

Article

Asymmetry in genitalia is in sync with lateralized mating behavior but not with the lateralization of other behaviors

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Abstract

Asymmetries in bilateral organisms attract a lot of curiosity given that they are conspicuous departures from the norm. They allow the investigation of the integration at different levels of biological organization. Here we study whether and how behavioral and asymmetrical anatomical traits co-evolved and work together. We ask if asymmetry is determined locally for each trait or at a whole individual level in a species bearing conspicuous asymmetrical genitalia. Asymmetric genitalia evolved in many species; however, in most cases the direction of asymmetry is fixed. Therefore, it has been rarely determined if there is an association between the direction of asymmetry in genitalia and other traits. In onesided livebearer fish of the genus *Jenynsia* (Cyprinodontiformes, Anablepidae), the anal fin of males is modified into a gonopodium, an intromittent organ that serves to inseminate females. The gonopodium shows a conspicuous asymmetry, with its tip bending either to the left or the right. By surveying 13 natural populations of *Jenynsia lineata*, we found that both genital morphs are equally common in wild populations. In a series of experiments in a laboratory population, we discovered asymmetry and lateralization for multiple other traits; yet, the degree of integration varied highly among them. Lateralization in exploratory behavior in response to different stimuli was not associated with genital morphology. Interestingly, the direction of genital asymmetry was positively correlated with sidedness of mating preference and the number of neuromasts in the lateral line. This suggests integration of functionally linked asymmetric traits; however, there is no evidence that asymmetry is determined at the whole individual level in our study species.

Key words: antisymmetry, behavioral lateralization, *Jenynsia lineata*, *Jenynsia multidentata*, phenotypic integration

Phenotypic asymmetries in otherwise bilaterally symmetric animals provide a great opportunity to better understand developmental processes, and if functionally integrated phenotypes are locally or globally determined (Bisazza et al. 2000a; Palmer 2004; Facchin

et al. 2009; Schilthuisen 2013; Palmer 2016). The simple dichotomous nature (left or right) allows for comparative studies across different traits within a species, as well as across species (Palmer 2016). Asymmetry has been divided into 3 categories (Neville 1976; Palmer

2005); the most common is fluctuating asymmetry, where developmental noise impedes the normal development of symmetric structures. This type of asymmetry is suggested to result in fitness costs associated with discrimination against asymmetric partners during mate choice, as asymmetry might be a proxy for the genetic quality of individuals (Van Valen 1962; Palmer and Strobeck 1986; Møller 1992). Yet, asymmetries can clearly be adaptive in some cases (Hori 1993; Windig and Nylin 1999; Hosoi et al. 2007; Palmer 2009). Here, adaptive asymmetry appears to evolve in response to different selection pressures. On one hand, there is adaptive random asymmetry, where left- and right-handed individuals are equally common within a species, and the trait is distributed in a bimodal fashion. On the other hand, in directional asymmetry, the direction of asymmetry is fixed or almost fixed, and all or most of the individuals of a species are either right- or left-handed (Palmer 2005).

Morphological asymmetry in functionally important traits has been shown to be associated with lateralization in behaviors to which they are functionally linked (Windig and Nylin 1999; Huber et al. 2007; Matsui et al. 2013). Asymmetric muscle size induces lateralization in the direction of escape responses in different fish species (Heuts 1999), and Endler's guppies that are asymmetric with regard to body coloration, preferentially show their most colorful side to females during courtship behavior (Řežucha and Reichard 2015). Quite often, though, behavioral lateralization is not obviously associated with morphological asymmetry. An increasing number of examples of behavioral laterality across vertebrates have supported the idea that these might be the result of hemispheric specialization of the brain (Wiper 2017). Under this assumption, a correlation in laterality among different behaviors would be expected (Facchin et al. 1999; Bisazza et al. 2000a), and its relationship (i.e., positive or negative correlation) would depend on the particular stimuli triggering the lateralized response (Facchin et al. 1999; Wiper 2017). If the 2 hemispheres of the brain are specialized in processing information from different types of stimuli-relevant versus irrelevant, familiar versus unfamiliar, or threatening versus unthreatening—then a negative correlation within individuals could be expected when exposed to these different stimuli (for a recent review see Güntürkün and Ocklenburg 2017). This seems to be the case in eye use preference in fish, which is often lateralized and dependent on familiarity, or the potential risk of the explored object (Dadda and Bisazza 2006; Wiper 2017). For example, several studies have found that fish tend to prefer their right eye; therefore, the left hemisphere of the brain, when exploring a potential predator (Bisazza et al. 1998; Facchin et al. 1999; Brown et al. 2004; Broder and Angeloni 2014), but their left eye, and thus their right brain hemisphere, when presented with non-threatening novel objects (Facchin et al. 1999; Brown et al. 2004) or conspecifics (Bisazza et al. 1998; Brown et al. 2007). When tested at the individual level, this pattern is maintained as a negative correlation between eye preference when exposed to a threatening and an unthreatening object (Facchin et al. 1999).

A functionally important trait that has been found to be asymmetric in several lineages of animal is male genitalia (Schilthuisen 2013). Genital morphology is surprisingly variable across the animal kingdom and its diversity had been studied mostly in regards to sexual selection (Eberhard 1985; Langerhans 2008; Leonard and Córdoba-Aguilar 2010; Schilthuisen 2013). A particularly interesting way in which animal genitalia diverge among closely related species is in their (a)symmetry (Schilthuisen 2013). Surprisingly, this aspect of genital evolution has been neglected and much of it remains unknown, including how genital asymmetry covaries with

asymmetry in other traits of the organism, which can inform on the relative contribution of sexual and natural selection on the evolution of these traits (Schilthuisen 2013). Only few studies that investigated such covariation found an association between lateralized mating behavior and genital asymmetry, mainly in the context of coercive mating and sexual conflict over the control of reproduction (Coker et al. 2002; Palmer 2006; Brennan et al. 2007; Huber et al. 2007; Langerhans 2011). These studies are nonetheless rare, because the direction of genital asymmetry is fixed in most species (i.e., directional asymmetry) and cases where both, right and left, morphs are equally common within populations (i.e., antisymmetry) are rare (Ueshima and Asami 2003; Huber et al. 2007; Schilthuisen 2007, 2013). Thus, addressing the question about covariance in asymmetry among traits relied mainly on comparisons across species or on rare mutants (e.g., Lang and Orgogozo 2012).

