

1 **Behavioural and life-history responses of mosquitofish to**
2 **biologically-inspired and interactive robotic predators**

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20 **ABSTRACT**

21 Invasive alien species threaten biodiversity worldwide and contribute to biotic
22 homogenization, especially in freshwaters where the ability of native animals to disperse is
23 limited. Robotics may offer a promising tool to address this compelling problem, but
24 whether and how invasive species can be negatively affected by robotic stimuli is an open
25 question. Here, we explore the possibility of modulating behavioural and life-history
26 responses of mosquitofish by varying the degree of biomimicry of a robotic predator, whose
27 appearance and locomotion are inspired by natural mosquitofish predators. Our results
28 support the prediction that real-time interactions at varying swimming speeds evoke a more
29 robust antipredator response in mosquitofish than simpler movement patterns by the
30 robot, especially in individuals with better body conditions that are less prone to take risks.
31 Through an information-theoretic analysis of animal-robot interactions, we offer evidence in
32 favour of a causal link between the motion of the robotic predator and a fish antipredator
33 response. Remarkably, we observe that even a brief exposure to the robotic predator of
34 fifteen minutes per week is sufficient to erode energy reserves and compromise the body
35 condition of mosquitofish, opening the door for future endeavours to control mosquitofish
36 in the wild.

37

38 **Keywords**

39 Animal personality; bioengineering; biomimetics; body condition; invasive species;
40 predation risk

41 1. INTRODUCTION

42 The presence of animal species in areas where they are not native is common across the
43 globe, with tremendous costs for both human activities and the ecological integrity of those
44 areas [1, 2]. Despite efforts from both governmental and academic institutions, existing
45 methods for eradicating invasive alien species (IAS) or mitigating their negative effects
46 remain labour-intensive, economically unviable, and, often, ineffective [3].

47 Freshwater animals are particularly vulnerable to IAS, whereby native species are
48 confined to smaller water bodies and their ability to disperse is limited compared to other
49 ecosystems [4]. Mosquitofish (*Gambusia affinis*, Baird and Girard, and *Gambusia holbrooki*,
50 Girard) are among the most widely diffused freshwater IAS in the globe, and their negative
51 impact on indigenous animal communities (via aggressive behaviours and/or predation [5-
52 8]) has been recognized by the International Union for Conservation of Nature that listed
53 mosquitofish among the world's hundred worst IAS [9].

54 Technical efforts to eradicate mosquitofish from water bodies and mitigate their negative
55 impact on the native fauna are, however, limited. For example, increasing the structural
56 complexity of the environment through artificial refugia was successful in reducing mortality
57 in barrens topminnow (*Fundulus julisia*, Williams and Etnier) exposed to mosquitofish under
58 laboratory settings, but beneficial effects from artificial refugia disappeared in the wild [10].
59 Similarly, the use of fish toxicants to combat the spread of invasive mosquitofish resulted in
60 detrimental consequences for native fish [11]. The utilization of floating traps to target
61 mosquitofish near the water surface has been shown to be a successful technique, but it is a
62 labour intensive process that can be pursued only in small sites and for short periods of time
63 [12].

64 Robotics constitutes a promising tool for addressing some of these challenges, by
65 offering a versatile, customizable, and consistent approach to modulate the behavioural
66 response of live animals [13-15]. Particularly relevant are experiments that have shown the
67 possibility of eliciting behavioural responses in freshwater fish through biologically-inspired
68 robots triggering a cost-benefit decision process [16-21]. The use of robotics in the study of
69 predator-prey interactions might afford the design of new hypotheses-driven studies that
70 could unfold the basis of fear and anxiety in prey fish [22-25] and illuminate the
71 evolutionary consequences of nonlethal exposure to predators [26, 27]. Just as robotics

72 might bring new scientific insight into predator-prey interactions, it also contributes to
73 ethics in animal experimentation by minimizing potential harm to live animals.

74 In particular, previous research efforts from our group indicate that a robotic fish can be
75 designed to repel mosquitofish [28] and simultaneously attract non-invasive fish under
76 laboratory settings [19]. The possibility to isolate fish from one species to another allows
77 safeguarding non-invasive species from the aggressive attitudes of mosquitofish, thereby
78 providing compelling evidence for the use of biologically-inspired robots as a possible
79 method for the selective control of mosquitofish in the wild. However, the technology to
80 deploy autonomous robotic fish in a complex ecological environment to control the
81 behaviour of mosquitofish is still in its infancy, calling for a scientifically-principled
82 understanding of how mosquitofish interact with biologically-inspired robotic stimuli.

83 Mosquitofish can adjust their behavioural and life-history strategies in response to
84 varying environmental conditions, especially in the attempt to minimize risks of predation
85 [29]. Mosquitofish are typically less prone to take risks [30] and invest less in reproduction
86 [31] and energy reserves [32] under predation risk than in more beneficial conditions, with
87 plastic adjustments associated with predation risks that can eventually result in the whole
88 body morphology of mosquitofish to be reshaped [33]. Visual cues represent the
89 predominant factor for predator recognition in most freshwater fish [34], especially
90 mosquitofish [28, 35], and a growing literature has provided convincing evidence that visual
91 cues from animated images [36-38] and biologically-inspired robots [19, 28] can be used to
92 influence mosquitofish behaviour.

93 While experiments comparing mosquitofish behavioural response to computer-animated
94 and robotic stimuli are presently lacking, evidence from other freshwater fish suggest that
95 visual stimuli associated with a biologically-inspired robotic predator might elicit a stronger
96 response than computer-animated images [23]. Experiments in [23] have compared the fear
97 response of zebrafish (*Danio rerio*, Hamilton) evoked by live predator fish, a robotic replica
98 of the predator fish, and computer-animated images of the predator fish, determining that:
99 (a) the robot caused a robust avoidance response in zebrafish that was comparable to that
100 observed for live predators, while computer-animated images did not, and (b) individual
101 responses were more consistent over time when zebrafish were exposed to the robot than
102 to live predators and computer-animated images. In addition to these methodological
103 observations, practical considerations toward future deployment in the wild favour the use

104 of robots over computer-animated images. In fact, practicality challenges the use of
105 computer-animated images in the wild, where it may be unfeasible to employ computer
106 screens or projectors. Based on these methodological and practical aspects, we favour the
107 use of robotic stimuli in place of computer-animated images.

108 In this study, we sought to test whether behavioural and life-history responses of
109 mosquitofish could be modulated through a robotic predator whose visual appearance and
110 locomotion were inspired by mosquitofish predators, the largemouth bass (*Micropterus*
111 *salmoides*, Lacépède; Figure 1). Largemouth bass coexist with mosquitofish in the wild and
112 constitute their most common predators [39, 40], with mosquitofish representing over 80%
113 of the fish consumed by juvenile largemouth bass in their native environments [41]. Our
114 biologically-inspired robotic predator was designed to take advantage of the innate
115 antipredator behaviour that largemouth bass induce on mosquitofish under laboratory
116 settings [31, 42]. We repeatedly exposed mosquitofish to robotic predators varying in their
117 degree of biomimicry to disentangle the relative contribution of the robot swimming and its
118 interactivity on both behavioural and life-history adjustments associated with antipredator
119 responses in mosquitofish. We hypothesized that: (a) visual stimuli from the robotic
120 predator would repel mosquitofish, as suggested in [28, 36], (b) increasing the degree of
121 biomimicry in the motion of the robot would increase antipredator behaviours and impact
122 life-history strategies (that is, energy reserves) in mosquitofish, and (c) individuals would
123 differ from each other in the extent of their antipredator responses [43], with individuals
124 with high future expectations (that is, individuals with high energy reserves) being more
125 risk-averse than others [44].

