

1 **Replication alert: behavioural lateralisation in a detour test is not repeatable in fishes**

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24 **Abstract**

25
26 Behavioural lateralisation, defined as the asymmetric expression of cognitive functions, is
27 reported to enhance key fitness-relevant traits such as predator escape performance,
28 multitasking abilities, and group coordination. Therefore, studies reporting negative effects on
29 lateralisation in fish due to environmental stressors such as ocean acidification, hypoxia, and
30 pollutants are worrisome. However, such studies have focussed on population-level measures,
31 without validating whether lateralisation is consistent within individuals across time. We
32 conducted a multi-species, international assessment of the repeatability (R) of lateralisation in
33 four previously studied fish species using the common detour test, and re-analysed a
34 published dataset (on guppies) using new statistical methods. We expected the three shoaling
35 species to exhibit greater within-individual consistency in lateralisation than their non-
36 shoaling counterparts given previous reports of stronger lateralisation in group-living fishes.
37 However, both absolute and relative lateralisation scores were highly non-repeatable in all
38 five species ($0.01 < R < 0.08$). Thus, the commonly used detour test does not appear to be
39 appropriate for quantifying behavioural lateralisation in fishes, calling into question
40 inferences drawn by many published studies, including our own. As a consequence, potential
41 anthropogenic effects on lateralisation as a proxy for adaptive brain functioning need to be
42 assessed with alternative paradigms.

43
44 **Keywords:** Behavioural plasticity, *Ctenolabrus rupestris*, *Danio rerio*, laterality,
45 lateralization, *Neopomacentrus azysron*, *Poecilia reticulata*, *Pomacentrus amboinensis*,
46 repeatability, T-maze

48 **Introduction**

49
50 Behavioural lateralisation, the asymmetric expression of cognitive functions, has emerged as
51 an important fitness correlate in numerous taxa including invertebrates [e.g. 1] and vertebrates
52 [reviewed in 2]. Indeed, cerebral lateralisation is believed to offer advantages in terms of
53 enabling multiple stimuli to be processed simultaneously by different sides of the brain [2].
54 Fitness benefits associated with high degrees of lateralisation are thought to include increased
55 cognitive performance [3, 4], multitasking [5, 6], spatial learning [7], predator recognition
56 learning [8], schooling performance [9], coordination of group behaviours [2], prey capture
57 success [10], foraging efficiency [11], and escape performance [12, 13]. Despite these
58 apparent advantages, most studies report considerable inter- and intra-specific variation in the
59 strength and direction of lateralisation. Several studies also report a high degree of within-
60 individual variation in the strength of lateralisation in individuals measured repeatedly across
61 different contexts, suggesting that this trait may be more labile than previously believed [e.g.
62 8, 14]. Yet, no studies have systematically measured lateralisation several times per
63 individual, across multiple species, without a change in context, to establish the baseline
64 repeatability of this behaviour (but see [15] for a single-species study, the data for which are
65 re-analyzed here). Such a validation of the repeatability of lateralisation is crucial to assess the
66 usefulness of this trait, particularly when differences in individual-level lateralisation strength
67 between ecologically relevant treatments are used to infer effects on fitness.

68
69 Behavioural lateralisation is commonly measured in the laboratory using a detour test (e.g. T-
70 maze), wherein an individual moves along a runway until it faces a barrier forcing it to make
71 a choice between turning left or right. This quick and simple test is now widely used in
72 studies of fish behaviour, evolutionary ecology and ecotoxicology (Electronic Supplementary
73 Material [ESM], Table S1), and is also used to assess lateralisation in various other taxa

74 including birds [16], reptiles [17], and molluscs [1]. In fishes, lateral bias in a detour test is
75 believed to arise from asymmetries in eye-use, a phenomenon widely documented in animals
76 with laterally-placed eyes and low binocular overlap [18]. For example, goldbelly
77 topminnows (*Girardinus falcatus*) reported to exhibit a strong side-bias in a detour test also
78 display an eye preference for viewing a neutral *versus* threatening stimulus [19]. Similarly,
79 left-right asymmetries in *G. falcatus* assessed in a detour test appear to be consistent with
80 lateral bias measured using other methods [20]. Furthermore, lateralisation score in a detour
81 test is reported to be heritable [21, 22], although lateralisation strength decreases rapidly
82 across generations in artificial selection lines [23, 24]. These results have led researchers to
83 assert that the detour test does, indeed, assess inherent asymmetry in an individual's brain
84 function that influences fitness-relevant behavioural strategies.

85
86 Given likely fitness-relevant effects of lateralisation and the relative ease of use of the detour
87 test, various studies have evaluated how environmental and anthropogenic stressors such as
88 pollutants and climate change affect lateralisation in fishes, several of which have produced
89 worrying results (ESM, Table S1). Most notably, ocean acidification is reported to decrease
90 lateralisation across a range of marine fishes, including tropical [25-27] and temperate species
91 [28-30]. Such effects are concerning given, for example, the important benefits of
92 lateralisation reported for fishes under high predation risk [e.g. 8, 31, 32]. Despite this strong
93 interest in behavioural lateralisation and growing use of the detour test in the ecological,
94 cognitive and behavioural sciences, there is a notable absence of studies assessing the
95 repeatability of individual lateralisation scores. Establishing the consistency of lateralisation
96 within individuals is essential for determining the relevance of this trait for assessing fitness
97 as well as the responses of animals to exogenous stressors.

98
99 To evaluate the repeatability of behavioural lateralisation in fishes, we studied four species
100 from tropical and temperate environments, including marine and freshwater habitats: two
101 coral reef damselfishes, the Ambon damsel (*Pomacentrus amboinensis*) and yellowtail
102 demoiselle (*Neopomacentrus azysron*), the tropical freshwater zebrafish (*Danio rerio*), and
103 the temperate marine goldsinny wrasse (*Ctenolabrus rupestris*). All of these species have
104 previously been used in published studies assessing behavioural lateralisation using a detour
105 test (T-maze) similar to the one used here [8, 25, 26, 33, 34]. We also used new (more
106 appropriate) statistical methods to re-analyse a published dataset that included multiple
107 lateralisation measurements for the tropical freshwater guppy (*Poecilia reticulata*) [15]. We
108 predicted a significant repeatability in lateralisation scores comparable in magnitude to that
109 observed for other behavioural traits [35]. Population-level lateralisation strength was also
110 predicted to be higher in the shoaling species (*N. azysron* and *D. rerio*) compared to territorial
111 species (*P. amboinensis* and *C. rupestris*) as this should help enhance school cohesion [36].
112 We had no *a priori* prediction about the direction (left or right) of lateralisation, as among-
113 species differences in response to the same stimulus have been shown previously [36]. We
114 also examined for an effect of habituation to the test arena across the four test trials because
115 (1) repeatedly measuring the same individuals in a similar apparatus can decrease
116 measurement error and thus be predicted to increase individual repeatability [37], and
117 conversely (2) habituation or desensitisation to a stimulus may reduce repeatability by
118 generating different results between trials within an individual [37].

119 **Materials and methods**

120 *Fish collection and husbandry*

124 Sixty *C. rupestris* (1.38-46.52 g) were collected with baited minnow traps from the wharfs at
125 the Sven Lovén Centre for Marine Infrastructure, Kristineberg, Sweden. Experiments on *C.*
126 *rupestris* were conducted between 22 June and 1 July 2017 (water temperature range in the
127 holding tanks and experimental setup: 14.9-16.1°C). Sixty *N. azysron* (0.74-6.40 g) and 60 *P.*
128 *amboinensis* (1.0-14.3 g) were collected by SCUBA divers with a barrier net and hand nets on
129 coral reefs nearby the Lizard Island Research Station, Northern Queensland, Australia.
130 Experiments on these two species were conducted between 24 July and 11 August 2017
131 (water temperature range: 24.3-25.5°C). Sixty *D. rerio* (0.26-1.07 g) were collected in West
132 Bengal, India, in August to September 2016 using hand nets and kept in the Norwegian
133 University of Science and Technology's (NTNU) aquarium facility, Trondheim, until the
134 experiments were conducted between 10 and 17 October 2017 (water temperature range:
135 26.9-28.9°C).

136

137 Fish from each species were equally divided into three to four holding tanks (density ~5.5
138 individuals L⁻¹), provided with artificial plants and/or cut PVC pipes as shelter, and fed *ad*
139 *libitum* twice daily. *C. rupestris* were fed blue mussels (*Mytilus edulis*) and bloodworms.
140 *Neopomacentrus azysron* and *P. amboinensis* were fed commercial tropical fish flakes. *Danio*
141 *rerio* were fed TetraPro fish flakes. Holding tanks were supplied with flow-through water
142 from the fish's natural habitat for *C. rupestris*, *N. azysron*, and *P. amboinensis*. *D. rerio* were
143 kept in flow-through aquaria at NTNU, with a water exchange rate of ~20% per day, and
144 water quality (conductivity and temperature) monitored daily. Light cycles followed natural
145 conditions at each location. Fish were individually marked using two-colour combinations of
146 Visible Implant Elastomer (Northwest Marine Technology Inc., Shaw Island, WA, USA)
147 implanted postero-dorsally, on both sides of the dorsal fin a minimum of two days prior to the
148 first test.

149

150 *Lateralisation test*

151

152 We used a standard detour test to assess behavioural lateralisation [12, 30, 38]. Experiments
153 were conducted in the morning between 9:00-12:30 and fish were returned to their respective
154 holding tank between test days. A single fish was introduced into a double-sided, opaque T-
155 maze, consisting of a tank with a runway down the middle and a barrier at both ends
156 (dimensions in Fig. S1). *Ctenolabrus rupestris* and *D. rerio* were tested in one maze and *N.*
157 *azysron* and *P. amboinensis* in another (see ESM Fig. S1). A symmetrical neutral stimulus
158 was affixed to the centre of the barrier to focus the eye-gaze of the fish. This neutral stimulus
159 was changed between each of the four trials (see below) to prevent habituation to the set-up (a
160 cross, two parallel black bars, a cross with a solid circle above it, a cross with a horizontal bar
161 below it). The water height was 10 cm. Experiments were conducted in a closed room and
162 care was taken to ensure that the surrounding environment and lighting were as uniform as
163 possible. We emptied and re-filled the maze with new water between each fish tested to
164 standardise the temperature, maintain normal levels of dissolved oxygen, and to avoid any
165 effect of conspecific stress hormones (e.g. cortisol) on fish behaviour.

166

167 To start the experiment, a fish was placed at one end of the runway (maintained in this
168 position by an acrylic divider blocking the entrance to the middle runway) for one minute
169 (ESM Fig. S1). The starting side was determined by flipping a coin and the fish was
170 transferred from its holding tank to the maze in a water-filled container. The divider was then
171 lifted from behind the tank; if the fish did not advance on its own, it was gently pushed with
172 an acrylic paddle to initiate movement down the runway [30, 39]. At the end of the runway,
173 the fish faced an opaque barrier forcing it to turn left or right. We ran 10 consecutive trials per

174 fish, recording its turning direction each time. One experimenter conducted any given series
175 of 10 trials, standing behind the fish, in a centred position, and walked from one side of the
176 maze to the other between trials when the fish was behind the barrier (Fig. 1). The side of the
177 maze walked on was random. Another experimenter recorded the observations on paper
178 except for *D. rerio*, where observations were typed directly into a computer. Experiments
179 were recorded on video with the exception of *C. rupestris*, which was the first species tested
180 and where no video camera was available. Four series of 10 trials were repeated for each
181 individual at intervals of 48 h. Four full series could not be obtained for some individuals as
182 fish occasionally did not swim down the runway for 10 consecutive runs.

183
184 We calculated the relative lateralisation index (L_R) for each individual for each series of 10
185 trials, where $L_R = ((\text{turns to the right} - \text{turns to the left}) / (\text{turns to the right} + \text{turns to the left}))$
186 $\times 100$ [18]. A score of -100 indicates that the fish turned left 10 times out of 10; a score of
187 100 indicates that the fish turned right 10 times out of 10. L_R can be examined at both the
188 individual and the population (i.e. mean) level, informing whether individuals and/or
189 populations have a side bias, respectively. Since several studies conduct analyses on the
190 absolute lateralisation index (L_A), rather than L_R , we also calculated each individual's L_A for
191 each series, where $L_A = |L_R|$ (ESM Fig. S3). Scores between 60 and 100 (i.e. 8 and 10 turns to
192 one direction, respectively) are indicative of strong lateralisation.

193

194 *Re-analysis of published data*

195

196 To our knowledge, the only published study testing the consistency of individual lateralisation
197 scores was performed on 40 female guppies (*P. reticulata*) [15]. Females were individually
198 marked and tested in a detour test with 24 h or more between trial series. Fish were tested
199 three times in each of two conditions: a barrier presenting a neutral stimulus (an empty
200 aquarium behind a barrier of vertical bars) or a social stimulus (a conspecific in an aquarium
201 behind a barrier of vertical bars) (Fig. 1 in [15]). For lack of a better statistical approach at the
202 time, the data were analysed using six Spearman rank correlations to assess the consistency of
203 L_R scores [15]. We re-analysed these data and computed a single intra-class correlation
204 coefficient (R) for each of the two test conditions (see *Statistical analysis*).