An interesting species to study integration between genital asymmetry and other lateralized traits is the South American livebearer fish *Jenynsia lineata* (Cyprinodontiformes, Anablepidae; Figure 1A). The common name of this species, onesided livebearer, derives from the conspicuous asymmetry of males' gonopodium, a modified anal fin used as intromittent organ to internally inseminate females (Neville 1976). Gonopodia are not limited to Anablepidae fish, having independently evolved in other fish families (e.g., Poeciliidae and Hemiramphidae; Meyer and Lydeard 1993). Particularly in Poeciliids, the sister family to Anablepidae, gonopodia display an impressive amount of morphological variation (Rosen and Bailey 1963; Langerhans 2011). In the onesided livebearer, the gonopodium is a tubular structure formed by the enlargement of some rays of the anal fin and the reduction of others (Parenti 1981). The conspicuous genital asymmetry is caused by ray number 6, the thickest and longest, which is laterally displaced and at the tip it bends either to the left or right, forming a hook (Figure 1B). This bending can occur on either side and thus both, left and right morphs are found within populations, although it is unclear if these are equally common (Miller 1979; Bisazza et al. 2000b). Females of the genus *Jenynsia*, but not *Anableps*, have symmetric genital openings (called gonopores), so males of both morphs can potentially mate randomly with females (Neville 1976; Bisazza et al. 2000b; personal observation). The genital asymmetry of onesided livebearers is interpreted as limitation of males' ability to fertilize females, since they only seem to be able to do so when they approach them from 1 side, the side to which the gonopodium is bent (Neville 1976; Miller 1979). This morphological asymmetry is expected to result in a strong lateralization of mating behavior although this has not been experimentally tested yet.

Interestingly, onesided livebearers were previously found to show lateralization in escape and exploratory behaviors (Bisazza et al. 1997a, 2000a). Females of this species were tested for exploratory behavior and lateralization at the individual level was found. Some individuals preferentially turned to the left and others to the right during predator inspection behavior (Bisazza et al. 2000a). As only females were tested, the covariation in direction between behavior and genitalia could not be tested, so it is unclear if males show this lateralized escape behavior as well and whether or not this is associated with genital asymmetry. A second experiment found onesided livebearer males to be lateralized in their fast-start escape response. But, no association with genital asymmetry was found (Bisazza et al. 1997a). This might be expected given that a correlation between these traits would make the direction of escape predictable and predators could take advantages of morphological induced behavioral biases (Ghirlanda and Vallortigara 2004;

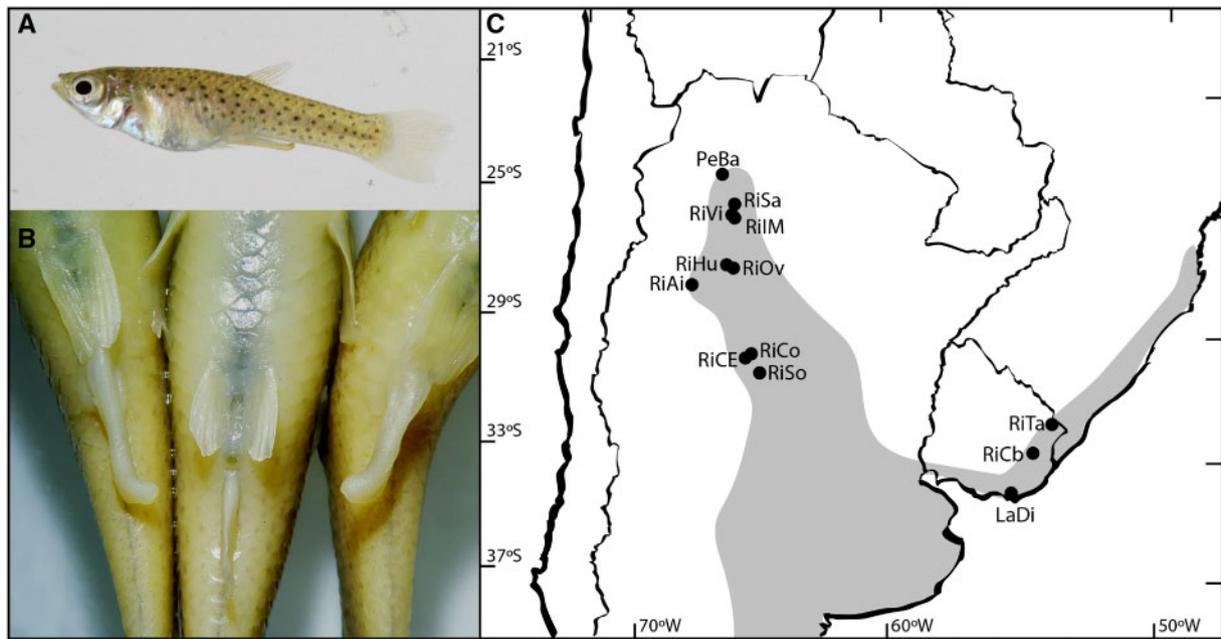


Figure 1. *Jenynsia lineata*, the onesided livebearer, is a South American fish with a wide distribution. Its common name comes from the peculiar genital asymmetry in males. (A) Onesided livebearer male showing the modified anal fin into a gonopodium. (B) Ventral view of the pelvic area of a female (in the middle) and 2 males of the onesided livebearer. The female shows the symmetrical gonopore directly anterior to the anal fin. To each corresponding side of the female, a left and a right male show the bending of the tip of their gonopodia in the direction of the female. (C) Map of part of South America, showing the estimated distribution of the *J. lineata* (gray shade) and the localities sampled in this study.

RiAi, Río Aimogasta; LaDi, Laguna de Diario; PeBa, Peña Baya; RiSo, Río Soto; RiCo, Río Cosquín; RiCE, Río Cruz del Eje; RiHu, Río Huacra; RiIM, Río India Muerta; RiOv, Río Ovanta; RiSa, Río Salí; RiVi, Río Vipos; RiTa, Río Tacuari; RiCb, Río Cebollati; Geographic coordinates and sample sizes are presented in [Supplementary Table S1](#).