126 From a methodological point of view, our study contributes to the state-of-the-art in
127 animal-robot interactions [13, 14, 45] along several research directions. First, we established
128 a robotic platform that allows for tailoring the degree of complexity of the interaction
129 through a closed-loop control system, integrating real-time tracking and high-precision
130 robotics. Through this platform, we successfully varied the degree of biomimicry of the
131 interactive robotic predator, by simulating random attacks toward the fish at either
132 constant or increasing speed. This experimental manipulation effectively allowed for the
133 quantification of the relative contributions of typical locomotory patterns of predators in
134 triggering antipredator responses in mosquitofish. Second, we shied away from a rigid
135 prototype, in favour of a soft robotic replica that incorporates a spine-like structure to

136 promote natural oscillation that are reminiscent of body undulations, which are known to
137 be critical for fish-robot interactions in the water [18, 21]. Third, we integrated traditional
138 means of behavioural analysis with modern elements of dynamical systems theory, through
139 the information-theoretic framework of transfer entropy [46]. Through the lens of transfer
140 entropy, we demonstrated an improved comprehension of the antipredator response of
141 mosquitofish, by testing for potential cause-and-effect relationships between the motion of
142 the robotic predator and mosquitofish antipredator response. Finally, although few recent
143 studies have considered behavioural response of animals repeatedly confronted with robots
144 [47, 48], a detailed study of individual variation in mosquitofish behaviour was lacking,
145 especially in the context of life-history consequences of the exposure to robotic stimuli.

146 Although focused on mosquitofish, the theoretical and methodological underpinnings of
147 this work could inform research on other IAS, whose presence in the environment is also a
148 threat to biodiversity and economy. For example, recent studies have demonstrated the
149 possibility of utilizing robots inspired by live predators to influence the behaviour of locusts
150 (*Locusta migratoria*, Linnaeus), a major pest for human agricultural economies and
151 ecosystems stability [49, 50]. Similarly, the peregrine falcon-like robot Robird has been
152 recently presented for deployment in the aviation industry to deter birds from flying in the
153 vicinity of aircrafts [51].

154

155 **2. MATERIALS AND METHODS**

156 **2.1 Study organism and maintenance**

157 A total of 150 wild-caught Western mosquitofish (*Gambusia affinis*, Baird and Girard) were
158 purchased from a commercial supplier (Carolina Biological Supply Co., Burlington, NC, USA)
159 and were acclimatized for one day in stock tanks. Then, 75 focal individuals (average body
160 length of 2.9 ± 0.3 cm) were randomly selected from stock tanks, with sick individuals and
161 fish showing physical and/or behavioural anomalies excluded *a priori*.

162 Focal fish were housed individually in transparent Plexiglas cylinders (10 cm diameter),
163 placed within a large housing tank (185 x 47 x 60 cm, length, width, and height) and
164 submerged in water per 10 cm, as in [29, 52]. The lateral surface of the transparent
165 cylinders was perforated to promote water circulation across separate cylinders, affording
166 visual and chemical interaction among individuals despite physical isolation. This housing
167 scheme prevented aggression, competition for resources, and sexual harassment among

168 mosquitofish, with each cylinder marked with a unique identification code to facilitate the
169 identification of individuals over time. The position of the cylinders was periodically
170 randomized to allow visual and chemical interactions among all fish. Fish were acclimatized
171 in the cylinders for one month before experiments, and they were housed in these cylinders
172 for the whole duration of the study (approximately three months).

173 Fish were kept under a 12h light/12h dark photoperiod and fed with commercial flake
174 food (Nutrafin max; Hagen Corp., Mansfield, MA, USA) once a day. Water parameters were
175 checked daily, with temperature and pH maintained at 26°C and 7.2 pH, respectively,
176 throughout the study.

177

178 **2.2 Experimental setup**

179 *2.2.1 Experimental arena for behavioural tests*

180 Behavioural trials were performed in an experimental arena (44 × 30 × 30 cm, length, width,
181 and height), filled with 10 cm of conditioned water (Figure 1A). The walls and the bottom
182 surface of the arena were covered with white opaque contact paper to control for external
183 disturbance and optimize automated computer tracking of fish motion during trials. Two 38
184 W fluorescent tubes (All-Glass Aquarium, UK) were mounted 130 cm above ground and
185 were used to provide homogeneous illumination to the apparatus. A high-resolution
186 webcam (Logitech C920 webcam, Lausanne, Switzerland) was mounted 140 cm above the
187 floor for a complete overview of the experimental arena.

188

189 *2.2.2 Robotic platform and predator replica*

190 The experimental arena was supported by aluminium T-slotted bars 29 cm above the
191 ground to allow the placement of the robotic platform underneath (Figure 1A). The platform
192 allowed for manoeuvring the robotic replica along the three degrees of freedom: two
193 degree of freedom were controlled for in-plane translational motion of the replica and one
194 degree of freedom served to adjust the predator body rotation. The replica was
195 magnetically connected to the platform through a 3D-printed base made of Polylactic Acid
196 filaments (3.2 cm x 1.0 cm x 0.6 cm length, width, and height) containing two circular
197 neodymium magnets (0.63 cm thick and 0.3 cm diameter) and an acrylic rod (4 cm length
198 and 0.62 cm diameter; Figures 1A, 1B, and 1C). The in-plane translational motion was based
199 on a Cartesian plotter (XY Plotter Robot Kit, Makeblock Co., Ltd, Shenzhen, China) and the

200 body rotation was controlled via a stepper motor (NEMA 14, Pololu Corp., Las Vegas, NV,
201 USA). Further details on the robotic platform are in the Supplementary Material. The
202 platform was originally designed in [53] to study zebrafish social behaviour and utilized in
203 [54] to examine zebrafish learning.

204 Locomotory patterns of the predator replica were inspired by pilot tests performed on
205 three juvenile largemouth bass (7.0 ± 0.5 cm), purchased from Teichwirtschaften Armin
206 Kittner in Quitzdorf am See, Germany (<https://www.teichwirtschaft-kittner.de/>), before the
207 beginning of the experiment (Figure 1D). Live bass were placed individually in the
208 experimental arena and their behaviour was recorded over 30 minutes. Swimming
209 trajectories and swimming speeds were then obtained through an offline tracking software
210 developed by our group [55]. Mean and maximum swimming speed measured in the pilot
211 tests and a swimming trajectory representative of the bass behaviour in the experimental
212 arena were used for the motion of the predator replica.

