



## Behavioural lateralization in a detour test is not repeatable in fishes

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Behavioural lateralization, the asymmetric expression of cognitive functions, is reported to enhance key fitness-relevant traits such as group coordination, multitasking and predator escape. Therefore, studies reporting negative effects on lateralization in fish due to environmental stressors such as ocean acidification, hypoxia and pollutants are worrisome. However, such studies tend to use a detour test and focus on population level measures, without validating whether lateralization is consistent within individuals across time. We conducted a multispecies, international assessment of the repeatability ( $R$ ) of lateralization in four previously studied fish species using a detour test (T-maze), a common method for testing lateralization. We also reanalysed a published data set on a fifth species using new statistical methods. We expected the three shoaling species to exhibit greater within-individual consistency in lateralization than their nonshoaling counterparts given previous reports of stronger lateralization in group-living fishes. Absolute and relative lateralization scores were highly nonrepeatable in all five species ( $0.01 < R < 0.08$ ), irrespective of their shoaling status. We carefully reviewed 31 published studies in which the detour test was employed to examine lateralization in fish and identified statistical issues in all of them. We develop and propose new statistical analyses to test for population and individual level lateralization. The commonly used detour test does not appear to be appropriate for quantifying behavioural lateralization in fishes, calling into question functional inferences drawn by many published studies, including our own. Potential fitness benefits of lateralization and anthropogenic effects on lateralization as a proxy for adaptive brain functioning need to be assessed with alternative paradigms.

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Behavioural lateralization, the asymmetric expression of cognitive functions, has emerged as an important fitness correlate

in numerous taxa including invertebrates (e.g. Domenici, Torres, & Manríquez, 2017) and vertebrates (reviewed in Vallortigara & Rogers, 2005). Indeed, cerebral lateralization is believed to offer advantages in terms of enabling multiple stimuli to be processed simultaneously by different sides of the brain (Vallortigara & Rogers, 2005). Fitness benefits associated with high degrees of lateralization are thought to include increased cognitive

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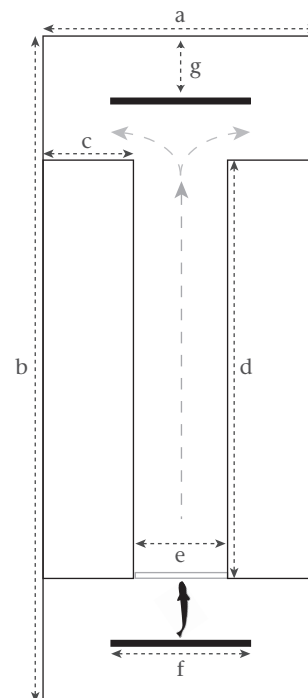
performance (Bibost & Brown, 2014; Magat & Brown, 2009), multitasking (Dadda & Bisazza, 2006b; Rogers, Zucca, & Vallortigara, 2004), spatial learning (Sovrano, Dadda, & Bisazza, 2005), predator recognition learning (Ferrari et al., 2017), schooling performance (Bisazza & Dadda, 2005), coordination of group behaviours (Vallortigara & Rogers, 2005), prey capture success (Kurvers et al., 2017), foraging efficiency (Güntürkün et al., 2000) and escape performance (Dadda, Koolhaas, & Domenici, 2010b; Lucon-Xiccato, Chivers, Mitchell, & Ferrari, 2016). Despite these apparent advantages, most studies report considerable inter- and intraspecific variation in the strength and direction of lateralization. Several studies also report a high degree of within-individual variation in the strength of lateralization in individuals measured repeatedly across different contexts, suggesting that this trait may be more labile than previously believed (e.g. Ferrari et al., 2017; Roche, Binning, Strong, Davies, & Jennions, 2013). Yet, no studies have systematically measured lateralization several times per individual, across multiple species, without a change in context, to establish the baseline repeatability of this behaviour (but see Irving & Brown, 2013, for a single-species study, the data for which are reanalysed here). Such a validation of the repeatability of lateralization assessed with a given test is crucial to establish the usefulness of a test for assessing this trait, particularly when differences in individual level lateralization strength between ecologically relevant treatments are used to infer effects on fitness.

Behavioural lateralization in fishes is commonly measured in the laboratory using a detour test (T-maze), wherein the animal moves along a runway until it faces a barrier forcing it to make a choice between turning left or right (Fig. 1). This quick and simple test is now widely used in studies of fish behaviour, evolutionary ecology and ecotoxicology (see Supplementary Material Table S1), and is also used to assess lateralization in various other taxa including birds (Vallortigara, Regolin, & Pagni, 1999), reptiles (Csermely, Bonati, & Romani, 2010) and molluscs (Domenici et al., 2017). In fishes, lateral bias in a detour test is believed to arise from asymmetries in eye use, a phenomenon widely documented in animals with laterally placed eyes and low binocular overlap (Bisazza, Pignatti, & Vallortigara, 1997a). For example, goldbelly topminnows, *Girardinus falcatus*, reported to exhibit a strong side bias in a detour test also display an eye preference for viewing a neutral versus a threatening stimulus (Facchin, Bisazza, & Vallortigara, 1999). Similarly, left–right asymmetries in *G. falcatus* assessed in a detour test appear to be consistent with lateral bias measured using other methods (Bisazza, Sovrano, & Vallortigara, 2001). Furthermore, lateralization score in a detour test is reported to be heritable (Bisazza, Facchin, & Vallortigara, 2000b; Brown, Western, & Braithwaite, 2007), although lateralization strength decreases rapidly across generations in artificial selection lines (Bisazza, Dadda, Facchin, & Vigo, 2007; Facchin, Argenton, & Bisazza, 2009). These results have led researchers to assert that the detour test does, indeed, assess inherent asymmetry in an individual's brain function that influences fitness-relevant behavioural strategies.