Blumstein et al. 2018). However, associations between morphological asymmetry and fast-start escape response have been observed in other fishes (Matsui et al. 2013). Thus, the relationship between genital asymmetry and behavioral lateralization in onesided livebearers is still unclear.

Our main objective is to understand how a functionally important morphological asymmetry (i.e., genital asymmetry) is associated with other asymmetric traits, including morphological asymmetry and behavior lateralization. Specifically, here we study multiple populations of the onesided livebearer *J. lineata* across most of its distribution (Figure 1C) to determine the pattern of genital asymmetry through space and time. Then, we use a laboratory population with a 1:1 ratio of left and right males to determine if the direction of asymmetry in the gonopodium is associated with 1) paired morphological traits (e.g., eye size and lateral line neuromast number); 2) lateralization in mating behavior; and/or 3) lateralization in exploratory behavior. As adult males spend a significant amount of their time budget attempting to force copulation (Bisazza et al. 2000b), we hypothesize that genital asymmetry would be strongly associated with brain lateralization and asymmetry in sensory organs.

Materials and Methods

Phenotyping

Genital asymmetry

To determine the relative abundance of left or right morphs of onesided livebearer males in natural populations, we analyzed the collection of the Fundación Miguel Lillo in Tucumán, Argentina, and

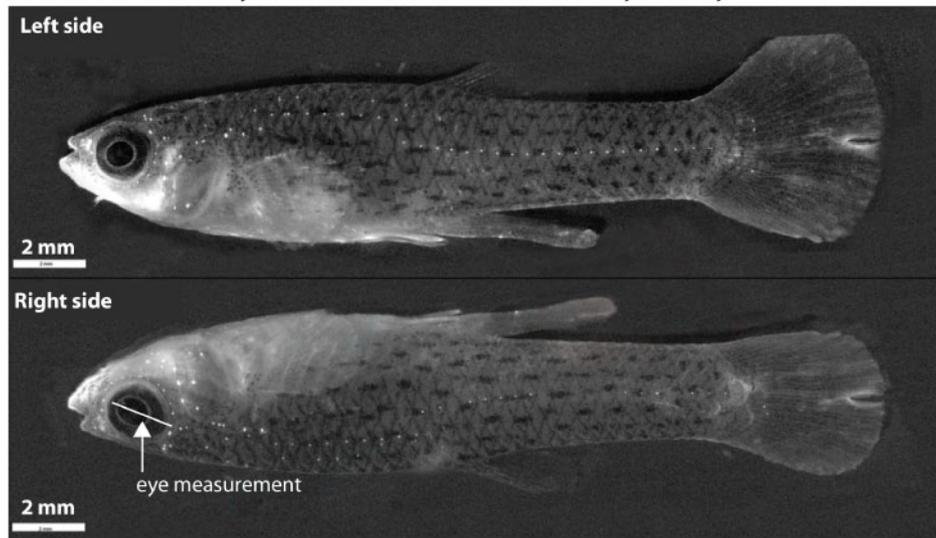
at Universidad de la República, Uruguay. In total, 409 males from 13 localities across Argentina and Uruguay were examined for gonopodial morphology (Supplementary Table S1). One of those localities was sampled 5 times within 2004, spanning 2 different breeding seasons (Goyenola et al. 2011), allowing us to determine not only spatial, but also temporal variation in morph frequency. The direction of the bending of the distal tip of ray 6 was used to classify males as left or right morph individuals.

A total of 52 experimental fish were used in the different experiments to determine the association between genital asymmetry, sensory organ asymmetry, and behavior laterality come from a laboratory stock that has been bred in captivity for multiple generations. Fish were reared in group-tanks until the anal fin started to elongate. At that point, males were isolated and reared in 2-L tanks in a recirculating aquarium facility. Males were kept at a 12:12 light:dark cycle at 24 degrees Celsius and fed twice daily with recently hatched brine shrimp and commercial flake food. At sexual maturity the distal tip of the gonopodium was used to classify males as left or right morph. In addition, standard length and absolute gonopodium length were measured from photographs using ImageJ[®] and relative gonopodium length was determined by calculating the percentage of gonopodium length relative to body size (Standard length; Supplementary Figure S1).

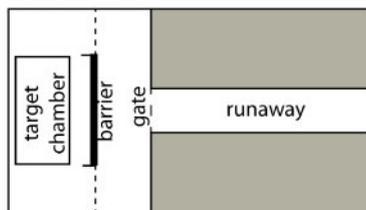
Morphological asymmetry: eye size and neuromasts in the lateral line

Asymmetry in eye size was determined as the difference in diameter (measured horizontally in the middle of the eye) between the right and the left eyes (Figure 2A). Asymmetry in lateral line was determined as the

A Quantification of eye size and neuromast number asymmetry



B Detour behavior arena



C Different stimuli used in the target chamber

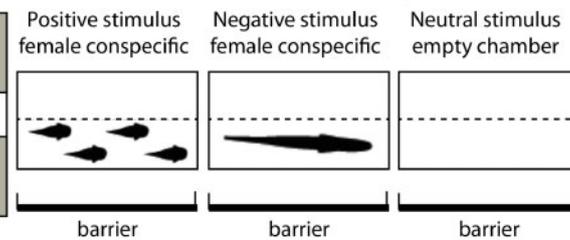


Figure 2. Experimental procedures used to determine morphological asymmetry and behavioral lateralization in the on-sided livebearer, *J. lineata*. **(A)** On-sided livebearer stained with a fluorescent DASPEI dye for visualization of the neuromasts of the lateral line. Measurement of the eye size was taken from both sides as the maximum horizontal length. **(B)** Schematic representation of the arena used for quantifying asymmetry in exploratory behavior. Subjects were released into the middle of the runway and allowed to pass a gate in order to examine a target behind a translucent barrier. The direction of detour around the barrier was recorded in 10 consecutive trials (dashed line next to barrier indicates virtual line which had to be crossed in order to accomplish detour). **(C)** Detailed view of stimuli presented to the subjects: a tank containing 4 females, a tank containing predator fish, and an empty tank (dashed line indicates divider).

