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ORIGINAL PAPER

Repeatability of lateralisation in mosquitofish *Gambusia holbrooki* despite evidence for turn alternation in detour tests

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Abstract
Akin to handedness in humans, some animals show a preference for moving to the left or right. This is often attributed to lateralised cognitive functions and eye dominance, which, in turn, influences their behaviour. In fishes, behavioural lateralisation has been tested using detour mazes for over 20 years. Studies report that certain individuals are more likely to approach predators or potential mates from one direction. These findings imply that the lateralisation behaviour of individuals is repeatable, but this is rarely confirmed through multiple testing of each individual over time. Here we quantify the repeatability of turning behaviour by female mosquitofish (*Gambusia holbrooki*) in a double sided T-maze. Each female was tested three times in each of six treatments: when approaching other females, males, or an empty space; and when able to swim freely or when forced to choose by being herded from behind with a net. Although there was no turning bias based on the mean population response, we detected significant repeatability of lateralisation in five of the six treatments ($R=0.251$–0.625). This is noteworthy as we also found that individuals tended to alternate between left and right turns, meaning that they tend to move back and forth along one wall of the double-sided T-maze. Furthermore, we found evidence for this wall following when re-analysing data from a previous study. We discuss potential explanations for this phenomenon, and its implications for study design.

Keywords Behavioural laterality · Cerebral lateralisation · Cognition · Poeciliidae · T-maze test

Introduction

Behavioural lateralisation is the asymmetrical performance of a particular function, and it is widely attributed to asymmetrical distribution of cognitive functions in brain hemispheres (Vallortigara et al. 2011). Possibly the most familiar example of behavioural lateralisation is handedness in humans. Brain hemisphere lateralisation and the resultant specialisation on different tasks is, despite early claims, not unique to primates (MacNeilage et al. 1987). Behavioural lateralisation has now been documented in other mammals (Versace et al. 2007; Blois-Heulin et al. 2012; Giljov et al. 2013), birds (Prior et al. 2004; Rogers et al. 2004; Koboroff et al. 2008; Magat and Brown 2009; Wilzeck et al. 2010), reptiles (Csermely et al. 2010; Sovrano et al. 2018), amphibians (Dill 1977; Robins and Rogers 2004; Sovrano 2007), and fishes (Fuss et al. 2019).

There are even studies reporting behavioural lateralisation in invertebrates (Taylor et al. 2010; Frasnelli et al. 2012; Rigosi et al. 2015; Schnell et al. 2019).

The occurrence of behavioural lateralisation in so many taxa suggests that it has adaptive benefits, despite some associated costs. Brain lateralisation might allow each hemisphere to specialise on different tasks without the need to increase brain size (Mutha et al. 2012). Laterality has, therefore, been suggested to enhance cognitive function (Rogers 2000) since brain lateralisation may allow an individual to simultaneously focus on two tasks if each is controlled by a different hemisphere (Rogers et al. 2004; Dadda and Bisazza 2006). For example, lateralised parrots perform better than non-lateralised individuals when solving foraging problems

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(Magat and Brown 2009). At the population level, if individuals are lateralised in the same direction this could help them evade predators when they act in a coordinated manner and turn in the same direction as a group, as seen in sheep herds (Versace et al. 2007) or fish shoals (Lopes et al. 2016). However, a tendency for everyone in a population to share the same direction of lateralisation also makes their actions more predictable, which might allow predators to more readily capture prey when they encounter isolated individuals (Vallortigara and Rogers 2005; Dadda et al. 2009; Chivers et al. 2017). In humans, handedness affects success in interactive, competitive sports (e.g. tennis, boxing): left-handers, being rarer in the population, have a less familiar style of play to their opponents, providing a competitive advantage (Loffing et al. 2012; Malagoli Lanzoni et al. 2019). Similarly, a recent study on cuttlefish suggested that males with left-eye dominance, which is more common, have lower fighting success than males with right-eye dominance (Schnell et al. 2019). The extent to which each individual in a population shows lateralised behaviour, alongside its direction, can, therefore, affect the outcome of interactions both within and between species.

In fishes, research on behavioural lateralisation has a long history (Aronson and Clark 1952), and it has been causally linked to eye dominance, with one eye specialized to detect certain types of stimulus, such as predators or mates (Bisazza et al. 1997a), leading to lateralisation in how stimuli are approached. Since behavioural lateralisation is likely to affect fitness, several studies have examined how biotic and abiotic factors shape its variation (e.g. Domenici et al. 2012; Lopes et al. 2016; Maulvault et al. 2018). Collectively this work has demonstrated differences in behavioural lateralisation across species (Bisazza et al. 2000a, b), between the sexes (Bisazza et al. 1998; Reddon and Hurd 2009), and even among populations (Brown et al. 2004). Behavioural lateralisation has also been shown to vary with: visual experiences when young (Brown et al. 2007; Dadda and Bisazza 2012), exposure to stressful conditions (e.g. ocean acidification; Domenici et al. 2012; Lopes et al. 2016; Maulvault et al. 2018; but see Clark et al. 2020), and genotype (Bisazza et al. 2000a, b). This variation is partly attributable to the relative importance of behavioural lateralisation in determining fitness when shoaling (Bibost and Brown 2013; Chivers et al. 2016), reorientating (Sovrano et al. 2005), performing cognitive tasks (Bibost and Brown 2014; Lucon-Xiccato and Bisazza 2017; Gatto et al. 2019), or avoiding predators versus catching prey (Cantalupo et al. 1995; Brown 2005; Takeuchi et al. 2012).

