustain until exhaustion, which is defined as U_{crit}, mostly to test physiological and ecological
479   hypotheses[11,32,33]. The method described in this study focuses, instead, on the rheotactic
480   behavior at low flow speeds. This choice was made because the objective of this study is to assess
481   the sensitivity to a subtle and weak cue like the magnetic field through the observation of a well-

known and robust orientation behavior of fish, the positive rheotaxis. In the representative results reported here, zebrafish displayed a very low rheotactic threshold (only a few centimeters per second). This observation could be ecologically relevant for this species, which inhabits environments where the speed of water currents can significantly vary. Zebrafish live both in turbulent rivers[34] and in water bodies where the water moves slowly, such as paddies, ponds, and floodplains[35]. When water moves slowly, the ability to detect and orient at low flow speeds (a low rheotactic threshold) could possibly be advantageous, as the rheotactic response increases the chances to intercept downstream-drifting prey[36] and provides directional stimuli for migration[37].

These observations could not be made using flows with high rates. These would elicit a strong locomotory response that would depend more on the body conditions and high swimming performances of the animals rather than external cues like the magnetic field. The protocol presented here was applied on zebrafish, but it is likely suitable for any freshwater or marine species that inhabit environments with moving water and can be handled in laboratory conditions.

However, this protocol presents some limitations. While it clearly highlights whether a species is sensitive or nonsensitive to magnetic fields, it cannot reveal the orientation mechanisms through which the animal uses magnetic fields for movement decisions. In order to investigate the magnetic orientation mechanisms in aquatic species, setups with circular arenas and still water[7,38,39] or mazes[13] are commonly used. However, fish (and aquatic animals in general) do not live in environments where currents are absent, and the presented method is a first attempt to investigate the integrative behavioral response to ubiquitous directional cues, like water flows and magnetic fields. Another limitation of this protocol is the manual video-tracking procedure. Integrating this setup with an automatic tracking software would improve the timing of the whole data analysis process.

The experimental protocol presented here is the first one designed to investigate the influence of animal personality on magnetic sensitivity and rheotaxis. This topic has been overlooked in the literature and needs to be further explored. Individuals from the same species, or even within a population or small group (such as a fish shoal), are characterized by different personality traits[22,40], which can be an important factor in studies on migratory, exploratory, navigation, and orientation behavior. Not all the individuals integrate the environmental cues in the same way. Thus, taking into account internal factors, such as personality, could help to reduce the data variability which is commonly observed in studies on movement ecology[16].

526

DISCLOSURES:

The authors have nothing to disclose.