213 The morphology and coloration of the replica were also chosen to capture salient
214 features of juvenile largemouth bass (Figures 1B, 1C, and 1D). Toward this aim, we took
215 photos of the live bass from different angles and estimated their body dimensions using a
216 dedicated software (ImageJ, National Institute of Health, Bethesda, Maryland, USA). The
217 body morphology of the replica was accordingly modelled in Solidworks (Dassault Systèmes
218 SolidWorks Corp., Waltham, Massachusetts, USA) to create a 3D design and, then, a solid
219 mould. A spine-like structure in Polylactic Acid filament material was 3D-printed and
220 integrated within the 3D printed mould of the predator replica together with two glass eyes,
221 relatively smaller than in live bass (Figure 1B). Then, the mould was filled with non-toxic and
222 aquarium safe silicone (Dragon Skin 10 Medium, Smooth-On, Macungie, PA, USA) and let
223 dry. The spine-like structure provided support to the weight of the silicone body of the
224 replica and facilitated body oscillations during swimming. Lastly, the silicone body of the
225 replica was hand-painted using non-toxic, aquarium safe, and silicone based light grey and
226 silver paints (Smooth-On, Inc., Macungie, PA, USA) to mimic the characteristic coloration
227 pattern of largemouth bass (Figure 1C). Colour reflectance comparison between live bass
228 and its robotic replica were not performed. However, non-toxic pigments utilised to paint
229 the body of the robotic replica have been shown to be effectively perceived as natural
230 pigments in bluefin killifish (*Lucania goodie*, Jordan; [20]), a freshwater fish with well-
231 developed vision like mosquitofish.

232 The moulded silicone body with glass eyes and spine-like structure was attached to a
233 clear acrylic rod, connected to 3D-printed base with magnets. The clear acrylic rod allowed
234 for setting the swimming depth of the biologically-inspired predator replica in the middle of
235 the water column, that is, where the antipredator response of mosquitofish is known to be
236 the strongest [28].

237

238 *2.2.3 Experimental conditions and live tracking*

239 We designed a series of experimental conditions with robotic replicas varying their motion
240 to proxy different degrees of biomimicry of live predators. In one control condition, the
241 experimental fish were tested in the absence of the replica (no predator: NP). In a second
242 control condition, the replica was motionless and positioned randomly within the arena
243 before each trial started (predator motionless: PM). In the four experimental conditions
244 where a swimming replica was employed, the replica swam on either the predetermined
245 trajectory inspired by live bass (open-loop: OL) or it alternated between the predetermined
246 trajectory and targeted real-time interactions (closed-loop: CL) with the focal fish. In two OL
247 conditions, the biologically-inspired predator replica followed the predetermined swimming
248 trajectory, either at a varying speed based on the motion of the live predator (OL1) or at a
249 constant speed (OL2). In condition OL2, the trajectory from the live bass was processed to
250 manoeuvre the replica at a constant speed. Specifically, we locally fitted the trajectory using
251 cubic splines (interparc, Copyright (c) 2012 John D'Errico) and placed equally-spaced
252 waypoints on the splines such that the replica would move at a constant speed. The
253 constant speed was chosen to be 6 cm/s to match the mean speed observed in juvenile
254 largemouth bass in our pilot tests and provide a dynamically rich visual stimulation for
255 mosquitofish. The same speed was used as the mean value of the speed profile in condition
256 OL1, consistently scaling experimental observations.

257 In the CL conditions, the replica, besides following swimming trajectories at a varying
258 speed, was programmed to interact in real-time with the focal fish and to perform simulated
259 attacks at random. However, the replica always performed an attack every minute of the
260 trial for a total of 15 attacks. During an attack, the replica either accelerated to attain a large
261 speed (20 cm/s; CL1) comparable to the maximum speed of live bass attacking a prey [56],
262 or swam at a constant speed toward the fish (6 cm/s; CL2). When the replica was
263 commanded to attack the focal fish, its motion was a function of the distance from the fish.

264 For CL1 condition, if the distance between the fish and replica was less than 1 cm, the
265 replica would only change its heading towards the direction of the focal fish and return to
266 the original heading; for distances between 1 and 10 cm (inspection zone in [57]), the
267 replica would change its heading, accelerate towards the fish at 20 cm/s^2 , and stop at
268 approximately 1 cm from it; and for distances larger than 10 cm, the replica would change
269 the heading, accelerate at 20 cm/s^2 until reaching a speed of 20 cm/s , and maintain this
270 speed until stopping at 1 cm from the fish. For CL2 condition, if the distance between the
271 fish and replica was less than 1 cm, the replica would only change its heading towards the
272 direction of the focal fish and return to the original heading. For any distance greater than 1
273 cm, the replica would change its heading, and attack the fish with a constant speed of 6
274 cm/s and stop at 1 cm from the fish.

275 After an attack was completed, the replica returned to its original position prior to the
276 attack and restarted swimming along the predetermined trajectory until the next attack.
277 Notably, the region in which the robotic replica swam was smaller than the actual size of the
278 experimental arena to allow at least 1 cm from the extremities of the replica's body (that is,
279 head and caudal fin) and the edges of the arena. This tolerance permitted smooth operation
280 of the robotic platform and avoided collision with the walls of the arena. Further details on
281 the real-time tracking system implemented for CL conditions are in the Supplementary
282 Material.

283 The custom-made software was calibrated on the exact size of an individual fish at each
284 trial (week) separately and utilized to calculate the following quantities: distance moved
285 (cm), time spent freezing (s), speed variance during swimming (cm^2/s^2), mean distance from
286 the predator replica (cm), predator inspection (counts), and time spent within one-body
287 length from the wall (s) – that is, thigmotaxis [58]. In particular, if a fish moved at a speed
288 less than half of its body length per second for two consecutive seconds, it was considered
289 as freezing [59]. Predator inspection was estimated according to standard protocols
290 developed for guppies (*Poecilia reticulata*, Peters; [57]), a poeciliid species closely related to
291 mosquitofish. In particular, we counted the number of events that a fish voluntarily
292 approached the predator replica by entering the 10 cm region around the replica while
293 actively swimming in its direction, that is, at an angle lower than ± 90 degrees from the
294 replica's head [57]. The distance from the wall used to estimate thigmotaxis was selected
295 based on pilot tests in which mosquitofish were exposed to the same robotic predator

296 replica utilized in this study. Details on data extraction and tracking system are in the
297 Supplementary Material.

298 Notably, reduced activity (in the form of short travelled distances and prolonged
299 freezing) and large number of predator inspections, hesitancy in exploring open spaces that
300 are unfamiliar and potentially dangerous (that is, high thigmotaxis), and erratic swimming
301 patterns dominated by high speed variance are typically associated to risk aversion and
302 fearful states in animals [43], including mosquitofish [28, 29, 36, 52, 59].

303

304 **2.3 Experimental procedure**

305 Once a week over seven consecutive weeks, fish were anesthetized in a solution of tricaine
306 methanesulfonate (MS-222; 168 mg per 1L H₂O), sexed, and their body length (to the
307 nearest 0.5 mm) and body weight (to the nearest 0.01 g) were measured. These
308 measurements were conducted before the experiment started (baseline body
309 measurements) and after the conclusion of each behavioural trial (week 1 to week 6). The
310 Fulton's condition factor K (weight length⁻³ 10⁴, g mm⁻³ 10⁴; [60]) was then calculated as an
311 index for the nutritional state (that is, body condition) of each fish at each week.

