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

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Abstract

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

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

Introduction

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

Behavioural lateralisation is commonly measured in the laboratory using a detour test (e.g. T-maze), wherein an individual moves along a runway until it faces a barrier forcing it to make a choice between turning left or right. This quick and simple test is now widely used in studies of fish behaviour, evolutionary ecology and ecotoxicology (Electronic Supplementary Material [ESM], Table S1), and is also used to assess lateralisation in various other taxa including birds [16], reptiles [17], and molluscs [1]. 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 [18]. 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* threatening stimulus [19]. Similarly, left-right asymmetries in *G. falcatus* assessed in a detour test appear to be consistent with lateral bias measured using other methods [20]. Furthermore, lateralisation score in a detour test is reported to be heritable [21, 22], although lateralisation strength decreases rapidly across generations in artificial selection lines [23, 24]. 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 lateralisation 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 lateralisation in fishes, several of which have produced worrying results (ESM, Table S1). Most notably, ocean acidification is reported to decrease lateralisation across a range of marine fishes, including tropical [25-27] and temperate species [28-30]. Such effects are concerning given, for example, the important benefits of lateralisation reported for fishes under high predation risk [e.g. 8, 31, 32]. Despite this strong interest in behavioural lateralisation and 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 lateralisation scores. Establishing the consistency of lateralisation within individuals is essential for determining the relevance of this trait for assessing fitness as well as the responses of animals to exogenous stressors.

To evaluate the repeatability of behavioural lateralisation in fishes, 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*). All of these species have previously been used in published studies assessing behavioural lateralisation using a detour test (T-maze) similar to the one used here [8, 25, 26, 33, 34]. We also used new (more appropriate) statistical methods to re-analyse a published dataset that included multiple lateralisation measurements for the tropical freshwater guppy (*Poecilia reticulata*) [15]. We predicted a significant repeatability in lateralisation scores comparable in magnitude to that observed for other behavioural traits [35]. Population-level lateralisation 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 [36]. We had no *a priori* prediction about the direction (left or right) of lateralisation, as among-species differences in response to the same stimulus have been shown previously [36]. We also examined for an effect of habituation to the test arena across the four test trials because (1) repeatedly measuring the same individuals in a similar apparatus can decrease measurement error and thus be predicted to increase individual repeatability [37], and conversely (2) habituation or desensitisation to a stimulus may reduce repeatability by generating different results between trials within an individual [37].

Materials and 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 setup: 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).

Fish from each species were equally divided into three to four holding tanks (density ~5.5 individuals L^{-1}), provided with artificial plants and/or cut PVC pipes as shelter, and fed *ad libitum* twice daily. *C. 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. Holding tanks were supplied with flow-through water from the fish's natural habitat for *C. rupestris*, *N. azysron*, and *P. amboinensis*. *D. 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 (Northwest Marine Technology Inc., Shaw Island, WA, USA) implanted postero-dorsally, on both sides of the dorsal fin a minimum of two days prior to the first test.

Lateralisation test

We used a standard detour test to assess behavioural lateralisation [12, 30, 38]. Experiments were conducted in the morning between 9:00-12:30 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 (dimensions in Fig. S1). *Ctenolabrus rupestris* and *D. rerio* were tested in one maze and *N. azysron* and *P. amboinensis* in another (see ESM Fig. S1). A symmetrical neutral stimulus was affixed to the centre of the barrier to focus the eye-gaze of the fish. This neutral stimulus was changed between each of the four trials (see below) 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. 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 re-filled the maze with new water between each fish tested to standardise the temperature, maintain normal levels of dissolved oxygen, and to 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 one minute (ESM Fig. S1). 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 [30, 39]. 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 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). The side of the maze walked on was random. 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. Four full series could not be obtained for some individuals as fish occasionally did not swim down the runway for 10 consecutive runs.

We calculated the relative lateralisation index (L_R) for each individual for each series of 10 trials, where L_R = ((turns to the right − turns to the left) / (turns to the right + turns to the left)) × 100 [18]. 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 lateralisation index (L_A), rather than L_R, we also calculated each individual's L_A for each series, where $L_A = |L_R|$ (ESM Fig. S3). Scores between 60 and 100 (i.e. 8 and 10 turns to one direction, respectively) are indicative of strong lateralisation.