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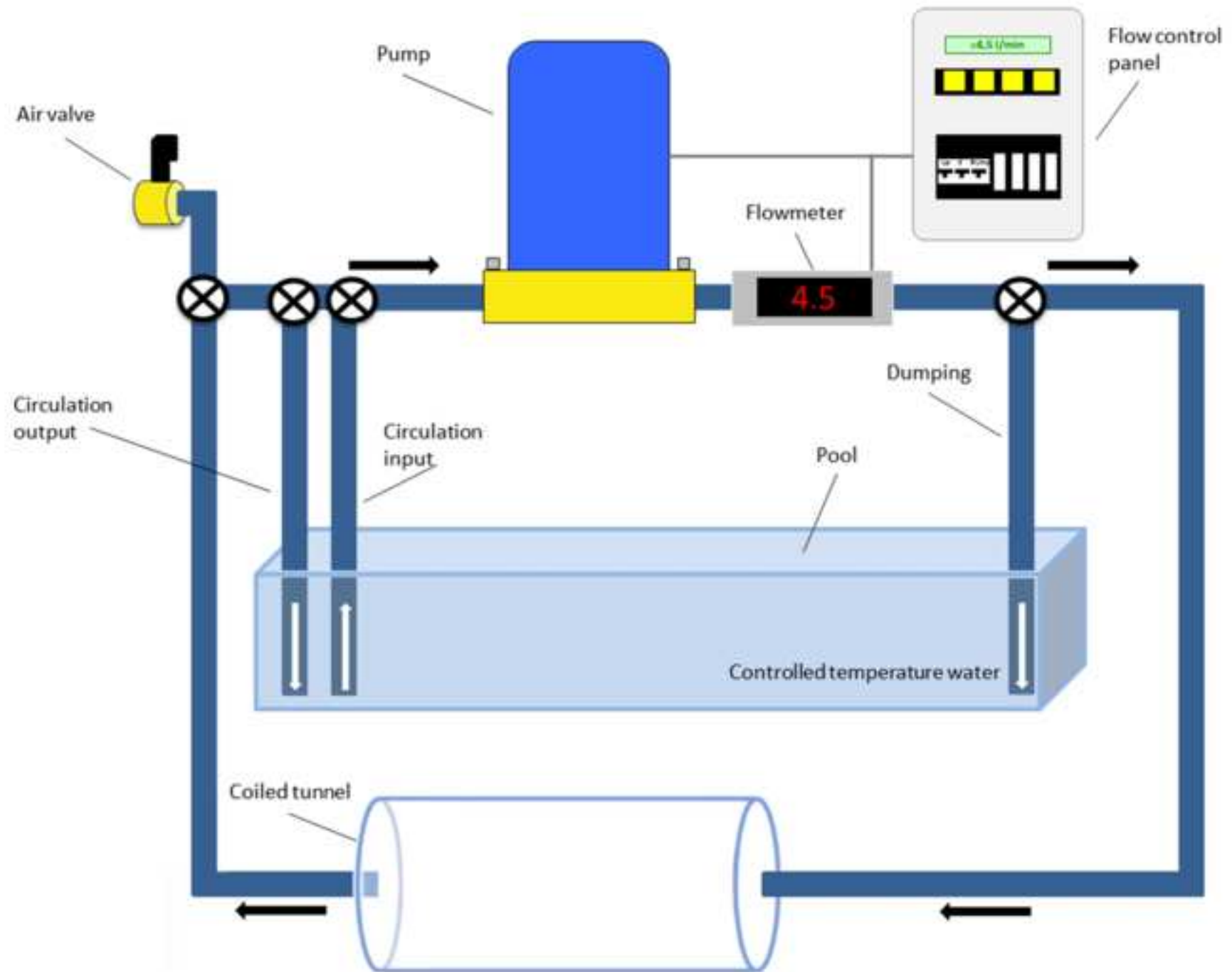