312 In each trial, a mosquitofish was gently hand-netted and placed into an opaque cylinder
313 in the experimental arena for 5 minutes to allow acclimatization to the setup. During
314 acclimatization, the motors of the robotic platform were turned off and fish had no visual
315 contact with the apparatus outside the opaque cylinder. Then, the opaque cylinder was
316 gently removed and the platform turned on, allowing the fish to explore the arena in either
317 absence (NP) or presence of the biologically-inspired predator replica (PM, OL1, OL2, CL1,
318 and CL2 conditions) for 15 minutes. After the trial was completed, the fish was transferred
319 back into its individual housing cylinder and the next trial was initiated.

320 The behaviour of each individual (n=75) was tested once a week over six consecutive
321 weeks, with individuals tested once per condition. No mortality was reported such that an
322 equal number of replicates were conducted for each condition. One week interval between
323 two consecutive behavioural measurements is commonly adopted when testing individual
324 variation in mosquitofish behaviour to minimize memory effects [29, 52]. Experimental
325 conditions were performed in a randomized order, but the NP condition was always
326 performed last to mitigate bias on fish baseline behaviour caused by individuals being
327 exposed to diverse degrees of predator threat, as observed in [42] for risk avoidance in

328 mosquitofish. Fish were tested in a randomized order to exclude consistent differences in
329 their behavioural outcome caused by hunger [61].

330

331 **2.4 Statistical analysis**

332 We initially tested whether body length, body mass, and Fulton's K were correlated by
333 estimating phenotypic correlations (that is, the overall correlation attributable to between-
334 and within-individual correlations) with bivariate linear mixed-effects models (LMMs), as
335 suggested by [62]. In these models, we specified the individual as the random effect (that is,
336 random intercepts) to account for repeated measures of the same individual across weeks.
337 Body size was correlated with both mass and K , while mass and K were not correlated with
338 each other (Table S1). Therefore, we included both body mass and Fulton's K as fixed effects
339 in the LMMs below, while body size was excluded from the models.

340 Since we were interested in testing whether mosquitofish antipredator response
341 increased with an increased degree of biomimicry of the replica, we measured individual
342 behaviour repeatedly across experimental conditions. We ran separate LMMs in which
343 distance moved, freezing, speed variance, mean distance from the replica, predator
344 inspection, and thigmotaxis were included one-by-one as the dependent variables. In each
345 model, individual identities were included as the random effect, while body mass, Fulton's
346 K , sex, week, and condition (that is, the degree of biomimicry of the robotic predator) were
347 entered as fixed effects. A significant effect of condition in a given model (or any other fixed
348 effect included in that model) would indicate that condition explained a significant portion
349 of the behavioural variance observed after accounting for the variation explained by the
350 other fixed effects. The significance of individual differences was tested using both
351 likelihood ratio tests (LRTs) and Akaike information criteria (AICs), where a full model
352 including individual as a random effect was compared with a reduced model in which the
353 random effect was excluded. Random intercepts represented the proportion of the total
354 phenotypic variance not attributable to fixed effects that was explained by among-individual
355 variance, that is, differences in personality traits among individuals.

356 Building upon our previous work [15], we implemented the information-theoretic notion
357 of transfer entropy to quantify the influence of the biologically-inspired predator replica on
358 the behaviour of the live fish and *vice versa*. Given two stochastic processes, transfer
359 entropy quantifies the reduction in the uncertainty in the prediction of the future of one of

360 the processes from its present due to additional knowledge about the other stochastic
 361 processes [63]. In this vein, a nonzero value of transfer entropy indicates a potential
 362 influence between the two processes [63]. Here, transfer entropy was computed on the
 363 time-series of the speed of the replica and the mosquitofish, which were first down sampled
 364 to 1 Hz to ensure that one time-step (1 s) would suffice to encode the response time of the
 365 fish to the replica and *vice versa*. Therefore, a total of 904 points (904 s) were used per each
 366 trial. Then, we converted the time series into symbols depending on whether the speed
 367 increased or decreased between two consecutive time-steps [64]. In agreement with [15],
 368 we computed the transfer entropy from the replica (R) to the fish (F) as follows:

$$369 \quad TE_{\text{Robot} \rightarrow \text{Fish}} = \sum_{F_{t+1}, F_t, R_t} \Pr(F_{t+1}, F_t, R_t) \log_2 \frac{\Pr(F_{t+1}/F_t, R_t)}{\Pr(F_{t+1}/F_t)}$$

370 where F and R are the down-sampled time-series of the speeds, and \Pr represents the
 371 probability mass function computed via plug-in estimation. By flipping F with R , we
 372 computed transfer entropy from the fish to the replica $TE_{\text{Fish} \rightarrow \text{Robot}}$. Across the five
 373 experimental conditions in which the predator replica was employed, transfer entropy could
 374 only be used in OL1, CL1, and CL2, since the speed of the replica was constant in PM and
 375 OL2 and, therefore, encoded no meaningful information.

376 We expected information flow in OL1 to be one-directional, since the replica swam
 377 irrespective of the fish, which should be influenced by the swimming pattern of the replica.
 378 On the other hand, the information flow in CL1 and CL2 was expected to be two-directional,
 379 with the fish responding to the replica and the replica adjusting its attacks as a function of
 380 the behaviour of the fish. For each of the three conditions (OL1, CL1, and CL2), we obtained
 381 surrogate data from all the possible shuffling (74×74) of the identities of the fish and the
 382 replica within each condition. For each of these shuffling, we randomly selected 74 values
 383 without repetitions to obtain a mean transfer entropy value; this process was repeated
 384 20000 times to obtain a surrogate distribution. To ascertain an influence through transfer
 385 entropy, we tested whether the corresponding experimental value was in the right tail of
 386 the distribution. This process was conducted six times, twice for each of the three
 387 conditions to examine information flow in either direction (fish to robot or robot to fish,
 388 similar to [65]).

389 We then tested whether transfer entropy differed across conditions and between
 390 directions ($TE_{\text{Fish} \rightarrow \text{Robot}}$ and $TE_{\text{Robot} \rightarrow \text{Fish}}$). Therefore, we built an LMM with transfer

391 entropy as the dependent variable, the direction of information flow, condition, and their
392 interaction as fixed effects, and both individual identities (fish identity) and pair identities
393 (fish and replica identities) included as random intercepts. As for the LMMs on behavioural
394 traits described above, the significance of random intercepts (both individual and pair
395 identities) was tested using LRTs and AICs.

396 Lastly, we were interested in testing whether fish energy reserves (Fulton's K) varied in
397 response to the exposure to robotic predator replicas. Toward this aim, we built an LMM
398 with Fulton's K as the dependent variable, including individuals' identity as the random
399 effect (that is, random intercepts) and sex, week, and condition (that is, the degree of
400 biomimicry of the robotic predator) as fixed effects. We then tested whether the
401 behavioural variation observed across conditions reflected variation in Fulton's K . Based on
402 our initial hypothesis and findings from behavioural analyses, experimental conditions were
403 consolidated in three categories: controls (K measured before the experiment started, after
404 tests performed in absence of the predator replica, and after the exposure to the predator
405 motionless; baseline, NP, and PM, respectively), low degree of biomimicry (OL1, OL2, and
406 CL2), and high degree of biomimicry (CL1). Variation in K was then tested with an LMM, in
407 which Fulton's K was the dependent variable, individuals' identities the random effect (that
408 is, random intercepts), and sex, weeks, and condition category the fixed effects.