205

206 *Statistical analysis*

207

208 We tested population-level lateralisation with generalised (binomial) linear random-effects
209 models, setting the intercept equal to the grand mean of the sample. Individual-level
210 lateralisation was examined with a chi-square test comparing the observed variance
211 (numerator) to the expected variance (denominator) assuming a normal approximation to the
212 binomial distribution. This is analogous to testing for overdispersion (i.e. are there more
213 observations in the tail ends of the distribution than expected by chance). See the ESM text
214 and Table S3 for details and an explanation of issues with tests of lateralisation employed in
215 previous studies.

216

217 We computed the repeatability (R) in the number of left and right turns (in a series of 10
218 trials) across trial series for each species using the 'rpt' function in the package 'rptR' [40].
219 This analysis on binomial data amounts to testing the repeatability of L_R . R values were
220 computed for primary data collected in this study and for the published data on *P. reticulata*
221 [15]. R ranges from 0 (non-repeatable) to 1 (fully repeatable) and provides a standardised
222 measure of the consistency of phenotypes across time or contexts [41]. We specified a
223 binomial error distribution and 1000 bootstrapping and permutation iterations to calculate

224 95% confidence intervals (CIs). We also computed an ‘adjusted repeatability’ by specifying
225 trial series (1 to 4), fish mass, and start-side of the maze as fixed effects in the models to
226 account for any confounding effects of these variables on R [41]. The repeatability of L_A was
227 calculated by specifying a Poisson error distribution. Finally, we tested the effect of
228 experimenter ID and arena start side on L_R using a generalised linear mixed-effects model
229 (GLMM) in the package ‘lme4’; species, start side, and their interaction were specified as
230 fixed factors in the model while fish ID and experimenter ID were included as random
231 factors. The importance of experimenter ID was tested with a likelihood ratio (LR) test.
232 Models were validated via diagnostic checks with the package ‘DHARMA’ [42]. Statistical
233 analyses were performed in R3.4.3 [43].

234

235 **Results**

236

237 Fish exhibited statistically significant individual-level lateralisation in two-thirds of trial
238 series, i.e. 15 of 22 trial series across the four species tested and the species for which data
239 were re-analysed (ESM Table S2 and Fig. S5-S9). Population-level lateralisation was
240 statistically apparent in five of the 22 trial series; two species exhibited a population side bias,
241 but this bias changed with test day for *D. rerio*, and stimulus type for *P. reticulata* (ESM
242 Table S2, Fig. S5-S9).

243

244 Importantly, however, L_R was highly variable within individuals and individual identity
245 explained less than 6% of the variance in relative lateralisation across the four species tested,
246 with repeatability estimates ranging from $R=0.006$ to $R=0.028$ (Table 1, Fig. 2, ESM Fig. S2).
247 Controlling for the order of trial series, fish mass, and start-side did not change these results
248 (Table 1); fixed factors accounted for less than 4% of the variance in L_R . Similar results were
249 obtained when considering L_A (ESM Fig. S3). The identity of the experimenter (LR test;
250 $\chi^2_{(1)}=0$, $P=0.999$) and the starting side of a series in the experimental arena had no effect on a
251 fish’s preference to turn left or right (GLMM; $\chi^2_{(1)}=0.01$, $P=0.917$), irrespective of the species
252 (GLMM; species \times start-side interaction; $\chi^2_{(3)}=2.00$, $P=0.575$); see also Table 1.

253

254 Similarly to the four species tested here, individual differences among female *P. reticulata*
255 [15] accounted for very little of the variance in L_R , both when fish were tested using a neutral
256 ($R=0.045$ [0.015-0.088]) and a social stimulus ($R=0.076$ [0.028-0.128]) (ESM Fig. S4).

257

258 The 95% CIs for the R estimates of L_R in two species (*N. azysron*, *D. rerio*) overlapped zero;
259 in the other three species, 95% CIs were narrow and close to zero, indicating high confidence
260 in very small (albeit non-zero) values of R (Table 1).

261

262 **Discussion**

263

264 Numerous studies on fishes and other taxa report that behavioural lateralisation is linked to
265 fitness-enhancing behaviours and can be severely impacted by environmental stressors such
266 as predation, parasitism, pollutants, drugs, and various abiotic parameters (see ESM Table S1
267 for examples in fishes). We assessed whether lateralisation is consistent in fish species with
268 differing ecologies to establish the baseline repeatability of this behavioural trait and broaden
269 our understanding of its ecological and evolutionary importance. Our validation exercise used
270 robust sample sizes, four species, multiple measurements through time (four repeats at 48 h
271 intervals), and included a re-analysis of data on a fifth species collected seven years ago.

272

273 Our results show that behavioural lateralisation as assessed by a detour test is not a repeatable
274 trait in fishes (Fig. 2, ESM Fig. S2-S4), and thus should be interpreted with great care. Even
275 though many individuals from all species displayed a strong side-bias (individual-level
276 lateralisation present in 68% of trial series; ESM Table S2, Fig. S5-S9), this turning
277 preference varied markedly across days (Fig. 2, ESM Fig. S4). Of the 69 individuals across all
278 five species displaying an absolute lateralisation (L_A) score of 80 or 100 (nine or 10 turns in
279 either direction) in at least one of the trial series, 52 also had at least one L_A score of 20 (four
280 or six turns in either direction) or zero (five turns each way) when tested again (ESM Fig. S3,
281 S4). Overall, across the five species examined, individual identity explained only a small
282 proportion of the phenotypic variance in lateralisation strength, ranging between 2.8 and
283 7.6%. These values contrast markedly with the results of a recent meta-analysis reporting that
284 individual differences account for a much higher proportion (approximately 35%) of the
285 variance in most behavioural traits [35]. A re-analysis of existing data (ESM Fig. S4) supports
286 our findings, and further suggests that individual decisions to turn left or right in the detour
287 test are random and do not provide accurate or precise estimates of eye-preference or cerebral
288 asymmetries in fishes. Our experiments were conducted by two or more researchers at any
289 one time and trials were video recorded except for *C. rupestris*. These recordings, lab
290 notebooks, and the raw data are publicly available [44]. The fact that multiple labs
291 collaborated to conduct this study across several geographic locations (Australia, Norway,
292 Sweden) further strengthens the robustness of our results [45].
293

294 Only two of the five species exhibited a mean L_R significantly different from zero in at least
295 one trial series, indicative of population-level lateralisation (ESM Table S2, Fig. S5-S9). This
296 population-level side-bias varied between days in the case of *D. rerio*, and stimulus type in
297 the case of *P. reticulata* (see methods in [15]). We anticipated a greater repeatability of L_R
298 and stronger evidence for a positive or negative mean L_R in the three shoaling species
299 examined (*N. azysron*, *D. rerio* and *P. reticulata*), as individual- and population-level
300 lateralisation are reported to help social individuals coordinate group behaviours and enhance
301 school cohesion [9, 36]. Bisazza & Dadda [9] used a detour test and reported that lateralised
302 poeciliids (*Girardinus falcatus*) exhibit greater school cohesion and coordination than non-
303 lateralised conspecifics; however, schools were composed of only two individuals, which
304 were females from a multi-generation laboratory strain. Bisazza et al. [36] also reported
305 significant population-level lateralisation in 10 of the 16 fish species they examined (n per
306 species: 7 to 18), yet only six of these were shoaling. Other studies have also reported such
307 population-level side biases in fishes [e.g. 15, 18, 19, 38, 46], yet their association with group
308 living appears ambiguous. For instance, Domenici et al. [26] and Lopes et al. [28] failed to
309 observe population-level lateralisation in shoaling *N. azysron* juveniles and *Atherina*
310 *presbyter* larvae, respectively, despite reporting L_A scores higher than random in their control
311 groups. Similarly, Chivers et al. [32] reported high L_R scores in some shoals of the schooling
312 fusilier *Caesio teres*, but lateralisation strength varied substantially both among and within the
313 four groups tested. Taken together, these results suggest that that the detour test is not
314 adequate for assessing lateralisation in fishes and that the benefits of lateralisation for
315 shoaling species might be overstated.
316

317 **Implications**

318
319 Given the large body of literature reporting significant effects of environmental stressors on
320 lateralisation in fishes (ESM Table S1), our results raise several questions that we address
321 below.
322

323 *Could methodological differences between ours and previous studies explain the lack of*
324 *repeatability in L_R ?*

325

326 Published studies have used a range of different obstacles to elicit eye-use preference when
327 fish arrive at the end of the runway in a detour test (Fig. S1). Some studies use a neutral
328 obstacle, such as an opaque barrier [e.g. 12, 14, 20, 26, 46] or a barrier of vertical bars [e.g.
329 15, 18, 47, 48]. Preferential eye-use is said to occur because fish must explore the unknown
330 space to the side or behind the barrier [19]. Other studies have used a non-neutral obstacle
331 with a stimulus, such as a conspecific or an object resembling a predator placed behind a
332 barrier of vertical bars [e.g. 5, 15, 19, 38]. The neutral stimuli (e.g. a cross on an opaque
333 barrier) used in our experiments may not have been valent enough to result in a strong,
334 consistent lateralised response in individuals. Indeed, some research suggests that
335 lateralisation direction and strength is stimulus-dependent [18, 49]. It is possible that a
336 predatory stimulus would have increased repeatability of measurements in this test since a
337 consistent behavioural response to a predator may be under stronger directional selection than
338 a neutral stimulus. However, previous studies using different methods for assessing
339 lateralisation in fishes (i.e. mirror tests or circular arena tests) have reported correlations in the
340 strength of individual lateralisation among tests, including between novel/neutral, predator,
341 and social stimuli [20, 50]. These results suggest that the specific stimulus used to focus gaze
342 should not significantly affect the strength of repeatability in the test. Our results provide
343 evidence for this: we found that L_R was not repeatable across time when experiments were
344 conducted with two different neutral stimuli (this study and re-analysis of [15]) as well as
345 with a non-neutral stimulus (re-analysis of [15]). The occurrence of high individual L_R scores
346 in all species in trial series 2, 3, and 4 of the experiment indicates that habituation to the test
347 arena is unlikely (Fig. 2). Importantly, studies have also reported a significant population-
348 level side-bias in species when only an opaque barrier with no stimulus was used [e.g. 31, 32,
349 38], suggesting that a neutral stimulus should not impede a strongly lateralised response in a
350 detour test. Interestingly, Ferrari et al. [8] found considerable variability in the lateralisation
351 scores of *P. amboinensis* tested twice on the same day using opaque barriers with no stimuli.
352 These results were interpreted as representing adaptive plasticity in lateralisation strength due
353 to different predation risk scenarios [8], yet these experiments lacked a control group
354 establishing the consistency of lateralisation strength in untreated individuals. Our results
355 suggest that such variability is likely due to the inherent randomness of turning choice
356 displayed by tested individuals rather than any adaptive behavioural decision. Similarly, a
357 recent reanalysis of a well-cited study on honeybee magnetoreception also revealed random
358 patterns rather than adaptive behaviour as originally suggested [51].

359

360 Numerous other methodological variations exist across published studies that are worth
361 considering in the context of our results. For example, studies differ in their maze design (e.g.
362 single T in [14]; Y- versus T-entry in [15, 30, 52]) and dimensions (ESM Table S1),
363 acclimation time before beginning a series (3 min in [14, 20]; 1 min in [39]), wait time
364 between trials within a series (3 min in [14]; no wait time in [34, 39]), and method for
365 encouraging fish to enter runway (e.g. [33] versus [34]), to name a few. Although subtle,
366 these differences in protocol or experimental apparatus could considerably influence the
367 results. For instance, Clark et al. (unpublished data) examined lateralisation behaviour in
368 juvenile *Acanthochromis polyacanthus* in a double T-maze and found that a slight asymmetry
369 in the barrier position at one end of their maze induced a strong side-bias in their tested fish.
370 This side-bias was not observed at the other end of the arena where the barrier was centrally
371 placed. Similarly, Sundin et al. (unpublished data) tested wild *P. reticulata* in a T-maze arena,
372 which they later discovered had a small crack in one corner, causing this area of the tank to be

373 slightly darker than the other side. Individuals consistently turned in the direction of this
374 darker area, but no consistent side bias was apparent at the other end of the maze, or in the
375 same individuals when tested in other identically constructed arenas. These observations
376 illustrate the care that must go into the construction of experimental apparatus designed to
377 assess side biases in individuals, as slight construction asymmetry or variations in protocol
378 may dramatically influence the results obtained. Such side biases generated through very
379 slight differences in arena construction or lighting may go overlooked, particularly in analyses
380 of absolute lateralisation.

381

382 *Could differences in species, sex, and/or life stage influence the repeatability of L_R ?*

383

384 Twenty-seven fish species have so far been tested in 31 published studies using a detour test
385 to either relate lateralisation to fitness-relevant traits or assess the effects of environmental
386 stressors on lateralisation strength (ESM Table S1). Several other fish species also feature in
387 studies simply examining whether individuals or populations are lateralised [e.g. 36, 53]. The
388 five species examined here (four original and one re-analysed) are phylogenetically diverse,
389 originating from tropical, temperate, marine, and freshwater habitats. Therefore, they
390 constitute a representative sample allowing our results to be generalised with a reasonable
391 degree of confidence.

