Sex differences in laterality are associated with reproduction in three-spine stickleback

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Figure S1: Diagrammatic representation of experimental methodology (detour test)

Table S1: The effects of sex, stage of reproduction and their interaction on a) L_R and b) L_A

Abstract

Laterality, the partitioning of information processing into specific brain hemispheres, is widespread across animal taxa. Substantial unexplained variation in this trait exists, particularly between the sexes, despite multiple identified advantages of lateralisation. Here, we demonstrate a relationship between laterality (measured as directional biases), reproduction and experience of mating and parenting. Using three-spine sticklebacks *Gasterosteus aculeatus*, a species with uniparental male care, we showed that individuals of the caring sex (males) were more strongly lateralised than the non-caring sex (females) during reproduction, and that laterality was reduced outside the breeding season in males. Additionally, males with experience of mating and parenting were more strongly lateralised than males without this experience. Our findings suggest that fitness related behaviours that vary between the sexes, such as reproductive behaviours including courtship, spawning and parenting, are significant but previously unidentified sources of variation in laterality.

Introduction

Cerebral lateralisation or 'laterality', the partitioning of cognitive functions into specific brain hemispheres, was originally thought to be a trait unique to humans due to its tight association with complex brain functions (Bisazza et al, 1998; Corballis, 2012). However, laterality is now recognised as a ubiquitous trait, exhibited by vertebrate (Walker, 1980; Güntürkün, 1997; Bisazza et al, 1998) and invertebrate taxa (Frasnelli et al, 2012; Niven & Bell, 2018) that is often observed at the behavioural level as preferential use of one side of a bilateral characteristic (Koboroff et al, 2008; Brown & Magat, 2011) or as side biases in behaviours (Bisazza et al, 2001). Such biases stem from cerebral lateralisation and are an indicator of the degree of lateralisation in an individual's brain (Vallortigara & Rogers, 2005; Reddon et al, 2009; Gutiérrez-Ibáñez et al, 2011; Jozet-Alves et al, 2012).

Several advantages of cerebral lateralisation have been identified, including an increased neural capacity (Pascual et al, 2004; Magat & Brown, 2009), enhanced ability to multi-task (Dadda & Bisazza, 2006a; 2006b), greater spatial and numerical discrimination abilities (Bisazza & Dadda, 2005; Sovrano et al, 2005; Dadda et al, 2015) and improved performance when using the preferred side in fitness related behaviours (Takeuchi et al, 2012). These advantages are thought to arise from lateralised individuals being better able to cope with divided attention resulting from simultaneous information processing (Rogers et al, 2004). However, lateralisation also has costs, including a reduced efficiency in tasks requiring hemispheric communication and cooperation (Dadda et al, 2009), increased predictability from consistent biases in behaviours (Cantalupo et al, 1995; Takeuchi, 2012) and reduced efficiency in responses to stimuli viewed in the non-preferred visual hemifield (Vallortigara & Rogers, 2005).

Substantial unexplained variation in laterality persists both within and between

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species, especially at the individual level (Bisazza et al, 1997). Sex is an increasingly recognised source of variation in laterality, with males and females often differing in the patterns of laterality they exhibit (*Lemur* spp, Milliken et al, 1991; *Gallus gallus domesticus*, Vallortigara & Andrew, 1991; *Archocentrus nigrofasciatus*, Reddon & Hurd, 2008). While in some cases this variation has been attributed to prenatal steroid hormones (Schaafsma & Groothuis, 2011) and intrinsic traits such as boldness (Irving & Brown, 2013) and aggression (Reddon & Hurd, 2008), the underlying drivers of sex-specific variation in laterality remain poorly understood.

The influence of reproductive experience on both brain and behaviour is well documented (Franssen et al, 2011; Reichert et al, 2012; Royle et al, 2012; Stein et al, 2016), yet it has rarely been considered in the context of laterality, which is surprising given it can be highly variable between the sexes. Some reproductive behaviours are lateralised including courtship *(*Ventolini et al, 2005*;* Vidal et al, 2018) and male mate choice (Templeton et al, 2012), but how reproductive experience could influence laterality is largely unknown. The performance of reproductive behaviours could select for different patterns of cerebral lateralisation in individuals or between the sexes, especially in cases where these behaviours are sex-specific. Courtship behaviours, for example, often involve auditory, visual and chemical signals and include, dance, posture and/or calls (Bastock, 1967), whereby individuals could have enhanced performance when these signals are processed in a specific brain hemisphere. Similarly parental care is a key reproductive behaviour in many taxa that is extremely diverse both between species and sexes (Royle et al, 2012). Caring parents must perform multiple functions simultaneously including, for example, predator avoidance or foraging while concurrently caring for offspring (Royle et al, 2012). The cognitive advantages of lateralisation, particularly an enhanced ability to perform simultaneous information processing (Dadda & Bisazza, 2006a; 2006b) could mitigate the cognitive costs of reproduction, ultimately driving variation in laterality that results from selection for different

patterns of cerebral lateralisation between sexes or between individuals with differing experience of reproduction due to the potential fitness benefits that could be obtained.

Here, we test the hypotheses that variation in laterality is driven by reproduction and the performance of reproductive behaviours. Specifically, we examine whether in a uniparental species i) the caring sex is more strongly lateralised than the non-caring sex, and ii) whether laterality is linked to the performance of reproductive behaviours including nest construction and courting, mating and parenting. Three-spine sticklebacks, *Gasterosteus aculeatus,* are a model system to examine these questions as they perform uniparental male care with a number of distinctive stages to the reproductive cycle, including territory defence, construction of a nest, attraction of a mate and care for the offspring pre and post hatching (Tinbergen, 1952). We predict that males should exhibit stronger laterality than females, reproductive males should be more strongly lateralised than non-reproductive males, and males that have experience of performing reproductive behaviours, including nest construction and courting, spawning and parenting should be more strongly lateralised than males absent in this experience due to the cognitive advantages associated with cerebral lateralisation.