Re-analysis of published data

To our knowledge, the only published study testing the consistency of individual lateralisation scores was performed on 40 female guppies (*P. reticulata*) [15]. 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) (Fig. 1 in [15]). For lack of a better statistical approach at the time, the data were analysed using six Spearman rank correlations to assess the consistency of L_R scores [15]. We re-analysed these data and computed a single intra-class correlation coefficient (R) for each of the two test conditions (see *Statistical analysis*).

Statistical analysis

We tested population-level lateralisation with generalised (binomial) linear random-effects models, setting the intercept equal to the grand mean of the sample. Individual-level lateralisation 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 ESM text and Table S3 for details and an explanation of issues with tests of lateralisation 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' [40]. This analysis on binomial data amounts to testing the repeatability of L_R. R values were computed for primary data collected in this study and for the published data on *P. reticulata* [15]. R ranges from 0 (non-repeatable) to 1 (fully repeatable) and provides a standardised measure of the consistency of phenotypes across time or contexts [41]. 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 to 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 [41]. The repeatability of L_A was calculated by specifying a Poisson error distribution. Finally, we tested the effect of experimenter ID and arena start side on L_R using a generalised linear mixed-effects model (GLMM) in the package 'lme4'; 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' [42]. Statistical analyses were performed in R3.4.3 [43].

Results

Fish exhibited statistically significant individual-level lateralisation 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 re-analysed (ESM Table S2 and Fig. S5-S9). Population-level lateralisation 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 stimulus type for *P. reticulata* (ESM Table S2, Fig. S5-S9).

Importantly, however, L_R was highly variable within individuals and individual identity explained less than 6% of the variance in relative lateralisation across the four species tested, with repeatability estimates ranging from R=0.006 to R=0.028 (Table 1, Fig. 2, ESM Fig. S2). 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 (ESM Fig. S3). 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 × start-side interaction; $\chi^2_{(3)}$=2.00, P=0.575); see also Table 1.

Similarly to the four species tested here, individual differences among female *P. reticulata* [15] 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]) (ESM Fig. S4).

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 non-zero) values of R (Table 1).

Discussion

Numerous studies on fishes and other taxa report that behavioural lateralisation is linked to fitness-enhancing behaviours and can be severely impacted by environmental stressors such as predation, parasitism, pollutants, drugs, and various abiotic parameters (see ESM Table S1 for examples in fishes). We assessed whether lateralisation is consistent 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 re-analysis of data on a fifth species collected seven years ago.

Our results show that behavioural lateralisation as assessed by a detour test is not a repeatable trait in fishes (Fig. 2, ESM Fig. S2-S4), and thus should be interpreted with great care. Even though many individuals from all species displayed a strong side-bias (individual-level lateralisation present in 68% of trial series; ESM Table S2, Fig. S5-S9), this turning preference varied markedly across days (Fig. 2, ESM Fig. S4). Of the 69 individuals across all five species displaying an absolute lateralisation (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 (ESM Fig. S3, S4). Overall, across the five species examined, individual identity explained only a small proportion of the phenotypic variance in lateralisation strength, ranging between 2.8 and 7.6%. These values contrast markedly with the results of a recent meta-analysis reporting that individual differences account for a much higher proportion (approximately 35%) of the variance in most behavioural traits [35]. A re-analysis of existing data (ESM Fig. S4) supports our findings, and further suggests that individual decisions to turn left or right in the detour test are random and 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 video recorded except for *C. rupestris*. These recordings, lab notebooks, and the raw data are publicly available [44]. The fact that multiple labs collaborated to conduct this study across several geographic locations (Australia, Norway, Sweden) further strengthens the robustness of our results [45].

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 lateralisation (ESM Table S2, Fig. S5-S9). This population-level side-bias varied between days in the case of *D. rerio*, and stimulus type in the case of *P. reticulata* (see methods in [15]). 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* and *P. reticulata*), as individual- and population-level lateralisation are reported to help social individuals coordinate group behaviours and enhance school cohesion [9, 36]. Bisazza & Dadda [9] used a detour test and reported that lateralised poecilids (*Girardinus falcatus*) exhibit greater school cohesion and coordination than non-lateralised conspecifics; however, schools were composed of only two individuals, which were females from a multi-generation laboratory strain. Bisazza et al. [36] also reported significant population-level lateralisation in 10 of the 16 fish species they examined (*n* per species: 7 to 18), yet only six of these were shoaling. Other studies have also reported such population-level side biases in fishes [e.g. 15, 18, 19, 38, 46], yet their association with group living appears ambiguous. For instance, Domenici et al. [26] and Lopes et al. [28] failed to observe population-level lateralisation 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. [32] reported high L_R scores in some shoals of the schooling fusilier *Caesio teres*, but lateralisation strength varied substantially both among and within the four groups tested. Taken together, these results suggest that that the detour test is not adequate for assessing lateralisation in fishes and that the benefits of lateralisation for shoaling species might be overstated.