392

393 We did not include sex in our analyses of repeatability. Some studies suggest that sex should
394 always be considered in studies of cerebral lateralisation because male and female brains are
395 organised differently [54]. A meta-analysis also suggests that there are important sex-
396 differences in the repeatability of many behavioural traits [35]. Indeed, several studies have
397 reported sex-specific differences in lateralisation strength in fishes (e.g. [15, 38, 55-57]),
398 although others have not (e.g. [33, 39, 47, 57]). While this is worth exploring in future studies,
399 it is unlikely that sex differences account for the dramatically low repeatability in
400 lateralisation strength measured in all four of our study species ($R=0.006$ to $R=0.028$). With
401 the exception of the protogynous *P. amboinensis*, where our sample is likely to be female-
402 biased based on the size distribution of the collected individuals [58], the remaining species
403 tested have distinct sexes and are not strongly sexually dimorphic. As a result, we assume a
404 roughly even sex-ratio in our samples. If lateralisation in one sex were highly repeatable, we
405 would still expect an R measure considerably higher than observed, with consistency in a
406 turning direction observed in approximately half of the population. This is clearly not
407 observable in our data (Fig. 2).

408

409 The effect of life stage on the strength of lateralisation is another consideration that was not
410 explicitly addressed in our study. Although Bell et al. [35] found no difference in the
411 repeatability of behaviours between juveniles and adults in general, they note that, among
412 ectotherms, juvenile behaviour is significantly more repeatable. We included a range of sizes
413 in our tested species, which, for *C. rupestris* and *D. rerio*, included juveniles and sub-adults.
414 We also included fish body mass as a fixed factor in our analyses. We found no effect of mass
415 on the strength of lateralisation in any of our tested species. Published studies of detour tests
416 in fishes have tested a range of life stages from pre-settlement larval fish to adults (ESM
417 Table S1). Although there does not seem to be a consistent trend in lateralisation strength
418 owing to life stage, this should be tested more systematically in future studies.

419

420 *What explains positive results in previous studies?*

421

422 The high intra-individual variation in lateralisation we observed across test days in all five
423 species examined (Fig. 2, ESM Fig. S1) suggests that individual L_R scores are random at any
424 point in time. Therefore, why do numerous studies report significant relationships between
425 lateralisation strength and other phenotypic traits or environmental stressors? Unfortunately,
426 many lateralisation studies (including previous publications by authors involved in this study)
427 suffer from low sample sizes (e.g. 20 or fewer individuals per group [8, 14, 25, 28, 30, 36, 39,
428 57]; ESM Table S3), which considerably increases the likelihood of spurious results [59-61].
429 In addition, all 35 tests (31 studies) identified in ESM Table S3 employed inadequate statistics
430 to test for the presence of lateralised individuals (see *Supplemental materials and methods* in
431 the ESM). Confirmation bias and poor research practices such as p-hacking and selective
432 reporting also contribute to false positives, which are published more readily than negative
433 results (i.e. the publication bias or file-drawer effect) [62, 63]. A recent survey of over 800
434 researchers revealed that such practices are rife in ecology and evolution, contributing to the
435 ongoing reproducibility crisis [64]. Improving our confidence in, and ability to replicate,
436 lateralisation studies requires the implementation of validated methodologies, appropriate
437 statistics, high powered designs [61], double-blinded protocols [65], video recordings [66],
438 open data [67], and other transparency measures advocated by the recent Transparency and
439 Openness Promotion (TOP) guidelines [68, 69].

440

441 **Conclusion**

442

443 Behavioural lateralisation is likely to be an ecologically important trait that should continue to
444 be tested and measured. However, the method with which to assess eye-use preference as a
445 proxy of cerebral asymmetry must be thoroughly validated for a species of interest before
446 drawing conclusions about the fitness-consequences of changes in lateralisation direction or
447 strength. Drawing inferences from non-repeatable measurements (or studies) not only
448 obfuscates our understanding of a species' evolutionary ecology but also risks misleading
449 policy and conservation efforts. Our study shows that the detour test as it has traditionally
450 been implemented does not provide accurate, precise, or repeatable estimates of behavioural
451 lateralisation in fishes. However, numerous other methods of assessing eye-use and side
452 preference, including mirror tests, swimming, feeding or attack direction preference, and
453 flume tests, have been applied in a range of species in both field and laboratory settings (see
454 [3, 10, 70-76]). Measurements using these methods and their cross-context repeatability
455 should be validated in accordance with TOP guidelines [69] to establish reproducible
456 protocols that inspire confidence.

457

458

459 **Animal ethics**

460 Field collections and experiments were approved by the Swedish Board of Agriculture (Dnr
461 103-2014), Great Barrier Reef Marine Park Authority (G14/36625.1), the James Cook
462 University Animal Ethics Committee in association with AIMS (A2314), and the Norwegian
463 Animal Research Authority (Permit Number:8578).

464

465 **Data, code, and materials**

466 The data, script, notebook copies, and videos for this study are archived in the repository
467 figshare following best practices [67] and were made available to editors and reviewers upon
468 initial submission: <https://doi.org/10.6084/m9.figshare.6881489.v1> [44].

469

470 **Data re-use**

471 Published data [15] were re-used for this study. The original authors were invited to
472 participate and offered co-authorship.

473

474 **Competing interests**

475 We have no competing interests.

476

477 **Author contributions**

478 DGR, MA, JS, TDC, FJ, TN, BSR, and SAB designed the experiments; DGR, MA, RM, JS,
479 AHA, MHF, FJ, MJL, EH, and SAB performed the experiments. JS, FJ and RB contributed
480 vital equipment and funding for the experiments. CB provided the data used in the re-analysis.
481 DGR analysed the data. DGR, MA, and SAB wrote the manuscript with input and approval
482 from all authors.

483

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497

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- 699

700 **Table 1.** Sample size (n), mass range (g), total length range (TL range, cm), and statistics for
701 five species of fish tested to examine the repeatability of behavioural lateralisation. P .
702 *reticulata* were tested with a neutral (¹) and a social (²) stimulus (see [15]). Estimates are
703 presented for agreement and adjusted repeatability of relative lateralisation (L_R) with 95% CIs
704 in parentheses. Statistics and P values are presented for the effect of trial series (1 to 4), body
705 size (total length for *P. reticulata* and mass for all other species), and start-side of the maze on
706 L_R .
707

| | <i>C. rupestris</i> | <i>N. azysron</i> | <i>P. amboinensis</i> | <i>D. rerio</i> | <i>P. reticulata</i> ¹ | <i>P. reticulata</i> ² |
|-----------------|---------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|
| n | 57 | 52 | 60 | 49 | 40 | 40 |
| mass range | 1.61-14.9 | 0.90-6.40 | 1.0-10.0 | 0.43-1.07 | NA | NA |
| TL range | 4.00-45.24 | 4.16-8.27 | 3.77-7.90 | 2.68-3.58 | 1.74-3.51 | 1.74-3.51 |
| R (agreement) | 0.028 (0.004-0.052) | 0.012 (0.000-0.033) | 0.027 (0.008-0.046) | 0.006 (0.000-0.023) | 0.045 (0.015-0.088) | 0.076 (0.028-0.128) |
| R (adjusted) | 0.028 (0.006-0.053) | 0.012 (0.000-0.028) | 0.022 (0.004-0.038) | 0.007 (0.000-0.023) | 0.046 (0.011-0.081) | 0.077 (0.030-0.126) |
| trial series | $\chi^2_{(1)} = 0.123$ $P = 0.725$ | $\chi^2_{(1)} = 2.379$ $P = 0.123$ | $\chi^2_{(1)} = 0.663$ $P = 0.415$ | $\chi^2_{(1)} = 3.078$ $P = 0.080$ | $\chi^2_{(1)} = 2.757$ $P = 0.097$ | $\chi^2_{(1)} = 1.696$ $P = 0.193$ |
| body size | $\chi^2_{(1)} = 0.084$ $P = 0.772$ | $\chi^2_{(1)} = 0.890$ $P = 0.345$ | $\chi^2_{(1)} = 0.458$ $P = 0.499$ | $\chi^2_{(1)} = 0.002$ $P = 0.963$ | $\chi^2_{(1)} = 3.050$ $P = 0.081$ | $\chi^2_{(1)} = 0.942$ $P = 0.332$ |
| start side | $\chi^2_{(1)} = 0.204$ $P = 0.651$ | $\chi^2_{(1)} = 0.849$ $P = 0.357$ | $\chi^2_{(1)} = 0.354$ $P = 0.552$ | $\chi^2_{(1)} = 0.020$ $P = 0.887$ | N/A | N/A |

708

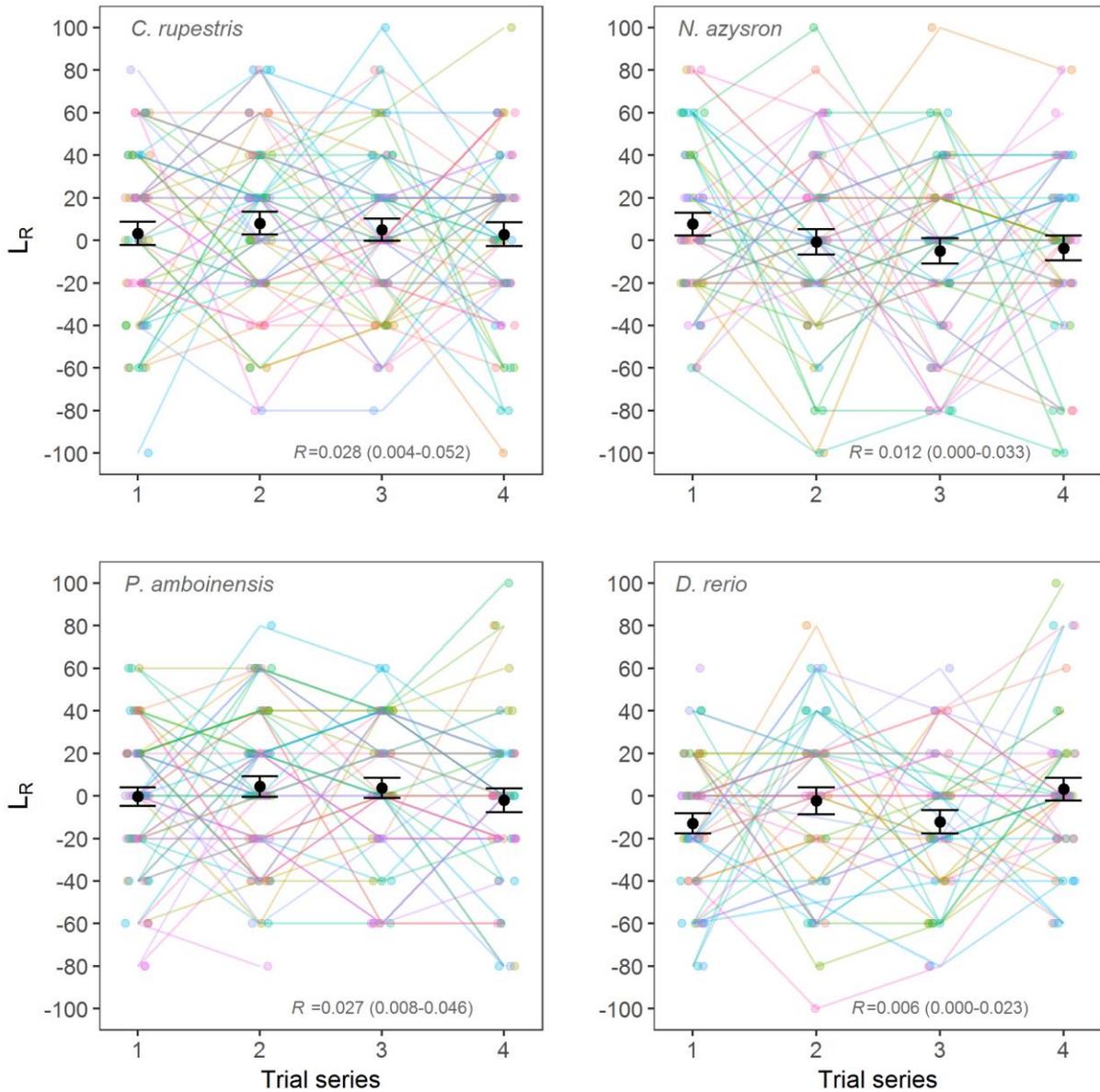
709 **Fig. 1** The four fish species from temperate, tropical, marine, and freshwater habitats tested to
710 determine the repeatability of lateralisation: *Ctenolabrus rupestris* (credit: F. Jutfelt),
711 *Neopomacentrus azysron* (credit: picture.world, <https://goo.gl/mTLphF>), *Pomacentrus*
712 *amboinensis* [77], and *Danio rerio* (credit: P.H. Olsen, NTNU).