difference in the neuromast count number between left and right sides. To measure these traits, 41 mature males were photographed under a Leica MZ10 F fluorescent microscope using a Leica DFC3000 G camera attached to the microscope. Previous to photographing, neuromasts were stained using a fluorescent 2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide (DASPEI) dye following the protocol by Wark and Peichel (2010) and modifications by Fischer et al. (2013). In short, the DASPEI dye was suspended in ddH₂O to prepare a 0.25% DASPEI stock solution that was later diluted to a final concentration of 0.025% by adding conditioned tank water. Fish were allowed to swim freely in the 0.025% DASPEI solution for 30 min before they were removed from the dye, rinsed in conditioned tank water and anaesthetized in 0.067% MS-222 until only shallow gill ventilation was detectable. For taking photographs, fish were placed in a Petri dish containing 0.0335% MS-222, and a green filter was used to capture images from both sides of each fish. As neuromasts were better visible under the microscope than in the captured images, these were counted during microscopy. Eye diameter was measured from photographs using ImageJ[®].

Sidedness of mating attempts

The 41 males examined for eye and lateral line asymmetry and an additional 11 males ($N = 52$) were individually tested for lateralization in mating behavior, by allowing each of them to interact with a female and determining the differences in the number of gonopodial

thrust performed from each side of the female. Males were tested in a circular arena (diameter: 25 cm) with white, opaque sides and filled with 6 cm of conditioned water. A video camera (Panasonic Full HD; HC-V110) was placed 44 cm above the arena to record the behaviors. A randomly selected male (genital morphology was checked after the trial was finished) was placed alone into the arena and it was allowed to freely explore it for 5 min. After this acclimation time, a female was gently introduced into the arena, and both fish were allowed to freely interact for 25 min while being filmed from above. Each male was tested twice, using a different female in each trial to verify that laterality in mating behavior was due to male's rather than female's morphological characteristics. The 2 trials were conducted at least 7 days apart. We found no differences between the 2 trials. Thus, only the results with the first female are reported. This is because in their first trial males were naïve to females, given that they were placed in individual tanks as juveniles when the anal fin started to form the gonopodium. Thus, it can be assumed that the measured behavior is innate rather than learned. A total of 5 different females were used in this experiment.

We counted the number and side of mating attempts from the recorded videos. A mating attempt was counted as such when the male gonopodium was moved toward the female's gonopore while both fish were swimming in close proximity. When we refer to mating attempts we cannot be sure of mating success, as we could not always verify that the gonopodium was successfully inserted into the female's gonopore.

Detour test for exploratory behavior

Ten left and 10 right morph males were tested for lateralization in exploratory behavior, referred to as detour behavior as fish are forced to detour to either the right or the left to explore a stimulus, and dependence of lateralization on the nature of the stimulus. Individuals were tested in an arena consisting of a large glass tank (35 cm × 101 cm) with a runway in the middle (10 cm × 69 cm) that connected to a testing chamber (35 cm × 32 cm) by a remote-controlled gate (Figure 2B). A funnel-like structure was placed in front of the gate connecting the runway and the testing chamber to ensure that the experimental subjects left the runway centered. In the testing chamber a translucent barrier (13.5 cm × 23.5 cm) forced the individuals to turn to the right or to the left to explore a smaller target chamber (12.5 cm × 23.5 cm) located behind this barrier. The target chamber was empty (e.g., neutral stimulus), contained 4 onesided livebearer females (e.g., positive stimulus) or a *Crenicichla regani*, a small predatory cichlid fish (e.g., negative stimulus). Pike cichlids of the genus *Crenicichla* spp., are common predators of onesided livebearer fish, at least in some parts of their distribution (Petry et al. 2016). The target chamber where the predator or the females were presented was reduced in space with a divider (Figure 2C) in order to force them to distribute across the front of the tank. This division reduced the probability that they occupy only 1 corner of the target chamber of our experimental setup. The testing chamber was isolated with a white opaque adhesive foil in order to reduce the influence of external stimuli on the subject during the experiment and 2 fluorescent lamps (KFB RB 218 N HF 5464, 18 W) were placed symmetrically above the experimental area in order to minimize differences in illumination between sides of the experimental arena. A video camera (Panasonic Full HD; HC-V110) was attached 95 cm above the whole setup and all trials were recorded. Water in the tank was 8 cm deep.

The experimental procedure was modified from Bisazza et al. (1997b). First, the fish was introduced into the middle of the runway, facing the open gate, and allowed to swim freely and explore the setup without any barrier or target present for 5 min. Then, the fish was transferred back to the runway and the gate was closed. At this point, the target and barrier were placed in the testing chamber and after this the fish was allowed to freely swim in the runaway chamber for 3 min with the gate closed. After this acclimation time, the gate was opened again and the fish was allowed to leave the runway and explore the stimulus. If the fish did not leave the runway on its own after 5 min, it was gently pushed toward its end with a fish-net. After the fish left the runway, the gate was closed slowly and the direction of detour around the barrier was recorded. A detour was regarded as accomplished when the fish completely crossed a virtual line extending from the barrier to the lateral sides of the arena (Figure 2B). A small number of fish did not complete the trail after 3 min, and in these cases it was transferred back to the runway and the trial was regarded as not completed. After each detour, the fish was given 30 s to examine the target chamber before it was transferred back to the runway and kept there again for 3 min before the next trial started. The experiment was conducted in 10 consecutive trials for each stimulus. If the fish refused to leave the runway, even when being gently pushed, the experiment was paused and continued later. Each subject was exposed to the 3 above-mentioned stimuli in a random order and with a separation of 7 days.

Data analysis

Distribution of left- and right-morph individuals in natural populations

To test if both genital morphs are equally abundant in wild populations (i.e., antisymmetry) or if 1 of the 2 morphs is consistently more common than the other (i.e., directional asymmetry) a Wilcoxon

signed-rank test was used (W). Males in the sampled populations were classified as left- or right-morph based on the direction of bending of the gonopodium's distal tip. Sampled populations were ranked based on the absolute difference in the number of right- and left-morph males (e.g., $|\#R_{\text{gon}} - \#L_{\text{gon}}|$). Then, rank values were signed based on the difference of the number of males of both morphs; positive values if there were an excess of right males and negative values if there was an excess of left males. Given that the probability distribution of the sum of the signed ranks (W) follows a normal distribution (Quinn and Keough 2002), a 2-tailed z -statistic with a significance level of 0.05 was used to test if there are differences in the relative abundance of both morphs. To determine the pattern of temporal variation in the relative abundance of both morphs of the onesided livebearer, we computed the proportion of right males collected at 5 different occasions within 2004 in Río Huacra, Santa Rosa, Catamarca (Supplementary Table S1). Due to the small sample size, no formal statistics were performed.