409 Data analyses were performed in R version 3.5.1 [66] using the "lme4", "nlme",
410 "lmerTest", and "MCMCglmm" packages [67-70], estimated marginal means (EMMs) based
411 on univariate models and post hoc comparisons were performed with "emmeans" adjusted
412 for simultaneous inference with the *mvt* method [71], while permutation tests for transfer
413 entropy analysis were conducted in MATLAB (R2018a; MathWorks, Natick, MA, USA [72]).
414 Prior to all analyses, speed variance was log-transformed to normalize error distribution in
415 the model's residuals. Except for the permutation test that is independent by error
416 distributions, we assumed Gaussian error distributions that were confirmed for all response
417 variables after visual inspection of model residuals. The significance level was set at $\alpha <$
418 0.05.

419

420 **3. RESULTS**

421 Behaviour was strongly dependent on the experimental condition in which mosquitofish
422 were tested after controlling for variation explained by week (see results from the LMMs in

423 Table 1). The distance between the fish and the replica decreased when the replica was
424 allowed to swim in the arena with respect to the condition PM where it was held in place
425 ($P < 0.001$ in pairwise comparisons between PM and any other experimental condition;
426 Figure S1). This was especially evident when attacks were performed in real-time by
427 accelerating toward the fish and the interactive nature of the replica buffered fish' attempts
428 to be away from it ($P < 0.001$ in pairwise comparisons between CL1 and OL1, OL2, and CL2;
429 Figure S1).

430 On the contrary, fish tendency to inspect the predator replica did not vary across
431 swimming replicas, that is, the number of inspections in CL1 was undistinguishable from
432 OL1, OL2, and CL2 (Figure 2A). Accordingly, fish swam on average longer distances, varied
433 their swimming speed more, and froze less when exposed to a swimming replica than in
434 control conditions ($P < 0.001$ in pairwise comparisons between NP and PM confronted with
435 any other experimental condition, except for speed variance and freezing between NP and
436 CL1 and between PM and CL1, respectively; Figure S1).

437 Thigmotaxis increased with increasing biomimicry in the replica's motion, whereby the
438 time interval spent in the proximity of the walls was longer when fish were exposed to a
439 replica varying its attacking speed in real-time (CL1) than other replicas ($P < 0.001$ in pairwise
440 comparisons between CL1 and any other experimental condition in which a robotic replica
441 was employed), with the shortest time observed in the presence of the motionless replica
442 (PM; Figure 2B). On the other hand, behavioural responses of fish exposed to an attacking
443 replica that swam at a constant speed (CL2) were comparable with those observed in open-
444 loop conditions (OL1 and OL2), consistently across all measured traits (Figures 2 and S1).

445 The variation in body condition (Fulton's K) among individuals was a significant predictor
446 for the variation in their behavioural response across conditions (see results from the LMMs
447 in Table 1). In particular, individuals with more energy reserves varied their swimming speed
448 more (that is, exhibited higher speed variance) in response to the replica and an analogous
449 role of K was also noted, albeit not significant, with respect to distance moved, distance
450 from the replica, predator inspection, and thigmotaxis (Table 1). Accordingly, individuals
451 with higher K tended to swim longer distances, maintained larger distances from the
452 replicas, inspected the replicas less, and spent more time in the proximity of the walls.
453 Nevertheless, we registered consistent among-individual variance in all traits after that
454 behavioural variation explained by the model predictors was accounted for, that is, fish

455 differed in personality traits (see results from the LMMs in Table 2), except for the mean
456 distance from the replica and the individual intercepts for the transfer entropy.

457 We failed to identify an information transfer flow in the open-loop condition OL1 in both
458 directions (that is, from the robot to the fish and *vice versa*; Figures 3A and 3B). On the
459 contrary, a significant information transfer was observed in both directions in CL1 (Figures
460 3C and 3D) and CL2 (Figures 3E and 3F). When comparing information transfers within
461 conditions, we observed that transfer entropy from the robot to the fish in the open-loop
462 condition OL1 was higher than from the fish to the robot ($P=0.003$; Figure 3G), in agreement
463 with our expectations on the one-directional nature of the interaction in OL1. Transfer
464 entropy in the closed-loop condition CL1 was also larger from the robot to the fish than in
465 the opposite direction ($P<0.001$), while transfer entropy in CL2 was comparable between
466 directions (Figure 3G). Importantly, the effect of the replica on fish behaviour was stronger
467 in CL1 than in CL2 ($P=0.042$; Figure 3G), while other pairwise comparisons were not
468 significant. In other words, the biologically-inspired robotic predator interacting with
469 mosquitofish in real-time and accelerating toward the fish (CL1) was more effective in
470 eliciting antipredator responses in mosquitofish than when it attacked at a constant speed
471 (CL2).

472 We also found that body condition (Fulton's K) varied across experimental conditions
473 (see results from the LMM in Table S2), with K significantly lower after fish faced the
474 predator replicas than after fish were tested in absence of the replica ($P<0.001$ in pairwise
475 comparisons between NP and any other experimental condition; Figure S2). The decrease in
476 K after exposure to the replica ($P<0.001$ in pairwise comparisons between controls and
477 replicas with either low and high biomimicry; Figure 4) appeared, however, to be
478 independent of the degree of biomimicry of the replica (non-significant pairwise comparison
479 between low and high biomimicry; Figure 4).

480

481 **4. DISCUSSION**

482 Here, we have disentangled the relative contributions of swimming pattern and closed-loop
483 control of an interactive robotic predator on the antipredator behavioural response and life-
484 history strategies in mosquitofish. Fish thigmotaxis increased with the degree of biomimicry
485 in the motion of the replica, suggesting that integrating real-time feedback from
486 mosquitofish position in the control of a replica interacting at increasing speed plays a key

487 role in eliciting antipredator response in mosquitofish. The quantification of the information
488 flow between the replica and fish supported the existence of a causal relationship between
489 fish antipredator response and the motion of the biologically-inspired replica. We also
490 observed that individual behaviour was relatively predictable, with variations in energy
491 reserves explaining a large portion of the behavioural variance observed among
492 mosquitofish. Notably, energy reserves decreased after fish were exposed to the
493 biologically-inspired robot only 15 minutes per week, but variation in energy reserves did
494 not depend on the degree of biomimicry in the motion of the replica.

495 After the initial detection of a potential predator, a fish typically identifies and assesses
496 the threat based on cues from its natural predators [73]. The extent of an antipredator
497 response is determined from the trade-off between minimizing risk of predation and energy
498 consumption toward survival and reproduction [74], such that greater threats produce
499 stronger avoidance [75]. Here, we provide experimental evidence that swimming patterns
500 represent a salient source of information for predator recognition in mosquitofish that
501 regulate the extent of their antipredator response. This evidence is based on highly-
502 controllable experiments that employ a state-of-the-art robotic predator replica, whose
503 visual appearance and swimming pattern were inspired by measurements on juvenile
504 largemouth bass, the main predator of mosquitofish in the wild [39-41]. Not only did the
505 robotic replica allowed for controlling the swimming speed and acceleration of the predator
506 stimulus, but also it afforded the implementation of controlled attacks toward mosquitofish
507 to study their antipredator response in real time. By opting for a robotics-based platform in
508 lieu of a live predator, we were able to exclude potential correlations between antipredator
509 response of mosquitofish and inherent biological variations in the predator behaviour (that
510 is, idiosyncrasies with focal individuals, fatigue, and hunger) that could confound
511 hypothesis-testing.