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721 **Fig. 2** Relative lateralisation index (L_R) across repeated trials (2 to 4) per individual for four
 722 fish species: *Ctenolabrus rupestris* ($n=57$), *Neopomacentrus azysron* ($n=52$), *Pomacentrus*
 723 *amboinensis* ($n=60$), and *Danio rerio* ($n=49$). Individual-level L_R is indicated by the coloured
 724 dots connected by lines; the mean (i.e. population-level) L_R and its 95% CI are indicated by
 725 black dots and error bars. The repeatability index (R) of L_R and its 95% CI are indicated for
 726 each species. Four series of 10 trials were conducted. Data points are jittered along the x-axis
 727 for presentation purposes.
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SUPPLEMENTARY MATERIAL

Replication alert: behavioural lateralisation in a detour test is not repeatable in fishes

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Supplementary materials and methods

Issues with tests of behavioural lateralisation in the literature

We systematically reviewed the statistical tests employed by studies in Table S1 to determine whether groups of fish exhibit population- and/or individual-level lateralisation. We found that authors often use an arbitrary cut-off or eyeball the distribution of lateralisation scores obtained to determine whether there is evidence of population- or individual-level lateralisation (e.g. see Tables S1 and S3 for a full list of studies). Many studies employ linear models to compare the mean lateralisation scores of control and treatment groups but without testing whether control and treatments groups actually exhibit lateralisation (at the population- or individual-level) (Table S3). This is problematic because populations might have significantly different lateralisation scores without statistically exhibiting lateralisation. Worryingly, the few studies that relied on statistics to explicitly test for lateralisation employed inadequate tests, such as a one-sample t-test or a G-test of goodness-of-fit (this includes previous studies by the authors) [e.g. 1, 2-6] (Table S3). Unfortunately, goodness-of-fit tests such as the G-test result in an inflated type-I error rate when the total sample size is less than 500 and/or an expected number (e.g. the number of right or left turns taken by an individual) is less than five ([7 p. 86-89], page 86-89). Given that, in a detour test, each individual is subjected to only 10 decisions whether to turn left or right, the probability of obtaining an expected number of right or left turns smaller than five is 90%. Therefore, using such tests considerably increases the probability of false positives (see [see 7 p. 87], page 87).

Testing for lateralisation is not straightforward because it involves multiple binomial experiments with structure. Since a binomial trial (a decision to turn left or right) is repeated 10 times per individual in the detour test, trials conducted on multiple individuals are non-independent. This data structure is different from a simpler situation, such as in a standard binomial experiment (e.g. a coin toss or a trial involving a decision to turn left or right), repeated multiple times by/on a single individual. Here, each trial can be treated as independent.

We have developed and examined the sensitivity of two statistical approaches for testing population- and individual-level lateralisation. A test for detecting lateralisation at the population level requires examining the mean lateralisation score across all individuals in the sample since population-level lateralisation is present when a group of individuals collectively exhibits a side-bias. Specifically, this test involves assessing whether the mean number of turns to the right (or to the left) is significantly different from five, given 10 trials per individual. In contrast, a test for detecting individual-level lateralisation requires examining the sample variance since individual-level lateralisation is present when more individuals exhibit a side-bias than expected by chance (irrespective of whether it is to the left or to the right). For explanations and examples of these two concepts, see Bisazza et al. [8], Domenici et al. [1], and Roche et al. [3].

A robust test to detect population-level lateralisation

Population-level lateralisation can be tested with a generalised linear random-effects model (glmer function in R) that sets the intercept equal to the grand mean of the data. This model can be written as:

```
g <- glmer(X~1+(1|ind), data=dat, family="binomial")
```

where ‘dat’ is a data frame containing three columns: individual ID (‘ind’); trial number (1 to 10); and turning side (column ‘X’) as right (1) or left (0). Specifying the error family as “binomial” sets

the probability of success (p) to 0.5. Here, a right turn is considered a success. One *expects* a mean number of 5 turns to the right across all individuals if $p = 0.5$ (i.e. there is a 50% chance of turning left or right). Testing whether the *observed* mean number of turns to the right differs from the *expected* mean under a binomial error distribution with $p = 0.5$ simply requires extracting the p-value for the model intercept as such:

```
summary(g)$coefficients[4]
```

If $P < 0.05$, there is statistical evidence that the population is lateralised (i.e. has a side-bias).

A robust test to detect individual-level lateralisation

Individual-level lateralisation can be tested with a chi-square test comparing the observed variance (numerator) to the expected variance (denominator) assuming a normal approximation to the binomial distribution. This is analogous to testing for overdispersion (i.e. are there more observations in the tail ends of the distribution than expected by chance), and is achieved as such:

```
chi_sq=((N-1)*var(X1)/(n*.5*.5))
pchisq(chi_sq,df=(N-1),lower.tail=F)
```

where N is the total number of individuals tested; n is the number of trials per individual (i.e. 10); and $X1$ is a column in a data frame corresponding to the number of right (or left) turns per individual (each row of the data frame represents one individual).

If $P < 0.05$, there is statistical evidence that some individuals are lateralised (i.e. more individuals have an extreme lateralisation score than expected by chance based on a normal approximation to the binomial distribution with $p = 0.5$).

Sensitivity analysis

We ran simulations to examine the tests' sensitivity and probability of type I and type II errors. Both tests were robust to a changes in N , n , and p across a wide range of values. The R code to carry out the tests and run the sensitivity analysis are publicly available on figshare: <https://figshare.com/s/b8a70d0f083e7741508f>.

Alternatives

Note that: 1) individual-level lateralisation can also be assessed with a GLMM rather than a chi-square test but the GLMM approach is less powerful; and 2) population-level lateralisation can also be assessed with a Z-test rather than a GLMM – both have similar power but the Z-test does not account for structure in the data (i.e. it ignores individual ID). We provide these alternatives but recommend the two tests detailed in the paragraphs above.

Table S1. Fish studies documenting links between phenotypic traits or environmental stressors and lateralisation assessed using a detour test. Studies that used a detour test only to assess whether individuals or populations are lateralised are not included. Similarly, studies that used methods other than a detour test to assess lateralisation are excluded. Information is provided on: the type of environmental stressor or phenotypic trait examined (stressor / trait) for each species; life stage; body size (T = treatment group, C = control group); sample size (*n*); environment (env.; FW = fresh water, SW = salt water); whether or not individuals are shoaling/schooling (shoal / school; yes or no); habitat (temperate or tropical); sex (♀ = females, ♂ = males, ♀♂ = both sexes); whether population-level lateralisation was assessed (pop. lat.; R = right biased, L = left biased); the effect of the environmental stressor on the strength of lateralisation or the relationship between lateralisation and a given trait (effect; ‘+’ = increase or positive, ‘-’ = decrease or negative); whether relative (L_R) and/or absolute (L_A) lateralisation were examined (L_R / L_A); the dimensions of the T-maze in cm (dimensions; L = length of the runway, W = width of the runway, B = length of the barrier); reference (ref.).

| stressor / trait | species | life stage | body size | <i>n</i> | env. | shoal / school | habitat | sex | pop. lat. | effect | L _R / L _A | dimensions | ref. |
|-----------------------|------------------------------------|------------|------------------------------|----------------|------|----------------|-----------|-----|-----------|-----------------------|---------------------------------|---------------------|------|
| aggression | <i>Archocentrus nigrofasciatus</i> | adult | NA | C=26 T=14 | FW | no | tropical | ♀♂ | no | ♀ + right ♂ + left | L _R + L _A | L=75, W=10, B=15 | [9] |
| aquatic acidification | <i>Acanthochromis polyacanthus</i> | juvenile | NA | C=105 T=250 | SW | yes | tropical | NA | yes (L) | - | L _R + L _A | L=25, W=3, B=12 | [10] |
| | <i>Argyrosomus regius</i> | juvenile | 6.8±0.6 cm 2.6±0.8 g | 10 | SW | | temperate | NA | yes (L) | - | L _R + L _A | NA | [11] |
| | <i>Atherina presbyter</i> | larval | 20.48±0.30 mm | C=46 T=60 | SW | yes | temperate | NA | yes (L) | - | L _R + L _A | L=50, W=3, B=NA | [12] |
| | <i>Ctenolabrus rupestris</i> | adult | C: 94.2±8.46 93.7±8.25 mm | C&T= 17-24 | SW | no | temperate | NA | no | none | L _R + L _A | L=33, W=9, B=NA | [13] |
| | <i>Danio rerio</i> | adult | NA | C&T= 7-11 | FW | yes | tropical | ♀♂ | no | + | L _R + L _A | L=33, W=9, B=NA | [14] |
| | <i>Gadus morhua</i> | juvenile | 5.8±0.3 to 13.0±2.4 g | C=21 T=17 | SW | yes | temperate | NA | yes (L) | none | L _R + L _A | L=33, W=9, B=NA | [15] |
| | <i>Gasterosteus aculeatus</i> | adult | ~1.6 - 1.9 g | C&T= 20-25 | SW | yes | temperate | ♀ | no | - | L _A | L=33, W=9, B=NA | [16] |

| | | | | | | | | | | | | | |
|------------------|------------------------------------|----------|----------------------------------|--------------------|----|-----|-------------|----|---------|----------|---------------------------------|----------------------|------|
| | <i>Gasterosteus aculeatus</i> | adult | 46.5±2.2 mm 1.3±0.2 g | C=11-12 T=12-13 | SW | yes | temperate | NA | no | – | L _A | L=33, W=9, B=NA | [17] |
| | <i>Gasterosteus aculeatus</i> | adult | C: 46.5±8.9 mm T: 46.2±6.8 mm | C=14 T=13 | SW | yes | temperate | ♀♂ | yes (R) | – | L _R + L _A | NA | [18] |
| | <i>Gobiusculus flavescens</i> | adult | 0.73-0.86 g | C=11-30 T=19-25 | SW | yes | temperate | ♀♂ | yes (R) | none | L _R + L _A | L=33, W=9, B=NA | [19] |
| | <i>Neopomacentrus azysron</i> | larval | ~12 mm | C=15-30 T=15-30 | SW | yes | tropical | NA | NA | – | L _A | L=25, W=3, B=NA | [5] |
| | <i>Neopomacentrus azysron</i> | larval | 12.1±0.0 mm | C=70 T=68 | SW | yes | tropical | ♀♂ | no | – | L _R + L _A | L=25, W=3, B=12 | [1] |
| | <i>Pomacentrus wardi</i> | juvenile | NA | 24-58 | SW | yes | tropical | ♀♂ | yes (R) | reversed | L _R + L _A | L=25, W=3, B=12 | [2] |
| boldness | <i>Archocentrus nigrofasciatus</i> | adult | NA | 100 | FW | no | tropical | ♀♂ | no | + | L _R + L _A | L=75, W=10, B=15 | [6] |
| | <i>Heterodontus portusjacksoni</i> | juvenile | ~30 cm | 17 | SW | no | tropical | NA | no | none | L _R + L _A | NA | [4] |
| | <i>Poecilia reticulata</i> | adult | ♀: 28.4±0.4 mm ♂: 19.2±0.2 mm | 80 | FW | yes | tropical | ♀♂ | yes (R) | none | L _R + L _A | L=25, W=10, B=7.5 | [20] |
| cognition | <i>Cyprinus carpio</i> | adult | 4.6-6.9 g 7.0-7.8 cm | C=8 T=8 | FW | no | temperate | NA | no | none | L _R | L=30, W=12, B=30 | [21] |
| | <i>Girardinus falcatus</i> | adult | NA | 12-16 | FW | yes | tropical | ♀ | no | + | L _R | NA | [22] |
| | <i>Girardinus falcatus</i> | adult | NA | 6-9 | FW | yes | tropical | ♀ | no | + | L _R | NA | [23] |
| | <i>Gambusia holbrooki</i> | adult | NA | 4-7 | FW | yes | subtropical | ♀ | no | + | L _R | L=40, W=7, B=NA | [24] |

| | | | | | | | | | | | | | |
|---------------------------|--------------------------------|----------|---|--|----|-----|-----------|----|---------|---|---------------------------------|---------------------|------|
| cortisol | <i>Pomacentrus amboinensis</i> | juvenile | NA | C=25 T=25 | SW | no | tropical | NA | no | - | L _R | L=25, W=3, B=12 | [25] |
| growth rate | <i>Geophagus brasiliensis</i> | adult | 7.0-14.4 cm | 26 | FW | no | tropical | ♀♂ | no | + | L _R + L _A | L=75, W=10, B=NA | [26] |
| hypoxia | <i>Leptocottus armatus</i> | adult | 12.4±0.3 cm (mean±SE) | C=42 T=42 | SW | no | temperate | NA | yes (R) | - | L _R + L _A | L=75, W=10, B=20 | [27] |
| parasitism | <i>Scolopsis bilineatus</i> | adult | T: 11.8±2.5 C: 14.8±1.5 cm (mean±SD) | C=25 T=16 | SW | no | tropical | NA | no | + | L _R + L _A | L=70, W=15, B=25 | [3] |
| pesticide exposure | <i>Acanthurus triostegus</i> | larval | NA ¹ | C=10 T=10 | SW | yes | tropical | NA | no | - | L _R | NA | [28] |
| Pharmaceuticals | <i>Argyrosomus regius</i> | juvenile | C: 5.4-6.8 T: 5.4-6.6 cm | C=10 T=10 | SW | | temperate | NA | yes (L) | - | L _R + L _A | NA | [11] |
| predation | <i>Brachyrhaphis episcopi</i> | adult | ~2.5 cm | T ₁ =32 T ₂ =27 | FW | no | tropical | ♀♂ | no | + | L _R + L _A | L=47, W=10, B=NA | [29] |
| | <i>Poecilia reticulata</i> | adult | NA | C=65 T=68 | FW | yes | tropical | ♂ | no | + | L _R + L _A | L=NA, W=8, B=15 | [30] |
| | <i>Caesio teres</i> | juvenile | 3.59±0.03 cm (mean±SE) | 6-35 | SW | yes | tropical | NA | yes (R) | + | L _R + L _A | L=25, W=3, B=12 | [31] |
| | <i>Pomacentrus amboinensis</i> | juvenile | NA | T ₁ =10 T ₂ =15 | SW | no | tropical | NA | no | + | L _R + L _A | L=25, W=3, B=12 | [25] |
| | <i>Pomacentrus chrysurus</i> | juvenile | NA ² | C=30 T=30 | SW | no | tropical | NA | NA | + | L _A | L=25, W=3, B=12 | [32] |
| schooling | <i>Girardinus falcatus</i> | adult | NA | 17-37 | FW | yes | tropical | NA | no | + | L _R | NA | [33] |
| temperature | <i>Pomacentrus wardi</i> | juvenile | NA | C=20 T=20 | SW | yes | tropical | ♀♂ | yes (R) | - | L _R + L _A | L=25, W=3, B=12 | [2] |

| | | | | | | | | | | | |
|---------------------------|----------|--------------------------------|--------------|----|-----------|----|---------|---|---------------------------------|----|------|
| <i>Argyrosomus regius</i> | juvenile | C: 5.4-6.8 T: 7.0-8.6 cm | C=10 T=10 | SW | temperate | NA | yes (L) | - | L _R + L _A | NA | [11] |
|---------------------------|----------|--------------------------------|--------------|----|-----------|----|---------|---|---------------------------------|----|------|