Materials and methods

Animals

153 adult *G.aculeatus* were purchased from CarpCo in February 2019, sourced from naturally breeding populations in clay-based ponds filled from the River Bourne in Hadlow, Kent, UK. The fish were held in mixed sex groups in two large stock tanks (75 x 75 x 40 cm) provided with enrichment (gravel substrate, plastic plants and shelter) and maintained on aerated fresh water under temperature and lighting conditions that encouraged reproductive development

(17 ± 1° C on a 16:8 hour light:dark photoperiod). Once individuals were in a reproductive state, identifiable by male nuptial colouration (an orange/red throat and blue eyes; Hiermes et al, 2016), the sexes were isolated into two single-sex stock tanks (75 x 75 x 40 cm); 69 males, 84 females) for one week prior to behavioural trials. All individuals were fed to excess three times daily frozen bloodworm (chironomid larvae) and *Daphnia* sp.

Males and females were assessed for behavioural laterality (see below) at multiple stages during a reproductive cycle outlined in Figure 1:

Stage i.   In a *reproductive state*, but before any reproductive behaviours were performed [males and females]

Stage ii.   After performing *pre-spawning* reproductive behaviours, but before courting and spawning [males only]

Stage iii.   After performing *post-spawning* reproductive behaviours including courting and spawning [males and females]

Stage iv.   In a *non-reproductive state* after a reproductive cycle [males and females]

Stages of reproduction

Stage i. Reproductive state

83 *G.aculeatus* (50 males and 33 females) were haphazardly selected from the stock population and initially assessed for laterality (see below) once they were in a reproductive state, males showing nuptial colouration and females showing evidence of being gravid (Figure 1i), but before any reproductive behaviours had been performed. Reproductive males were then assigned to one of two treatment groups, 'breeding' or 'non-breeding' males, using a random number generator, but ensuring an equal distribution across treatments (n per treatment=24). The breeding treatment represents males performing reproductive behaviours including nest construction, courtship, spawning and parenting (Kynard, 1978), while non-breeding males are a control for males performing such behaviours (Figure 1).

Stage ii. Pre-spawning

Breeding males were transferred to individual nesting aquaria (n=24) comprising a plastic tank (45 x 30 x 15 cm) with gravel substrate, a plastic plant, and nesting materials: a small plastic dish (18cm diameter) filled with sand and 200 x 6cm long black polyester threads. Sticklebacks readily use polyester threads as nesting materials in laboratory studies since they resemble unicellular algae and wild vegetation (Barber et al, 2001; Johannesen et al, 2012). To encourage nest construction each breeding male was provided with visual access to a different heavily gravid female (confined to a small area 28 x 14 x 14 cm of the nesting aquaria by a clear, perforated plastic divider), for 30 minutes, twice daily, until a nest with a visible entrance, representative of completion (Van Iersel, 1953), was present (~1 day). Breeding males were then measured for laterality (Figure 1ii).

Non-breeding (control) males were transferred to individual nesting aquaria (n=24) identical to those of breeding males but absent of nesting materials. Control males were also given visual access to a different heavily gravid female for 30 minutes twice daily for one day

(average no. of days to complete nest construction by breeding males) thus, the only difference between treatments is nest construction. Following visual exposure to gravid females non-breeding males were measured for laterality (Figure 1ii).

Stage iii. Post-spawning

Breeding males with a constructed nest were then given access to the heavily gravid female, which they were previously exposed during nest building (n=24). Once females had successfully laid eggs and males had spawned, females were isolated and measured for laterality within 24 hours (Figure 1iii). Males were allowed to perform parental care behaviours (territory defence, nest maintenance and egg fanning and guarding) until free-swimming larvae were observed, before being measured for laterality (Figure 1iii).

Non-breeding control males were provided with nest materials (a small 18cm diameter plastic dish filled with sand and 200 x 6cm long black polyester threads) and visual access to a gravid female to encourage nest construction. Once nest construction was complete control males were measured for laterality (Figure 1iii). Thus, our experimental treatment differed from our control treatment in mating (courting and spawning with a female) and parenting experience. We recognise that our experiment does not separate mating and parenting experience. However, disentangling the two effects would have involved i) experimental destruction and removal of the nests and fertilised eggs of control males and ii) removal of breeding males from their breeding territory after spawning to perform measurements of laterality, both of which could influence the subsequent behaviour of these individuals.

Stage iv. Non-reproductive state

Following a reproductive cycle, breeding and non-breeding males were individually housed within a large stock tank (75 x 75 x 40 cm) to allow for individual identification, while a stock population of females were kept separately from males but in a group to ensure they could be sexed in a non-reproductive state. Temperature and photoperiod were adjusted over a period of 7 days to be representative of UK winter conditions when this species is not reproductive (10° ± 1°C on a 12:12 hour light:dark cycle and fed once daily ad libitum). Fish were maintained under winter conditions for six weeks, by which time males and females showed no signs of nuptial colouration and egg production respectively, before both sexes (males: n=48, females; n=32) were measured for laterality (Figure 1iv).

Assessing behavioural laterality

Behavioural laterality was measured in a standard detour test (Bisazza et al, 1997; Figure S1), which assesses directional preferences in detour behaviour. The set-up consisted of a large glass tank (90 x 50x 40 cm; water depth 12cm maintained at 17 ± 1° C (reproductive) and 10° ± 1°C (non-reproductive)), lit evenly from above, that contained a central runway joining two t-shaped compartments (Figure S1). Each compartment could be partially obscured by a barrier (10x16cm) made of plastic cylindrical bars (0.25cm diameter) placed 0.25cm apart, designed to hinder but not eliminate viewing of a stimulus placed behind the barrier, here a shelter consisting of half a plant pot (7.5cm diameter) placed behind an artificial plant (13cm tall), since three-spine sticklebacks often seek refuge before performing ecologically relevant behaviours, e.g. foraging, in new environments (Krause et al, 1998). Fish have laterally placed eyes with little frontal overlap, thus each eye receives an independent view of the surrounding environment with slow and incomplete inter-ocular information transfer (McClearly, 1960). As a result information obtained from each eye is sent almost exclusively to the contralateral

brain hemisphere (Irving & Brown, 2013) thus, the direction detoured represents a proxy for preferences in eye use and associated hemispheric processing of the visual stimulus.