Patterns of morphological asymmetry and behavioral laterality

Onesided livebearer males were staged for morphological asymmetry in the gonopodia, eyes size, and number of neuromasts in the lateral line and for laterality in mating and detour behavior using 3 different stimuli. Genital asymmetry was considered as a binary variable and males in the experimental population were classified as left or right morph as described for natural populations. For all traits, a laterality index (LI) was calculated as $LI = [(R-L)/(R+L)]$ (Bisazza et al. 1997b), where R represent the number of events on the right side (for eye size, we used the diameter of the right eye) and L number of events on the left (for eye size, the diameter of the left eye). LI can take values from +1 to -1, with a value of zero representing no asymmetry or lateralization, positive values indicating larger right eyes, more neuromast at the right side, or more mating attempts or detours to the right, and negative values an excess of these to the left.

In order to determine the pattern of asymmetry or laterality in the studied traits, we conducted 2 sequential tests. First, a Hartigan's DIP-test for unimodality was conducted for the LI scores of the different traits. If unimodality (Hartigan and Hartigan 1985) was rejected for a trait, the kernel density function for that trait's LI was plotted to visually inspect for the presence of a bimodal pattern. Bimodality is expected in the case of an antisymmetric pattern or if there is behavioral lateralization at the individual, but not at the population level (Palmer 2005; Wiper 2017). Second, if there was no evidence to reject unimodality based on the DIP-test, a 1-sample t -test was used to determine if LI for each trait was significantly different from zero. A significant departure from zero is expected in the case of directional asymmetry or if there is behavioral lateralization both, at the individual and population level (Palmer 2005; Wiper 2017). Traits for which neither the DIP-test, nor the 1-sampled t -test were significant, were considered to be not lateralized or morphologically symmetric or showing fluctuating asymmetry (Palmer 2005; Wiper 2017).

Pattern of association between genital asymmetry and asymmetry-laterality in other traits

Males in this study were selected based on the direction of asymmetry of their gonopodium to include an equal number of left and right morph individuals. This provided us with the opportunity to test if left- and right-morph males differ in the direction of asymmetry in other traits. LI of eye size, number of neuromast in the

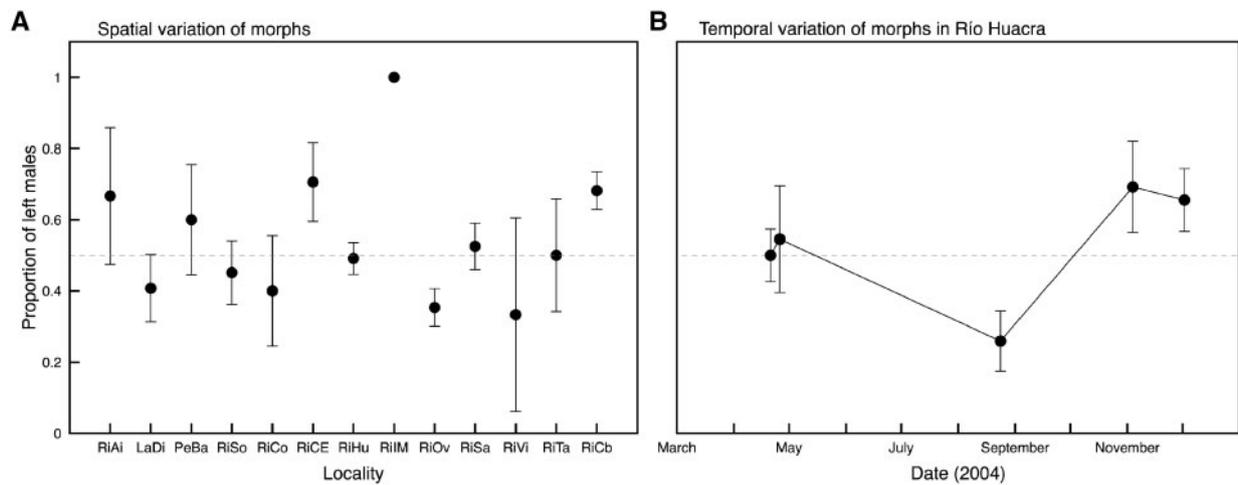


Figure 3. Spatial and temporal variation in the relative abundance of left- and right-handed males in wild populations of the onesided livebearer, *J. lineata*. **(A)** Proportion (\pm s.e.) of the left morph in 11 wild populations of the onesided livebearer. Locality names as in Figure 1. **(B)** Proportion of left males in Río Huacra in Catamarca, Argentina at 5 different time points in 2004.

lateral line, and detour behaviors were analyzed using a *t*-test for each trait, with direction of bending of the gonopodial tip as the explanatory variable ($N=20$). Mate choice was not analyzed in this way, as it was perfectly associated with direction of genital asymmetry (see “Results” section). Hence, there was no residual variance for this trait. We also tested if there were correlations in the *LI* of the different examined traits using Spearman’s correlational coefficients. All analyses in this study were conducted in R (R Core Team 2013), using the DIP-test (Maechler 2013) and Hmisc (Harrell Jr 2019) packages.

Results

Frequency distribution of left- and right-morph individuals in natural populations

Onesided livebearer males showed a pattern of antisymmetry or random asymmetry, where both male morphs have equal probability of being found in the wild ($W=9$, $z=0.33$, $P=0.74$, $N=12$; Figure 3A). Samples at 5 times across a year, including the end of 1 breeding season and the beginning of the next, were obtained from 1 locality. These data show, showing that the probability of capturing males of each morph varies with time, but oscillate around a 1:1 ratio (Figure 3B).