Detour tests are a staple method in animal cognition studies (Kabadayi et al. 2017). They are, for example, used to quantify behavioural lateralisation in dogs (Pongrácz et al. 2001), horses (Rørvang et al. 2015), mice (Juszczak and Miller 2016), birds (Regolin et al. 1995; Vallortigara 1999), lizards (Csermely et al. 2010), and frogs (Munteanu et al. 2016). A variation of a detour test, a double-sided T-maze, was developed by Bisazza et al. (1997a, b) to study lateralisation in fishes. The testing apparatus consists of two identical chambers connected by a narrow corridor. A transparent or partially see-through barrier is placed at each end of the corridor, and a "stimulus" is placed behind each barrier. The focal individual then has to move around the barrier from either the left or right to approach the stimulus. Each individual's relative lateralisation index (L_R) is calculated based on a pre-determined number of turning decisions, typically 10. Due to its simple design and cheapness, the standard detour test has become the foundation of behavioural studies to quantify behavioural lateralisation and link it to factors that affect its variation among individuals. This implicitly assumes that L_R is repeatable, but Roche et al. (2020) recently reported nonrepeatable lateralisation in five fish species tested with a neutral stimulus in a double-sided T-maze.

Here we investigated the repeatability of behavioural lateralisation in female eastern mosquitofish (*Gambusia holbrooki*) using a detour test (double-sided T-maze). Our first aim was to quantify the repeatability of L_R. To increase statistical power, we recorded more turns per individual than the standard 10, rather than testing more individuals. Statistical confidence in the extent to which each individual is lateralised increases with the number of observations *per individual*. Our second aim was to test whether two key design elements of the T-maze affect repeatability: (1) stimulus type: no conspecifics (neutral), female conspecifics (social stimulus), or male conspecifics (could be perceived as either a social stimulus, or as a negative stimulus due to the harassment cost males impose on females in *G. holbrooki*; Agrillo et al. 2006) and (2) movement (*forced:* individual "forced" into a turning decisions by being herded with a net; *roaming:* individual was free to swim around the test apparatus and approach the stimuli). We, therefore, had a 3 × 2 experimental design. Each focal individual was tested in all six treatments on three separate days in a randomised block design.

Roche et al. (2020) recently reported no detectable repeatability of lateralisation in four species of fish when tested with a neutral stimulus and forced movement. We, therefore, predicted that *G. holbrooki* would show no, or low, repeatability of behavioural lateralisation when presented with a neutral stimulus, but higher repeatability with social stimuli which are more likely to affect fitness in the wild (Agrillo et al. 2006; Ward 2012). We further hypothesized that fish in "forced" trials would more often exhibit lateralisation than those in "roaming" trials, because the investigator might introduce an unintentional directional bias. Finally, we examined the sequence of turns made by each individual to determine if the direction of a turn predicts that of the next turn. This could be due to individual lateralisation (i.e. repeatedly turning left or right), a tendency to move along the same corridor wall (i.e. to alternate left and right turns in a double-sided T-maze), or short-term memorization of the maze (i.e. consistently using the same path in a maze).

Methods

Origin and maintenance of fish

We randomly selected 36 adult female *G. holbrooki* from laboratory stocks of fish that had previously been collected in Canberra, Australia and housed in single-sex groups in 90L aquaria (< 50 individuals per tank). The 36 focal females were transferred to individual 1-L tanks for the purposes of identification. We also randomly selected 16 adult males and 16 adult females for us as stimulus fish in the experiment. These individuals were held in larger 7.5-L tanks in same-sex groups of four fish. Water temperature in all tanks was 27 °C (± 1 °C). Fish were housed under a light/dark day cycle of 14/10 h, and fed brine shrimp twice daily.

Experimental setup

Behavioural experiments were conducted in six aquaria (60 × 42 × 40 cm high). The detour apparatus was similar to that of Bisazza et al. 1997a, b, but with slightly different dimensions (Fig. 1). Each tank had a pair of transparent plastic containers to house stimuli fish (no chemical exchange was possible between the container and the main tank), a pair of transparent plastic barriers that the focal fish had to swim around, and white PVC plastic walls that separate the tank into two chambers with a narrow corridor between them. The tank walls were covered with white corflute to prevent external disturbance. The water was changed daily and filled to a depth of 10 cm. LED strip lighting was installed directly above and parallel to the corridor. This ensured that the tank was lit symmetrically with respect to movement around the barriers. A single CCTV camera (5.0 megapixel, model: NCDOTIR21) was placed above each tank to record fish behaviour. Six experimental tanks were used, and we ensured that each tank was perfectly symmetrical with respect to swimming right or left around a barrier to approach the stimulus.