Given likely fitness-relevant effects of lateralization (e.g. Whiteside et al., 2018) and the relative ease of use of the detour test, various studies have evaluated how environmental and anthropogenic stressors such as pollutants and climate change affect lateralization in fishes, several of which have produced worrying results (Table S1). Most notably, ocean acidification is reported to decrease lateralization across a range of marine fishes, including tropical (Domenici, Allan, McCormick, & Munday, 2012; Nilsson et al., 2012; Welch, Watson, Welsh, McCormick, & Munday, 2014) and temperate species (Jutfelt, Bresolin de Souza, Vuylsteke, & Sturve, 2013; Lopes et al., 2016; Maulvault et al., 2018). Such effects are concerning given, for example, the important benefits of

lateralization reported for fishes under high predation risk (e.g. Chivers et al., 2016; Ferrari et al., 2015; Ferrari et al., 2017). Despite this strong interest in behavioural lateralization and the growing use of the detour test in the ecological, cognitive and behavioural sciences, there is a notable absence of studies assessing the repeatability of individual lateralization scores. Establishing the consistency of lateralization within individuals is essential for determining the relevance of this trait for evaluating fitness as well as the responses of animals to exogenous stressors (see Roche, Bennett et al., 2019).

To evaluate the short-term repeatability of behavioural lateralization in fishes using a detour test, we studied four species from tropical and temperate environments, including marine and freshwater habitats: two coral reef damselfishes, the Ambon damsel, *Pomacentrus amboinensis*, and yellowtail demoiselle, *Neopomacentrus azysron*, the tropical freshwater zebrafish, *Danio rerio*, and the temperate marine goldsinny wrasse, *Ctenolabrus rupestris* (Fig. 2). All of these species have previously been used in published studies assessing behavioural lateralization using a detour test (T-maze) similar to the ones used here (Domenici et al., 2012; Ferrari et al., 2017; Nilsson et al., 2012; Sundin & Jutfelt, 2016; Vossen, Jutfelt, Cocco, Thörnqvist, & Winberg, 2016). We also used new (more appropriate) statistical methods to reanalyse a published data set that included repeated lateralization measurements for the tropical freshwater guppy, *Poecilia reticulata* (Irving & Brown, 2013). We predicted a significant repeatability in lateralization scores comparable in magnitude to that observed for other behavioural traits (Bell, Hankison, & Laskowski, 2009). Population



**Figure 1.** Schematic representation of a double T-maze used to test lateralization in fishes. A focal fish is acclimated in one side of the arena for 1 min 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*: (a) 500, (b) 500, (c) 200, (d) 330, (e) 80, (f) 100 and (g) 40; wall height = 150; water level = 100. Dimensions (mm) of the maze used for *Neopomacentrus azysron* and *Pomacentrus amboinensis*: (a) 350, (b) 640, (c) 125, (d) 400, (e) 80, (f) 150 and (g) 50; wall height = 370; water level = 100.

level lateralization strength was also predicted to be higher in the shoaling species (*N. azysron* and *D. rerio*) compared to territorial species (*P. amboinensis* and *C. rupestris*) as this should help enhance school cohesion (Bisazza, Cantalupo, Capocchiano, & Vallortigara, 2000a). We had no a priori prediction about the direction (left or right) of lateralization, as among-species differences in response to the same stimulus have been shown previously (Bisazza et al., 2000a). We also examined whether individuals became habituated to the test arena across the four trial series because (1) repeatedly measuring the same individuals in a similar apparatus

can decrease measurement error and thus be predicted to increase individual repeatability (Martin & Réale, 2008), and conversely (2) habituation or desensitization to a stimulus may reduce repeatability by generating different results between trials within an individual (Martin & Réale, 2008). Finally, we carefully reviewed the statistical methods in 31 published studies that examined lateralization in fishes using the detour test. We develop and propose new statistical approaches to test for population and individual level lateralization.