Patterns of morphological asymmetry and behavioral laterality

Based on DIP-tests, there was significant evidence of departure from unimodality for 2 behavioral traits, sidedness of mating attempts and detour behavior with females as a stimulus (Table 1, 2nd column). Plots of the kernel function for the *LI* of mating behavior showed complete separation, where some individuals only attempted to mate from the right side whereas others only attempted it from the left side (Figure 4A). There was also evidence for laterality at the individual level for the detour behavior with females as stimulus (i.e., female inspection), where some specimens consistently turned to the right whereas others consistently turned to the left. However, there was much more variation at the individual than for sidedness of mating attempts (Figure 4B).

For traits that showed a unimodal distribution (i.e., non-significant DIP-test), we tested for patterns of directional asymmetry or population level lateralization using 1-sampled *t*-test ($H_0: LI=0$). Predator inspection behavior showed a pattern of population level lateralization, where most tested individuals turned preferentially toward the left, using the right eye for inspection of the predator (Figure 4C). In no other trait *LI* departed significantly from zero (Table 1, 3rd column).

Pattern of association between genital asymmetry and asymmetry in other traits

There was a perfect association between the direction of bending of the gonopodial tip and sidedness of mating attempts. Right-morph males only attempted copulation from their right side and left morph males only attempted copulation from their left side. In addition, the number of neuromast in the lateral line significantly differed between right- and left-morph males (Table 1, 4th column). Males with the tip of the gonopodium bending to the right had on average 1 more neuromast on the right side of their body ($Mean_{R-L} = 1.10 \pm 0.56$ standard error (SE), sample size ($N=20$)). Left-morph males showed a trend to have more neuromasts on their left side, although the count difference is not different from zero ($Mean_{R-L} = -0.57 \pm 0.63$ SE, $N=21$). No other trait showed an association with the direction of bending of the gonopodial tip, including those that showed patterns of antisymmetry (Table 1). No significant correlations were observed among any of the studied traits (Supplementary Table S2).

Discussion

Antisymmetric genitalia and mating behavior in the onesided livebearer

Jenynsia lineata is known as onesided livebearer due to the asymmetry in male genital morphology. Although this asymmetry has been known for more than a century (e.g., Regan 1913) it had not been investigated before in natural populations. Some publications described a pattern consistent with directional asymmetry (all right males; Neville 1976), but others reported a pattern consistent with antisymmetry, with both morphs being commonly found (Palmer

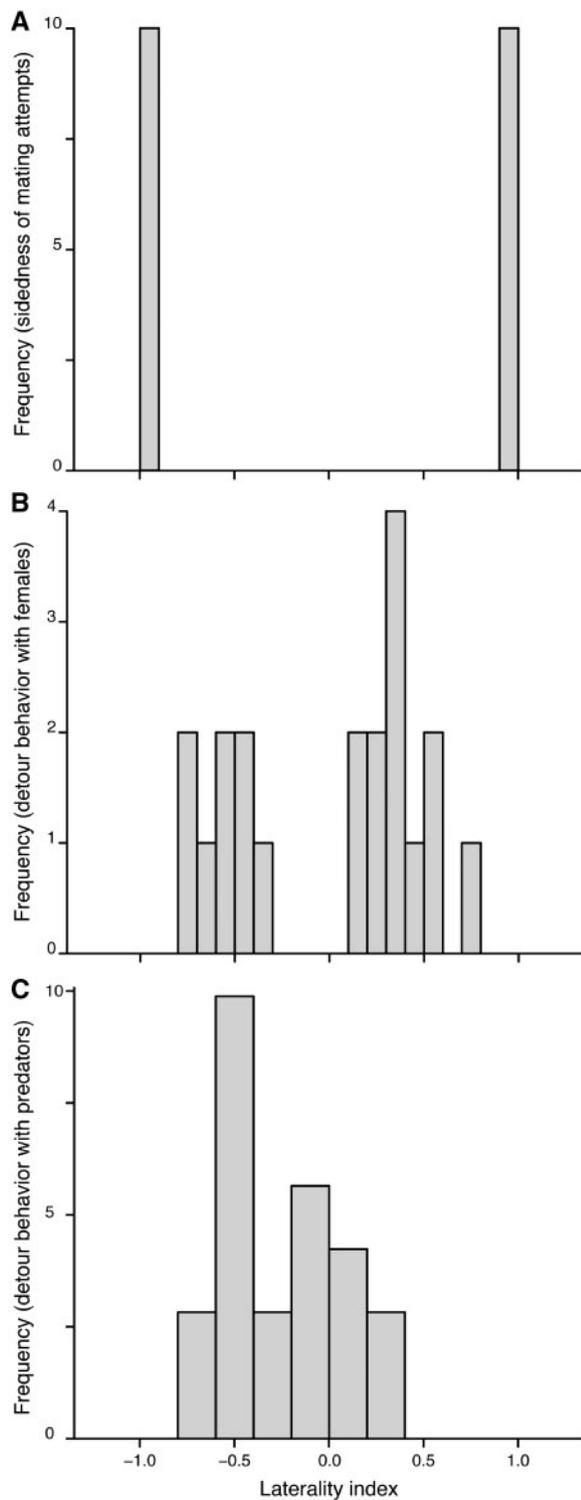


Figure 4. Frequency plots for the individual laterality indices of 3 different behaviors of the onesided livebearer, *J. lineata*. **(A)** Sidedness in mating attempts and **(B)** detour behavior using conspecific females as a stimulus show patterns consistent with lateralization at the individual, but not at population, level. **(C)** The distribution of turns when inspecting a predator as the stimulus is consistent with a pattern of lateralization at the population level. Positive values of the laterality index denote an excess of right turns—and negative values an excess of left turns—than those expected by chance.