Implications

Given the large body of literature reporting significant effects of environmental stressors on lateralisation in fishes (ESM 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 (Fig. S1). Some studies use a neutral obstacle, such as an opaque barrier [e.g. 12, 14, 20, 26, 46] or a barrier of vertical bars [e.g. 15, 18, 47, 48]. Preferential eye-use is said to occur because fish must explore the unknown space to the side or behind the barrier [19]. 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. 5, 15, 19, 38]. The neutral 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 lateralised response in individuals. Indeed, some research suggests that lateralisation direction and strength is stimulus-dependent [18, 49]. 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 neutral stimulus. However, previous studies using different methods for assessing lateralisation in fishes (i.e. mirror tests or circular arena tests) have reported correlations in the strength of individual lateralisation among tests, including between novel/neutral, predator, and social stimuli [20, 50]. These results suggest that the specific stimulus used to focus gaze 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 re-analysis of [15]) as well as with a non-neutral stimulus (re-analysis of [15]). 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. 2). 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. 31, 32, 38], suggesting that a neutral stimulus should not impede a strongly lateralised response in a detour test. Interestingly, Ferrari et al. [8] found considerable variability in the lateralisation 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 lateralisation strength due to different predation risk scenarios [8], yet these experiments lacked a control group establishing the consistency of lateralisation 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 [51].

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 in [14]; Y- *versus* T-entry in [15, 30, 52]) and dimensions (ESM Table S1), acclimation time before beginning a series (3 min in [14, 20]; 1 min in [39]), wait time between trials within a series (3 min in [14]; no wait time in [34, 39]), and method for encouraging fish to enter runway (e.g. [33] *versus* [34]), to name a few. Although subtle, these differences in protocol or experimental apparatus could considerably influence the results. For instance, Clark et al. (unpublished data) examined lateralisation 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. (unpublished data) 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 lateralisation.

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 lateralisation to fitness-relevant traits or assess the effects of environmental stressors on lateralisation strength (ESM Table S1). Several other fish species also feature in studies simply examining whether individuals or populations are lateralised [e.g. 36, 53]. The five species examined here (four original and one re-analysed) are phylogenetically diverse, originating from tropical, temperate, marine, and freshwater habitats. Therefore, they constitute a representative sample allowing our results to be generalised with a reasonable degree of confidence.

We did not include sex in our analyses of repeatability. Some studies suggest that sex should always be considered in studies of cerebral lateralisation because male and female brains are organised differently [54]. A meta-analysis also suggests that there are important sex-differences in the repeatability of many behavioural traits [35]. Indeed, several studies have reported sex-specific differences in lateralisation strength in fishes (e.g. [15, 38, 55-57]), although others have not (e.g. [33, 39, 47, 57]). While this is worth exploring in future studies, it is unlikely that sex differences account for the dramatically low repeatability in lateralisation strength measured in all four of our study species (R=0.006 to R=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 [58], 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 lateralisation 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 not clearly observable in our data (Fig. 2), and likely reflects the weakness of the experimental method (i.e. the detour test) rather than a failure to account for a confounding variable such as sex in our analyses.

The effect of life stage on the strength of lateralisation is another consideration that was not explicitly addressed in our study. Although Bell et al. [35] found no difference in the repeatability of behaviours between juveniles and adults in general, they note 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 sub-adults. We also included fish body mass as a fixed factor in our analyses. We found no effect of mass on the strength of lateralisation in any of our tested species. Published studies of detour tests in fishes have tested a range of life stages from pre-settlement larval fish to adults (ESM Table S1). Although there does not seem to be a consistent trend in lateralisation strength owing to life stage, this should be tested more systematically in future studies.

What explains positive results in previous studies?