## METHODS

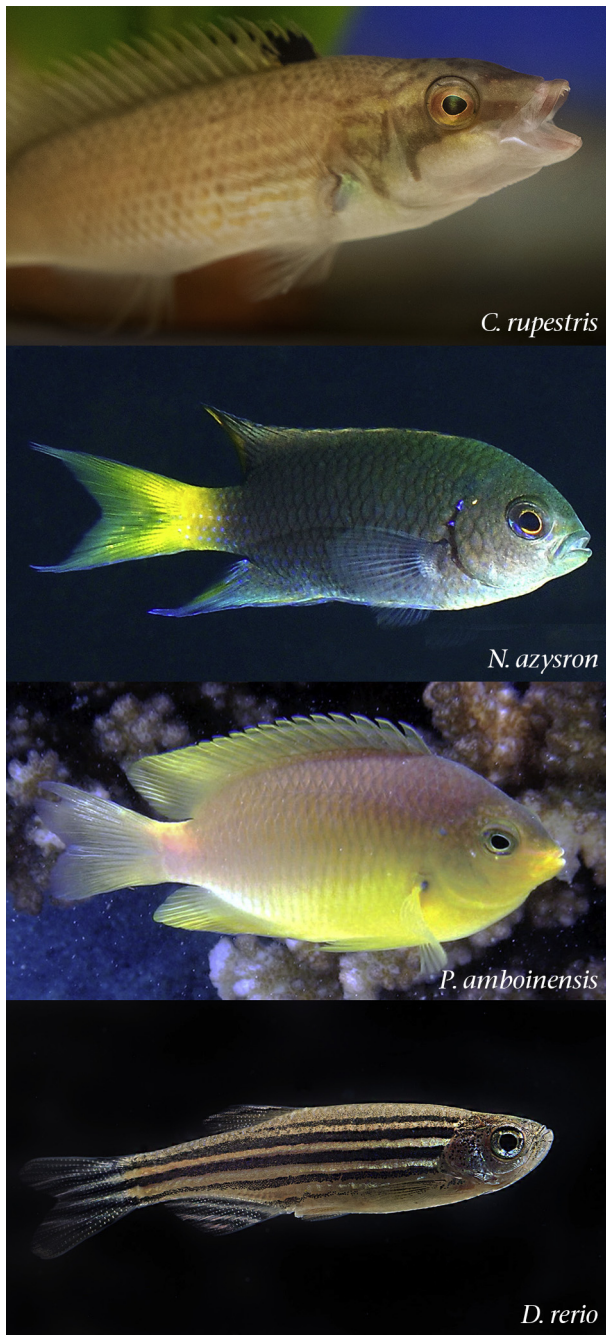
### Fish Collection and Husbandry

Sixty *C. rupestris* (1.38–46.52 g) were collected with baited minnow traps from the wharfs at the Sven Lovén Centre for Marine Infrastructure, Kristineberg, Sweden. Experiments on *C. rupestris* were conducted between 22 June and 1 July 2017 (water temperature range in the holding tanks and experimental set-up: 14.9–16.1 °C). Sixty *N. azysron* (0.74–6.40 g) and 60 *P. amboinensis* (1.0–14.3 g) were collected by SCUBA divers with a barrier net and hand-nets on coral reefs nearby the Lizard Island Research Station, Northern Queensland, Australia. Experiments on these two species were conducted between 24 July and 11 August 2017 (water temperature range: 24.3–25.5 °C). Sixty *D. rerio* (0.26–1.07 g) were collected in West Bengal, India, in August to September 2016 using hand-nets and kept in the Norwegian University of Science and Technology's (NTNU) aquarium facility, Trondheim, until the experiments were conducted between 10 and 17 October 2017 (water temperature range: 26.9–28.9 °C; for details on collection, import and holding conditions, see Morgan et al., 2019).

Fish from each species were equally divided into three to four holding tanks (density ~5.5 individuals/litre), provided with artificial plants and/or cut PVC pipes as shelter, and fed ad libitum twice daily. *Ctenolabrus rupestris* were fed blue mussels, *Mytilus edulis*, and bloodworms. *Neopomacentrus azysron* and *P. amboinensis* were fed commercial tropical fish flakes. *Danio rerio* were fed TetraPro fish flakes (Tetra, Blacksburg, VA, U.S.A.). Holding tanks were supplied with flow-through water from the fish's natural habitat for *C. rupestris*, *N. azysron* and *P. amboinensis*. *Danio rerio* were kept in flow-through aquaria at NTNU, with a water exchange rate of ~20% per day, and water quality (conductivity and temperature) monitored daily. Light cycles followed natural conditions at each location. Fish were individually marked using two-colour combinations of visible implant elastomer (VIE, Northwest Marine Technology Inc., Shaw Island, WA, U.S.A.) implanted posterodorsally, on both sides of the dorsal fin a minimum of 2 days prior to the first test.

### Lateralization Test

We used a standard detour test to assess behavioural lateralization (Bisazza, Facchin, Pignatti, & Vallortigara, 1998a; Dadda et al., 2010b; Jutfelt et al., 2013). Experiments were conducted in the morning during 0900–1230 hours, and fish were returned to their respective holding tank between test days. A single fish was introduced into a double-sided, opaque T-maze, consisting of a tank with a runway down the middle and a barrier at both ends (Fig. 1). *Ctenolabrus rupestris* and *D. rerio* were tested in one maze and *N. azysron* and *P. amboinensis* in another (dimensions in Fig. 1). A symmetrical stimulus was affixed to the centre of the barrier to focus the eye gaze of the fish. This stimulus was changed between each of the four trial series to prevent habituation to the set-up (a cross, two parallel black bars, a cross with a solid circle above it, a cross with a horizontal bar below it). The water height was 10 cm.



**Figure 2.** The four fish species from temperate, tropical, marine and freshwater habitats tested to determine the repeatability of lateralization: *Ctenolabrus rupestris* (credit: F. Jutfelt), *Neopomacentrus azysron* (credit: picture.world, <https://goo.gl/mTLpbF>), *Pomacentrus amboinensis* (Gagliano & Depczynski, 2013), and *Danio rerio* (credit: P. H. Olsen, Norwegian University of Science and Technology).

Experiments were conducted in a closed room and care was taken to ensure that the surrounding environment and lighting were as uniform as possible. We emptied and refilled the maze with new water between each fish tested to standardize the temperature, maintain normal levels of dissolved oxygen and avoid any effect of conspecific stress hormones (e.g. cortisol) on fish behaviour.

To start the experiment, a fish was placed at one end of the runway (maintained in this position by an acrylic divider blocking the entrance to the middle runway) for 1 min (Fig. 1). The starting side was determined by flipping a coin and the fish was transferred from its holding tank to the maze in a water-filled container. The divider was then lifted from behind the tank; if the fish did not advance on its own, it was gently pushed with an acrylic paddle to initiate movement down the runway (Jutfelt et al., 2013; Sundin & Jutfelt, 2018). At the end of the runway, the fish faced an opaque barrier forcing it to turn left or right. We ran 10 consecutive trials per fish, recording its turning direction each time. One experimenter conducted any given series of 10 consecutive trials, standing behind the fish, in a centred position, and walked from one side of the maze to the other between trials when the fish was behind the barrier (Fig. 1). A fish was considered to have completed a turn when its head (to the opercula) passed the end of the barrier (black bar in Fig. 1). Once a fish had completed a turn, the experimenter blocked re-entry to the runway with a paddle; the next trial was initiated as soon as the experimenter was in position, behind the fish. The side of the maze walked on was haphazardly decided by the experimenter (see Supplementary Material, Effect of experimenter movement on turning direction in the detour test). Another experimenter recorded the observations on paper except for *D. rerio*, where observations were typed directly into a computer. Experiments were recorded on video with the exception of *C. rupestris*, which was the first species tested and where no video camera was available. Four series of 10 trials were repeated for each individual at intervals of 48 h, allowing us to evaluate the short-term repeatability of this behaviour. Four full series could not be obtained for some individuals ( $N = 23$  of 218), as fish occasionally did not swim down the runway for 10 consecutive runs.