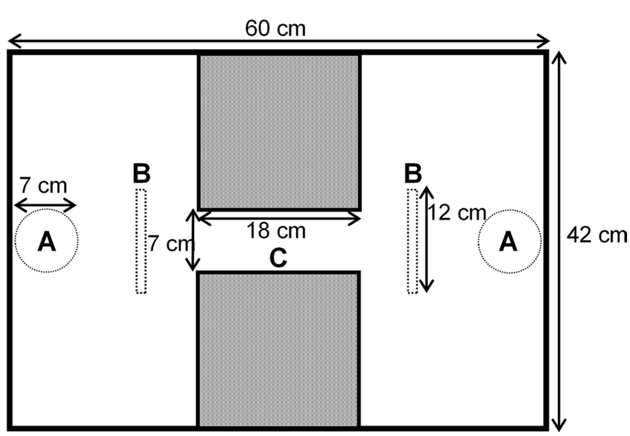

Fig. 1 Diagram of a detour test apparatus inside a glass tank (60 × 42x40 cm). The apparatus comprises a pair of transparent plastic cylinders housing stimulus fish, $d = 7$ cm, $h = 15$ cm (**a**); a pair of transparent plastic barriers 12 × 15 cm (**b**); and a corridor connecting the two ends of the tank(**c**)

Experimental procedure

All trials were conducted between 07.00 and 15:00 h. Focal fish were fed 30–50 min before the trial, as hunger levels affect behaviour in fish (Hansen et al. 2015). Each female was tested with each of the three stimuli with 72 h between trials. This procedure was repeated twice until each female had conducted three trials with each stimulus. Females were split into six blocks of six females. Females in each block were presented with the three stimuli in the same unique order in each replicate. There are six possible orders (3!) so the design was perfectly balanced.

To start the experiment, we prepared each stimulus container: empty for the neutral stimulus and four conspecifics for female and male stimulus groups. We then added a focal female to a randomly selected side of the tank and videoed her movements for 3 h. The first 10 min of each recording was an acclimation period and excluded from our analyses. Immediately following the "roaming" trial, we conducted a "forced" trial where we repeatedly chased the focal female with a handheld net into the corridor so that she had to choose to go around the barrier (see Roche et al. 2020). "Forced" trials were recorded for 5 min, during which at least 10 turning decisions were made per female. The experimenter was always positioned on the same side of the tank, perpendicular to the runway to minimize any side bias. All test fish were then returned to their individual tank.

Turning decisions were recorded from the video footage, with all data collected by I.V. We defined a turning decision as occurring when the female crossed the perpendicular line joining the end of the barrier to the end of the corridor, either on the left or right side. After re-entering the corridor, the focal female had to reach half-way along its length for her next turn to be recorded. We also recorded the side of the tank where the turning decision was made. Trials where individuals made fewer than 10 turns were excluded from the analysis.

Statistical analyses

Relative lateralisation indices (L_R) were calculated for each female for each trial as follows: L_R = (Right turns − Left turns) / (Right turns + Left turns) × 100. A score of 100 indicates that she always turned right, and a score of -100 that she always turned left. A score of 0 indicates no lateralisation. To determine the consistency of turning behaviours over time, we calculated the repeatability (R) of L_R using the 'rptR'package in R v. 3.5.2 (Stoffel et al. 2017), which compares variance within individuals to variance among individuals. Repeatability was calculated separately for each of the six treatments (neutral, female, and male stimuli in forced and roaming trials). We then tested for population-level lateralisation using a generalised linear random-effects model with a binomial error distribution with turn direction (left = 0, right = 1) as the dependent variable and fish ID as a random factor (as proposed by Roche et al. 2020). A separate model was run for each treatment in each replicate trial. The model intercept indicates the average turning direction in the population (i.e. 0.5 indicates no population level bias), and the associated *Z* statistic and *P* value were computed using the 'GLRE' function.

To test for lateralisation of individuals, we ran a chi-square goodness of fit test, assuming an equal propensity to turn left or right, for each trial for the six treatments. We then summed the chi square values for each individual and tested if it deviated significantly from the expected value (df = number of individuals − 1). It should be noted that a significant result can arise even if only one individual is lateralised if its deviation from 50:50 is extreme. We, therefore, additionally examined individual chi-squared values (df = 1) to determine how many individuals per trial were significantly lateralised: by chance we expect 1 in 20 when alpha is set at 0.05.

To test for the effect of stimulus, movement type, and the direction of the previous turn on the direction in which a fish turned we took a conditional probability approach. We ran a generalized linear mixed-effects model with stimulus group (neutral, male, or female), movement type (forced or roaming), 'previous turn direction', tank end, presentation order of stimuli (6 levels) and replicate (first to third) as fixed factors, and fish ID as a random factor. We also included fish body size as a fixed covariate. We then computed estimated marginal means for these factors to compare the strength of their effect, while taking into account that the number of observations varied among treatments as the number of turns per trial was free to vary. To test the generality of the effect of 'previous turn direction' on subsequent turning behaviour (see Results), we ran an analysis on datasets of turning in an I-maze generously provided by Roche et al. (2020) for four fish species (*Ctenolabrus rupestris, Neopomacentrus azysron, Pomacentrus amboinensis, Danio rerio*). For each species we ran a generalised linear mixed-effects model similar to that described above, to test for an effect of previous turn direction.