An individual fish, captured using a small dip net, was allowed to acclimatise to the empty experimental set-up for three minutes prior to commencing a behavioural trial. The individual was then confined to one of the t-shaped compartments using an opaque plastic door while the barrier and visual stimulus were placed in the alternative t-shaped compartment at the opposite end of the runway. Assessment of laterality commenced when the door was lifted allowing the individual access to the runway. Each individual was given 30 seconds to independently approach the runway after which they were gently encouraged from behind with a small dip net. Fish then swam down the runway towards the barrier forcing them to detour left or right. For each individual this procedure was repeated for 10 consecutive detours (1 behavioural trial), on alternating ends of the runway to account for any asymmetry in the set-up, and the direction detoured was recorded. Water changes were conducted between trials since changes in temperature and dissolved oxygen levels are known to affect behavioural laterality (Domenici et al, 2014).

For each individual at each reproductive stage, a relative lateralisation index (L_R) was calculated using the formula: [(right detours – left detours) / (right detours + left detours) x 100]. L_R assesses directional biases of fish at the population level and ranges from -100 to +100, representing a population that detoured consistently leftward or rightward respectively (Bisazza et al, 1997). An absolute laterality index (L_A) was also calculated to determine the strength of laterality at the individual level. L_A was calculated as $|L_R|$ and ranges from 0 (individuals that turned left and right an equal number of times) to 100 (individuals that turned consistently in one direction; Bisazza et al, 1997). Additionally, we also calculated a

measurement of body size (standard length, cm) for each individual using ImageJ (Schneider et al, 2012).

Data analyses

Data analyses were conducted using R version 3.3.2 (R Core Team, 2019).

Is there evidence for laterality in sticklebacks?

One-sample t-tests were used to determine whether the L_R of male and female populations at each reproductive stage differed significantly from a random expectation of no laterality (0). To test for individual level laterality we examined the sample variance using a chi-squared test that compared the observed variance to the expected variance to determine if more male and female individuals exhibited extreme L_A scores than expected by chance, and thus exhibited significant laterality, based on a normal approximation to the binomial distribution at p=0.5 (Roche et al, 2020; McLean & Morrell, 2020). χ^2 was calculated as $((N - 1) \times var(X1)/(n \times 0.5 \times 0.5)$, where N is the number of individuals, n is number of trials per individual and X1 is the number of right (or left) detours per individual (see Roche et al 2020 supplementary information for a detailed description of the methodology).

Are there sex differences in laterality when reproductive and not?

Next, we used a linear mixed effects model (LMM), fitted using lme4 (Bates et al, 2015) to assess the effects of sex, reproductive stage and their interaction on both L_R and L_A. Only individuals in a reproductive (stage i) and non-reproductive state (stage iv) were included as only these are common states across both sexes. Body size was included as a covariate and

individual ID as a random effect to account for the repeated measures design for males across all reproductive stages and females before and after egg laying. Females in a non-reproductive state (stock population) were assigned a unique ID also included in the model. The model was fitted using a gaussian error distribution following previous work on laterality (Bisazza et al, 1997) and assumptions of normality and homoscedasticity of residuals were assessed using visual inspection of residual-fit plots and Q-Q plots.

Is laterality linked to the performance of reproductive behaviours?

Finally, L_A data were split by sex to examine the effect of reproductive stage, fitted as a categorical predictor variable, and body size within each sex separately using LMMs (Bates et al 2015) including individual ID as a random effect and assuming guassian errors. Assumptions of normality and homoscedasticity of residuals were assessed as before, but for females these assumptions were violated. Consequently, we used a generalised linear model (GLMER) with a binomial error distribution (fitted with lme4; Bates et al, 2015) to assess if the reproductive behaviours performed by females influenced the proportion of turns to the preferred side. The fitted GLMER included individual ID as a random factor and was not over-dispersed (Harrison, 2014). For males we incorporated *a priori* planned comparisons into our LMM to test hypotheses about the performance of reproductive behaviours on laterality (Sokal & Rohlf 1995; Ruxton & Beauchamp, 2008). Specifically, whether there were differences in L_A between males who were reproductive, had performed nest construction and had experience of mating and parenting relative to their respective control treatments.

Results

Is there evidence for laterality in sticklebacks?

There was no evidence for a population-level directional bias in either sex at any stage of reproduction (Table 1a, p>0.05 in all cases). However, at an individual level, significant turning preferences were evident in males (caring sex) but not in females (non-caring sex) across several stages of reproduction (Table 1b). In four of the six stages examined in males (reproductive state, pre-spawning: built nest, pre-spawning: no nest and post-spawning: mating and parenting experience) individuals consistently detoured in a specific direction, regardless of directional preference, more often than expected by chance.

Are there sex differences in laterality when reproductive and not?

There was no effect of body size, sex, stage of reproduction or their interaction on directional biases in laterality at the population level (L_R; Table S1a) however, there was a significant interaction between sex and reproductive state on the strength of laterality (L_A; Sex:Stage interaction: $F_{1, 154} = 5.499$, p =0.020; Table S1b). Males were more strongly lateralised than females when reproductive however, no variation was evident between the sexes in non-reproductive state (Figure 2b).

Is laterality linked to the performance of reproductive behaviours?

In males, the strength of laterality exhibited differed with experience of reproduction (Male L_A LMM stage main effect: $F_{5,183} = 2.551$, p = 0.029; Figure 3a), while in females it did not (Female L_A LMM Stage main effect: $\chi^2 = 0.257$, df = 2, p = 0.879; Figure 3b). Specifically, reproductive males were more strongly lateralised than non-reproductive males (t = 2.540, df = 141, p = 0.012; Figure 3) and males that had experience of mating and parenting (Figure 3 - post spawning behaviours: mated and parented) were more strongly lateralised than males

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who had built nests but had no experience of mating or parenting (t = 1.966, df = 183 , p = 0.050; figure 3 – post spawning behaviours: not mated or parented). However, there were no differences between males who had constructed nests and those who had not (t = -1.039, df = 183, p = 0.300; Figure 3 – pre spawning behaviours: built nest vs. no nest).

Discussion

Our results provide the first evidence of a link between laterality, reproduction and experience of mating and parenting. Individuals of the caring sex (males) were more strongly lateralised than the non-caring sex (females) in a reproductive state, but not outside the breeding season in a non-reproductive state. Furthermore, males that had experience of mating and parenting were more strongly lateralised than males absent in this experience and laterality was reduced outside of the breeding season in males. Together, these findings suggest that the benefits and costs of laterality may be modified by asymmetries in reproductive investment between the sexes and between individuals, which could in turn influence the expression of laterality.