We calculated the relative lateralization index ( $L_R$ ) for each individual for each series of 10 trials, where  $L_R = ((\text{turns to the right} - \text{turns to the left}) / (\text{turns to the right} + \text{turns to the left})) \times 100$  (Bisazza et al., 1997a). A score of  $-100$  indicates that the fish turned left 10 times out of 10; a score of 100 indicates that the fish turned right 10 times out of 10.  $L_R$  can be examined at both the individual and the population (i.e. mean) level, informing whether individuals and/or populations have a side bias, respectively. Since several studies conduct analyses on the absolute lateralization index ( $L_A$ ), rather than  $L_R$ , we also calculated each individual's  $L_A$  for each series, where  $L_A = |L_R|$  (Supplementary Fig. S2).  $L_A$  scores of 80 and 100 (i.e. 9 and 10 turns to one direction) are indicative of lateralization since this number of turns to one side is statistically different from random based on a two-tailed binomial test.

#### Reanalysis of Published Data

To our knowledge, the only published study testing the repeatability of individual lateralization scores in a detour test was performed on 40 female guppies (*P. reticulata*) (Irving & Brown, 2013). Females were individually marked and tested in a detour test with 24 h or more between trial series. Fish were tested three times in each of two conditions: a barrier presenting a neutral stimulus (an empty aquarium behind a barrier of vertical bars) or a social stimulus (a conspecific in an aquarium behind a barrier of vertical bars) (Figure 1 in Irving & Brown, 2013). For lack of a better statistical approach at the time, the data were analysed using six Spearman rank correlations to assess the repeatability of  $L_R$  scores

(Irving & Brown, 2013). We reanalysed these data and computed a single intraclass correlation coefficient ( $R$ ) for each of the two test conditions (see Statistical Analysis).

#### Statistical Analysis

We tested population level lateralization with generalized (binomial) linear random-effects models, setting the intercept equal to the grand mean of the sample. Individual level lateralization was examined 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). See the Supplementary Material and Table S3 for details and an explanation of issues with tests of lateralization employed in previous studies.

We computed the repeatability ( $R$ ) in the number of left and right turns (in a series of 10 trials) across trial series for each species using the 'rpt' function in the package 'rptR' (Stoffel, Nakagawa, & Schielzeth, 2017). This analysis on binomial data amounts to testing the repeatability of  $L_R$ . We stress that the statistical analyses were conducted on true Bernoulli responses (left or right turn) rather than  $L_R$  scores (used in data visualization) because the latter have much less favourable statistical properties.  $L_R$  scores are shown in figures for comparison with previous studies.  $R$  values were computed for primary data collected in this study and for the published data on *P. reticulata* (Irving & Brown, 2013).  $R$  ranges from 0 (nonrepeatable) to 1 (fully repeatable) and provides a standardized measure of the consistency of phenotypes across time or contexts (Nakagawa & Schielzeth, 2010). We specified a binomial error distribution and 1000 bootstrapping and permutation iterations to calculate 95% confidence intervals (CIs). We also computed an 'adjusted repeatability' by specifying trial series (1–4), fish mass and start-side of the maze as fixed effects in the models to account for any confounding effects of these variables on  $R$  (Nakagawa & Schielzeth, 2010). The repeatability of  $L_A$  was calculated by specifying a Poisson error distribution. Finally, we tested the effect of experimenter identity (ID) and arena start-side on  $L_R$  using a generalized linear mixed-effects model (GLMM) in the package 'lme4' (Bates, Mächler, Bolker, & Walker, 2014); species, start-side, and their interaction were specified as fixed factors in the model while fish ID and experimenter ID were included as random factors. The importance of experimenter ID was tested with a likelihood ratio (LR) test. Models were validated via diagnostic checks with the package 'DHARMA' (Hartig, 2017). Statistical analyses were performed in R3.4.3 (R Core Team, 2017).

#### Ethical Note

Field collections and experiments were approved by the Swedish Board of Agriculture (Dnr 103–2014), Great Barrier Reef Marine Park Authority (G14/36625.1), the James Cook University Animal Ethics Committee in association with AIMS (A2314) and the Norwegian Animal Research Authority (Permit Number: 8578). Fish were collected in the wild and returned to their site of capture at the conclusion of the experiments (except for *D. rerio*, which were kept in the laboratory). Fish from each species were equally divided into three to four holding tanks (density  $\sim 5.5$  individuals/litre), provided with artificial plants and/or cut PVC pipes as shelter, and fed ad libitum twice daily. *Ctenolabrus rupestris* were fed blue mussels (*M. edulis*) and bloodworms. *Neopomacentrus azyron* and *P. amboinensis* were fed commercial tropical fish flakes. *Danio rerio* were fed TetraPro fish flakes. Holding tanks were supplied with flow-through water from the fish's natural habitat for *C. rupestris*,

*N. azysron* and *P. amboinensis*. *Danio rerio* were kept in flow-through aquaria at NTNU, with a water exchange rate of ~20% per day, and water quality (conductivity and temperature) monitored daily. Light cycles followed natural conditions at each location.

## RESULTS

Fish exhibited statistically significant individual level lateralization in two-thirds of trial series, i.e. 15 of 22 trial series across the four species tested and the species for which data were reanalysed (Supplementary Table S2, Figs S4–S8). Population level lateralization was statistically apparent in five of the 22 trial series; two species exhibited a population side bias, but this bias changed with test day for *D. rerio* and with stimulus type for *P. reticulata* (Supplementary Table S2, Figs S4–S8).

Importantly, however,  $L_R$  was highly variable within individuals, and individual identity explained less than 6% of the variance in relative lateralization across the four species tested, with repeatability estimates ranging from  $R = 0.006$  to  $R = 0.028$  (Table 1, Fig. 3, Supplementary Fig. S1). Controlling for the order of trial series, fish mass and start-side did not change these results (Table 1); fixed factors accounted for less than 4% of the variance in  $L_R$ . Similar results were obtained when considering  $L_A$  (Supplementary Fig. S2). The identity of the experimenter (LR test:  $\chi^2_1 = 0$ ,  $P = 0.999$ ) and the starting side of a series in the experimental arena had no effect on a fish's preference to turn left or right (GLMM:  $\chi^2_1 = 0.01$ ,  $P = 0.917$ ), irrespective of the species (GLMM: species  $\times$  start-side interaction:  $\chi^2_3 = 2.00$ ,  $P = 0.575$ ); see Table 1 and archived analysis script for details.