¹ 0.21-0.25 cm at capture; ² settlement stage

Table S2. Statistics for tests of individual- and population-level lateralisation for five species of fish: *Ctenolabrus rupestris* ($n=57$), *Neopomacentrus azysron* ($n=52$), *Pomacentrus amboinensis* ($n=60$), *Danio rerio* ($n=49$), and *Poecilia reticulata* ($n=40$). Individuals were tested on four occasions (trials 1-4), except for *P. reticulata*, which were tested three times using both a neutral and a social stimulus (see [20]). A chi-square statistic (ind. χ^2) and P -value (ind. P) are presented for tests of individual-level lateralisation; $P < 0.05$ indicates lateralisation. A z-value (pop. z) and P -value (pop. P) are presented for tests of population-level lateralisation; $P < 0.05$ indicates lateralisation. Statistical significance is indicated in bold ($\alpha=0.05$).

| Species | trial | ind. χ^2 | ind. P | \bar{X} | pop. z | pop. P |
|--|-------|---------------|--------------|-------------|--------------|--------------|
| <i>C. rupestris</i> | 1 | 89.42 | 0.002 | 5.16 | -0.60 | 0.546 |
| | 2 | 91.88 | 0.002 | 5.40 | -1.53 | 0.126 |
| | 3 | 81.80 | 0.011 | 5.25 | -0.99 | 0.322 |
| | 4 | 93.14 | 0.001 | 5.14 | -0.52 | 0.603 |
| <i>N. azysron</i> | 1 | 70.31 | 0.025 | 4.98 | -1.45 | 0.149 |
| | 2 | 92.70 | 0.000 | 5.22 | 0.15 | 0.885 |
| | 3 | 93.50 | 0.000 | 5.19 | 0.87 | 0.386 |
| | 4 | 84.55 | 0.001 | 4.90 | 0.64 | 0.521 |
| <i>P. amboinensis</i> | 1 | 68.39 | 0.189 | 5.38 | 0.08 | 0.939 |
| | 2 | 80.05 | 0.029 | 4.96 | -0.93 | 0.354 |
| | 3 | 60.05 | 0.207 | 4.75 | -0.82 | 0.412 |
| | 4 | 73.80 | 0.010 | 4.82 | 0.37 | 0.712 |
| <i>D. rerio</i> | 1 | 51.59 | 0.299 | 4.35 | 2.71 | 0.007 |
| | 2 | 70.57 | 0.004 | 4.88 | 0.39 | 0.696 |
| | 3 | 49.50 | 0.144 | 4.39 | 2.24 | 0.025 |
| | 4 | 58.36 | 0.072 | 5.16 | -0.59 | 0.557 |
| <i>P. reticulata</i> (neutral stimulus) | 1 | 78.04 | 0.000 | 3.65 | -3.72 | 0.000 |
| | 2 | 49.56 | 0.120 | 4.05 | -3.35 | 0.001 |
| | 3 | 38.79 | 0.479 | 4.23 | -3.09 | 0.002 |
| <i>P. reticulata</i> (social stimulus) | 1 | 58.84 | 0.022 | 4.65 | -1.16 | 0.245 |
| | 2 | 74.04 | 0.001 | 5.15 | 0.47 | 0.642 |
| | 3 | 78.55 | 0.000 | 5.12 | 0.37 | 0.709 |

Table S3. Statistical analyses used by studies listed in Table S1.

| |
|--|
| <p>[9] Reddon & Hurd 2008 – aggression Tested the effect of sex and aggressor status on relative and absolute lateralisation (stated as laterality index). Statistical methods are not described, but R² and F-statistic are given.</p> |
| <p>[10] Welch et al. 2014 – aquatic acidification Tested juveniles across nine treatment groups. Differences between treatment groups (parents and offspring exposed to control, intermediate and high CO₂ in a full factorial design) in absolute lateralisation, was tested using a general linear mixed-effects model (LMM); LA ~ Parental treatment * Offspring treatment + various random effects). Auhors state that relative lateralisation was used to compare turning bias among the nine treatments using Pearson's Chi-square test but no test statistics are presented for L_R in the main text or the supplementary material.</p> |
| <p>[11] Maulvault et al. 2018 – aquatic acidification, warming, and pharmaceuticals The effects of venlafaxine (in water and in feed), high temperature, and CO₂ in various combinations on absolute and relative lateralisation were tested using generalised linear mixed-effects models (GLMMs) with a Gaussian error distribution. Tank was specified as random factor (not specified given whether fish from 2 or 3 tanks were used in the test) and the authors employed a model selection approach using AIC.</p> |
| <p>[12] Lopes et al. 2016 – aquatic acidification Tested the effect of high CO₂ exposure for 7 and 21 days on relative and absolute lateralisation with GLMMs. Treatment and time were entered as fixed effects and tank as a random effect. Model selection was performed using AIC.</p> |
| <p>[13] Sundin & Jutfelt 2016 – aquatic acidification Tested control and fish exposed to high CO₂, at exposure day 9, 19, and 21 (all fish tested in control water for the last run). Used generalised linear models (GLMs) with a binomial error structure and relative and absolute lateralisation as response variables (number of turns to the left over total number of left and right turns (corresponding to relative lateralisation), and maximum number of turns to the preferred side over total number of left and right turns (corresponding to absolute lateralisation), and treatment, test run (for run 1 and 2, run 3 was analysed separately), and the interaction between them as fixed effects.</p> |
| <p>[14] Vossen et al. 2016 – aquatic acidification Tested eight treatment groups (control CO₂, no gabazine females; control CO₂, no gabazine males; high CO₂, no gabazine females; high CO₂, no gabazine males; control CO₂, gabazine females; control CO₂, gabazine males; high CO₂, gabazine females; and high CO₂, gabazine males). Relative lateralisation (proportion of right turns) analysed using a GLMM with binomial errors with CO₂ exposure level, gabazine treatment, and sex as fixed effects, and exposure tank and number of days exposed as random effects.</p> |
| <p>[15] Jutfelt & Hedgärde 2015 – aquatic acidification Tested juveniles after 29-30 days exposure to control or high CO₂. Used a nested ANOVA (tank nested under treatment) on relative and absolute lateralisation to test for differences among treatment groups.</p> |
| <p>[16] Jutfelt et al. 2013 – aquatic acidification</p> |

| |
|---|
| <p>Tested control and high CO₂ treatments at 20 and 40 days of exposure. Among-treatment effects on relative and absolute lateralisation were tested at two time points using independent samples t-test with unequal variances.</p> |
| <p>[17] Lai et al. 2015 – aquatic acidification</p> <p>Tested control and high CO₂ treatments at 40 and 50 days of exposure (following 30 minutes of gabazine treatment). Absolute lateralisation between control and high-CO₂ fish on day 40 was analysed using a two-tailed t-test. A two-way ANOVA was used to examine the effects of high CO₂ and gabazine on day 50 followed by Sidak’s multiple comparison test as the post-hoc test.</p> |
| <p>[18] Näslund et al. 2015 – aquatic acidification</p> <p>Tested control and high CO₂ at 20 days of exposure. Absolute lateralisation between the two groups was analysed using a permutation test using Boss Permutations Tester.</p> |
| <p>[19] Sundin & Jutfelt 2018 – aquatic acidification</p> <p>Tested the effect of CO₂ exposure on relative lateralisation across two years. Individuals were tested at 12-19 days of exposure. The effect of CO₂ on relative lateralisation was tested using a GLMM with binomial errors. Wilcoxon-signed rank tests were used to test against an expected value of 0 to investigate whether the fish were significantly left- or right-biased.</p> |
| <p>[5] Nilsson et al. 2012 – aquatic acidification</p> <p>Tested the effect of CO₂ and gabazine exposure in a full factorial design. It appears that separate within-treatment tests of absolute lateralisation were performed using G-tests (i.e. separate tests were performed for the group treated with control or CO₂ water). Differences before <i>versus</i> after gabazine treatment were tested using t-tests (also separately for each control/CO₂ treatment).</p> |
| <p>[1] Domenici et al. 2012 – aquatic acidification</p> <p>Tested the effect of four days of CO₂ exposure, using relative and absolute lateralisation. “In addition, a random simulation (RS) was generated based on 10 random binary choices (i.e. left or right) per individual (n = 70). This simulation was generated in order to test if any of the samples yielded left–right proportions that were not different from that expected by random choice.” Within-group preference for left <i>versus</i> right turns was tested using one sample t-tests, among-group relative and absolute lateralisation was tested using Kruskal–Wallis tests (testing all three groups: control, CO₂, and hypothetical random choice group) followed by Dunn’s multiple comparison tests. The distributions of the control and the elevated-CO₂ individuals were compared with a theoretical binomial distribution using a G-test.</p> |
| <p>[2] Domenici et al. 2014 – aquatic acidification</p> <p>Tested effect of seven days of CO₂ exposure and elevated temperature on fish from four treatments (control CO₂/control temperature, control CO₂/high temperature, high CO₂/control temperature, high CO₂/high temperature), using both relative and absolute lateralisation. Within-group preference for left <i>versus</i> right turn (relative lateralisation) was tested using one sample t-tests, among-group relative and absolute lateralisation was tested using two-way ANOVAs followed by Tukey HSD tests.</p> |
| <p>[6] Reddon & Hurd 2009 – boldness</p> <p>Tested for laterality index (LI) and absolute LI (corresponding to relative and absolute lateralisation). Both were analysed using one-sample t-tests.</p> |

[4] Byrnes et al. 2016 – boldness

Tested for correlation between laterality index and laterality strength (corresponding to relative and absolute lateralisation) and personality. Population-level departures from random choice in laterality direction and overall non-lateralisation were examined using **two-tailed and one-tailed Wilcoxon signed rank tests**, respectively. General **linear models (LMs)** were used to examine the influence of sex, population, time in captivity, total length, boldness, and stress reactivity on lateralisation direction and strength.

[20] Irving & Brown 2013 – boldness

Used **repeated measures ANOVA** to examine the effect of stimulus (neutral or control; repeated measure) and sex on relative lateralisation. **Regression** was used to examine relationships between relative (and absolute) lateralisation and boldness, activity, and sociability.

[21] Garina et al. 2016 – cognition

Tested fish divided among three experimental groups (serotonin-modulating anticonsolidation protein and two controls). Fish were tested on four different days, five times per day. The median number of right turns out of all five turns was used to assess lateralisation. Changes in turning direction and latency were analysed using **Friedman's test and coefficient of concordance**. The median number of right turns was compared pair-wise between days within each experimental group, as well as on the same day between different experimental groups using **Mann–Whitney U tests**. The median values for each experimental group were calculated across days and compared pair-wise using the same test.

[22] Dadda & Bisazza 2006 – cognition

Tested fish from three lines that had been selected for lateralisation (left detour fish, right detour fish, and non-lateralised fish). Data were analysed using **two-way ANOVAs** where laterality type and presence/absence of the male were included as the independent factors, and average time to retrieve food was included as the dependant variable. Lateralisation was not tested directly in the paper; therefore, no statistics on absolute or relative laterality are presented.

[23] Sovrano et al 2005 – cognition

Tested fish from lines that had been selected for lateralisation: right detour (RD) fish, left detour (LD) fish, and non-lateralised (NL) fish. Data were analysed using **ANOVAs** firstly to see if laterality affected the proportion of correct choices and, secondly, to see if laterality affected the frequency of choice corner. Laterality type (RD, LD, and NL) was first included as a between-subjects factor, and trial as within-subjects factor. The RD and LD were then grouped into lateralised fish (L) and reanalysed using an **ANOVA**. Lateralisation was not tested directly in the paper; therefore, no statistics on absolute or relative laterality are presented.