1996; Bisazza et al. 1997a, 2000b, Schilthuisen 2013). However, all these quantifications were based on one and only a few populations that, in some cases, had been kept under laboratory conditions for several generations. Across 11 wild populations, spanning most of the distribution of the onesided livebearer fish, and at 5 different collections over a time span of 2 breeding seasons within a population, we have found no evidence for a significant deviation from an even 1:1 left: right abundance in the frequency of the 2 male genital morphs (Figure 3). The temporal oscillation around a 1:1 abundance of morphs in Río Huacra, although preliminary, is interesting as it could imply some kind of frequency-dependent mating success (Ghirlanda and Vallortigara 2004). Given that samples collected at close time points show very similar morph proportions, suggests that the oscillation observed does reflect variation in morph abundance around equal proportion of both morphs (Figure 3B). Two observations point into this direction. First, we found a strong association between gonopodial asymmetry and gonopodial thrust direction (Figure 4A and Table 1); thus, males could only insert their gonopodium into the female's gonopore from 1 side. Second, mating in this species is coercive, males attempt gonopodial thrusts on unaware females. Females typically avoid males by escaping from them or even attacking them (Bisazza et al. 2000b). Thus, it is possible that an excess of 1 morph in a population makes females more aware of males' gonopodial thrusting from 1 particular side and less aware of their other side. By comparing males of different sizes, it has been previously found that less conspicuous onesided livebearer males are more successful attaining unsolicited mating (Bisazza et al. 2000b).

However, some aspects need to be clarified before frequency-dependence mating success is proposed to explain the maintenance of this polymorphism in nature. This is because the time scale of sampling might have been short, including 2 breeding seasons (females produce multiple broods in each season; Turner 1957; Goyenola et al. 2011). Hence, the change in frequency observed might not necessarily reflect a shift in mating success between morphs. Also, it is yet unknown if and how strong the direction of genital asymmetry is heritable in this species. Although the direction of some asymmetric aspects has been found to be heritable in a few species showing antisymmetry (e.g., Jesson and Barrett 2002; Hori et al. 2007; Raffini et al. 2017; but see Palmer 2010) in other species this is not the case (e.g., Edelaar et al. 2005; reviewed in Palmer 2004). Future work will be necessary to elucidate these issues.

Asymmetry in lateral line: functional or fluctuating asymmetry?

Most departures from bilateral symmetry are not adaptive but rather a result of the inability of organisms to develop completely symmetrical along the left–right axis during their ontogeny (Klingenberg 2003; Van Dongen 2006). These non-adaptive morphological asymmetries are known as fluctuating asymmetry (Palmer 2005; Van Dongen 2006), and are characterized by subtle variation around symmetry (i.e., $LI \approx 0$). It has been shown that high levels of fluctuating asymmetry might be costly in terms of fitness, as it is interpreted as a sign of genetic quality and very important in mate choice (reviewed in Andersson 1994). For example, asymmetric males experience a decrease mating opportunity due to females' preference for more symmetrical males (Møller 1992; Van Dongen 2006; Koshio et al. 2007; Pflüger et al. 2012; reviewed in Andersson 1994). Asymmetry in sensory organs might also have important

Table 1. Sequential tests for determining pattern of asymmetry or lateralization

Trait	DIP-test	<i>t</i> -test (<i>df</i>)	<i>t</i> -test (<i>df</i>)	Interpretation
	Ho: unimodal	Ho: $\mu=0$	Ho: $L_{\text{morph}}=R_{\text{morph}}$	
Eye size	0.038	0.178 (40)	-1.650 (27)	Fluctuating asymmetry?
Neuromast number	0.057	0.337 (40)	2.054* (38.97)	Fluctuating asymmetry?
Mating behavior	0.250*	–	–	Individual lateralization
Detour behavior: stimulus				
Females	0.109*	–	-0.614 (17.64)	Individual lateralization
Predator	0.081	-2.720* (19)	0.189 (17.94)	Population lateralization
Empty tank	0.075	-0.137 (19)	1.387 (17.36)	No lateralization

* $P < 0.05$; values in bold to facilitate visualization.

consequences for fitness, although only few studies have evaluated asymmetry in eye size (Pouilly and Miranda 2003; Raffini et al. 2018) and lateral line (Almeida et al. 2008). Cave fish have been shown to have increased asymmetry in eye size compared with epigeic species (Wilkens 2001; Pouilly and Miranda 2003), suggesting that there is selection to maintain symmetry under photic conditions. However, asymmetry in sensory organs could be beneficial when it is associated with lateralized behaviors (Burt de Perera and Braithwaite 2005; Werner and Seifan 2006; Raffini et al. 2018).

In onesided livebearer males, we discovered subtle, but probably functionally important, departures from symmetry in the number of neuromasts on the right and left sides of the lateral lines. The pattern recovered does not depart from unimodality, suggesting fluctuating asymmetry, but the association between direction of the departure from symmetry and the genital asymmetry is intriguing. From a functional view, it is plausible that the larger number of neuromasts on the side of mating aids in positioning and gauging distance of the male during copulation (Engelmann et al. 2000). It has been suggested that in livebearer fish with coercive copulatory behavior, long gonopodia are favored as they allow their bearers to visually guide them toward the females' gonopore (Langerhans 2011). However, onesided livebearer males have a rather short gonopodium ($\leq 25\%$ of the standard length) compared with poeciliid fishes also showing coercive copulatory behavior ($> 35\%$, Langerhans 2011; Supplementary Figure S1). In addition, we found no evidence of functional asymmetry in the eye morphology that might increase the field of view (Table 1). Thus, it is unlikely that males can visually guide their gonopodium; and thus, the lateral line might play an important role in helping males to position themselves during copulation. From a developmental view, it is intriguing how this association occurs as both, the lateral line and the gonopodium, develop at different times during development. The lateral line in fish forms early during development (Webb 1989; Sapède et al. 2002), whereas the gonopodium develops at the onset of maturity (Bisazza et al. 2000b). Thus, it is unlikely that the number of neuromasts is plastically affected by male behavior associated with gonopodium morphology.

Behavioral lateralization is—mostly—dependent of genital asymmetry in the onesided livebearer

Three out of the 4 behaviors we tested showed evidence of lateralization, either at the individual (sidedness of mating attempts and female inspection) or the population level (predator inspection; Figure 4). Yet, there was little to no association with genital asymmetry. The only behavior that was lateralized and was—perfectly—correlated with male genital asymmetry was sidedness of mating

attempts. This association is expected for traits that show a functional link (e.g., Lee et al. 2015, 2017; Řežucha and Reichard 2015). The hook formed by the lateral bending of the tip of the gonopodium is inserted in a back-to-front direction into the gonopore of females (personal observation). Thus, potential attempts from the “wrong” side would result in failed copulation. Males of our laboratory stock have been seen moving the gonopodium in both directions (personal observations); hence, we infer that the limitation on the sidedness is indeed due to the direction of the gonopodial tip, rather than just due to the modifications in the suspensory of the modified fin (Parenti 1981) that would restrict movement.