Similarly to the four species tested here, individual differences among female *P. reticulata* (Irving & Brown, 2013) accounted for very little of the variance in  $L_R$ , both when fish were tested using a neutral ( $R = 0.045$  [0.015–0.088]) and a social stimulus ( $R = 0.076$  [0.028–0.128]) (Supplementary Fig. S3).

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

## DISCUSSION

Numerous studies on fishes and other taxa report that behavioural lateralization is linked to fitness-enhancing behaviours and can be severely impacted by environmental stressors such as predation, parasitism, pollutants, drugs and various abiotic parameters, some of which have relied on the detour test methodology (see Supplementary Table S1 for examples in fishes). We evaluated whether lateralization assessed using a detour test is consistent in the short-term in fish species with differing ecologies to establish the baseline repeatability of this behavioural trait and broaden our

understanding of its ecological and evolutionary importance. Our validation exercise used robust sample sizes, four species, multiple measurements through time (four repeats at 48 h intervals) and included a reanalysis of data on a fifth species collected 7 years ago.

Our results show that behavioural lateralization as assessed by a detour test is not a repeatable trait in fishes over short timescales (Fig. 3, Supplementary Figs S1–S3). Even though many individuals from all species displayed a strong side bias (individual level lateralization present in 68% of trial series; Supplementary Table S2, Figs S4–S8), this turning preference varied markedly across days (Fig. 3, Supplementary Fig. S1). Of the 69 individuals across all five species displaying an absolute lateralization ( $L_A$ ) score of 80 or 100 (nine or 10 turns in either direction) in at least one of the trial series, 52 also had at least one  $L_A$  score of 20 (four or six turns in either direction) or zero (five turns each way) when tested again (Supplementary Figs S2, S3). Overall, across the five species examined, individual identity explained only a small proportion of the phenotypic variance in lateralization strength, ranging between 2.8 and 7.6%. These values contrast markedly with the results of several meta-analyses, which reported much higher repeatability estimates in traits such as behaviour ( $R = 0.37$ : Bell et al., 2009;  $R = 0.41$ : Holtmann, Lagisz, & Nakagawa, 2017), cognition ( $R = 0.15$ – $0.28$ : Cauchoix et al., 2018), metabolic rates ( $R = 0.45$ : Holtmann et al., 2017) and hormone levels ( $R = 0.15$ : Holtmann et al., 2017). A reanalysis of existing data (Supplementary Fig. S3) supports our findings and further suggests that individual decisions to turn left or right in the detour test do not provide accurate or precise estimates of eye preference or cerebral asymmetries in fishes. Our experiments were conducted by two or more researchers at any one time and trials were videorecorded except for *C. rupestris*. These recordings as well as the laboratory notebooks and raw data are publicly available (Roche, Amcoff et al., 2019). The fact that multiple laboratories collaborated to conduct this study across several geographical locations (Australia, Norway, Sweden) further strengthens the robustness of our results (Voelkl, Vogt, Sena, & Würbel, 2018).

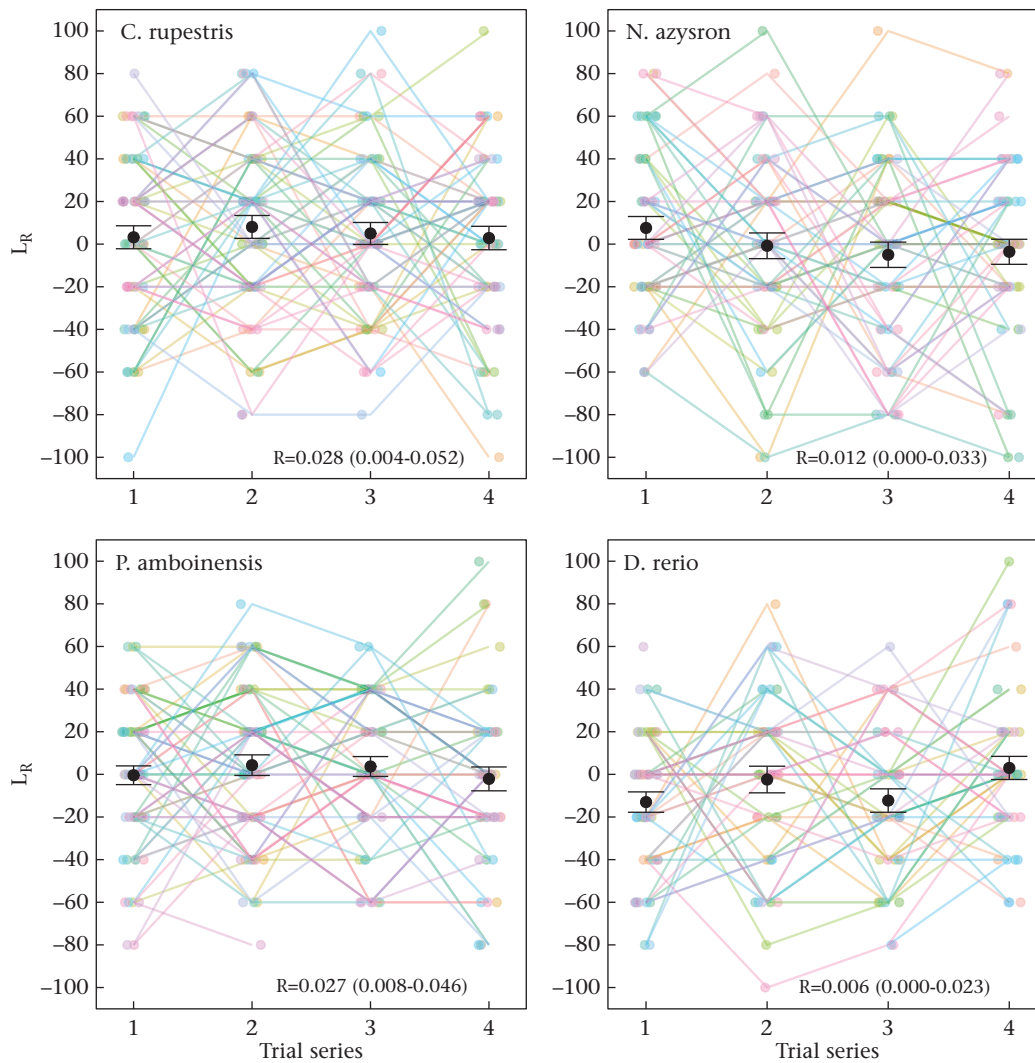
Only two of the five species exhibited a mean  $L_R$  significantly different from zero in at least one trial series, indicative of population level lateralization (Supplementary Table S2, Figs S4–S8). This population level side bias varied between days in the case of *D. rerio* and between stimulus types in the case of *P. reticulata* (see methods in Irving & Brown, 2013). We anticipated a greater repeatability of  $L_R$  and stronger evidence for a positive or negative mean  $L_R$  in the three shoaling species examined (*N. azysron*, *D. rerio*, *P. reticulata*), as individual and population level lateralization are reported to help social individuals coordinate group behaviours and enhance school cohesion (Bibost & Brown, 2013; Bisazza & Dadda, 2005; Bisazza et al., 2000a). Bibost and Brown (2013) used a mirror test and found that individual level lateralization could influence the geometry of school formation depending on the