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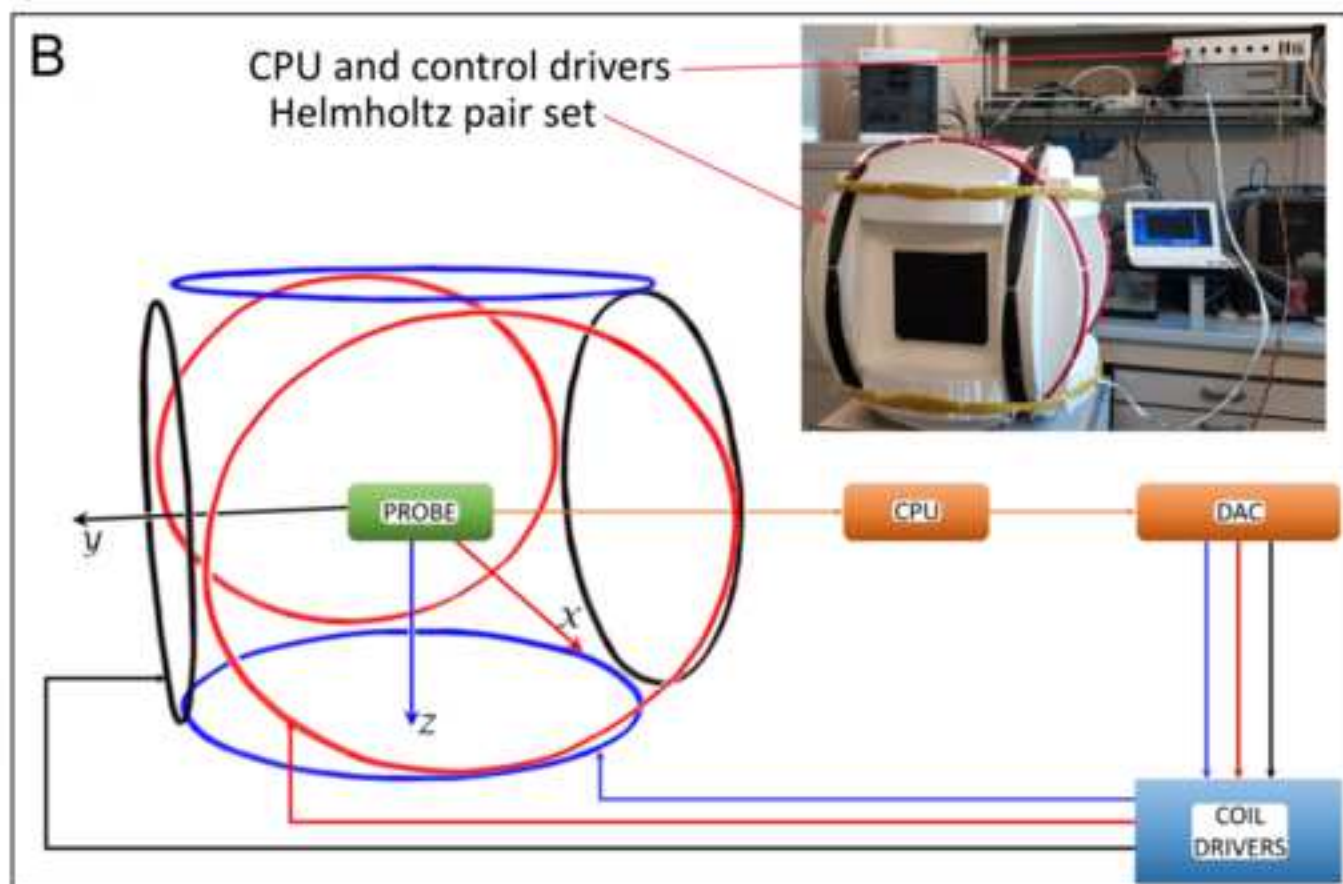