[24] Dadda & Bisazza 2006 – cognition

Tested fish from lines that had been selected for lateralisation (right detour fish, left detour fish, and non-lateralised fish). Data were analysed using **ANOVAs** (e.g. to test if eye-preference differed between lateralisation groups) and **t-tests** (e.g. to test eye-preference within each lateralisation group). Lateralisation was not tested directly in the paper; therefore, no statistics on absolute or relative laterality are presented.

[25] Ferrari et al. 2017 – cortisol

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| <p>Tested absolute lateralisation using a two-way blocked ANOVA, testing the effect of cortisol (sham <i>versus</i> cortisol), and blocking for testing day (random factor).</p> |
| <p>[26] Reddon et al. 2009 – growth rate</p> <p>Tested fish for absolute and relative lateralisation. Population- and individual-level lateralisation were tested using one sample t-tests.</p> |
| <p>[27] Lucon-Xiccato et al. 2014 – hypoxia</p> <p>Tested relative and absolute lateralisation. Both measurements were analysed after an arcsine transformation. Relative lateralisation was tested within each group using one sample t-tests. Independent sample t-tests were used to compare mean relative and absolute lateralisation indices between treatment groups.</p> |
| <p>[3] Roche et al. 2013 – parasitism</p> <p>Tested two groups (unparasitised and parasitised) for relative and absolute lateralisation. Used goodness-of-fit G-tests to test within-group relative lateralisation and a GLM to test for differences between groups. Between-group absolute lateralisation was tested using a Mann–Whitney U test and between parasitised and parasite-removed fish, using a Wilcoxon paired-sample test. A GLM was used to test turning direction with respect to the side the parasite was attached on parasitised and parasite-removed fish.</p> |
| <p>[28] Besson et al. 2017 – pesticide exposure</p> <p>Tested eye-use in fish exposed to one of four stimuli, for relative lateralisation. Within-stimulus relative lateralisation was tested using two-tailed Mann-Whitney U tests, differences between stimuli were tested using non-parametric tests (Wilcoxon rank sum test and Kruskal-Wallis test). Additionally, fish were tested for the effect of lateralisation on predator avoidance, using Kruskal-Wallis test (lateralisation was tested and fish were divided into three groups: left tending, right tending, and unbiased – it is not specified which criterion was used). Finally, relative lateralisation was tested for fish divided among two treatments (chlorpyrifos and control) and four different stimuli using Mann-Whitney U test as above.</p> |
| <p>[11] Malvault et al. 2018 – pharmaceuticals</p> <p>See above.</p> |
| <p>[29] Brown et al. 2007 – predation</p> <p>Tested first generation descendants, from either a high-predation population, or a low-predation population, as well as wild-caught adult females from high-predation or low-predation population, as representatives of the respective wild populations. Fish were tested for relative and absolute lateralisation when sequentially presented with three different stimuli: control, novel object, and unfamiliar same-sex conspecific. Repeated measures ANOVA was used to test the response of wild-caught female fish to the three treatments, the difference between the wild-caught females and the female laboratory-reared offspring, and the differences between males and females in the laboratory-reared fish only.</p> |
| <p>[30] Broder & Angeloni 2014 – predation</p> <p>Tested males from four different populations (high predation populations and low predation populations) exposed to one of two treatments (predator or no predator exposure). Relative and absolute lateralisation were tested using ‘mixed-effects’ ANOVAs.</p> |
| <p>[31] Chivers et al. 2016 – predation</p> <p>Tested fish from two treatments (low-risk and high-risk). Relative and absolute lateralisation were tested using a two-way nested ANOVA where fish were nested within</p> |

tanks, and tanks within risk group. Lateralisation scores of the low- and high-risk fish were also compared to pre-treatment scores using a **one-way ANOVA** followed by post hoc **Tukey tests**.

[25] Ferrari et al. 2017 – predation

Tested fish from two treatment groups differing in perceived predation risk pattern for relative and absolute lateralisation using a **two-way repeated measures ANOVA**, investigating the effect of risk peak time (noon *versus* evening) on the absolute lateralisation of fish tested both at noon and in the evening.

[32] Ferrari et al. 2015 – predation

Tested fish divided among three treatment groups (no treatment [‘before’ treatment], high-risk environment, and low-risk environment) for absolute lateralisation using a **one-way ANOVA**.

[33] Bisazza & Dadda 2005 – schooling

Tested fish originating from lines selected for lateralisation: left detour fish, right detour fish, a mixture of right and left detour fish, and non-lateralised fish. No additional tests on laterality are included in the paper; therefore, no statistics on absolute or relative laterality are presented.

[2] Domenici et al. 2014 – temperature

See above.

[11] Malvault et al. 2018 – temperature

See above.

Fig. S1 Schematic representation of a double T-maze used to test lateralisation in fishes. A focal fish is acclimated in one side of the arena for one minute behind a barrier (in grey). The barrier is then lifted and the fish is made to swim back and forth in the arena. As it approaches the end of the corridor, the fish faces a barrier and must choose to turn left or right. Decisions to turn left or right are recorded 10 times. Dimensions (mm) of the maze used for *Ctenolabrus rupestris* and *Danio rerio*: 500 (a), 500 (b), 200 (c), 330 (d), 80 (e), 100 (f), and 40 (g). Dimensions (mm) of the maze used for *Neopomacentrus azysron* and *Pomacentrus amboinensis*: 350 (a), 640 (b), 125 (c), 400 (d), 80 (e), 150 (f), and 50 (g).

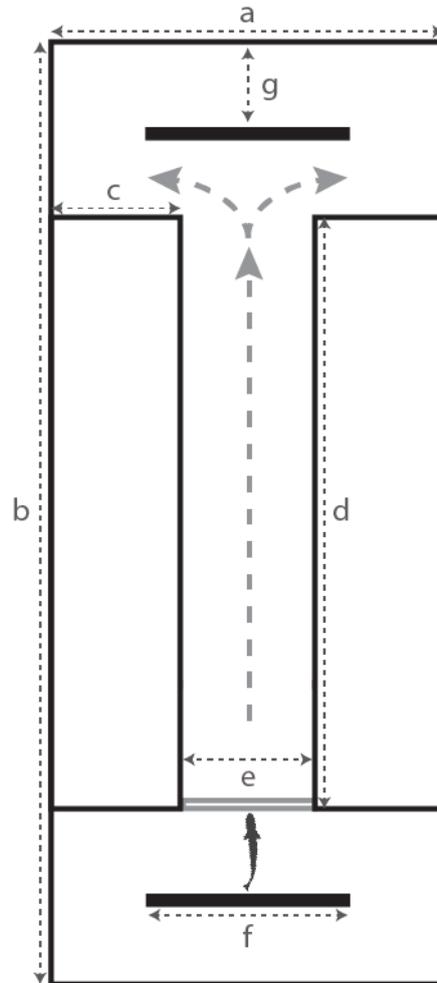


Fig S2. Relative lateralisation index (L_R) across repeated trials per individual for four fish species: *Ctenolabrus rupestris* ($n=57$), *Neopomacentrus azysron* ($n=52$), *Pomacentrus amboinensis* ($n=60$), and *Danio rerio* ($n=49$). Individuals are in ascending order of their mean L_R along the x-axis. The repeatability index (R) of L_R and its 95% CI are indicated for each species.

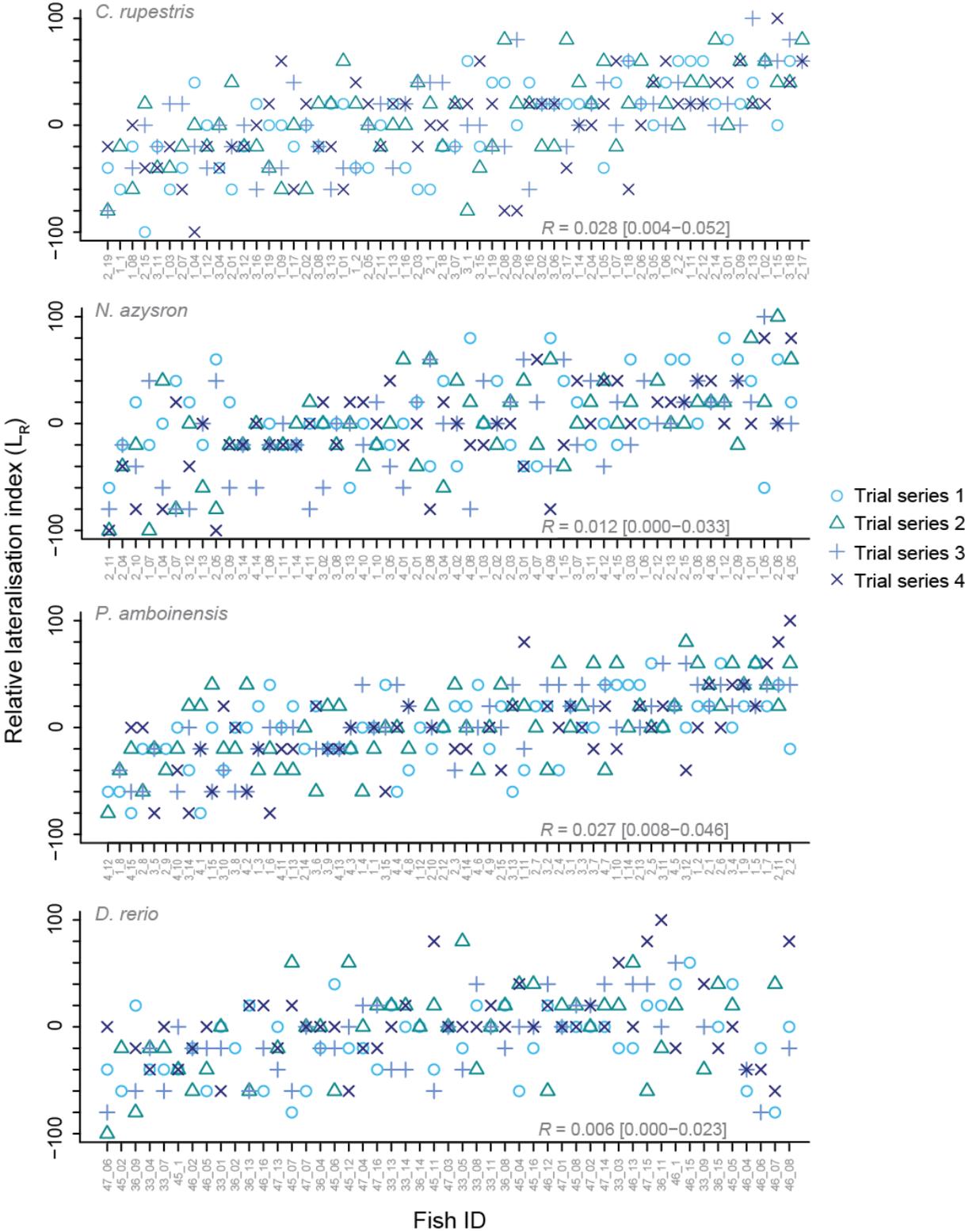


Fig. S3 Absolute lateralisation index (L_A) across repeated trials for four fish species: *Ctenolabrus rupestris* ($n=57$), *Neopomacentrus azysron* ($n=52$), *Pomacentrus amboinensis* ($n=60$), and *Danio rerio* ($n=49$). Individual-level L_A is indicated by the coloured dots connected by lines; the mean (i.e. population-level) L_A and its 95% CI are indicated by black dots and error bars. The repeatability index (R) of L_A and its 95% CI are indicated for each species. R was computed using the ‘rpt’ function in the package ‘rptR’ [34], with a Poisson error distribution. Four series of 10 trials were conducted. Data points are jittered along the x-axis for presentation purposes.