**Table 1**

Sample size ( $N$ ), mass range (g), total length range (TL range, cm) and statistics for five species of fish tested to examine the repeatability of behavioural lateralization

	<i>Ctenolabrus rupestris</i>	<i>Neopomacentrus azysron</i>	<i>Pomacentrus amboinensis</i>	<i>Danio rerio</i>	<i>Poecilia reticulata</i> <sup>1</sup>	<i>P. reticulata</i> <sup>2</sup>
$N$	57	52	60	49	40	40
Mass range	1.4–46.5	0.7–6.4	1.0–14.3	0.26–1.07	–	–
TL range	4.4–14.9	4.2–8.3	3.8–8.8	2.7–3.6	1.7–3.5	1.7–3.5
$R$ (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	$\chi^2_1 = 0.204$ $P = 0.651$	$\chi^2_1 = 0.849$ $P = 0.357$	$\chi^2_1 = 0.354$ $P = 0.552$	$\chi^2_1 = 0.020$ $P = 0.887$	–	–

*Poecilia reticulata* were tested with a neutral (<sup>1</sup>) and a social (<sup>2</sup>) stimulus (see Irving & Brown, 2013). Estimates are presented for agreement and adjusted repeatability of relative lateralization ( $L_R$ ) with 95% CIs in parentheses. Statistics and  $P$  values are presented for the effect of trial series (1–4), body size (total length for *P. reticulata* and mass for all other species) and start-side of the maze on  $L_R$ .



**Figure 3.** Relative lateralization index ( $L_R$ ) across repeated trials (2–4) per individual for four fish species: *Ctenolabrus rupestris* ( $N = 57$ ), *Neopomacentrus azysron* ( $N = 52$ ), *Pomacentrus amboinensis* ( $N = 60$ ), and *Danio rerio* ( $N = 49$ ). 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 for each species. Four series of 10 trials were conducted. Data points are jittered along the  $X$  axis for presentation purposes.

sex and species of the school. Bisazza and Dadda (2005) used a detour test and reported that lateralized poeciliids (*Girardinus falcatus*) exhibit greater school cohesion and coordination than non-lateralized conspecifics; however, schools were composed of only two individuals, which were females from a multigeneration laboratory strain. Bisazza et al. (2000a) also used a detour test and reported significant population level lateralization in 10 of the 16 fish species they examined ( $N$  per species: 7–18), yet only six of these were shoaling. Other studies have also reported such population level side biases in fishes (e.g. Bisazza et al., 1997a; Bisazza et al., 1998a; Domenici, Allan, Watson, McCormick, & Munday, 2014; Facchin et al., 1999; Irving & Brown, 2013), yet their association with group living appears ambiguous. For instance, Domenici et al. (2012) and Lopes et al. (2016) failed to observe population level lateralization in shoaling *N. azysron* juveniles and *Atherina presbyter* larvae, respectively, despite reporting  $L_A$  scores higher than random in their control groups. Similarly, Chivers et al. (2016) reported high  $L_R$  scores in some shoals of the schooling fusilier *Caesio teres*, but lateralization strength varied substantially both among and within the four groups tested. Taken together, these

results suggest that the detour test is not adequate for assessing lateralization in fishes and that possible benefits of lateralization for shoaling species should be investigated using other means.

#### Implications

Given the large body of literature reporting significant effects of environmental stressors on lateralization in fishes (Supplementary Table S1), our results raise several questions that we address below.

#### Could Methodological Differences between Ours and Previous Studies Explain the Lack of Repeatability in $L_R$ ?

Published studies have used a range of different obstacles to elicit eye use preference when fish arrive at the end of the runway in a detour test. Some studies use a neutral obstacle, such as an opaque barrier (e.g. Bisazza et al., 2001; Dadda et al., 2010b; Domenici et al., 2012; Domenici et al., 2014; Roche et al., 2013) or a barrier of vertical bars (e.g. Bisazza et al., 1997a; Dadda & Bisazza, 2016; Irving & Brown, 2013; Reddon & Hurd, 2009a). Preferential