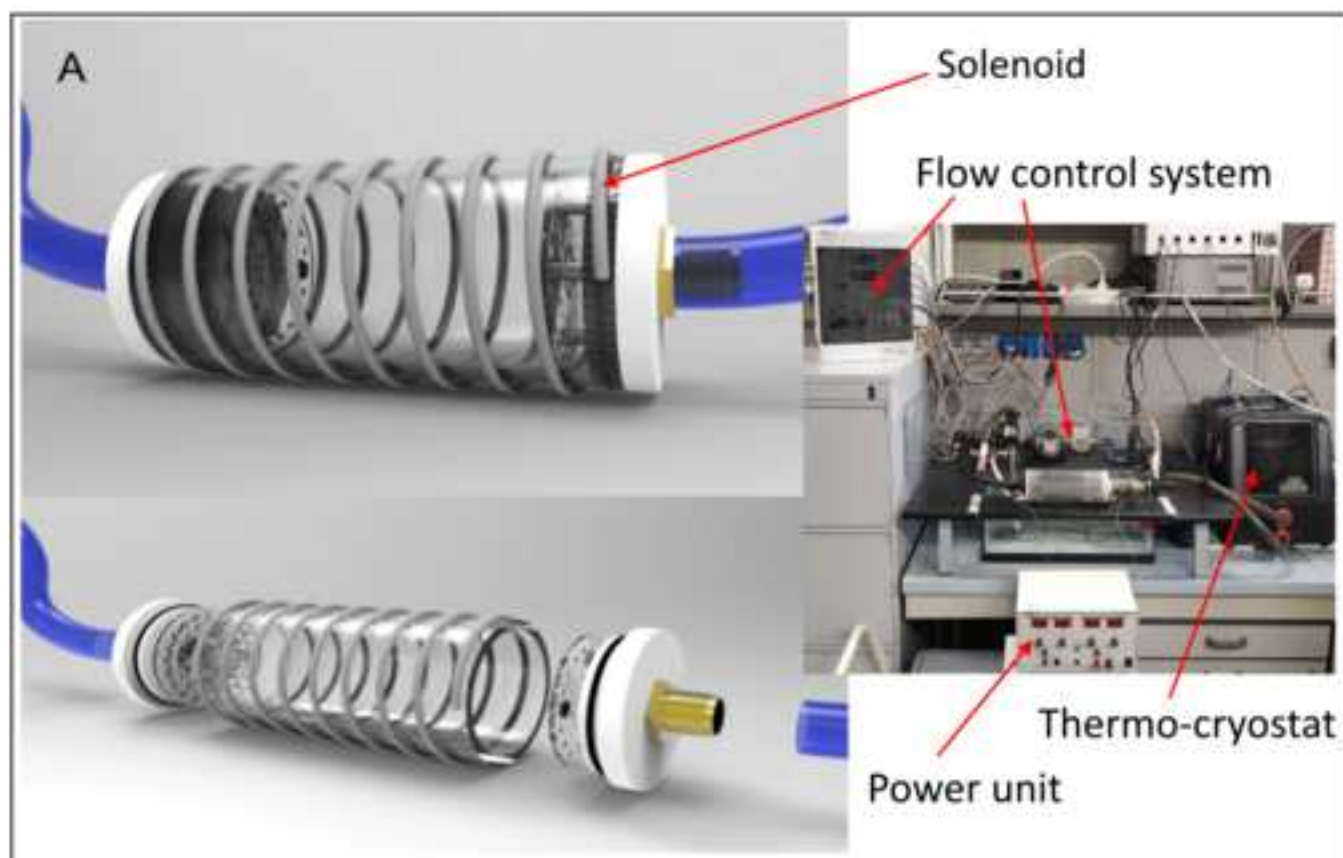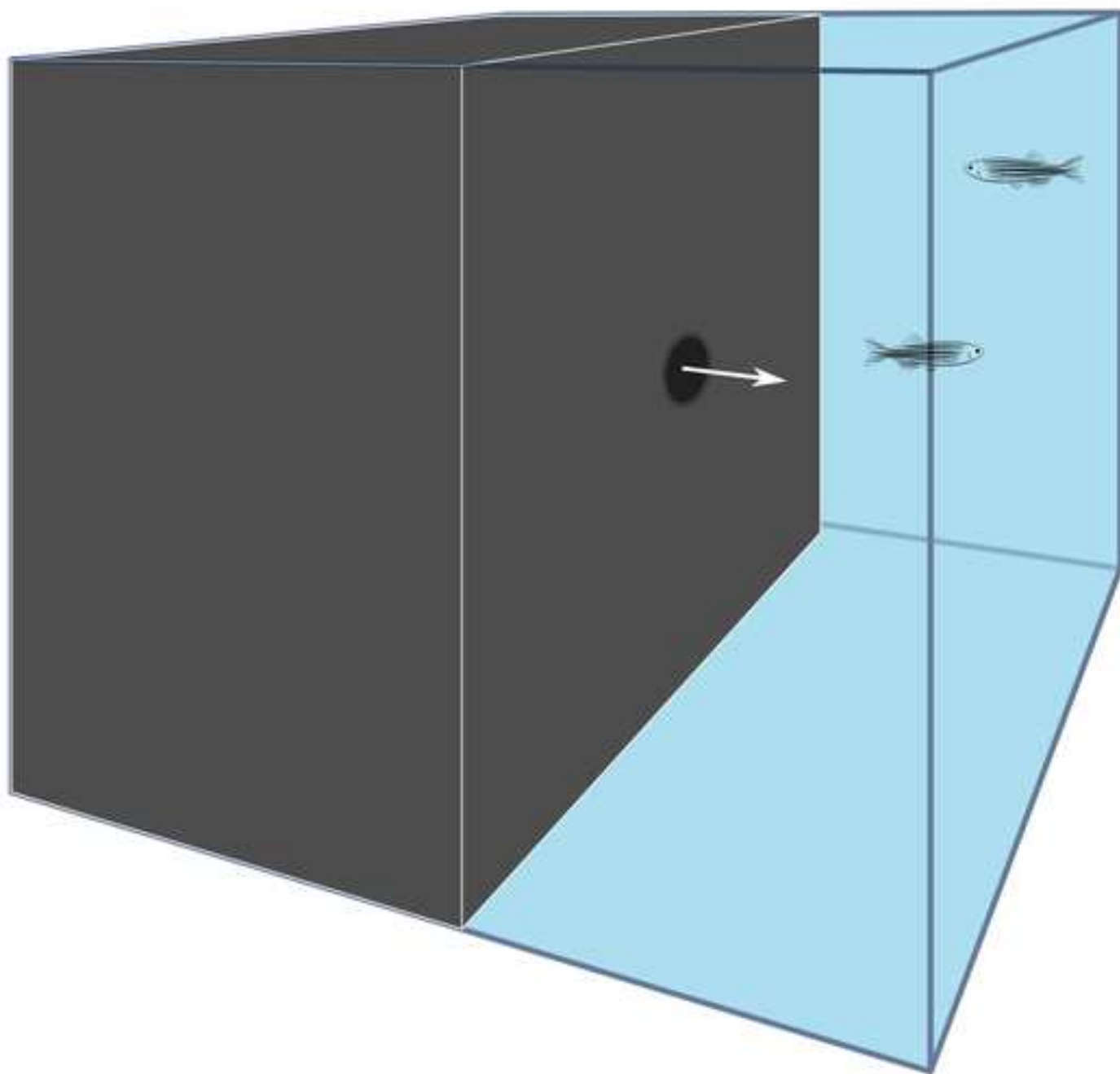
Figure 1