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

Conclusion

Behavioural lateralisation 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 lateralisation direction or strength. Drawing inferences from non-repeatable 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 lateralisation in fishes. However, 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 [3, 10, 70-76]). Measurements using these methods and their cross-context repeatability should be validated in accordance with TOP guidelines [69] to establish reproducible protocols that inspire confidence.

Animal ethics

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).

Data, code, and materials

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

Data re-use

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

Competing interests

We have no competing interests.

Author contributions

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

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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 lateralisation. *P. reticulata* were tested with a neutral (^{1}) and a social (^{2}) stimulus (see [15]). Estimates are presented for agreement and adjusted repeatability of relative lateralisation (L_R) with 95% CIs in parentheses. Statistics and *P* values are presented for the effect of trial series (1 to 4), body size (total length for *P. reticulata* and mass for all other species), and start-side of the maze on L_R.

	C. rupestris	***N. azysron***	***P. amboinensis***	***D. rerio***	***P. reticulata***^{1}	***P. reticulata***^{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

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

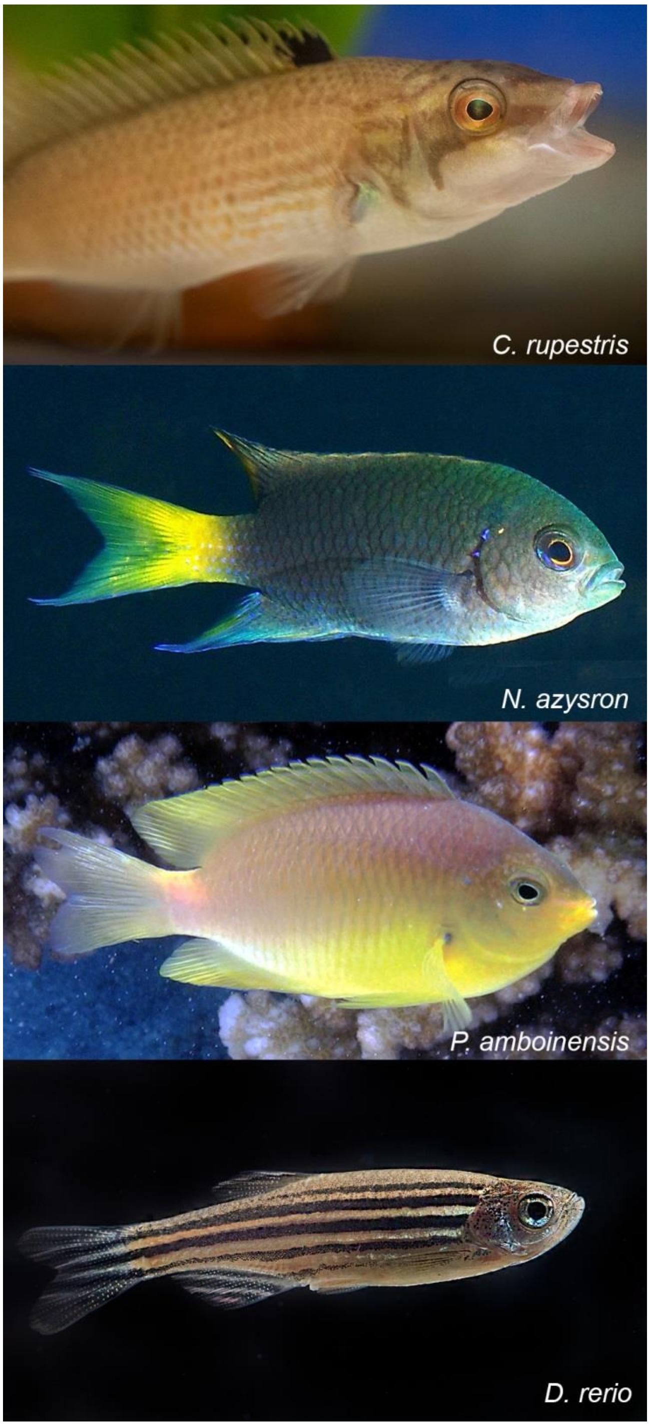

Fig. 2 Relative lateralisation index (L_R) across repeated trials (2 to 4) per individual for four fish species: *Ctenolabrus rupestris* (*n*=57), *Neopomacentrus azysron* (*n*=52), *Pomacentrus amboinensis* (*n*=60), and *Danio rerio* (*n*=49). Individual-level L_R is indicated by the coloured dots 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.