Lateralization was also observed in detour behavior when exposed to relevant stimuli (positive: females and negative: predator). Detour tests are commonly used to determine lateralization of fish behavior, both at the individual and population level (Wiper 2017). The results are often interpreted as a division of function between brain hemispheres and the associated use of sensory organs, mainly the eye, when exposed to biologically relevant or irrelevant stimuli (Bisazza et al. 1998; Facchin et al. 1999; Dadda and Bisazza 2012; Wiper 2017). When onesided livebearer males were exposed to a small group of conspecific females, they showed significant, although no perfect, lateralization at the individual level (Figure 4B). Because females were forced to spread in front of the target tank (Figure 2B), we reason that this lateralization is biologically significant. Yet, the direction of turn was independent of the direction of the gonopodial tip bending (Table 1). This dissociation might have several causes. The development of the gonopodium from the anal fin occurs late during development, at the onset of maturity (Bisazza et al. 2000b). We have not tested if brain lateralization occurs in juvenile onesided livebearers, but it does occur early in development in other species (Rogers and Sink 1988; Güntürkün 2002; Burns et al. 2009; Dadda and Bisazza 2016; Güntürkün and Ocklenburg 2017). Thus, development of brain lateralization and genital asymmetry might occur at very different time points in the life of the fish and they might be controlled by independent genetic mechanisms. However, genetically unlinked traits could nonetheless be linked if there is a functional benefit (Matsui et al. 2013). For example, males of Endler's guppies *Poecilia wingei*, which show asymmetry in courtship behavior, present females their most colorful flank to increase mating success (Řežucha and Reichard 2015). In the onesided livebearer, lateralization in inspection behavior might not directly influence the sidedness of mating; hence, there might be no selection to link these traits.

We found lateralization at the population level when evaluating predator inspection behavior, with most males preferentially turning to the left and using their right eye to inspect the predator (Figure 4C), independently of the direction of genital asymmetry.

Population-level lateralization in predator inspection has been found in many other fish species (Bisazza et al. 2000a), and has been suggested to be an adaptive response to elevated predation risk (reviewed in Wiper 2017). This is supported by empirical findings showing that fish with an evolutionary history of high predation pressure have higher lateralization than fish of the same species evolving under a release of predation pressure (Brown et al. 2004) and fish experiencing high predation risk due to the presence of alarm cues tend to be more lateralized than those experiencing an environment without alarm cues (Broder and Angeloni 2014; Chivers et al. 2016). Curiously, the onesided livebearer has been evaluated previously for predator inspection, finding lateralization at the individual, but not at the population level (Bisazza et al. 2000a). This difference could have different causes. As mentioned above, the historical level of predation exposure could affect the evolution of population level lateralization (Brown et al. 2004). Unfortunately, we lack information on the origin of the population examined by Bisazza et al. (2000a) and about the predation risk level in the wild population of our laboratory stock. It is also possible that the different results reflect differences between sexes. Male and female onesided livebearer have dramatically different behaviors, as males spent most of their time attempting to inseminate females, whereas foraging constitute the bulk of time budget for females (Bisazza et al. 2000b). These behavioral differences have been hypothesized to explain the difference in rates of mortality between sexes, with males experiencing higher predation risk (Mai et al. 2007; Goyenola et al. 2011). Thus, there could be stronger selection on males to evolve population level lateralization if males perform cooperative predator inspection (De Santi et al. 2000). However, studies in *Poecilia reticulata*, a poeciliid species with similar behavior as the onesided livebearer, show the opposite pattern. Females are performing more cooperative predator inspection than males (Magurran and Nowak 1991). Further studies comparing sexes and populations will be needed to better understand lateralization in predator inspection in this species.

Besides the lack of association between detour behaviors and genital asymmetry, we found no evidence of correlation in detour behavior between the 3 types of stimuli used. Correlations, either positive or negative, were expected under the hypothesis that hemispheric specialization evolved due to selection to respond to multiple ecological relevant stimuli in complex environments, with different hemispheres of the brain dealing with stimuli of a different nature (e.g., threatening vs. unthreatening; Dadda et al. 2012; Rogers et al. 2013; Wiper 2017). Facchin et al. (1999) found that fish inspected objects with 1 eye if it was ecologically relevant (a dummy predator) but used the other for irrelevant stimuli (a ball). Our data provide no evidence that different hemispheres are specialized for opposite tasks. Yet, fish did show lateralization in 3 out of the 4 behaviors measured, suggesting that lateralization could occur but might not need to result in an integrated response across behaviors.

Low level of integration among asymmetric morphologies and lateralized behaviors

Asymmetric morphologies and lateralized behaviors are unusual biological features that deserve special explanations, as they are rare exceptions from the near universal rule of bilateral symmetry of almost all animals. Such asymmetries involve genetic and/or functional separation among traits that usually require an adaptive explanation. The dichotomous nature of asymmetry facilitates the determination of the covariance among traits (Schilthuisen 2013; Palmer 2016; Wiper 2017). We discovered new asymmetry and

lateralization for multiple traits in the onesided livebearer fish, and, interestingly, the degree of integration varied highly among those traits. Lateralization in exploratory behavior in response to different stimuli were not correlated nor showed any associations to genital morphology. Thus, we found no evidence that hemispheric specialization of the brain results in a constraint in the pattern of behavioral lateralization in the studied species. However, some interesting associations were observed between genital asymmetry and sidedness of mating attempts as well as between genital asymmetry and the number of neuromasts in the lateral line. Sexual selection might have acted in the integration of these traits to maximize copulatory success in the context of the coercive mating behavior of this species (Bisazza et al. 2000b).

Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

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Authors' Contributions

J.T-D., S.J.R., and A.M. developed the project. G.A. provided the data on the wild caught populations from Argentina and G.G. collected the corresponding data from Uruguay. S.J.R. and J.T-D. conducted the laboratory experiments and analyzed the data. J.T-D. wrote the manuscript with revisions from all authors.

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