Stronger laterality in reproductive males may be linked to the benefits associated with lateralisation, specifically the performance of simultaneous information processing, which enables 'multitasking' (Rogers et al, 2004; Dadda & Bisazza, 2006a; 2006b). In sticklebacks, the cost, complexity and cognitive demand of reproduction is greater for males than females. Females perform mate choice and invest heavily in egg production, while males invest in several cognitively demanding behaviours including territory defence, nest construction, male-male competition, courtship behaviours and parental care (Bell & Foster, 1994). Multi-tasking would allow for males to simultaneously perform reproductive behaviours including parental care while concurrently performing tasks necessary for survival such as foraging and

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predator avoidance. Consequently, reproductive-breeding males would presumably benefit from a more strongly lateralised brain (expressed as the stronger behavioural laterality seen here; Figure 3a) than either females or non-breeding males.

Lateralisation is associated with costs including a reduced efficiency in tasks requiring inter-hemispheric communication (Rogers, 2000). Female sticklebacks perform mate choice whereby males may present in either visual hemisphere, thus inter-hemispheric communication is likely beneficial to females especially during reproduction (Facchin et al, 1999). As a result, the costs of laterality may outweigh the benefits, explaining the absence of laterality in this sex (Figure 3b). Additionally, three-spine sticklebacks exhibit sexual dimorphism in brain size where males have larger brains, associated with the cognitive demands of reproduction and parental behaviours in this species (Kotrschal et al, 2012; Samuk et al, 2014), than females. Larger brains could allow for greater cerebral lateralisation however, to date only asymmetries in brain structure have been linked to behavioural laterality in fish (Reddon et al, 2009), but correlations between brain size and laterality are evident in mice (Cassels et al, 1990). Furthermore, when individuals are repeatedly tested, learning may occur (Kieffer & Colgan, 1992; Kabadayi et al, 2018), and thus the observed sex difference in laterality could result from sex-specific variation in learning. However, if learning occurred, we would anticipate an increase in L_A over time as individuals learned to detour in a particular direction and for both sexes to show equivalent levels of L_A upon initial testing (when reproductive), neither of which were observed in the current study.

Males absent in mating and parenting experience were less strongly lateralised than males with this experience, and laterality was reduced outside of the reproductive cycle in the caring sex (Figure 3a), indicating that laterality may be a plastic trait. Side biases in behaviours, especially those tightly associated with fitness (Rogers, 2000), could be detrimental for males

when not combined with reproduction. For example, individuals exhibiting consistent side biases may suffer from increased predictability/vulnerability, especially where biases can be learned and/or exploited by predators (Cantalupo et al, 1995; Takeuchi, 2012). Thus, plasticity in behavioural laterality, as a result of changing environmental cues, would allow individuals to maximise their fitness during reproduction.

The observed difference in L_A between males with and without mating and parenting experience could have arisen from a loss of reproductive motivation in non-breeding males however, this is unlikely given all males had visual access to females and males during the entirety of the experiment representative of mating opportunities and male-male competition respectively. Whether or not laterality and motivation are linked and whether this could influence learning in laterality are fruitful avenues for further research. Although we are unable to determine whether the experience of courtship, mating or parenting independently or in combination are responsible for driving stronger lateralisation in breeding males, no differences were observed between breeding and non-breeding males that had and had not performed nest construction respectively, highlighting the influence of mating and performance of post spawning reproductive behaviours on L_A. Future research would benefit from identifying which specific aspect(s) are responsible for driving this variation, but methods adequate to disentangle mating experience from parenting experience that minimise or eliminate effects on subsequent behaviour are necessary.

Despite being an established method of assessing laterality (Bisazza et al, 1997; 1998; Reddon et al, 2009; Domenici et al, 2012), the detour test has recently been criticised (Roche et al 2020), as laterality measured using this technique was found to be variable within individuals under repeated testing in the same conditions. In contrast, McLean & Morrell (2020), using the same test, found that individual female guppies were consistent in both the

direction and strength of laterality, while males were consistent in direction. In the present study we expect low within-individual consistency, particularly as we are measuring individuals with different reproductive experience/in different reproductive states, and thus variation may be expected as these individuals could respond differently to stimuli in the detour test (Roche et al 2020; McLean & Morrell 2020). In addition to the overall changes in laterality observed between the caring and non-caring sex within and outwith the reproductive season, we found that 78% of males had an equivalent or lower L_A when non-reproductive relative to their L_A when reproductive, and 71% of males had an equivalent or increased L_A following mating and parental care experience relative to the same males after nest construction.

The mechanisms driving the observed variation in laterality are unknown, however changes in hormones and gene expression, which influence both the motivation and performance of behaviours simultaneously by regulating and controlling the brain and muscles (Garland et al, 2016) could be influential. Reproductive male sticklebacks exhibit an increase in plasma levels of 11-ketotestosterone, an androgen produced by the testes that is responsible for secondary sexual characters such as nuptial colouration, and nest construction (Borg & Mayer, 1995; Páll et al, 2002). Thus, levels of this androgen are higher in males than females during reproduction (Borg & Mayer, 1995). Postnatal exposure to related hormones, testosterone, have been associated with the expression of laterality in male *Aequidens rivulatus* (Schaafsma & Groothuis, 2011). Thus 11-Ketotestosterone levels could be important in the expression of laterality however; this hormone is down-regulated during the parental phase in sticklebacks (Páll et al, 2002). Additionally, Bukhari et al (2019) have shown that patterns of gene expression during the parenting period for caring and non-caring male stickleback differ. Specifically, oxytocin, important for social affiliation and parental care in mammals and fish is up-regulated when male stickleback are caring for eggs in a nest, but

whether this gene is linked to laterality is currently unknown. The relationship between hormone regulation, gene expression and laterality remains poorly understood particularly with regard to sex and reproduction.