Figure 1
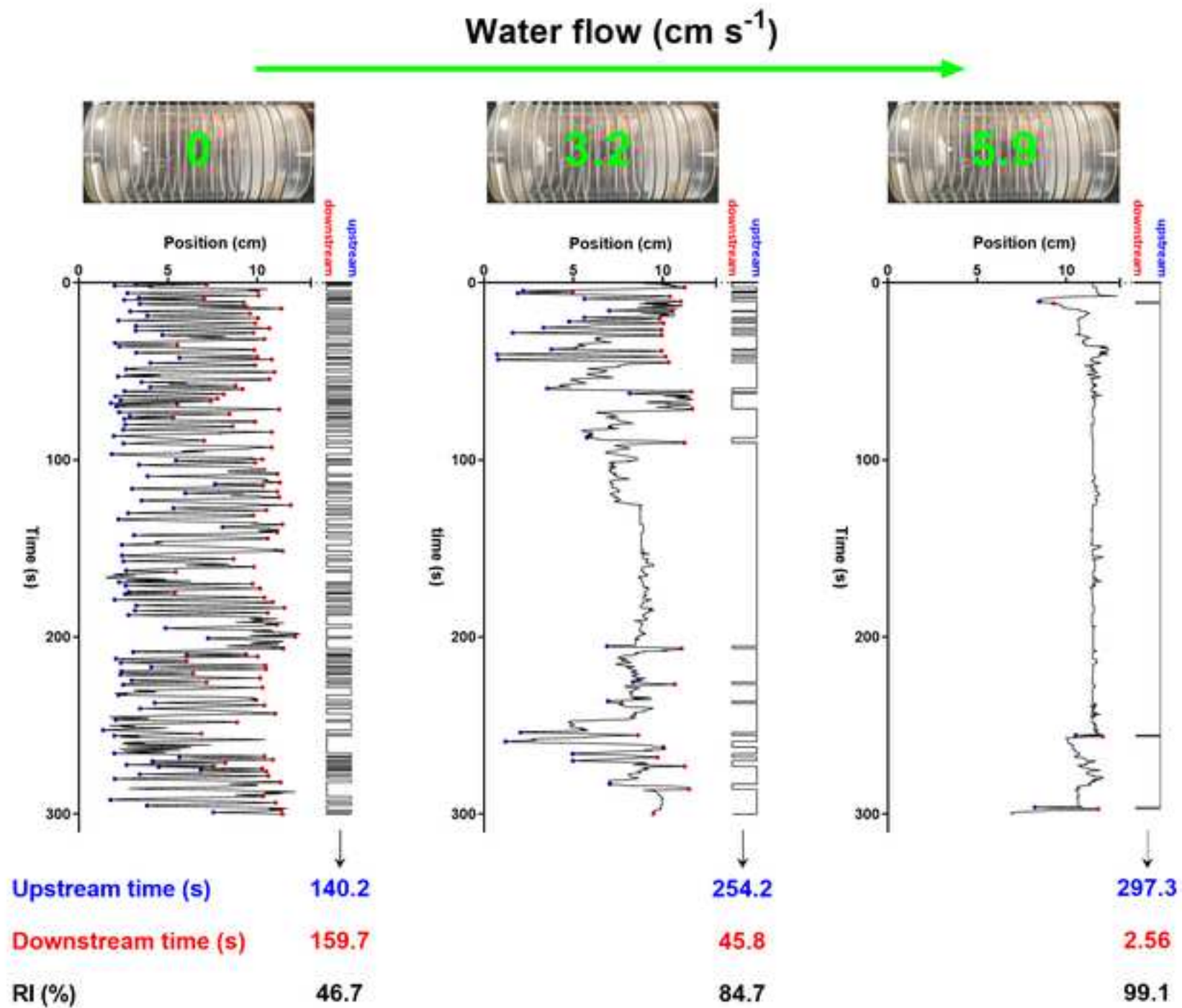
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