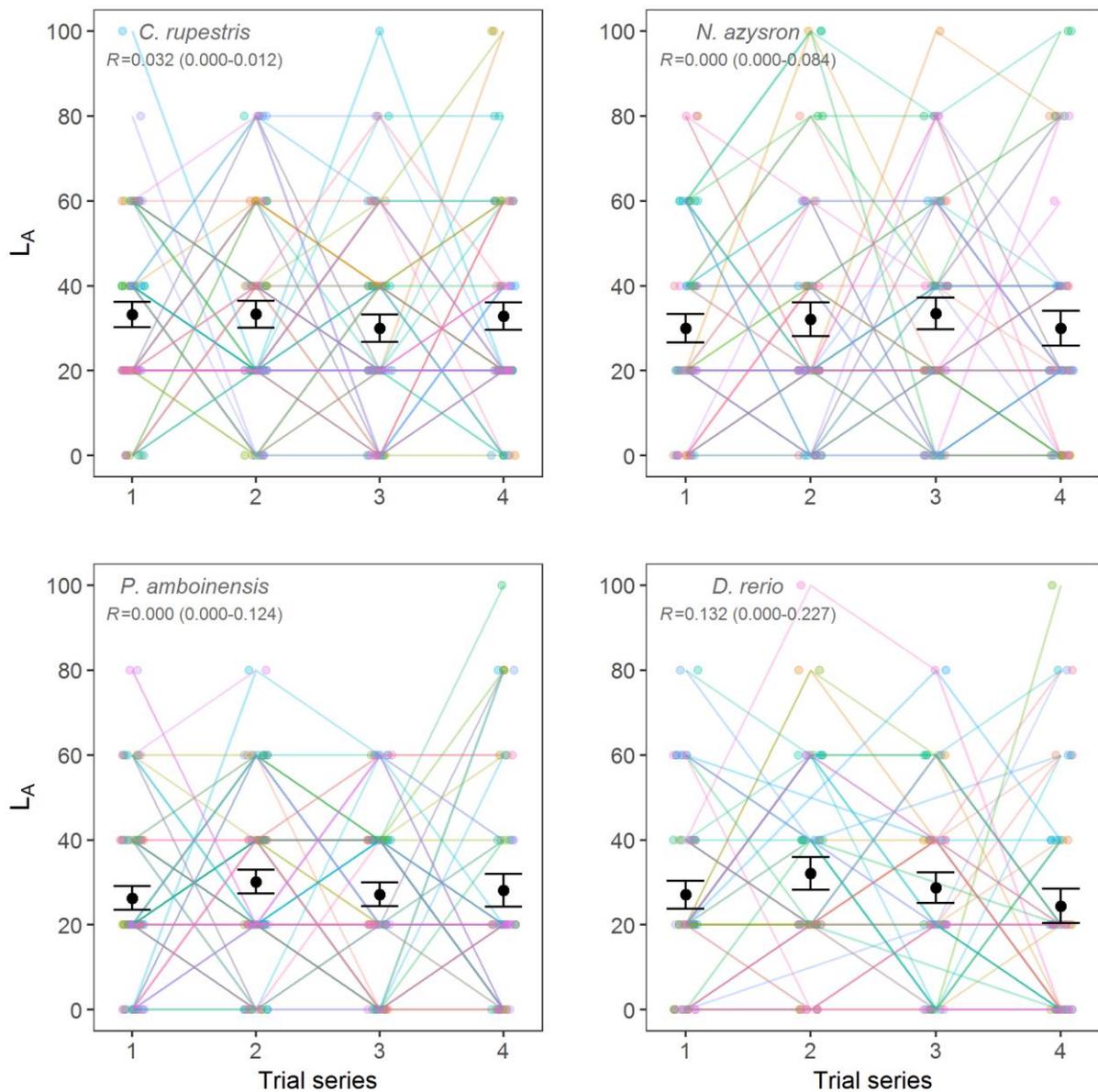


Fig. S4 Relative lateralisation index (L_R) of female guppies (*Poecilia reticulata*) ($n=40$) across three repeated trial series per individual in two different contexts: **(A)** the barrier of the T-maze consists of a neutral stimulus (an empty aquarium behind barrier of vertical bars); **(B)** the barrier of the T-maze consists of a social stimulus (an aquarium containing a conspecific behind a barrier of vertical bars); see Fig. 1 in [17]. Data are from Irving & Brown [20]. Individual-level L_R is indicated by the coloured dots connected by lines; the mean (i.e. population-level) L_R and its 95% CI are indicated by black dots and error bars. The repeatability index (R) of L_R and its 95% CI are indicated at the bottom of each panel. Three series of 10 trials were conducted on each individual. Data points are jittered along the x-axis for presentation purposes.

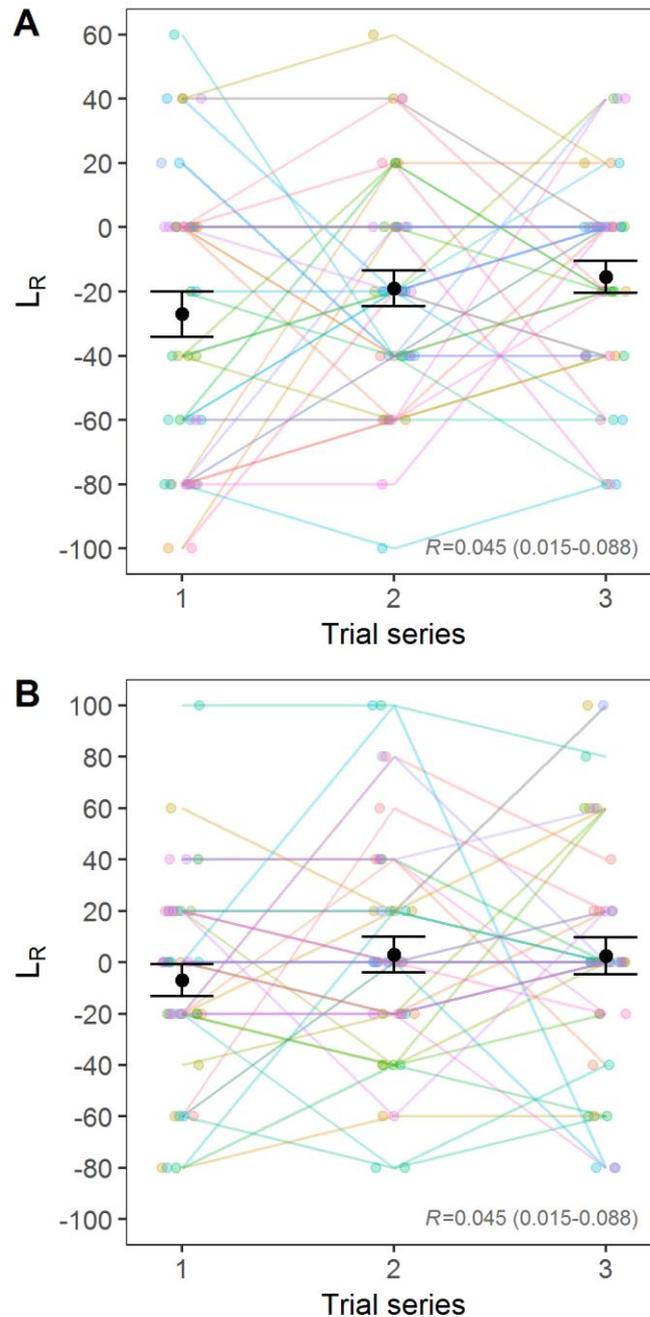


Fig. S5 Frequency distribution of the number of right turns taken by *Ctenolabrus rupestris* ($n=57$) in a detour test (total of 10 turns per individual). The same individuals were tested on four occasions (A, B, C, D) at 48 h intervals. The dashed line represents the mean number of right turns. See Table S2 for statistics.

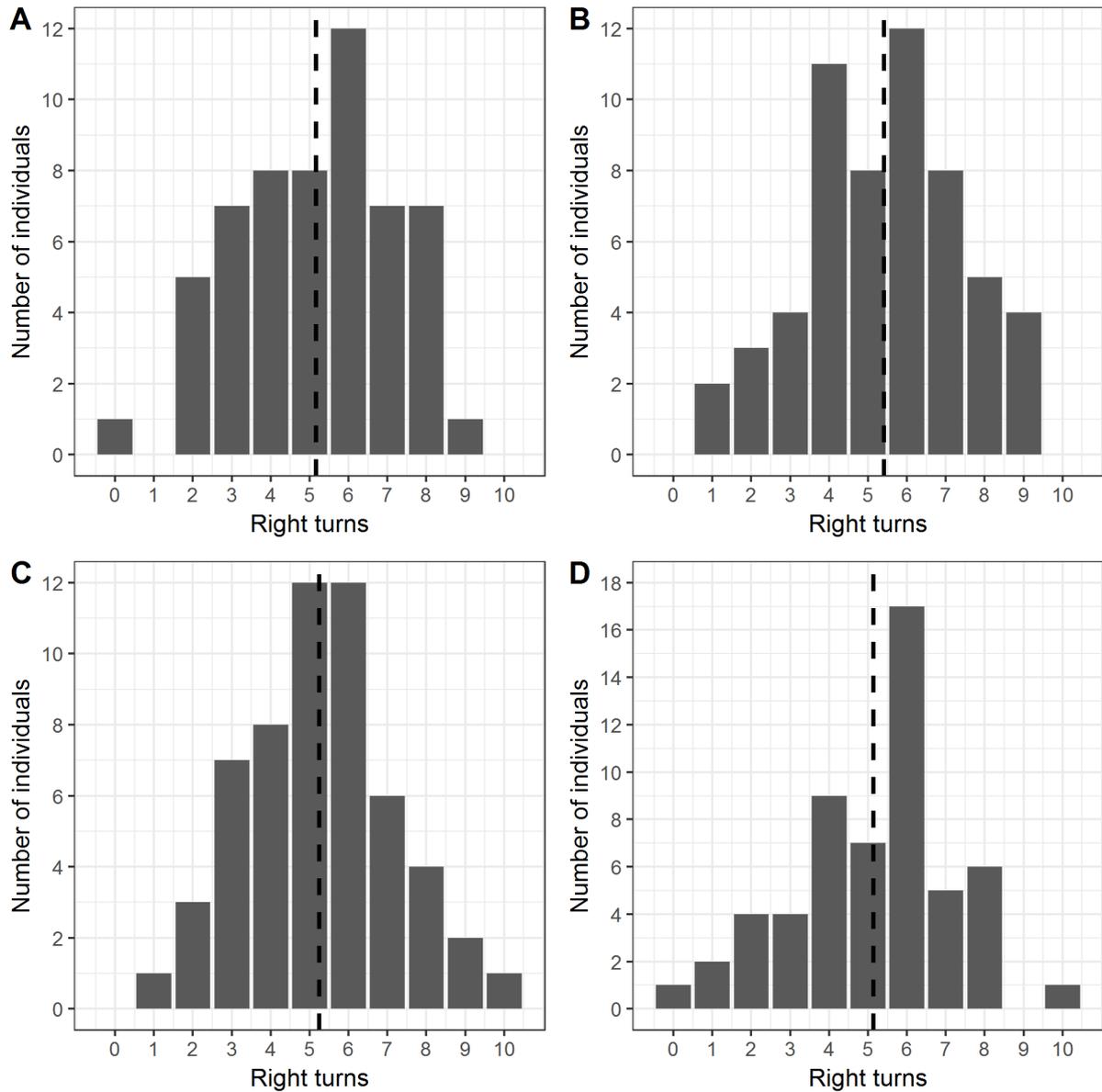


Fig. S6 Frequency distribution of the number of right turns taken by *Neopomacetrus azysron* ($n=52$) in a detour test (total of 10 turns per individual). The same individuals were tested on four occasions (A, B, C, D) at 48 h intervals. The dashed line represents the mean number of right turns. See Table S2 for statistics.

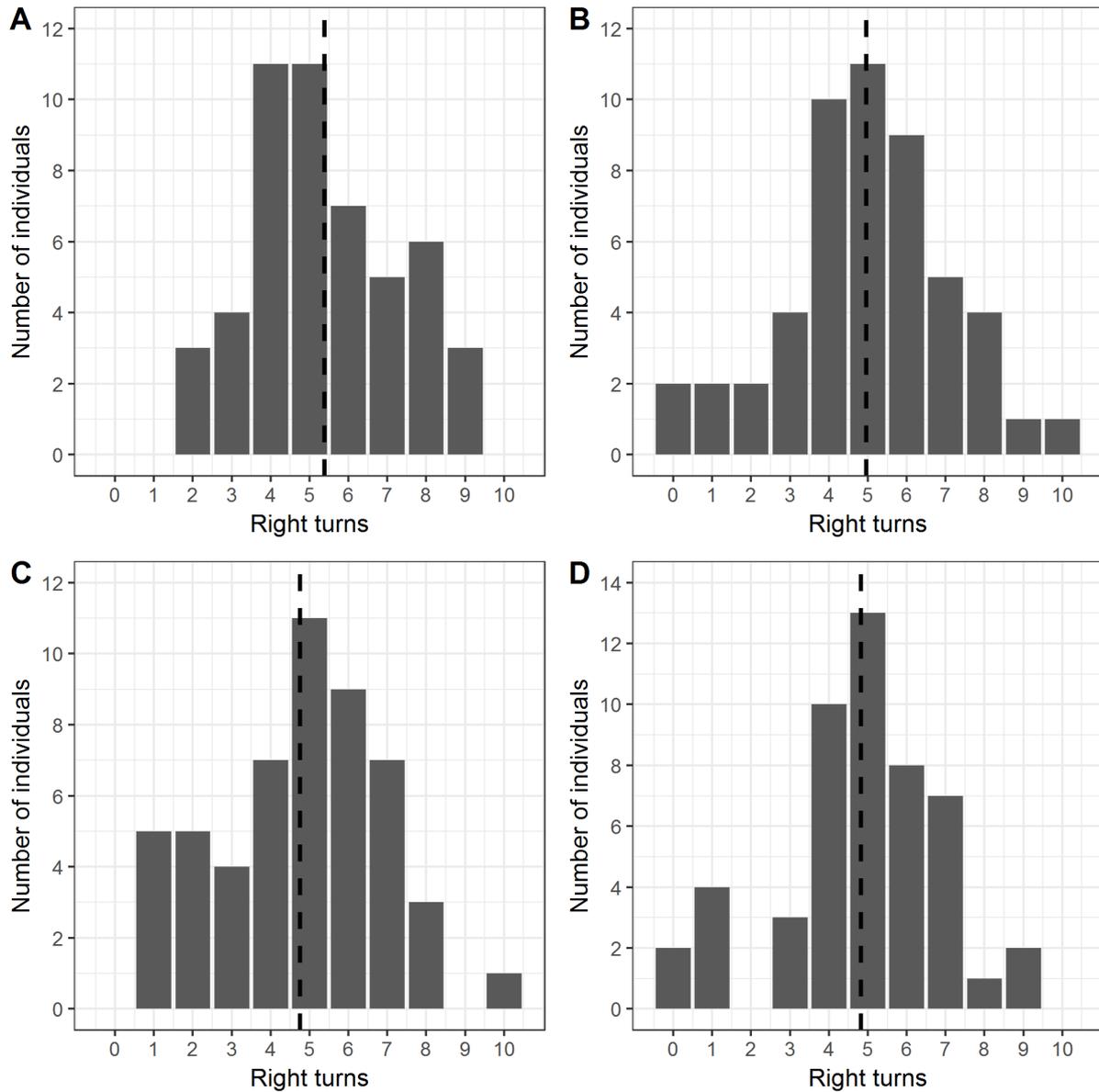


Fig. S7 Frequency distribution of the number of right turns taken by *Pomacentrus amboinensis* ($n=60$) in a detour test (total of 10 turns per individual). The same individuals were tested on four occasions (A, B, C, D) at 48 h intervals. The dashed line represents the mean number of right turns. See Table S2 for statistics.