Unless otherwise stated, summary statistics are presented as mean ± s.d. The alpha value is set at 0.05 and tests are two-tailed unless otherwise specified.

Results

The number of turning decisions made by female *G. holbrooki* in the roaming trials ranged from 10 to 134 (48.8 ± 25.5) with the neutral stimulus, 10 to 92 (39.2 ± 19.8) with the female stimulus, and 6 to 132 (39.5 ± 22.6) with the male stimulus. The number of turning decisions made by female in forced trials ranged from 10 to 57 (27.3 ± 6.5) with the neutral stimulus, from 11 to 37 (25.4 ± 5.9) with the female stimulus, and from 10 to 70 (26.7 ± 8.0) with the male stimulus.

The lateralisation index of individuals (i.e. L_R) was significantly repeatable in five of the six treatments (Table 1). Only 'roaming' females presented with a male stimulus showed no significant repeatability of L_R. Repeatability was higher in forced than roaming trials (Fig. 2).

Based on the pooled data from the three replicate trials and six treatments, the variance among individuals ($\sigma^2 = 0.216$) was greater than that within individuals ($\sigma^2 = 0.098$).

Using the full data set on each turn made, the population level mean L_R values did not differ from zero in any of the 18 treatment-trial combinations (all $P > 0.17$, Table 2). There was no population level tendency for fish to show a shared bias to turn in one direction. There was, however, evidence for significant lateralisation of one or more individuals for 17 of the 18 treatment-trial combinations. On average, the number of individuals that showed a significant turning bias was 9 (range 3–16) of the 29–36 females tested in each

Table 1 Repeatability of lateralisation (LR) of female Gambusia holbrooki in each of six treatments measured using a detour test

Treatment	Repeatability	SE	CI	*P*
Neutral roaming	0.280	0.111	0.059, 0.490	**0.007**
Female roaming	0.390	0.127	0.112, 0.590	**0.001**
Male roaming	0.036	0.081	0, 0.264	0.400
Neutral forced	0.623	0.085	0.432, 0.768	**< 0.001**
Female forced	0.477	0.098	0.27, 0.65	**< 0.001**
Male forced	0.442	0.103	0.211, 0.624	**< 0.001**

The table shows repeatability (R), standard error (SE), confidence interval (CI), and *P* value for each of the following six treatments: neutral, female, and male stimuli, in roaming or forced trials. Statistically significant results are represented with bold text ($P < 0.05$)

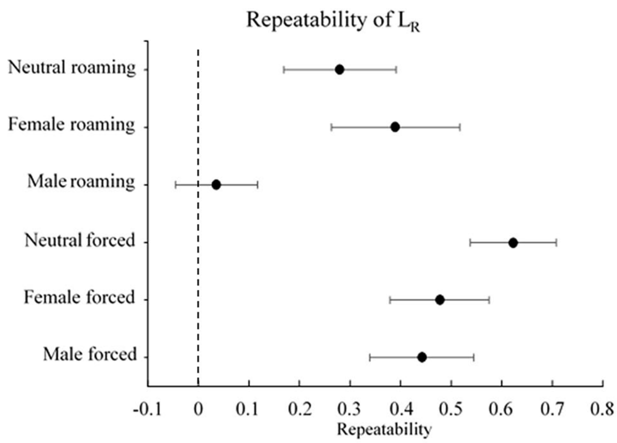

Fig. 2 Repeatability of behavioural lateralization index (L_R) of *Gambusia holbrooki* in a detour test for each of the six treatments. Repeatability is calculated based on the relative lateralization index L_R measured in three tests per individual. Data are presented as means ± standard error (SE). Sample sizes (trials) for each treatment are: neutral-roaming (n = 105), Female-roaming (n = 92), Male-roaming (n = 96), Neutral-forced (n = 105), Female-forced (n = 104), Male-forced (n = 104)

treatment-trial combination (Table 2). This is far higher than the two or fewer cases expected by chance alone. There was no obvious pattern between the number of turns a female made and the deviation of her L_R value from 0 (Figs. 3, 4). This suggests that significant lateralisation is not attributable to greater sampling error when examining females that made fewer turns.

There was a significant effect of "previous turn" on the direction of the subsequent turn ($\chi2 = 1257.3$, $P < 0.0001$) in all six treatments (Table S1, Supplementary Material). The estimated probability of turning to the right, given a previous left turn ranged from 0.6 to 0.7 (odds ratio = 3.06), which was significantly greater than the expected 0.5 ($P = 0.001$). This finding is consistent with a fish moving back and forth along the same wall of the corridor when swimming between stimuli at opposite ends of the tank. The movement factor (forced versus roaming trials) also had a significant effect on the turning direction ($\chi2 = 15.6$, $P = 0.0001$): individuals in roaming trials were significantly more likely to turn left, but the effect size was very small (odds ratio = 1.13).