We found a link between laterality, reproduction and experience of mating and parenting, indicating reproduction and its associated behaviours as key but previously unidentified sources of variation in laterality both between the sexes and within a single sex. The caring sex, and individuals that had mating and parenting experience, were more strongly lateralised than both the non-caring sex and individuals of the caring sex with no mating or parenting experience. These differences could arise from the benefits of lateralisation outweighing the costs across different stages of reproduction, but currently these costs and benefits are unknown. Future work should focus on disentangling the specific aspect(s) of mating and/or parenting that drive variation in laterality, the mechanistic basis of this variation and its implications for performance in both reproduction and parenting behaviours, and performance in other behavioural domains that will ultimately impact fitness. This information is critical to understand how variation in cerebral lateralisation evolved and is maintained**.**

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Statement of Authorship

S.M. and L.J.M. conceived and designed the study. S.M. performed data collection, S.M. and L.J.M. performed statistical analyses, S.M. drafted the manuscript and L.J.M. critically revised the manuscript. All authors gave final approval for publication and are accountable for the work performed therein.

Data and Code Accessibility

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Table 1: Population L_R (a) and individual level L_A (b) lateral biases of males and females across varies stages of reproduction.

Sex	Stage	df	a) L_R		b) L_A	
			t	p	X^2	p
Male	Reproductive	49	1.695	0.867	92.192	0.002**
	Pre-spawning – built nest	23	0.092	0.927	45.183	0.001**
	Pre-spawning – no nest	23	1.193	0.506	52.733	0.008**
	Post-spawning – mated & parented	23	1.175	0.453	54.333	0.001**
	Post spawning – not mated or parented	23	1.533	0.416	31.933	0.152
	Non-reproductive	43	0.637	0.678	47.154	0.345
Female	Reproductive - gravid	32	1.629	0.509	32.872	0.424
	Post-spawning – laid eggs	23	0.414	0.768	35.733	0.079
	Non-reproductive	31	0.820	0.627	36.800	0.280

Note: Results of a) one-sample t-tests examining if L_R differed from random expectation (0), thus, representing that greater than 50% of sampled individuals exhibited aligned directional biases at the population level (L_R), and b) chi-squared tests examining if L_A values were more extreme than expected, thus representing individual level consistent directional biases (L_A) in the detour test, for males and females at each stage of reproduction. P-values are corrected for multiple testing using the false discovery rate method (Benjamini & Hochberg, 1995). * $p < 0.05$. ** $p < 0.01$.

Figure 1: diagrammatical representation of the reproductive stages when males (♂) and females (♀) were measured for laterality: (i) reproductive state: showing nuptial colouration ♂ or gravid ♀, (ii) pre-spawning: nest construction ♂, (iii) post-spawning: courtship, spawning and performance of parental care behaviours ♂ or egg laying ♀, and (iv) non-reproductive: no signs of nuptial colouration ♂ or egg production respectively ♀.

Figure 2: L_A, of males (purple) and females (blue) in a reproductive and non-reproductive state.

Figure 3: L_A of a) males and b) females at each stage of reproduction. Asterisks indicate significant differences in planned comparisons of L_A for individuals performing different reproductive behaviours: * represents $p \leq 0.05$ and ** represents $p \leq 0.01$.