eye use is said to occur because fish must explore the unknown space to the side or behind the barrier (Facchin et al., 1999). Other studies have used a non-neutral obstacle with a stimulus, such as a conspecific or an object resembling a predator placed behind a barrier of vertical bars (e.g. Bisazza et al., 1998a; Dadda & Bisazza, 2006b; Facchin et al., 1999; Irving & Brown, 2013). The stimuli (e.g. a cross on an opaque barrier) used in our experiments may not have been valent enough to result in a strong, consistent lateralized response in individuals. Indeed, some research suggests that lateralization direction and strength is stimulus dependent (Bisazza et al., 1997a; Sovrano, 2004). It is possible that a predatory stimulus would have increased repeatability of measurements in this test since a consistent behavioural response to a predator may be under stronger directional selection than a nonthreatening stimulus. However, previous studies using different methods for assessing lateralization in fishes (i.e. mirror tests or circular arena tests) have reported correlations in the strength of individual lateralization among tests, including between novel/neutral, predator and social stimuli (Bisazza et al., 2001; Brown, Gardner, & Braithwaite, 2004). These results suggest that the specific stimulus used to focus gaze could affect the direction of lateralization (i.e. depending on eye use preference to focus on different stimuli) but should not significantly affect the strength of repeatability in the test. Our results provide evidence for this: we found that  $L_R$  was not repeatable across time when experiments were conducted with two different neutral stimuli (this study and reanalysis of Irving & Brown, 2013) as well as with a non-neutral stimulus (reanalysis of Irving & Brown, 2013). The occurrence of high individual  $L_R$  scores in all species in trial series 2, 3 and 4 of the experiment indicates that habituation to the test arena is unlikely (Fig. 3). Importantly, studies have also reported a significant population level side bias in species when only an opaque barrier with no stimulus was used (e.g. Bisazza et al., 1998a; Chivers et al., 2016; Ferrari et al., 2015), suggesting that a neutral stimulus should not impede a strongly lateralized response in a detour test. Interestingly, Ferrari et al. (2017) found considerable variability in the lateralization scores of *P. amboinensis* tested twice on the same day using opaque barriers with no stimuli. These results were interpreted as representing adaptive plasticity in lateralization strength due to different predation risk scenarios (Ferrari et al., 2017), yet these experiments lacked a control group establishing the consistency of lateralization strength in untreated individuals. Our results suggest that such variability is likely due to the inherent randomness of turning choice displayed by tested individuals rather than any adaptive behavioural decision. Similarly, a recent reanalysis of a well-cited study on honeybee magnetoreception also revealed random patterns rather than adaptive behaviour as originally suggested (Baltzley & Nabity, 2018).

Numerous other methodological variations exist across published studies that are worth considering in the context of our results. For example, studies differ in their maze design (e.g. single T: Roche et al., 2013; Y- versus T-entry: Irving & Brown, 2013; Jutfelt et al., 2013; Vila Pouca, Gervais, Reed, & Brown, 2018) and dimensions (Supplementary Table S1), acclimation time before beginning a series (3 min: Bisazza et al., 2001; Roche et al., 2013; 1 min: Sundin & Jutfelt, 2018), wait time between trials within a series (3 min: Roche et al., 2013; no wait time: Sundin & Jutfelt, 2016, 2018) and method for encouraging fish to enter runway (e.g. no encouragement: Vossen et al., 2016; encouragement: Sundin & Jutfelt, 2016), to name a few. Although subtle, these differences in protocol or experimental apparatus could considerably influence the results. For instance, Clark et al. (2020) examined lateralization behaviour in juvenile *Acanthochromis polyacanthus* in a double T-maze and found that a slight asymmetry in the barrier position at one end of their maze induced a strong side bias in their tested fish. This side bias was not observed at the other end of the

arena where the barrier was centrally placed. Similarly, Sundin et al. (n.d.) tested wild *P. reticulata* in a T-maze arena, which they later discovered had a small crack in one corner, causing this area of the tank to be slightly darker than the other side. Individuals consistently turned in the direction of this darker area, but no consistent side bias was apparent at the other end of the maze, or in the same individuals when tested in other identically constructed arenas. These observations illustrate the care that must go into the construction of experimental apparatus designed to assess side biases in individuals, as slight construction asymmetry or variations in protocol may dramatically influence the results obtained. Such side biases generated through very slight differences in arena construction or lighting may go overlooked, particularly in analyses of absolute lateralization.

#### *Could Differences in Species, Sex and/or Life Stage Influence the Repeatability of $L_R$ ?*

Twenty-seven fish species have so far been tested in 31 published studies using a detour test to either relate lateralization to fitness-relevant traits or assess the effects of environmental stressors on lateralization strength (Supplementary Table S1). Several other fish species also feature in studies simply examining whether individuals or populations are lateralized (e.g. Bisazza, Pignatti, & Vallortigara, 1997b; Bisazza et al., 2000a). The five species examined here (four original and one reanalysed) are phylogenetically diverse, originating from tropical, temperate, marine and freshwater habitats. Therefore, they constitute a representative sample allowing our results to be generalized with a reasonable degree of confidence.

We did not include sex in our analyses of repeatability because *C. rupestris* were juveniles, *P. amboinensis* were predominantly female (given the size distribution of tested fish) and sex is difficult to assess noninvasively in the other species. Some studies suggest that sex should always be considered in studies of cerebral lateralization because male and female brains are organized differently (Bianki & Filippova, 2001). Meta-analyses also suggest that there are important sex differences in the repeatability of many behavioural traits (Bell et al., 2009), and that including determinants such as participant age and sex increased estimates of temporal repeatability slightly (Cauchoix et al., 2018). Indeed, several studies have reported sex-specific differences in lateralization strength in fishes (e.g. Bisazza et al., 1998a; Byrnes, Pouca, & Brown, 2016; Irving & Brown, 2013; Reddon & Hurd, 2008, 2009b), although others have not (e.g. Byrnes et al., 2016; Reddon & Hurd, 2009a; Sundin & Jutfelt, 2018; Vossen et al., 2016). While this is worth exploring in future studies, it is unlikely that sex differences account for the dramatically low repeatability in lateralization strength measured in all four of our study species ( $R = 0.006\text{--}0.028$ ). With the exception of the protogynous *P. amboinensis*, where our sample is likely to be female-biased based on the size distribution of the collected individuals (McCormick, 2016), the remaining species tested have distinct sexes and are not strongly sexually dimorphic. As a result, we assume a roughly even sex ratio in our samples. If lateralization in one sex were highly repeatable, we would still expect an  $R$  measure considerably higher than observed, with consistency in a turning direction observed in approximately half of the population. This is clearly not observable in our data (Fig. 3, Supplementary Fig. S1).