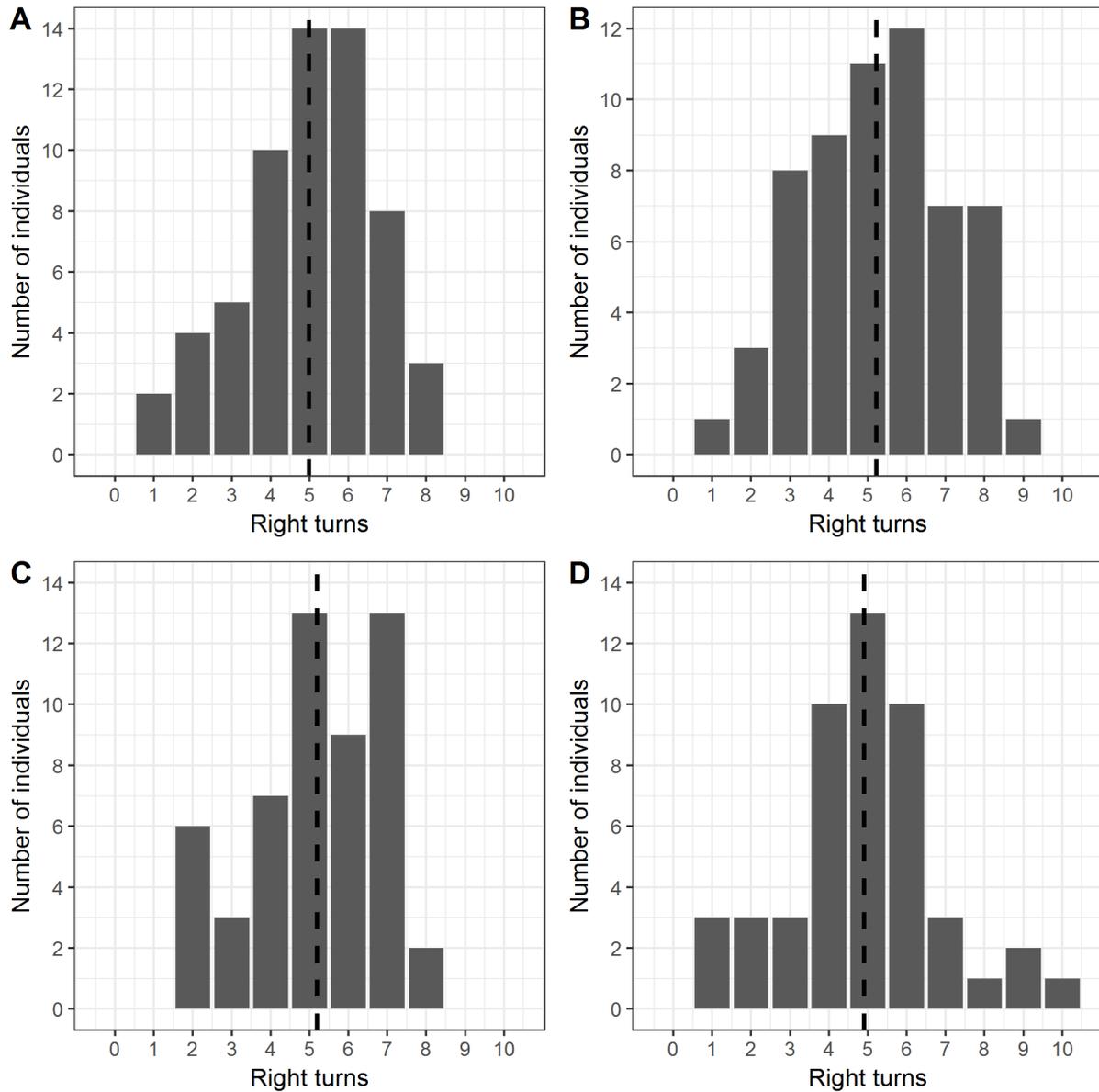


Fig. S8 Frequency distribution of the number of right turns taken by *Danio rerio* ($n=49$) in a detour test (total of 10 turns per individual). The same individuals were tested on four occasions (A, B, C, D) at 48 h intervals. The dashed line represents the mean number of right turns. See Table S2 for statistics.

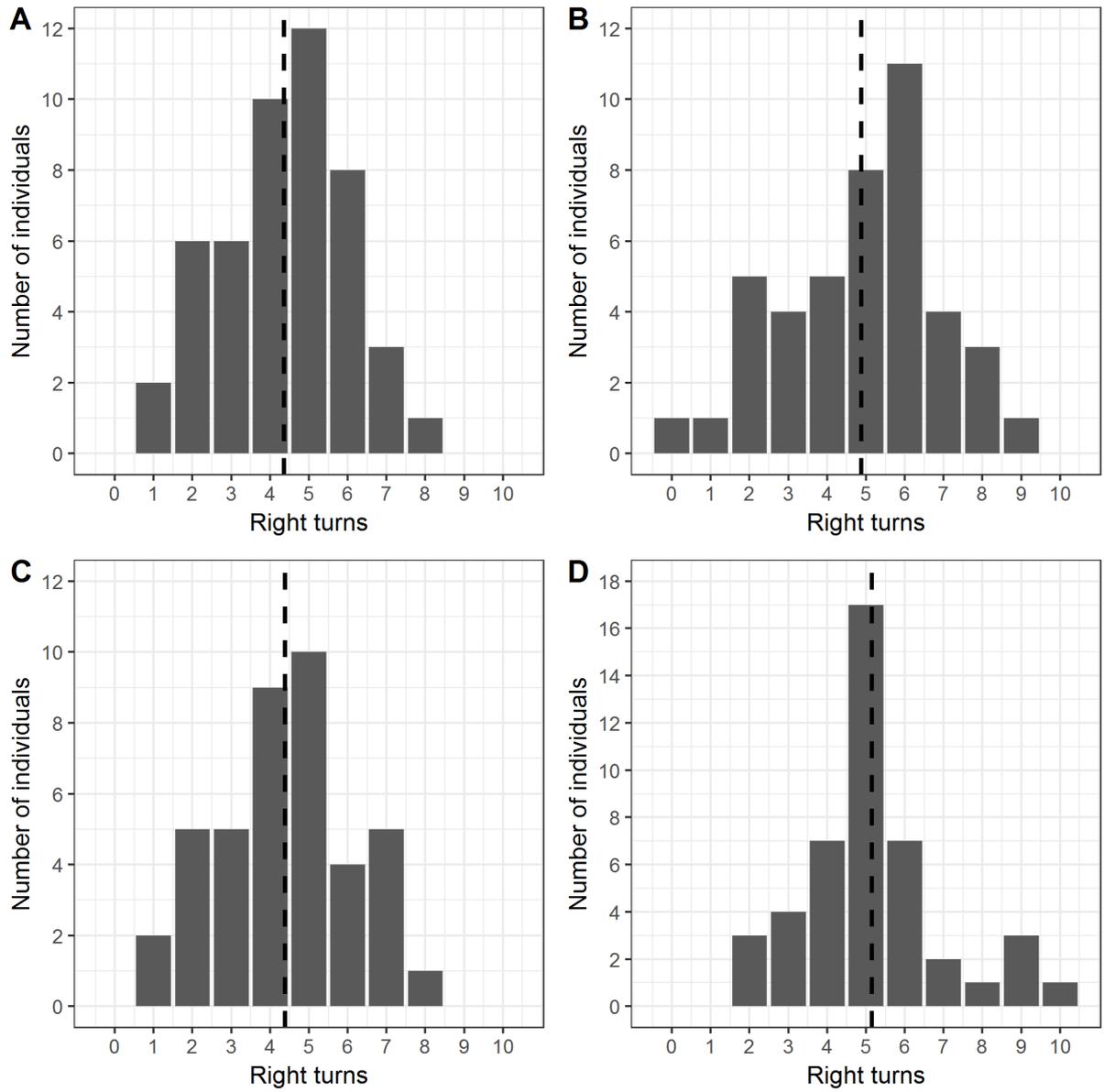
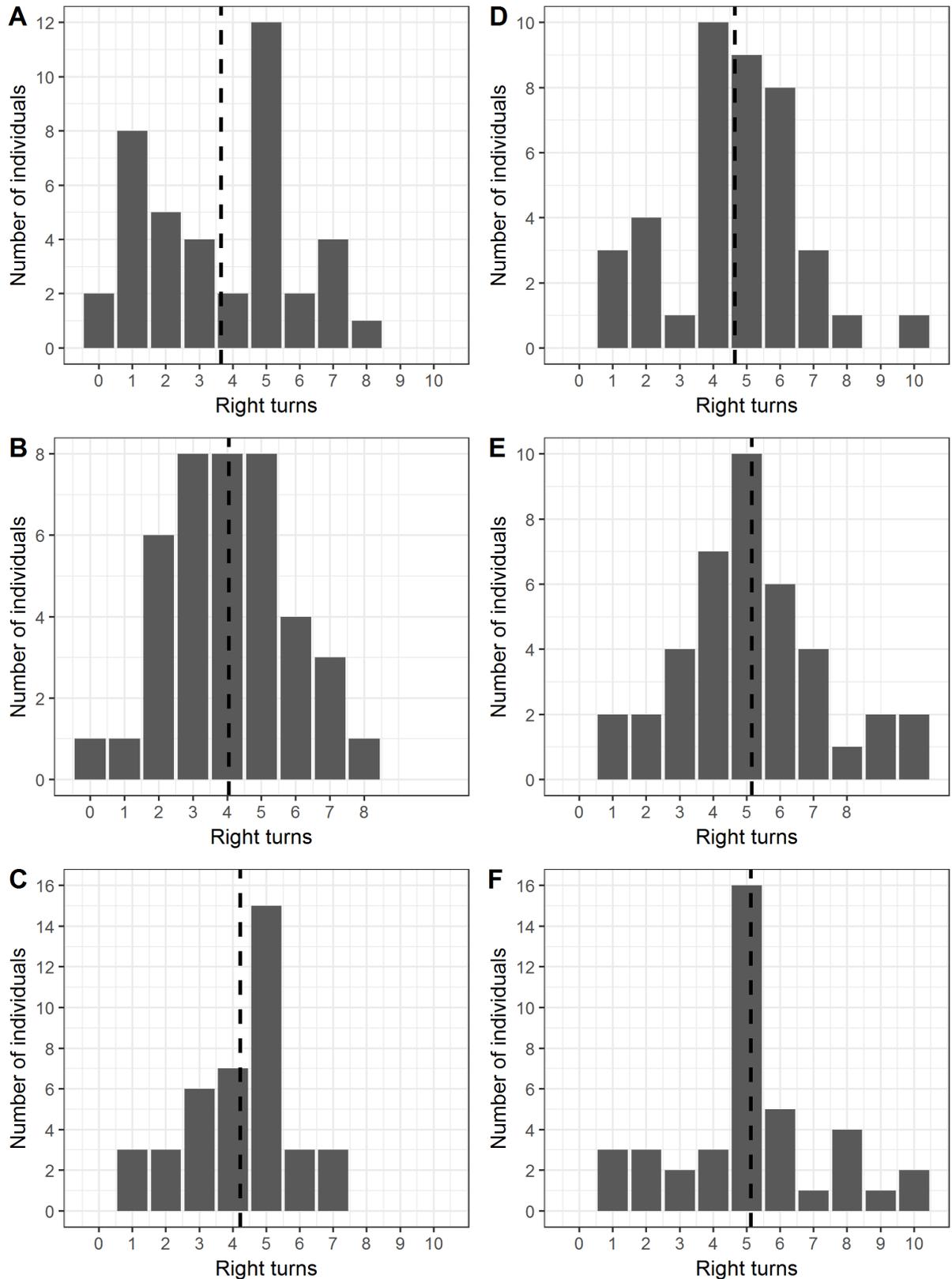


Fig. S9 Frequency distribution of the number of right turns taken by *Poecilia reticulata* ($n=40$) in a detour test (total of 10 turns per individual). The same individuals were tested on three occasions using either a neutral (A, B, C) or a social (D, E, F) stimulus. The dashed line represents the mean number of right turns. See Table S2 for statistics.



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