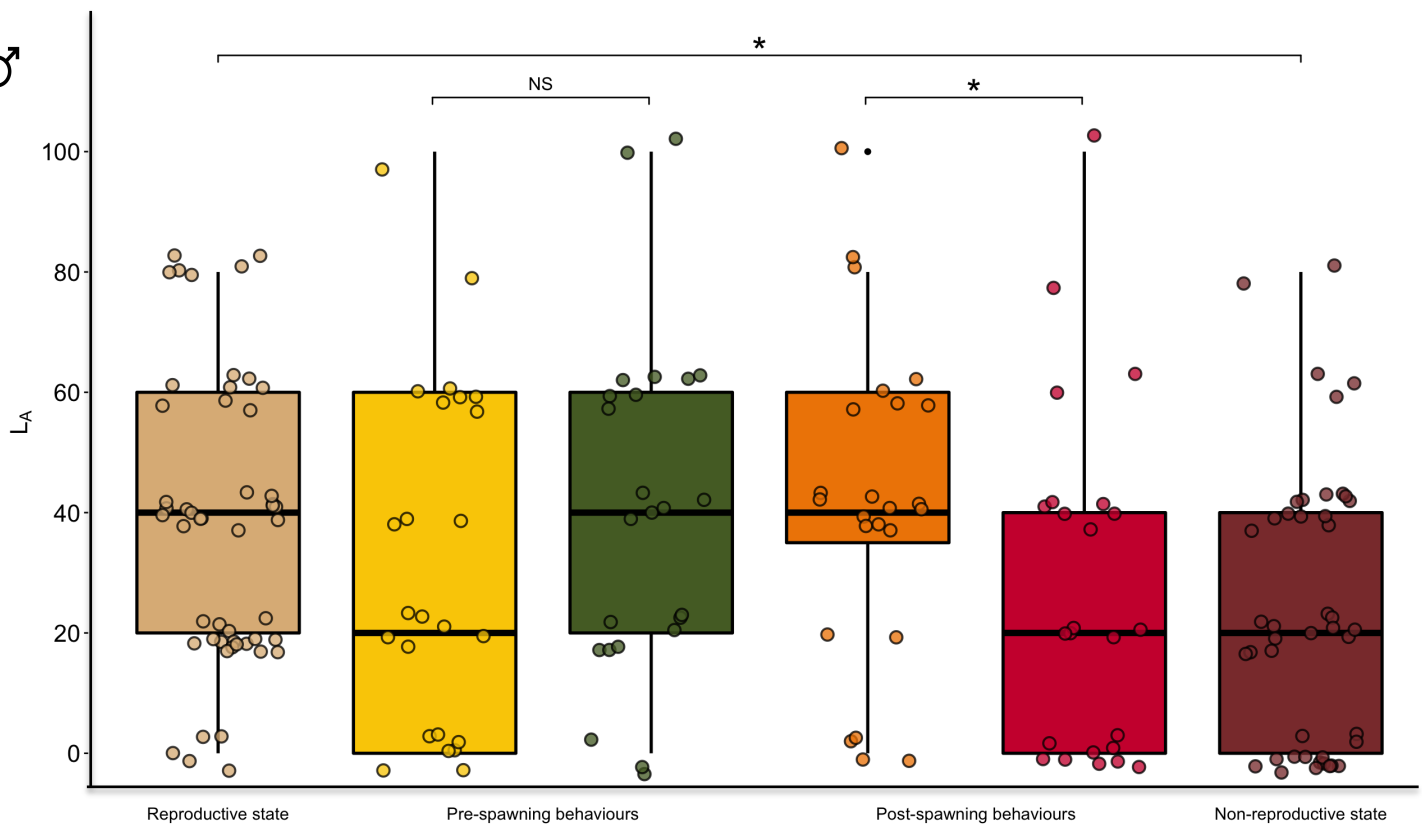| (i) Reproductive state | (ii) Pre-spawning behaviours | (iii) Post-spawning behaviours | (iv) Non-reproductive state |
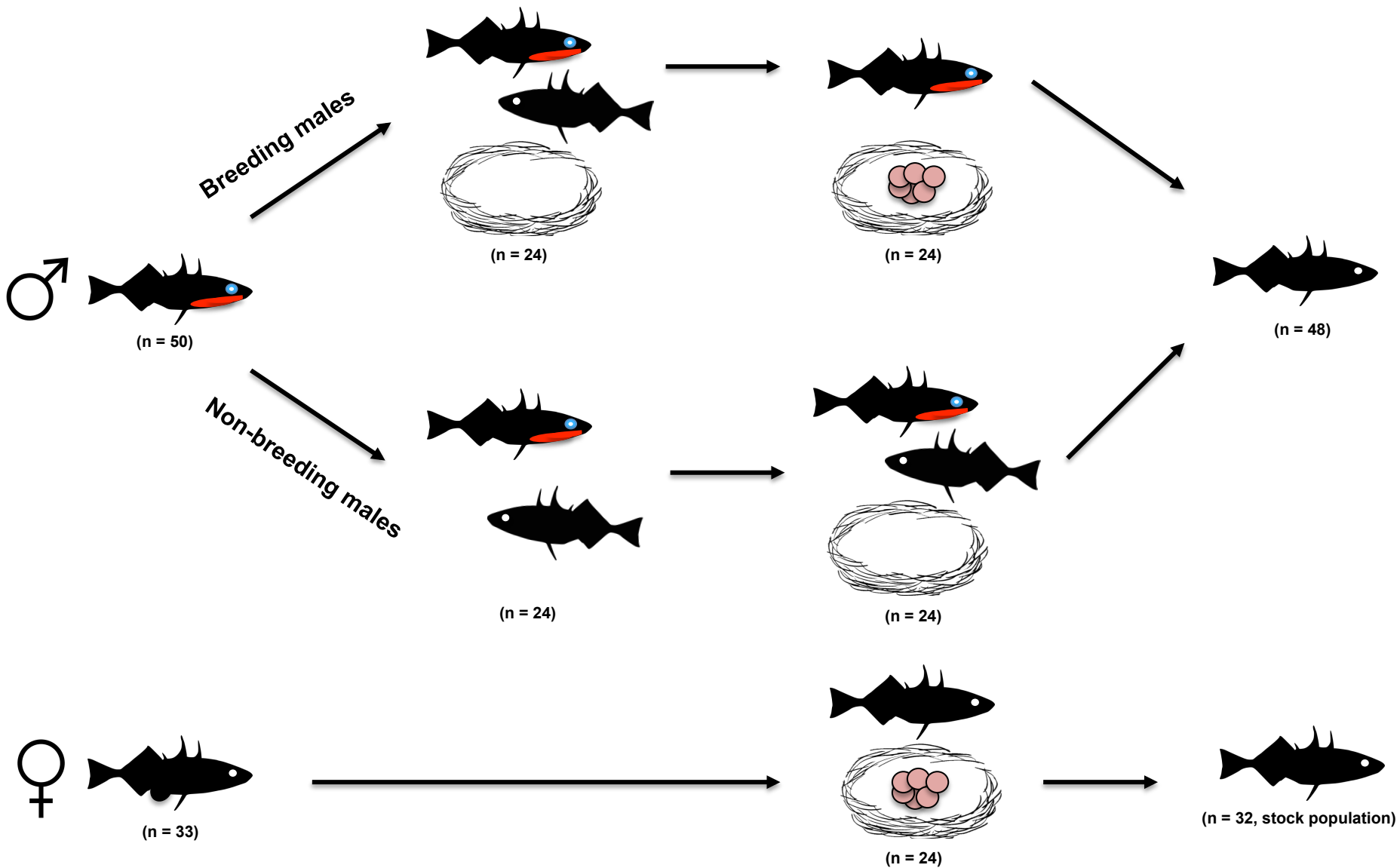