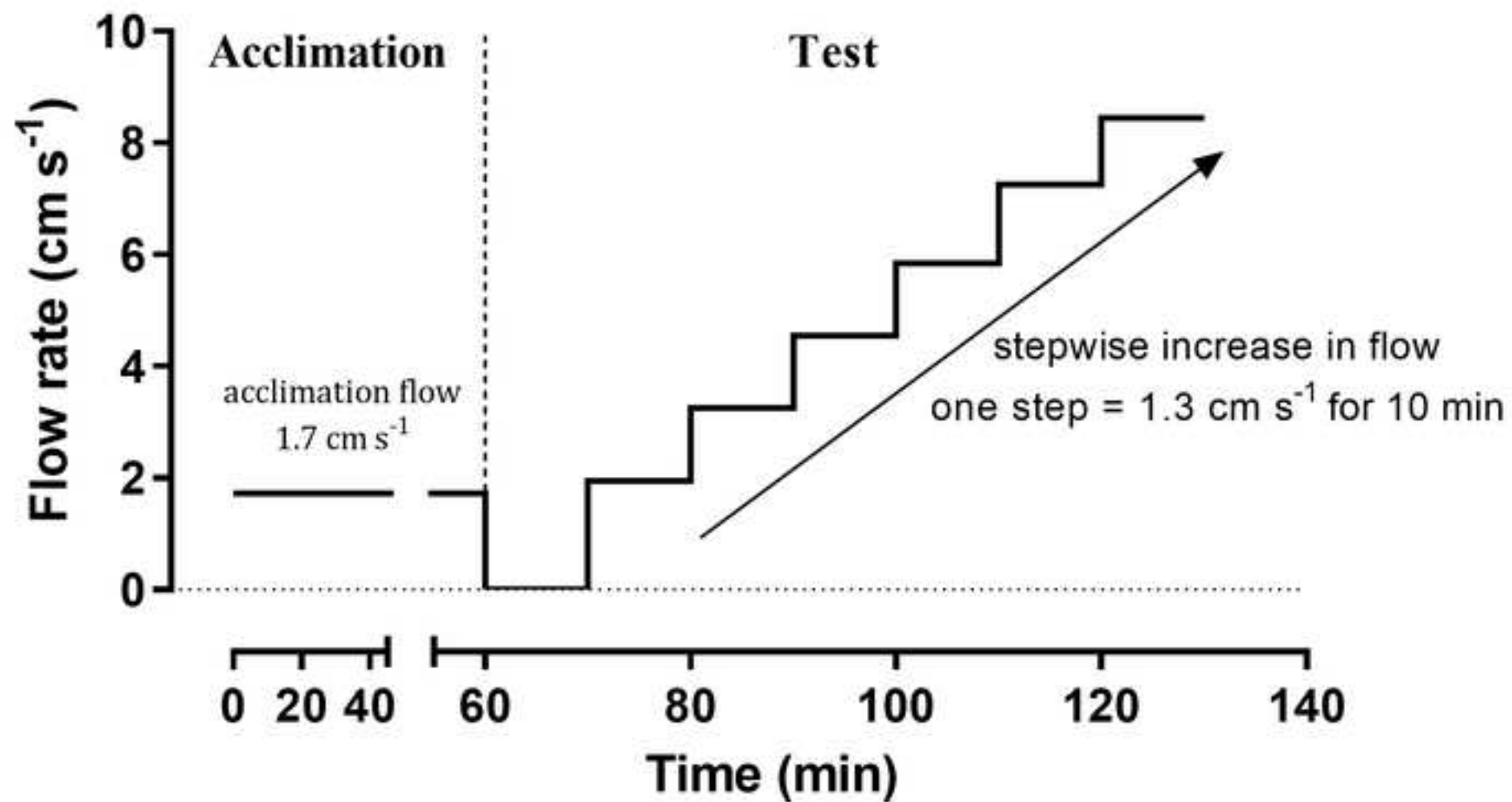
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A