Stimulus type had no detectable effect on the mean turning direction ($\chi2 = 1.6695$, $P = 0.4340$). There was also no significant effect of fish size, replication number, or stimulus presentation order on the turning direction (Table S1, Supplementary Material).

We found a significant effect of previous turn on subsequent turning direction for three of the four species examined by Roche et al. (2020): *Ctenolabrus rupestris* ($\chi2 = 93.836$, $P < 0.001$), *Neopomacentrus* azysron ($\chi2 = 71.197$,

Table 2 Measures of individual-level and population-level lateralisation in female *Gambusia holbrooki* obtained from detour tests in six treatments

Group	Trial	# lateralised individuals	Σ $\chi2$	Ind. P	Mean L_R	*Z* score	Pop P
Neutral stimulus roaming	1	4/32	69.55	**< 0.001**	− 1.77	− 0.60	0.548
	2	3/35	39.47	0.239	− 3.44	− 1.01	0.313
	3	8/36	78.75	**< 0.001**	− 3.14	− 0.79	0.425
Female stimulus roaming	1	6/25	52.52	**< 0.001**	4.48	1.345	0.179
	2	7/31	80.59	**< 0.001**	− 5.88	− 1.36	0.175
	3	6/30	70.14	**< 0.001**	− 5.33	− 1.07	0.283
Male stimulus roaming	1	5/29	45.85	**0.018**	4.50	1.134	0.257
	2	5/30	55.29	**0.002**	− 3.49	− 0.66	0.506
	3	5/32	55.83	**0.004**	1.03	0.37	0.710
Neutral stimulus forced	1	16/35	277.90	**< 0.001**	1.42	0.33	0.740
	2	7/33	103.19	**< 0.001**	− 1.36	− 0.44	0.659
	3	16/36	240.38	**< 0.001**	5.42	0.41	0.684
Female stimulus forced	1	12/33	220.00	**< 0.001**	9.65	1.06	0.289
	2	16/36	237.95	**< 0.001**	− 3.37	− 0.29	0.772
	3	9/35	123.74	**< 0.001**	− 0.31	0.11	0.915
Male stimulus forced	1	13/36	180.81	**< 0.001**	6.23	0.78	0.436
	2	10/34	138.67	**< 0.001**	− 2.61	− 0.53	0.598
	3	16/33	225.01	**< 0.001**	− 6.13	− 1.02	0.307

Treatments are as follows: neutral, female, and male stimuli in roaming or forced trials. For individual level lateralisation, the number of significantly lateralised individuals out of the total tested, sum of chi-squares for all individuals, and the associated *P* value are shown. For population level lateralisation, the mean relative lateralization (L_R), *Z* score and associated *P* value are shown. Statistically significant results are represented with bold text ($P < 0.05$)