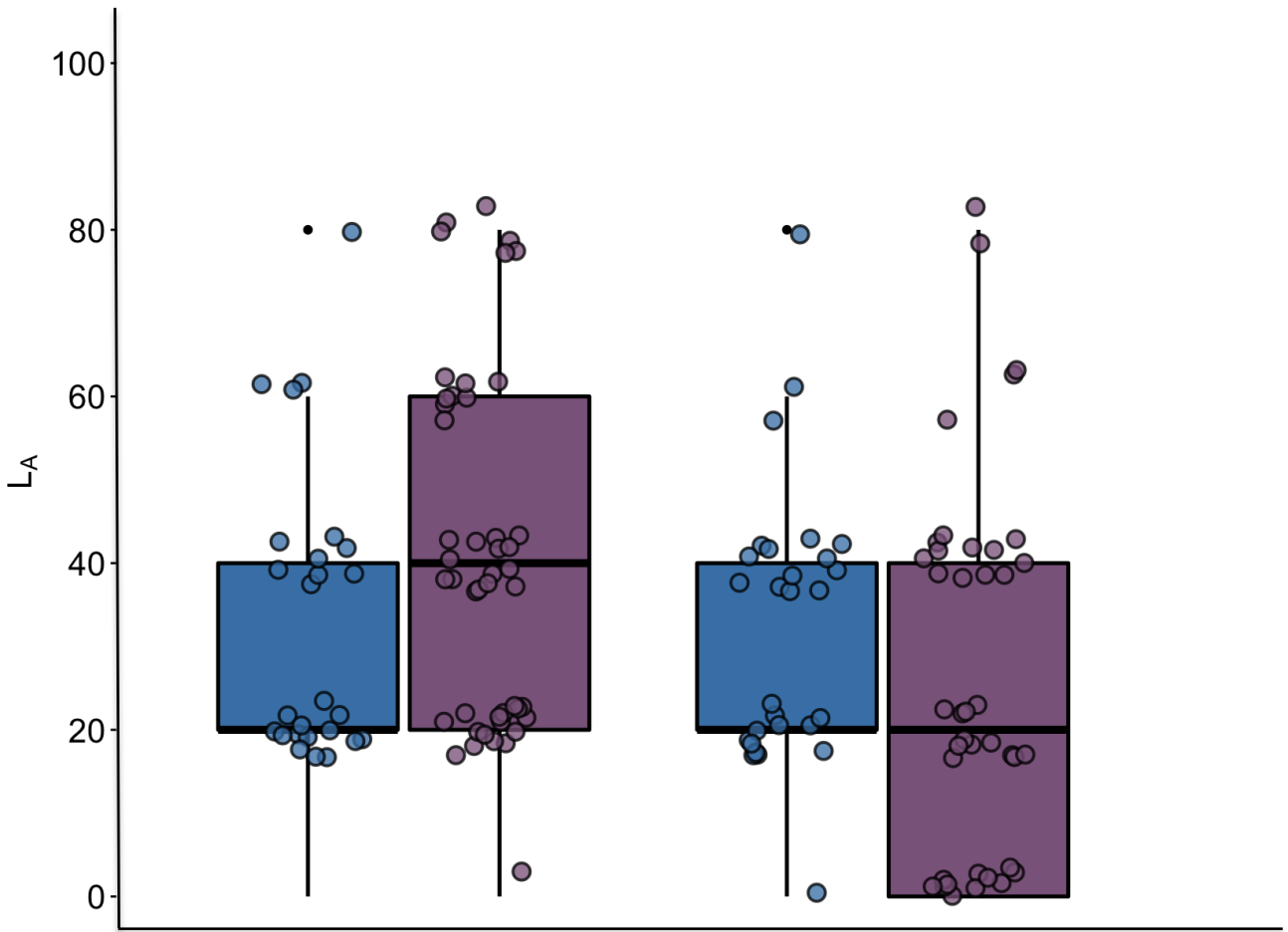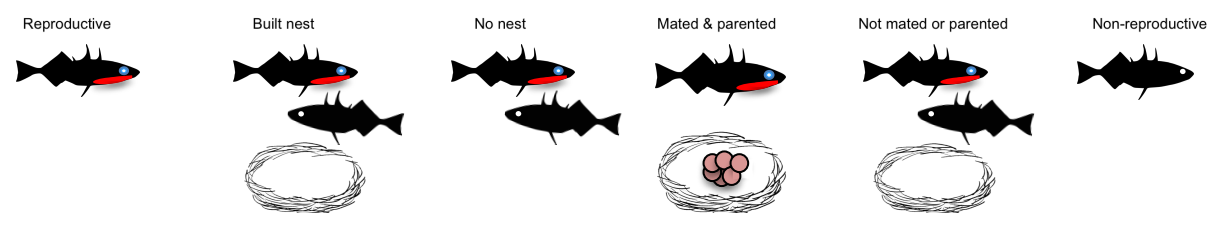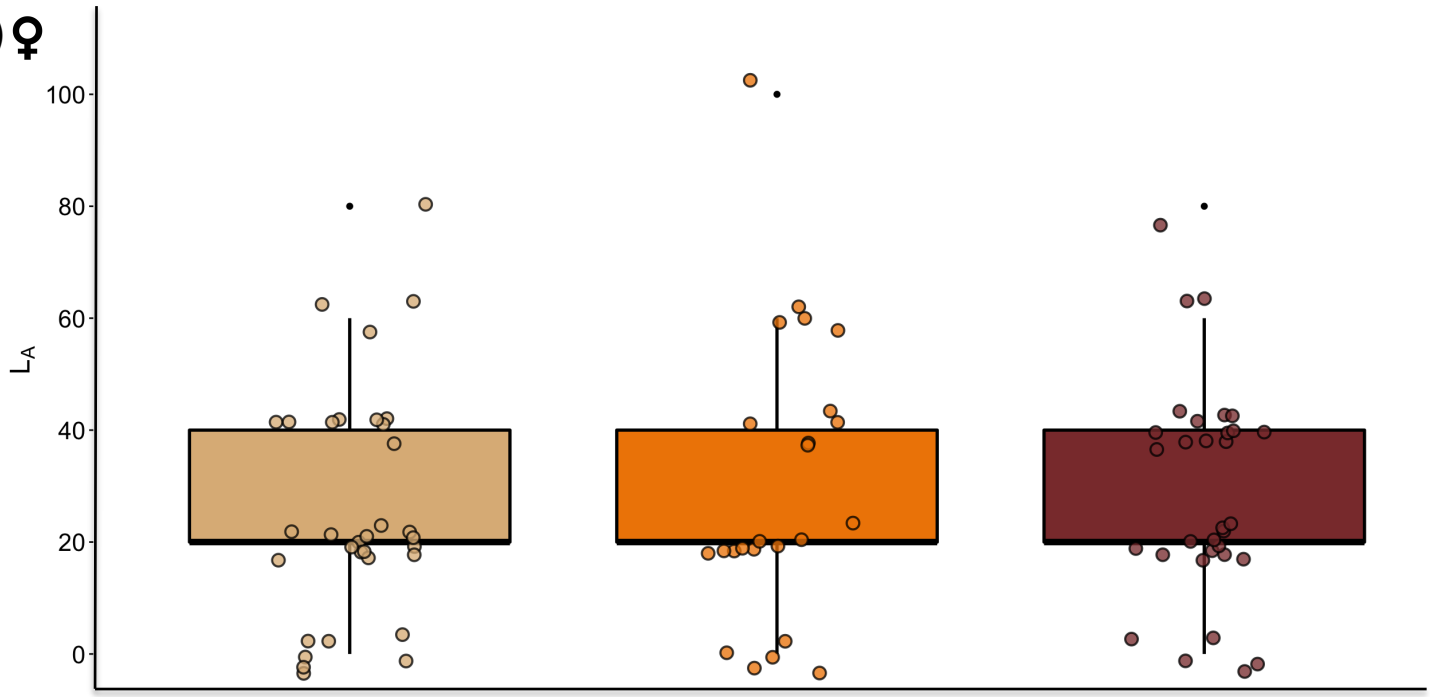
Sex differences in laterality are associated with reproduction in three-spine stickleback