Solenoid

Flow control system

Thermo-cryostat

Power unit

B

CPU and control drivers
Helmholtz pair set

PROBE

CPU

DAC

COIL DRIVERS

Figure 3

Figure 5

Click here to access/download;Figure;Figure 5.tif ⬇

Water flow (cm s⁻¹)

	140.2	254.2	297.3
Upstream time (s)	140.2	254.2	297.3
Downstream time (s)	159.7	45.8	2.56
RI (%)	46.7	84.7	99.1

Figure 6
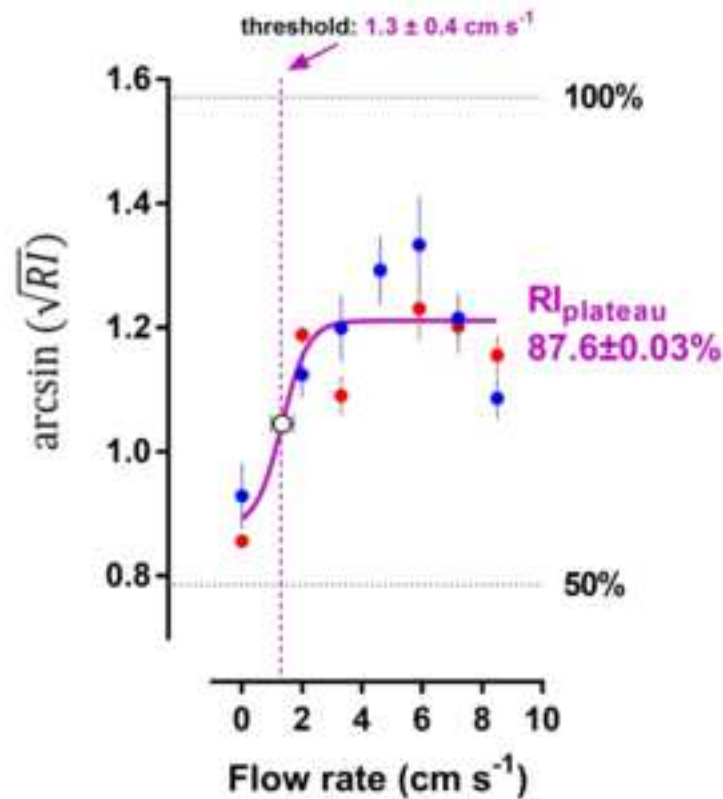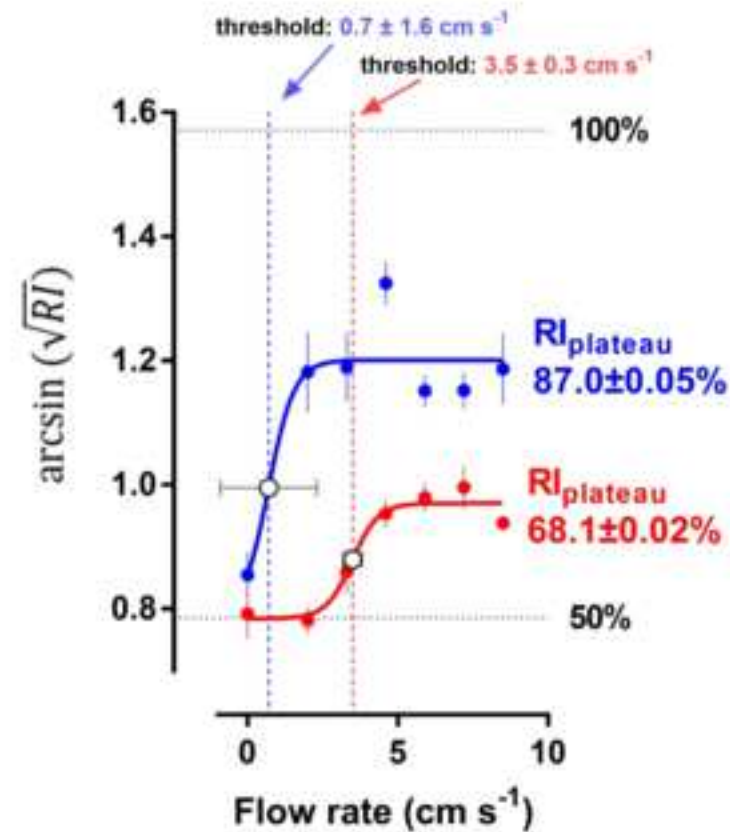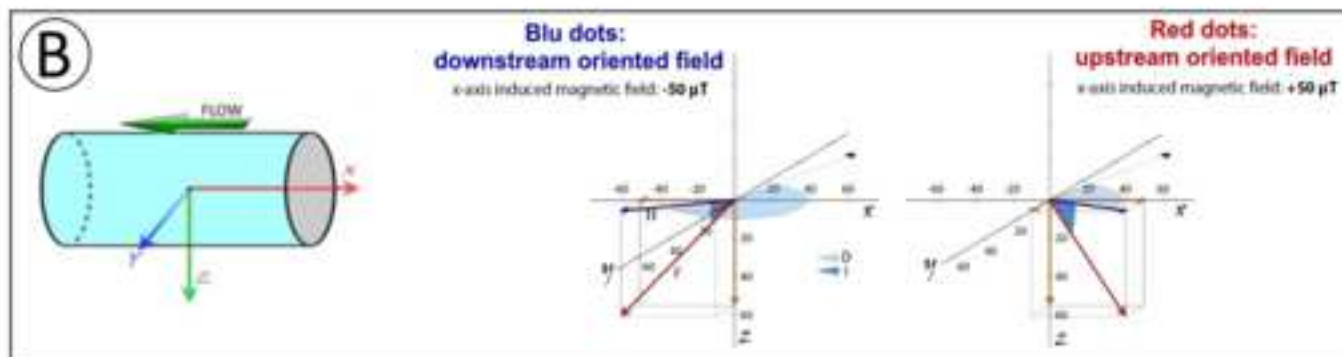
Table of Materials

