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The direction of genital asymmetry is expressed stochastically in internally fertilizing anablepid fishes

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Animal genitalia vary considerably across taxa, with divergence in many morphological traits, including striking departures from symmetry. Different mechanisms have been proposed to explain this diversity, mostly assuming that at least some of the phenotypic variation is heritable. However, heritability of the direction of genital asymmetry has been rarely determined. Anablepidae are internally fertilizing fish where the anal fin of males has been modified into an intromittent organ that transfers sperm into the gonopore of females. Males of anablepid fishes exhibit asymmetric genitalia, and both left- and right-sided individuals are commonly found at similar proportions within populations (i.e. antisymmetry). Although this polymorphism was described over a century ago, there have been no attempts to determine if genital asymmetry has a genetic basis and whether the different morphs are accumulating genetic differences, as might be expected since in some species females have also asymmetric gonopores and thereby can only be fertilized by compatible asymmetric males. We address this issue by combining breeding experiments with genome-wide data (ddRAD markers) in representative species of the two anablepid genera with asymmetric genitalia: *Anableps* and *Jenynsia*. Breeding experiments showed that all offspring were asymmetric, but their morphotype (i.e. right- or left-sided) was independent of parental morphotype, implying that the direction of asymmetry does not have a strong genetic component. Consistent with this conclusion, association analyses based on approximately 25 000 SNPs did not identify markers significantly associated with the direction of genital asymmetry and there was no evidence of population structure between left- and right-sided individuals. These results suggest that the direction of genital asymmetry in anablepid fishes might be stochastic, a commonly observed pattern in species with antisymmetry in morphological traits.

1. Introduction

The genitalia of animals with internal fertilization are meant to perform, in principle, a simple task: to transfer sperm to the place where fertilization of eggs occurs. Yet, in many species, genitalia are extremely elaborated, much beyond what this apparently simple function requires. The complexity of animal genitalia is fascinating, and several sexual selection evolutionary hypotheses have been proposed to explain their degree of elaboration [1–4]; but generalizations across taxa have been challenging due to the great diversity in structure and function [5]. Apart from size and shape, in some cases, genitalia have an additional layer of complexity: they can be asymmetric [6]. In males, and more rarely females, of some animals—including arthropods (e.g. [7,8]), most snakes (e.g. [9]), most ruminant mammals [10], waterfowl (e.g. [11]) and a few fish lineages with internal fertilization (e.g. [12–14])—the structures that make direct contact with the sexual partner during copulation and that are involved in sperm transfer

are structurally asymmetric (reviewed in [5]). Genital asymmetry is an unusual trait that is interesting at many different levels of biological organization and is particularly important for evolutionary biologists because of its potential to drive speciation if mating success is reduced between opposite asymmetric morphs and thereby contributes to population divergence [15,16] (but see [17]). Moreover, the simple left–right nature of asymmetries has the advantage of permitting comparisons, not only between sexes within a species, but also across species, thus allowing for generalization beyond individual cases [5,18]. Nonetheless, there are still several aspects of the evolution of genital asymmetries that are not well understood, in particular the genetic basis of the direction of departure from symmetry has been hardly studied [5,19]. The great majority of species with asymmetric genitalia are monomorphic in the direction in which their genitalia bends [5] (i.e. directional asymmetry *sensu* [20]). Species where both right and left morphs are found at nearly equal proportions (i.e. antisymmetry *sensu* [20]) are rare exceptions [5,7]. That most species show directional genital asymmetry has been interpreted as evidence that the bending direction is inherited [5]. If direction of asymmetry indeed has a large genetic component, then a polymorphism could be maintained if inter-morph breeding is favoured over intra-morph breeding (e.g. [21]), or the success of one morph is dependent on the relative frequency of both morphs (e.g. negative-frequency-dependent selection [22]). Alternatively, it could be expected that mating between different asymmetric morphs might be less successful (i.e. results in fewer effective copulations) than those between compatible asymmetric morphs (e.g. [16,23]), and that this could lead to the accumulation of genetic differences between morphs and potentially result in speciation [15,16,24]. In turn, this might explain the observed high abundance of species showing directionally asymmetric genitalia [5]. However, studies on other asymmetric morphologies have shown that the genetic contribution varies significantly among species (reviewed in [25]). Whereas in some cases there is a large genetic component underlying the direction of asymmetry [16,26,27], in other cases there is a strong environmental effect (e.g. [28]) or it is mostly stochastic (e.g. [29]). Moreover, the overwhelming excess of species with directional genital asymmetry suggests that sidedness of asymmetry has a large genetic component, but no statement can be made about its heritability due to the lack of variation in the trait in question (i.e. there is only one asymmetric morph). Heritability (in its broad sense) is understood as the proportion of the phenotypic variation in a population that is due to genetic variation among individuals [30]; thus, if there is no phenotypic variation to explain (e.g. all individuals are left-sided), no conclusions can be drawn about the heritability of the trait. Consequently, the rare polymorphic species showing antisymmetry in the sidedness of genitalia become key to address this question [25].