512 The more robust antipredator behavioural response was registered when mosquitofish
513 were exposed to a replica swimming at a varying speed and performing targeted, fast
514 attacks. Reducing the degree of biomimicry toward a replica that performed attacks in real-
515 time at a constant speed resulted into a weaker antipredator behavioural response, similar
516 to that registered with non-interactive replicas that followed predetermined swimming
517 trajectories. This evidence aligns with prediction from the literature positing that speed and
518 acceleration should play a key role on prey-predator interactions in fish [76]. Our

519 information-theoretic analysis of the interaction between the robotic replica and the fish
520 suggests the presence of a cause-effect relationship underlying the antipredator behavioural
521 response of mosquitofish, which confirms the expected link between a predator's attacking
522 speed and fish behavioural response [77]. More specifically, we determined that the
523 uncertainty in the prediction of the future speed of mosquitofish from its present speed was
524 reduced due to additional knowledge about the speed of the replica, such that the motion
525 of the replica encoded valuable information about the behaviour of mosquitofish.

526 Beyond the analysis of the mean behavioural response at the population level, we
527 discovered that a relatively short exposure to the biologically-inspired robotic predator (only
528 15 minutes per week) resulted in a substantial reduction in the whole body condition of
529 mosquitofish (index of fat reserves for a given body size; condition factor K) that did not
530 depend on the swimming pattern of the robot. Recent evidence from multiple populations
531 of mosquitofish in the wild has shown that the condition factor K in mosquitofish decreased
532 on average of 5.8% over a five months period in response to severe environmental
533 challenges associated with water pollution [78]. Here, we observed that the body condition
534 declined of 3.1% over a week, after mosquitofish were exposed once to a predator replica,
535 thereby suggesting a hidden effect of the robot on mosquitofish life-history adjustments.
536 This finding aligns with evidence of nonlethal effects of predator-prey interactions [26],
537 whereby costs of antipredator responses extend to ecologically-relevant traits beyond
538 behaviour, such as physiology and body condition [79].

539 In fact, theory predicts that stress responses affect the way animals allocate resources to
540 fuel emergency functions [79], with animals investing relatively more energy in survival (that
541 is, escaping from the predator) and relatively less in future reproduction (that is, energy
542 reserves) with increasing predation risk [80]. With respect to mosquitofish, nonlethal effects
543 of predator exposure have been found to lower their body condition, ultimately leading to
544 lower fertility and fecundity rates [31]. Under this perspective, evidence from this study
545 indicates that a relatively brief exposure to a biologically-inspired robotic predator
546 compromised the body condition of mosquitofish. Notably, the body condition increased
547 again when mosquitofish were tested in the arena in the absence of the replica, indicating
548 that variation in body condition resulted from the exposure to the robotic predator than
549 other factors (for example, time, exposure to the arena, and handling of the fish).

550 At the individual level, we found that fish differed consistently from each other in the
551 extent of their antipredator response across six repeated exposures to robotic predators
552 varying in their degree of biomimicry (that is, fish differed in personality traits; [43]). While
553 the presence of personality variation among mosquitofish is well documented in the
554 literature (see for example [29, 52, 59] and references therein), this study offers evidence
555 that meaningful variation in antipredator response among mosquitofish can be successfully
556 captured using robotic stimuli. Interestingly, a large portion of the variance in the
557 antipredator response observed among mosquitofish was explained by variation in their
558 body condition. In particular, individuals in better body conditions varied their swimming
559 speed more in response to the robotic predator, tended to swim longer distances,
560 maintained larger distances and inspected less the replicas, and spent more time in the
561 proximity of the wall than mosquitofish in poorer body conditions. Individuals can trade-off
562 survival at the cost of future reproduction, but the antipredator behavioural response of an
563 individual should also depend on its body condition [81] as the reproductive value is
564 condition-dependent. In this vein, our results are in agreement with predictions from the
565 life-history theory that individuals with high future expectations (that is, individuals with
566 high energy reserves) should systematically be more risk-averse than others [81]. Therefore,
567 our findings suggest that antipredator behavioural response toward robotic predator fish
568 differs at the individual level in a relatively predictable manner.

569 This study contributes to the state of the art on the modulation of the behaviour of
570 invasive and pest species through the use of predator-mimicking robotic fish [19, 28],
571 supporting the technological evolution of pest control agents, along similar line of
572 development as insects [50] and birds [51]. Specifically, we aimed at the precise
573 quantification of granular features of predator locomotion on antipredator responses of
574 invasive mosquitofish through the development of a state-of-the-art robotic predator
575 whose swimming characteristics can be controlled across a continuum range of biomimicry.
576 Our findings build on previous research efforts on the modulation of mosquitofish
577 behaviour through biologically-inspired robots, shedding light on the role of the robot
578 morphology on mosquitofish behaviour [28] and addressing the differential response of
579 mosquitofish and zebrafish to robots [19]. In particular, we demonstrated that a biologically-
580 inspired robotic predator swimming at a varying speed and performing targeted attacks
581 elicits a strong antipredator behavioural response that erodes energy reserves and

582 compromises the body condition of mosquitofish. We propose that further efforts should
583 test whether biologically-inspired robots can effectively represent a novel, autonomous, and
584 effective solution to contrast the negative impact of invasive mosquitofish on freshwater
585 ecosystems worldwide [5-9].

586

587 **AUTHOR CONTRIBUTIONS**

588 GP and MP conceived the research question and supervised the research. GP designed the
589 experiment. GP, VRS, and CS developed the experimental setup. MK, VRS, and CS conducted
590 the experiments. GP and MK analysed the data and all authors discussed the results. GP and
591 MK wrote a first draft of the Materials and Methods section. GP and MP wrote the
592 manuscript. All the authors reviewed the final draft.

593

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598

599 **DATA ACCESSIBILITY**

600 All data can be found at <https://figshare.com/s/1e755aeba5b781f753a9>.

601

602 **ETHICS**

603 Experiments were performed in accordance with relevant guidelines and regulations and
604 were approved by the University Animal Welfare Committee (UAWC) of New York University
605 under the protocol number 13-1424. Notably, pilot tests on predator fish were approved
606 through an animal care permit (G 0074/15) granted by the Landesamt für Gesundheit und
607 Soziales Berlin (LAGeSo) and performed in Germany.