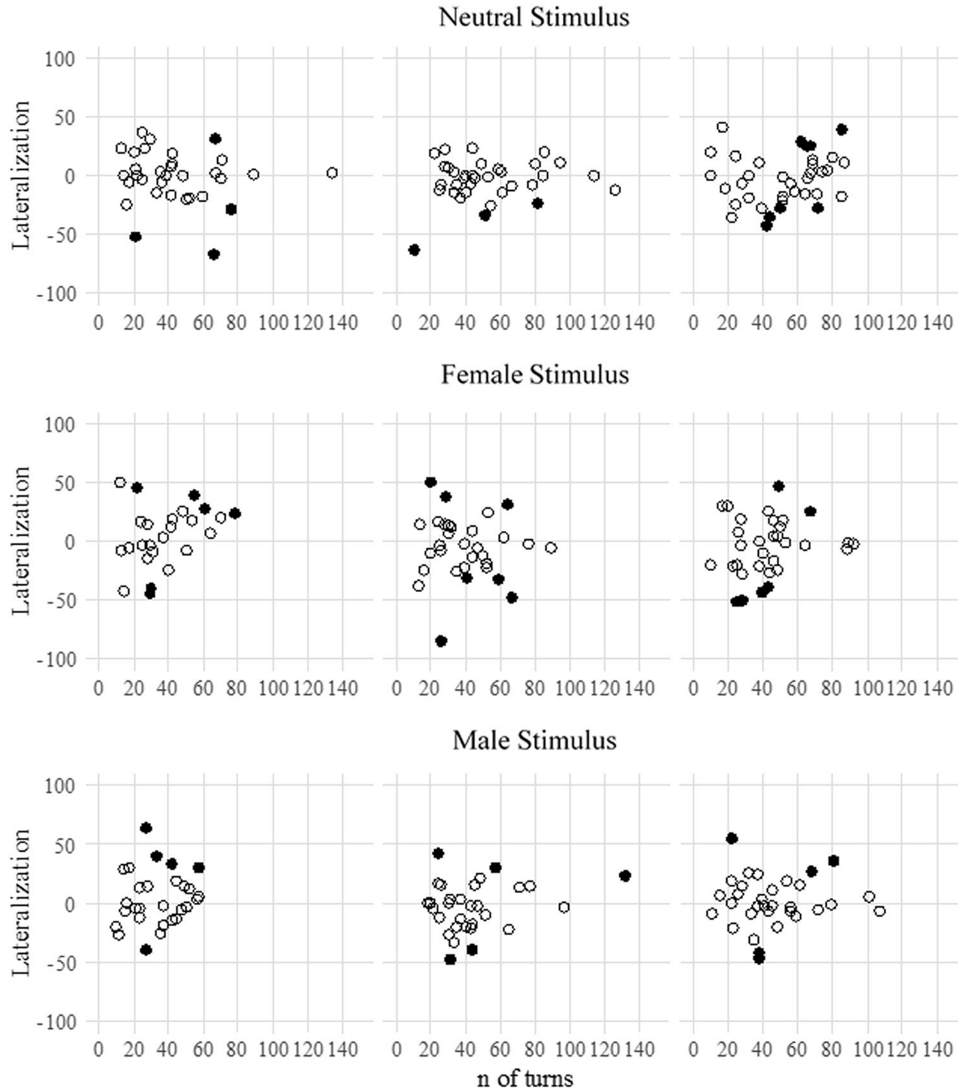

Fig. 3 Relative lateralization *versus* number of turns taken by *Gambusia holbrooki* females in roaming trials in a detour test. Results are shown for neutral, female, and male stimuli groups across three replicates. Lateralization ranges from − 100 (always turn left) to 100 (always turn right). Black circles indicate individuals that are significantly lateralized

$P<0.001$), and *Pomacentrus amboinensis* ($\chi 2=78.469$, $P<0.001$). The probability that an individual fish turned right given that the previous turn was left ranged between 0.57 and 0.63. This is again suggestive of a tendency towards alternating turns. There was, however, no effect of previous turn on subsequent turn direction for zebrafish, *Danio rerio* ($\chi 2=11.192$, $P=0.369$).

Discussion

In our study we revisited the use of the detour test (i.e. double-sided T-maze, or I maze) to measure behavioural lateralisation in fishes. We tested the repeatability of the relative lateralisation (L_R) of female mosquitofish (*G. holbrooki*) presented with three different stimuli (nothing, males or females) under two different conditions (roaming versus forced movement) (i.e. six treatments). We also directly tested for the presence of both individual and population level lateralisation. In five of the six treatments there was significant repeatability of behavioural lateralisation. Although an average of 25% (range 9–48%) of females in each treatment showed a significant turning bias, there was no bias at the population level. This suggests that: (a) the direction of behavioural lateralisation is unaffected by whether the stimulus females were approaching was neutral or social; and (b) there are equal numbers of females with a left and right bias. When we analysed the full dataset and the sequence of turns we found females tend to alternate between left and right turns, which is consistent with fish moving back and forth along the same wall in an I-maze. This alternation effect could either be due to a preference to remain close to the wall structure of the apparatus (wall following) or to short-term memorisation of a particular path through the apparatus. Either way, turn alternation results in the potential to underestimate the true level of laterality