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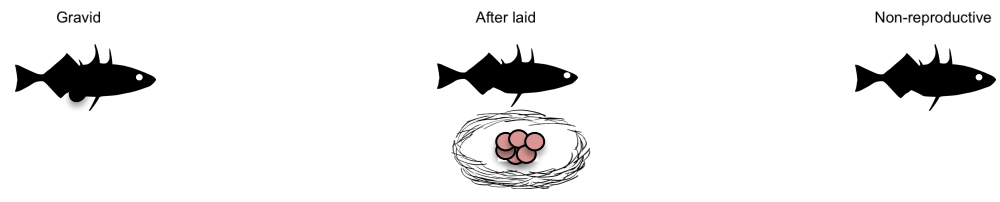
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Sex differences in laterality are associated with reproduction in three-spine stickleback

a)

50cm

40cm

90cm

b)
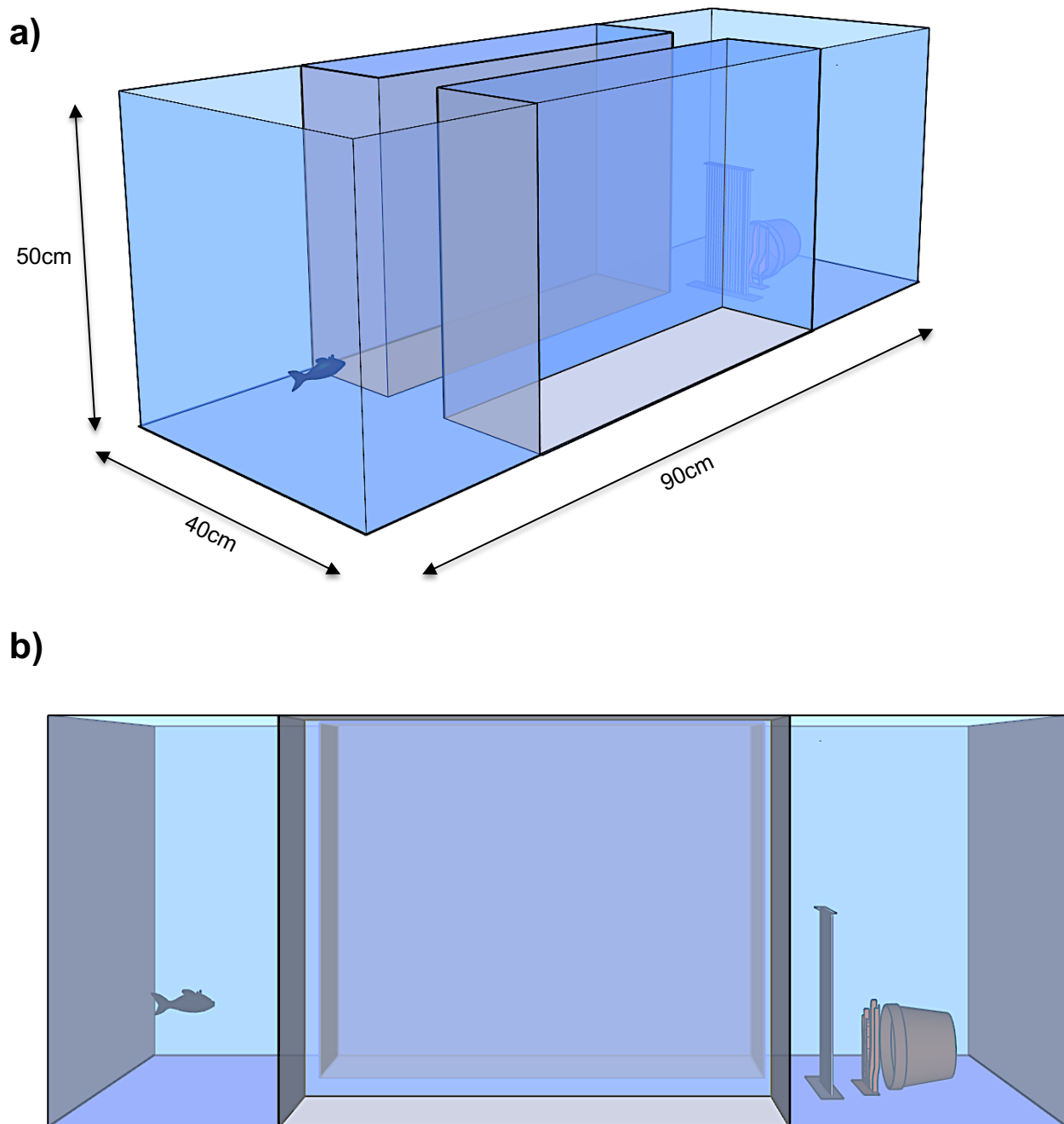
Figure S1: Diagrammatic representation of the detour test used to examine behavioural laterality: a) diagonal view and b) side view. Fish swam along a runway (alternatively in opposite directions) to detour around a barrier while viewing a visual stimulus (here a plant pot and artificial plant representative of a shelter).

Table S1: The effects of sex, stage of reproduction and their interaction on a) L_R and b) L_A.

	estimate	s.e.	df	t	p
a) Direction of laterality (L_R)					
Intercept	-3.553	34.400	154	-0.103	0.918
Sex (male)	2.020	8.475	154	0.238	0.812
Stage (non-reproductive)	-4.115	9.136	154	-0.450	0.653
Body size (cm)	2.673	7.146	154	0.374	0.709
Sex (male) * Stage (non-reproductive)	-3.060	11.893	154	-0.257	0.797
b) Strength of laterality (L_A)					
Intercept	59.388	20.198	154	2.940	0.003**
Sex (male)	9.665	4.976	154	1.942	0.053
Stage (non-reproductive)	2.754	5.364	154	0.513	0.608
Body size (cm)	-7.046	4.196	154	-1.679	0.095
Sex (male) * Stage (non-reproductive)	-16.376	6.983	154	-2.345	0.020*

Note: For all models, individual ID was fitted as a random factor and body size as a covariate. The intercept represents females in a reproductive state. * $p < 0.05$. ** $p < 0.01$.