Name of Material/ Equipment	Company	Catalog Number
9500 G meter	FWBell	N/A
AD5755-1	Analog Devices	EVAL-AD5755SDZ
ALR3003D	ELC	3760244880031
BeagleBone Black	Beagleboard.org	N/A
Coil driver	Home made	N/A
Helmholtz pairs	Home made	N/A
HMC588L	Honeywell	900405 Rev E
MO99-2506	FWBell	129966
Swimming apparatus	M2M Engineering Custom Scientific Equipment	N/A
TECO 278	TECO	N/A

Comments/Description

Gaussmeter, DC-10 kHz; probe resolution: 0.01 µT

Quad Channel, 16-bit, Digital to Analog Converter

DC Double Regulated power supply

Single Board Computer

Amplifier based on commercial OP (OPA544 by TI)

Coils made with standard AWG-14 wire

Digital three-axis magnetometer

Single axis magnetic probe

Swimming apparatus composed by peristaltic pump and SMC
Flow switch flowmeter with digital feedback

Thermo-cryostat

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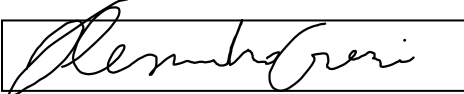
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Replies to comments

We thank the editor for the helpful comments. We followed the comments step by step and modified the manuscript accordingly, especially the protocol section. We updated the order of the figures, which is now different compared to the previous version of the manuscript.

All the edits in the manuscript are visible as we left the option "Track changes" active in the .docx document.

Please find our replies to the comments in blue.

Sincerely,

Alessandro Cresci

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1. The editor has formatted the manuscript to match the journal's style, please retain the same.
We retained the same format as the version of the manuscript provided by the editor.

2. Please address all the specific comments marked in the manuscript.
We addressed every comment we found in the manuscript. In case the comments from the editor are no longer visible it is because we had to erase that specific part of text. However, all the edits applied to the manuscript follow the instructions provided by the comments of the editor.

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The protocol now contains only short action items. All the additional information is provided in the introduction, the results or as NOTE.

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All the action items are now in imperative form.

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We moved all the text between sections to the introduction, to the results or as a note.

6. Please ensure you answer the "how" question, i.e., how is the step performed?
We now answer to "how" in almost every step.

7. Once done please ensure that the protocol is no more than 10 pages and highlighted step is no more than 2.75 pages including headings and spacing.
The protocol is less than 10 pages and the highlighted text is 2 pages long.

8. Please format all units as g/ml and not g ml-1. Please change this for the figures as well.

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