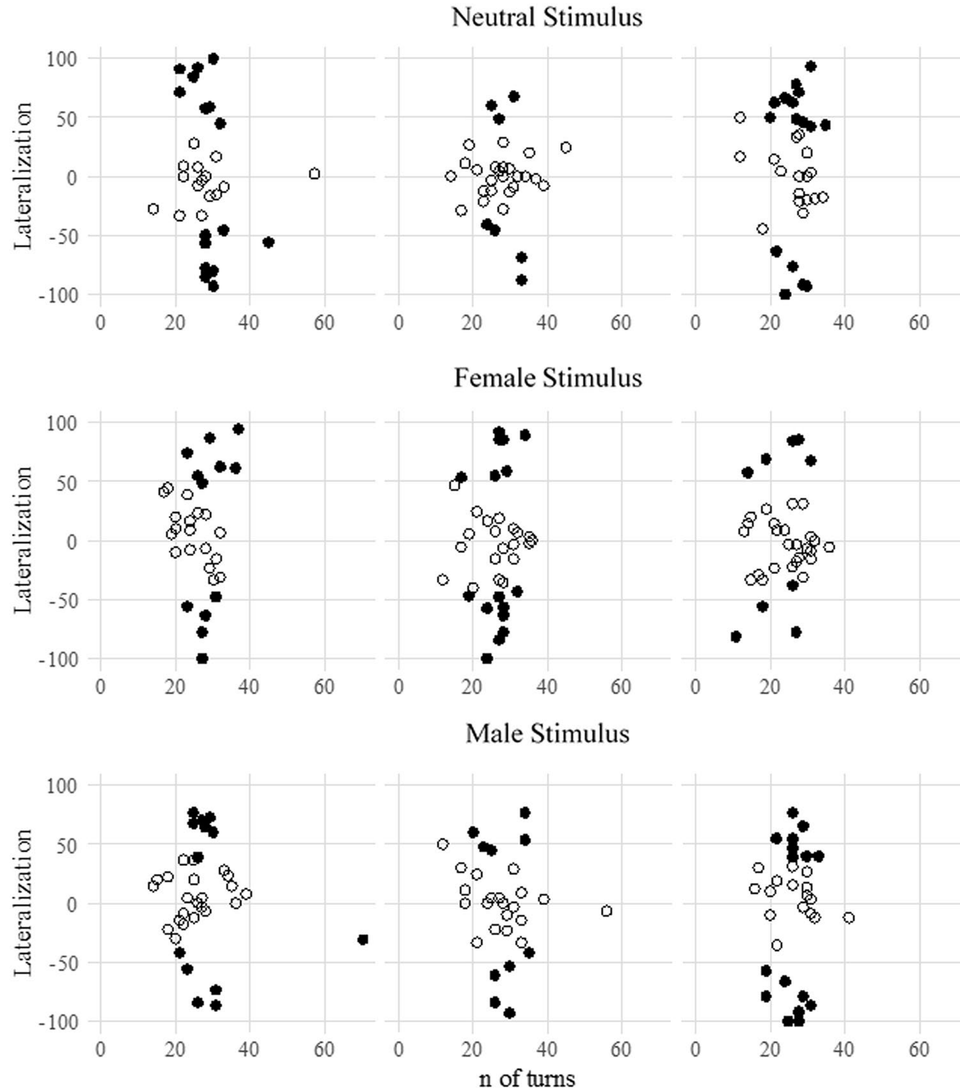

Fig. 4 Relative lateralization *versus* number of turns taken by *Gambusia holbrooki* females in forced trials in a detour test. Results are shown for neutral, female, and male stimuli groups across three replicates. Lateralization ranges from − 100 (always turn left) to 100 (always turn right). Black circles indicate individuals that are significantly lateralized

for any particular individual. Lateralisation is only detectable when fish break a period of wall following and independently choose which way to turn. The evidence that the I-maze set-up induces turn alternation was corroborated by our reanalysis of data from Roche et al. (2020). We found evidence of alternation between left and right turns for three of the four species they tested. In our study, the deliberate methodological decision to record a greater number of turning decision per individual (rather than test more individuals) meant that we had the power to detect repeatability of turning decisions in *G. holbrooki* despite the potential of a 'wall-effect' to mask behavioural lateralisation.

Repeatability of lateralisation

The repeatability of behavioural lateralisation in fishes has recently been called into question by Roche et al. (2020), but surprisingly few studies have attempted to verify the repeatability of laterality. Irving and Brown (2013) reported highly repeatable behavioural lateralisation in guppies, but Roche et al. (2020) concluded that this claim was unsubstantiated if the data was reanalysed using a different approach. In addition, Roche et al. (2020) found no evidence of repeatability of behavioural lateralisation in four fish species. Most recently, McLean and Morrell (2020) found that individual male and female guppies show consistency in their turning bias, although the lateralisation of males was more predictable than that of females. Our finding that behavioural lateralisation in female *G. holbrooki* in detour test is repeatable, therefore, represents an important contribution to this debate. Our positive findings can potentially be explained by the fact that we increased the power of our tests by making more observations per fish to better estimate within individual variation. Most previous studies calculate L_R based on only 10 turns per fish (e.g. Gatto et al. 2019; Roche et al. 2020; Torres-Dowdall et al. 2020), while our L_R estimates were, depending on the treatment, based on an average of 25–49 turns per fish. It is clearly important to establish the repeatability of a lateralisation measure like L_R, prior to the commencement of experiments designed to identify factors that affect lateralisation behaviour (see McLean and Morrell 2020).

We found that the repeatability of behavioural lateralisation was higher when fish were forced to move around the maze (chased with a hand-net), than when they could swim freely before turning. We offer three potential explanations. First, we could have introduced a bias due to the handedness of the experimenter, where hand movements that varied among trials could have pushed some fish in a consistent direction. There was, however, no evidence for a population level bias in forced treatments. Second, chasing a fish with a net might stress it in ways similar to those experienced when being chased by a predator (Stier et al. 2013; Ferrari et al. 2017). Stress is known to amplify cognitive performance (Koolhaas et al. 1999), including behavioural lateralisation (Byrnes et al. 2016), which might increase the repeatability of turning decisions. Third, individuals in roaming trials were excluded from the analyses if they made fewer than 10 turns. This might have reduced the reported variation in turning bias if the excluded fish were a non-random sample of the natural variation in turning bias.

Turn alternation and its relationship to wall following

Our analysis of the sequence of turning decisions made by each individual showed that fish tended to alternate between left and right turns. It is worth noting that in an I-maze, an individual must enter the corridor between each turn. If an individual consistently alternates between left and right turns it could simply reflect a preference to stay close to a wall. Wall following is a well-known behaviour in rodents (Simon et al. 1994), amphibians (Hänzi and Straka 2018), cavefish (Patton et al. 2010), and some invertebrates (Creed and Miller 1990; Basil and Sandeman 2000). We speculate that wall following in fish might arise from a preference for structured environments (Kistler et al. 2011; Davis and Smith 2017) or shadows (Maximino et al. 2010). A second potential explanation for the observed sequences of alternating left–right turns by individuals involves short-term memorization of the maze, where fish randomly choose an initial path and then simply repeat this pathway when moving between the two chambers of the tank. We consider this explanation unlikely, however, given that our model predicts individuals have a $>60\%$ probability ($P<0.05$ compared to 50%) of making a turn in the opposite direction to their previous one (compared to random choice between left and right second turns on which the short-term memorisation hypothesis is based).