608

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612

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815

816 TABLES

817 **Table 1 Analysis of variance with Satterthwaite's method from linear mixed models with**
 818 **distance moved, freezing, speed variance, mean distance from replica, predator**
 819 **inspection, thigmotaxis, and transfer entropy as dependent variables.**

Fixed factors	Mean Square	<i>df</i>	<i>F</i>	<i>P</i>
Distance moved (cm)				
<i>K</i>	2801434	1, 424	3.567	0.059
Mass	239961	1, 121	0.3055	0.581
Sex	2306162	1, 78	2.936	0.091
Week	82525615	1, 379	105.080	<0.001***
Condition	32234416	5, 367	41.044	<0.001***
Freezing (s)				
<i>K</i>	11392	1, 340	0.359	0.549
Mass	57781	1, 96	1.821	0.180
Sex	85910	1, 75	2.708	0.104
Week	3161017	1, 386	99.631	<0.001***
Condition	735543	5, 367	23.183	<0.001***
Speed variance (cm ² /s ²)				
<i>K</i>	3.376	1, 234	7.044	0.008**
Mass	0.047	1, 85	0.099	0.754
Sex	0.027	1, 74	0.056	0.814
Week	3.881	1, 389	8.099	0.005**
Condition	8.935	5, 368	18.644	<0.001***
Distance from replica (cm)				
<i>K</i>	62.46	1, 186	3.784	0.053
Mass	0.390	1, 77	0.024	0.878
Sex	0.06	1, 73	0.003	0.954
Week	0.59	1, 315	0.036	0.850
Condition	343.970	4, 291	20.837	<0.001***
Predator inspection (counts)				
<i>K</i>	199.50	1, 288	3.025	0.083

Mass	66.50	1, 80	1.008	0.318
Sex	212.90	1, 74	3.227	0.076
Week	441.80	1, 314	6.698	0.010*
Condition	5539.30	4, 291	83.977	<0.001***
Thigmotaxis (s)				
<i>K</i>	59307	1, 404	2.944	0.087
Mass	23814	1, 107	1.182	0.279
Sex	104813	1, 75	5.202	0.025*
Week	232953	1, 380	11.562	<0.001***
Condition	291277	5, 365	14.457	<0.001***
Transfer entropy (bits)				
Condition	<0.001	2, 146	0.514	0.599
Direction	<0.001	1, 219	49.516	<0.001***
Condition*Direction	<0.001	2, 219	5.262	0.006**

820

821 Fulton's *K*, body mass, sex, week, and condition are included in all models as fixed factors,
822 except for transfer entropy in which condition, direction, and their interaction were
823 included as fixed factors. The individual is included as a random effect (that is, random
824 intercepts) in all models, while pair (fish-robot) is included as a second random effect in the
825 transfer entropy model, to account for repeated measures. The significance was set at
826 $\alpha < 0.05$, and significant results are indicated with * (< 0.05), ** (< 0.01), and *** (< 0.001).

827 **Table 2 Results from general linear mixed models with distance moved, freezing, speed**
828 **variance, mean distance from replica, predator inspection, thigmotaxis, and transfer**
829 **entropy as dependent variables.**

Variance components	V_{within}	V_{among}	Repeatability	ΔAIC	χ^2_1	P
Distance moved (cm)	785359	599257	0.433	118.770	120.769	<0.001***
Freezing (s)	31727	9038	0.222	33.978	35.977	<0.001***
Speed variance (cm ² /s ²)	0.479	0.035	0.069	2.322	4.322	0.038*
Distance from replica (cm)	16.507	0.482	0.028	1.461	0.539	0.463
Predator inspection (counts)	65.960	19.270	0.226	25.019	27.019	<0.001***
Thigmotaxis (s)	20148	11185	0.357	80.800	82.800	<0.001***
Transfer entropy – individual (bits)	<0.001	<0.001	0.172	1.7	3.734	0.053
Transfer entropy – pair (bits)	<0.001	<0.001	0.455	42.100	44.068	<0.001***

830

831 Fulton’s K , body mass, sex, week, and condition are included in all models as fixed factors,
832 except for transfer entropy in which condition, direction, and their interaction were
833 included as fixed factors. Random intercepts are included for each individual in all models,
834 while random intercepts for each pair (fish-robot) are included for transfer entropy only,
835 which allowed variance decomposition. Within-individual variance (V_{within}), among-individual
836 variance (V_{among}), and repeatability are shown for each behavioural trait. Test statistics (χ^2_1)
837 and significant levels of the random effects (that is, intercepts) were estimated using LRTs
838 (P) and AICs between the full and the null model. Note that ΔAIC corresponds to the
839 difference in AIC between the null models minus the AIC from the full model. The
840 significance was set at $\alpha < 0.05$, and significant results are indicated with * (<0.05) and ***
841 (<0.001).

842 **FIGURE CAPTIONS**

843 **Figure 1** Schematic for (A) the overview of experimental apparatus, (B) the 3D
844 representation of the biologically-inspired predator replica, (C) the biologically-inspired
845 predator replica used for experiments, and (D) a picture of a juvenile largemouth bass.

846

847 **Figure 2** Estimated marginal mean (EMMs) differences represent adjusted mean differences
848 (+ SE) in predator inspection (A) and thigmotaxis (B) across conditions once the contribution
849 of fixed effects included in the model (that is, Fulton's K , body mass, sex, week) is accounted
850 for, except sex that was excluded in EMMs for predator inspection to preserve positive
851 values in PM condition and favour the interpretation while not altering results. White
852 histograms correspond to control conditions (NP and PM), light grey histograms to open-
853 loop conditions (OL1 and OL2), and dark grey histograms to closed-loop conditions (CL1 and
854 CL2). NP condition is not shown for predator inspection (A) since fish were tested in absence
855 of the predator replica. Means not sharing a common superscript are significantly different.
856 The significance was set at $\alpha < 0.05$.

857

858 **Figure 3** Transfer entropy between fish and robotic replicas. Transfer entropy from fish to
859 robot are represented in panels A, C, and E and from robot to fish in panels B, D, and F with
860 respect to conditions OL1 (first row), CL2 (second row), and CL1 (third row), respectively.
861 Transfer entropy in both directions (fish-to-robot and robot-to-fish) for each of the three
862 conditions is represented in panel G (+ SE). Means not sharing a common superscript are
863 significantly different. The significance was set at $\alpha < 0.05$.

864

865 **Figure 4** Estimated marginal mean (EMMs) differences represent adjusted mean differences
866 (+ SE) in Fulton's condition factor K across conditions once the contribution of fixed effects
867 included in the model (that is, sex and week) is accounted for. The white histogram
868 corresponds to controls (baseline, NP, and PM), the light grey histogram to replicas with low
869 biomimicry (OL1, OL2, and CL2), and the dark grey histogram to replicas with high
870 biomimicry (CL1). Means not sharing a common superscript are significantly different. The
871 significance was set at $\alpha < 0.05$.