The effect of life stage on the strength of lateralization is another consideration that was not explicitly addressed in our study. Although Bell et al. (2009) found no difference in the repeatability of behaviours between juveniles and adults in general, they noted that, among ectotherms, juvenile behaviour is significantly more repeatable. We included a range of sizes in our tested species,

which, for *C. rupestris* and *D. rerio*, included juveniles and subadults. We also included fish body mass as a fixed factor in our analyses. We found no effect of mass on the strength of lateralization in any of our tested species. Published studies of detour tests in fishes have tested a range of life stages from presettlement larval fish to adults (Supplementary Table S1). Although there does not seem to be a consistent trend in lateralization strength owing to life stage, this should be tested more systematically in future studies.

#### What Explains Positive Results in Previous Studies?

The high intraindividual variation in lateralization we observed across test days in all five species examined (Fig. 1, Supplementary Fig. S3) suggests that individual  $L_R$  scores in a detour test are random at any point in time. Therefore, why do numerous studies using detour tests report significant relationships between lateralization strength and other phenotypic traits or environmental stressors? Unfortunately, many lateralization studies (including previous publications by authors involved in this study) suffer from low sample sizes (e.g. 20 or fewer individuals per group: Bisazza et al., 2000a; Byrnes et al., 2016; Ferrari et al., 2017; Jutfelt et al., 2013; Lopes et al., 2016; Nilsson et al., 2012; Roche et al., 2013; Sundin & Jutfelt, 2018; Supplementary Table S1), which considerably increases the likelihood of spurious results (Button et al., 2013; Colquhoun, 2014; Halsey, Curran-Everett, Vowler, & Drummond, 2015). In addition, all 35 tests (31 studies) identified in Supplementary Table S3 employed inadequate statistics to test for the presence of lateralized individuals (see Supplementary Material). Confirmation bias and poor research practices such as *p*-hacking and selective reporting also contribute to false positives, which are published more readily than negative results (i.e. the publication bias or file-drawer effect) (Nuzzo, 2015; Parker et al., 2016). A recent survey of over 800 researchers revealed that such practices are rife in ecology and evolution, contributing to the ongoing reproducibility crisis (Fraser, Parker, Nakagawa, Barnett, & Fidler, 2018). Improving our confidence in, and ability to replicate, lateralization studies requires the implementation of validated methodologies, appropriate statistics, high-powered designs (Button et al., 2013), double-blinded protocols (Holman, Head, Lanfear, & Jennions, 2015), video recordings (Clark, 2017), open data (Roche, Kruuk, Lanfear, & Binning, 2015) and other transparency measures advocated by the recent Transparency and Openness Promotion (TOP) guidelines (Clark et al., 2016; Nosek et al., 2015).

#### Conclusion

Behavioural lateralization is likely to be an ecologically important trait that should continue to be tested and measured. However, the method with which to assess eye use preference as a proxy of cerebral asymmetry must be thoroughly validated for a species of interest before drawing conclusions about the fitness consequences of changes in lateralization direction or strength. Drawing inferences from nonrepeatable measurements (or studies) not only obfuscates our understanding of a species' evolutionary ecology but also risks misleading policy and conservation efforts. Our study shows that the detour test as it has traditionally been implemented does not provide accurate, precise or repeatable estimates of behavioural lateralization in fishes. Future studies could evaluate whether repeatedly assessing behavioural lateralization in a detour test over a longer time frame (i.e. weeks or months) yields higher repeatability estimates for this trait. Furthermore, numerous other methods of assessing eye use and side preference, including mirror tests, swimming, feeding or attack direction preference, and flume tests, have been applied in a range of species in both field and

laboratory settings (see Bibost & Brown, 2014; Bibost, Kydd, & Brown, 2013; Broder & Angeloni, 2014; Brown & Bibost, 2014; Dadda & Bisazza, 2006a; Dadda, Domenichini, Piffer, Argenton, & Bisazza, 2010a; Forsatkar, Dadda, & Nematollahi, 2015; Kurvers et al., 2017; Takeuchi, Hori, Myint, & Kohda, 2010). Measurements using these methods and their cross-context repeatability should be validated in accordance with TOP guidelines (Nosek et al., 2015) to establish reproducible protocols that inspire confidence. We also encourage the development of more studies that explicitly examine the link among structural differences between brain hemispheres and individual behaviour in fishes to more concretely validate the link between cerebral and behavioural lateralization in lower vertebrates (see Bisazza, Rogers, & Vallortigara, 1998b).

#### Data, Code and Materials

The data, script, notebook copies and videos for this study are archived in the repository figshare following best practices (Roche et al., 2015) and were made available to editors and reviewers upon initial submission: <https://doi.org/10.6084/m9.figshare.6881489> (Roche, Amcoff et al., 2019).

#### Data Reuse

Published data (Irving & Brown, 2013) were reused for this study. The original authors were invited to participate and offered co-authorship.

#### Author Contributions

D.G.R., M.A., J.S., T.D.C., F.J., T.N., B.S.R. and S.A.B. designed the experiments; D.G.R., M.A., R.M., J.S., A.H.A., M.H.F., F.J., M.J.L., E.H. and S.A.B. performed the experiments. J.S., F.J. and R.B. contributed vital equipment and funding for the experiments. C.B. provided the data used in the reanalysis. D.G.R. analysed the data. D.G.R. and S.A.B. wrote the manuscript with input and approval from all authors.

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#### Conflict of Interest

We declare no competing interests.

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## Supplementary Material

Supplementary methods and results to this article can be found online at <https://doi.org/10.1016/j.anbehav.2020.06.025>.

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