The effect of wall following behaviour is to decrease the precision of measurements of lateralisation since the mean L_R of an individual expressing this behaviour is zero, i.e. no bias. Lateralisation can, therefore only be detected when an individual breaks a spell of wall following and independently chooses its turning direction. Given a strong wall-effect in our study, why did we still find significant repeatability of behavioural lateralisation in *G. holbrooki*? First, it is possible that some individuals stay closer to the walls (i.e. wall following), while others do not and show behavioural lateralisation. Second, certain individuals may switch between wall following and exhibiting a turning bias. In the full data set we had a large number of observations per individual, and controlled for previous turn direction (via a lag effect term), still giving us the statistical power to detect significantly lateralised individuals based on the 'subset' of case where they expressed their turning bias. Even so, the proportion of lateralised individuals might be higher than reported based on L_R, because this index does not correct for the wall-effect. To our knowledge, ours is the first analysis to present evidence for wall following in an I-maze, both in *G. holbrooki* and three other species studied by Roche et al. (2020). Further experiments should now be conducted to investigate the basis of this alternation effect, the extent to which it can be explained by wall following, and potential triggers of wall following in mosquitofish. Future studies should strive to eliminate the wall-effect to obtain better measures of lateralisation. We predict that moving through a very narrow gate before entering an intersection (such as when leaving the central tunnel in an I-maze) will put a focal fish in a position where it is equally close to both left and right walls, thus "resetting" the wall following.

The effect of stimulus type

Neither the extent of individual lateralisation nor the mean of the population differed significantly when fish were presented with a neutral, female, or male stimulus. This suggests that the presence of conspecifics did not elicit strong behavioural lateralisation in *G. holbrooki* females. A recent study of male and female guppies (*Poecilia reticulata)* similarly found no population level bias in response to either a neutral or an opposite sex stimulus (McLean and Morrell 2020). Previous studies suggested, however, that laterality is often stimulus-specific in fish, including *G. holbrooki* (e.g. Bisazza et al. 1997a; Sovrano 2004; Dale Broder and Angeloni 2014; Ferrari et al. 2017; Fuss et al. 2019). Notably, predators elicit the strongest lateralisation behaviours; and predator-specific lateralisation also occurs in other taxa, including amphibians (Lucon-Xiccato et al. 2017) and reptiles (Robins et al. 2005). It is possible that the conspecific stimuli we used were insufficient to elicit a behavioural response, or that behavioural lateralisation diminished as fish habituated to the stimulus (Nepomnyashchikh and Izvekov 2006; Blois-Heulin et al. 2012). Although we initially expected a group of male conspecifics to act as a negative stimulus due to the harassment costs that impose on female mosquitofish, male conspecifics may instead be perceived as a social group (similarly to a female stimulus), especially when males cannot directly harass a focal female. Previous studies on *G. holbrooki* using the detour test have demonstrated a left turning bias with a female or predator stimulus (Bisazza et al.1997b, 1998), clockwise bias in a circular arena test (Bisazza and Vallortigara 1997), and right eye usage (i.e. turning left) in aggressive responses to a mirror image (Bisazza and de Santi 2003). The discrepancy between these findings and ours could be due an inflated type I error rate in previous studies that did not account for consecutive trials being non-independent, or that applied inappropriate goodness-of-fit tests to small samples (Roche et al. 2020). And, of course, fine-scale aspects of our experimental apparatus (e.g. tank size, barrier type) might have reduced the degree of stimulus-specific behavioural lateralisation, although it is hard to say why this would be the case.

Conclusion

In sum, we provide robust evidence that behavioural lateralisation is significantly repeatable in female eastern mosquitofish, *G. holbrooki*. We also highlight the potential for wall following behaviour in detour tests, which has not been accounted for in previous studies. It has the potential to mask the true strength of lateralisation. Our results also suggest that forcing fish to move around the tank might increase behavioural lateralisation and the repeatability of measures. It is, however, unclear whether this is due to experimenter bias or enhanced lateralisation. We suggest that greater consideration be given to the effects of different stimuli, how fish are allowed to choose, and ways to reduce any effect of wall following on lateralisation measures.

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Data availability The datasets and R code generated during the current study are available in supplementary materials. Video footage of the experimental procedure is available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval All experimental procedures were carried out under approval from ANU Animal Ethics Committee (Approval #A2018/27) and complied with existing laws regulating the treatment of vertebrates in Australia**.** The collection of animals was conducted under a Scientific License from the Australian Capital Territory (ACT) Government, granted under Section 21 of the Fisheries Act 2000, license number FS20188.

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