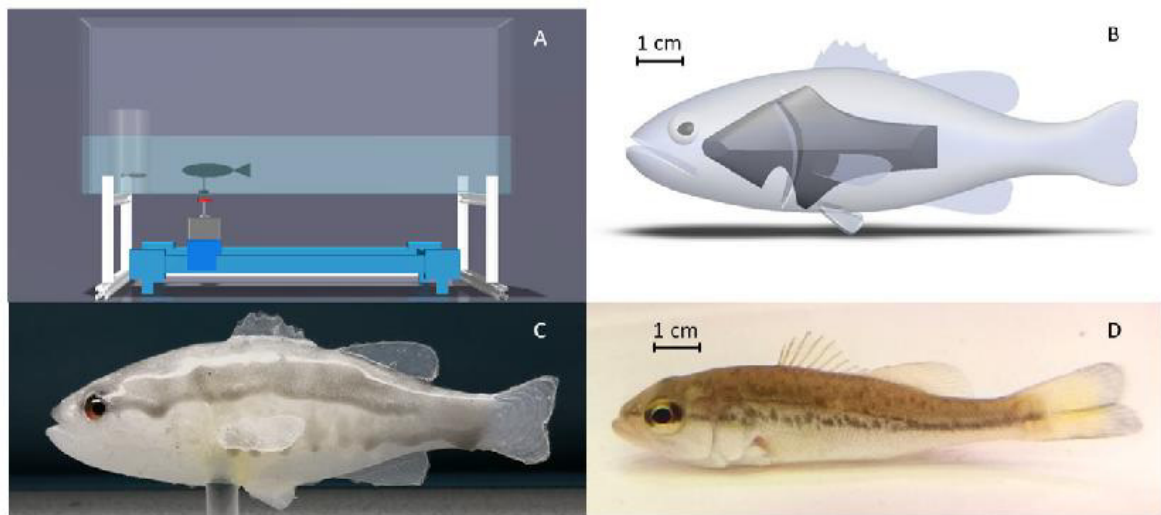
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874 **FIGURES**

875 **Figure 1**

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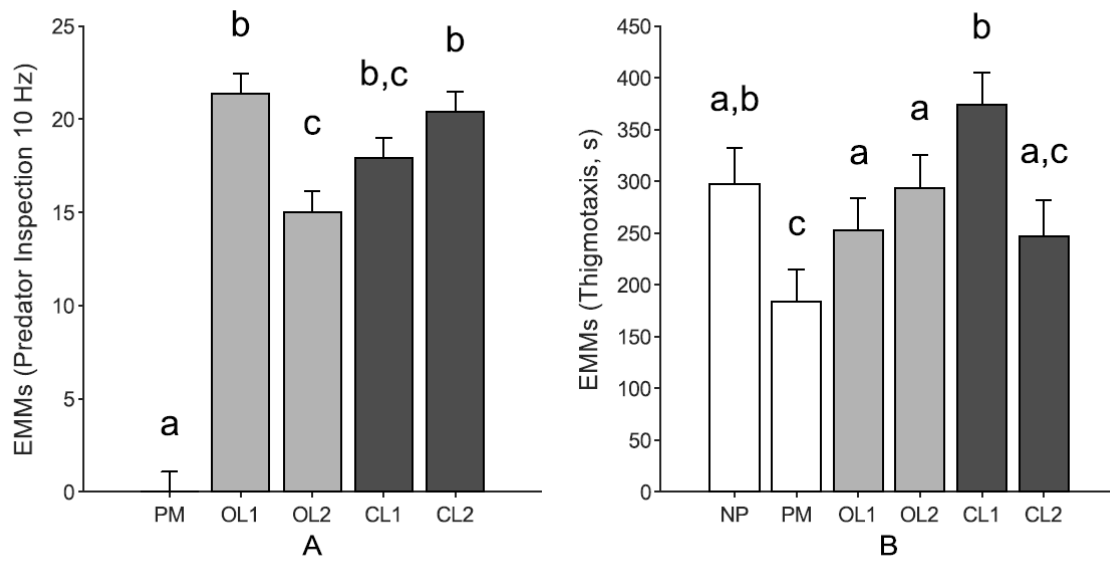
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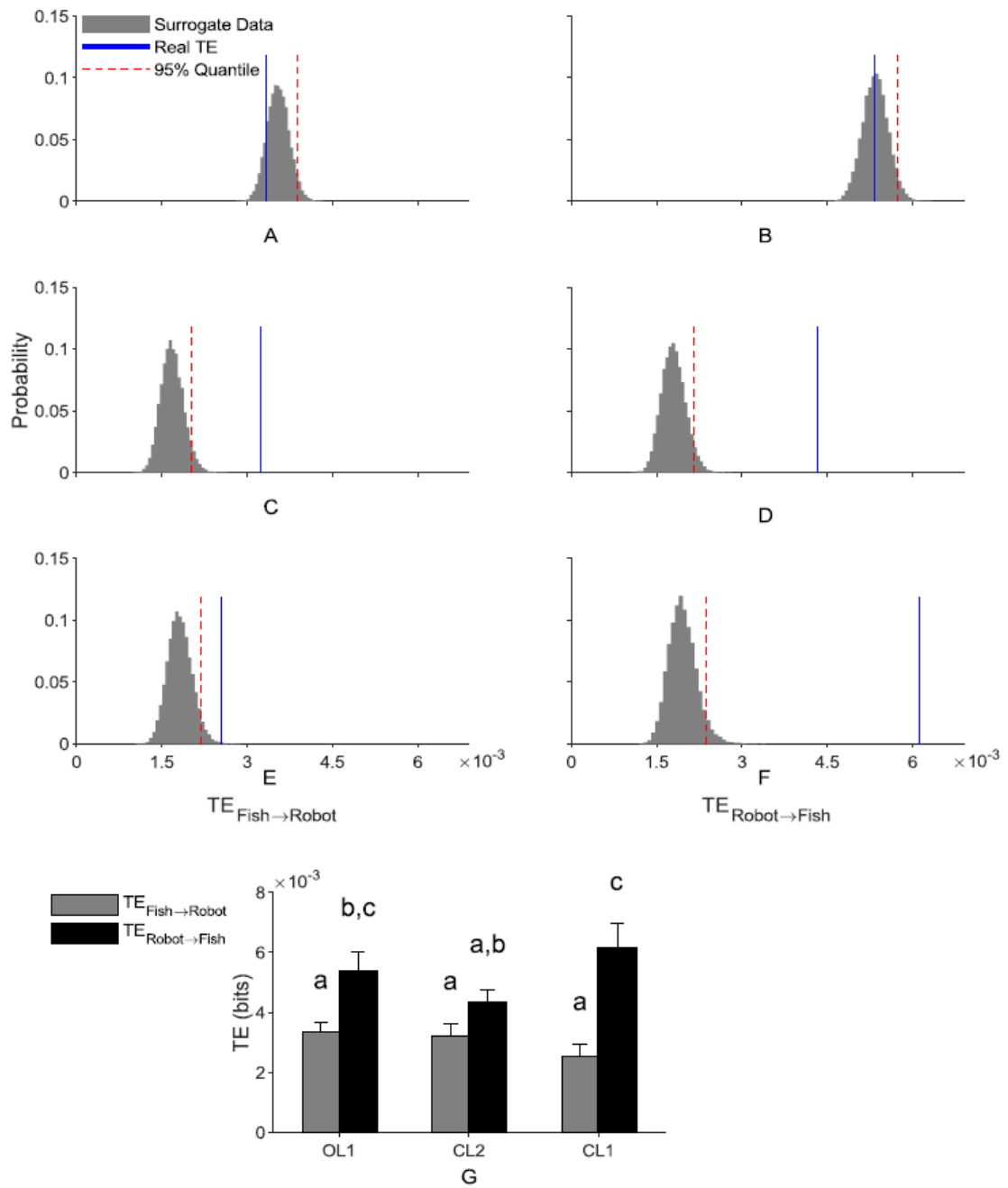
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892 **Figure 2**



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913 **Figure 3**



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