One of these rare cases are the livebearing fishes of the family Anablepidae [5,14,31–34]. This small family of Neotropical fishes comprises only three genera: *Oxyzygonectes* (the white-eye, 1 species), *Anableps* (four-eyed fish, 3 species) and *Jenynsia* (one-sided live-bearers, 15 species). Anablepids have attracted the attention of naturalists for centuries (first mentioned as early as 1608 [35]) as these fish combine a set of highly peculiar characteristics, including a ‘split’ eye that confers aerial and aquatic vision (only in species of *Anableps*, but not of *Jenynsia* or *Oxyzygonectes* [36,37]), being live-bearers

(except for *Oxyzygonectes*) by the retention of embryos during development in the ovarian follicles [38], and the feature we are focusing on here: conspicuous asymmetry in their genitalia (sexual rights and lefts, *sensu* [14]). Whereas *Oxyzygonectes* is an oviparous species, all species in the genera *Anableps* and *Jenynsia* are viviparous and males have a modified anal fin, called gonopodium, that serves as an intromittent organ that transfers sperm into the female genital tract [39]. The conspicuous genital asymmetry is caused mostly by ray number six of the anal fin, the thickest and longest, which is laterally displaced and bends at the tip forming a hook (figure 1). This bending occurs to either side and thus, left and right morphs are found within populations [14,31–33]. Females in the genus *Jenynsia* always have symmetrical genital openings and both male morphs can, therefore, potentially copulate with all females [31,40]. However, females of the genus *Anableps* also show an asymmetrical genital opening (the gonopore), which is laterally covered by one or two large scales forming a foricula [14,39]. Within populations of *Anableps* species, females with left- and right-sided foriculae openings are found [14,32,33]. Anablepid fish copulate in a lateral, side-by-side position with their heads pointing in the same direction [32,41]. Males position themselves at either side of the females and laterally move their gonopodium forward in an attempt to insert its tip into the female’s gonopore to transfer sperm for internal fertilization. A consequence of genital asymmetry exhibited by both sexes in *Anableps* species is that only males whose gonopodium bends to the left can copulate with females that have the foricula opening to the right and vice versa [33,39,42].

Despite the long interest in the ‘sexual rights and lefts’ of anablepids and the general interest in the genetics of genital polymorphisms, there had so far been no attempts to determine the heritability of the direction of genital asymmetry in these fish. However, this information is crucial for understanding the evolution and maintenance of this polymorphism and its potential for generating population structure (e.g. [15]). In his classic paper about the genital asymmetry of anablepids, Garman [14] discusses two potential scenarios. Under the first, the direction of asymmetry is mostly heritable, and it should be possible to establish monomorphic populations by artificially selecting for (selectively breeding) only one morph. Under the second scenario, the direction is determined solely by environmental factors and establishing monomorphic populations should not be possible (at least not if the environment is allowed to change). He favoured the second possibility, and subsequently, this was taken as evidence for the lack of a genetic basis of the trait by others (e.g. [43]), yet no formal tests of these hypotheses had been conducted so far.

Here, we study two polymorphic species of the genera *Anableps* and *Jenynsia* with the aim of contributing to our understanding of the evolutionary stability of this genital polymorphism. Determining the genetic basis of this trait would help to hypothesize if the polymorphism in the direction of genital asymmetry represents a stable state or an intermediate stage in the transition to uniform directional asymmetry and potentially speciation. First, we conducted captive breeding experiments in a common garden environment with *J. lineata* and *A. anableps* to determine the heritability of gonopodium direction. We complemented this approach by conducting RAD-sequencing in two wild-caught population samples of *J. lineata* and a second species of *Anableps* (*A. dowei*) to see if there are alleles that segregate with the two genital morphs

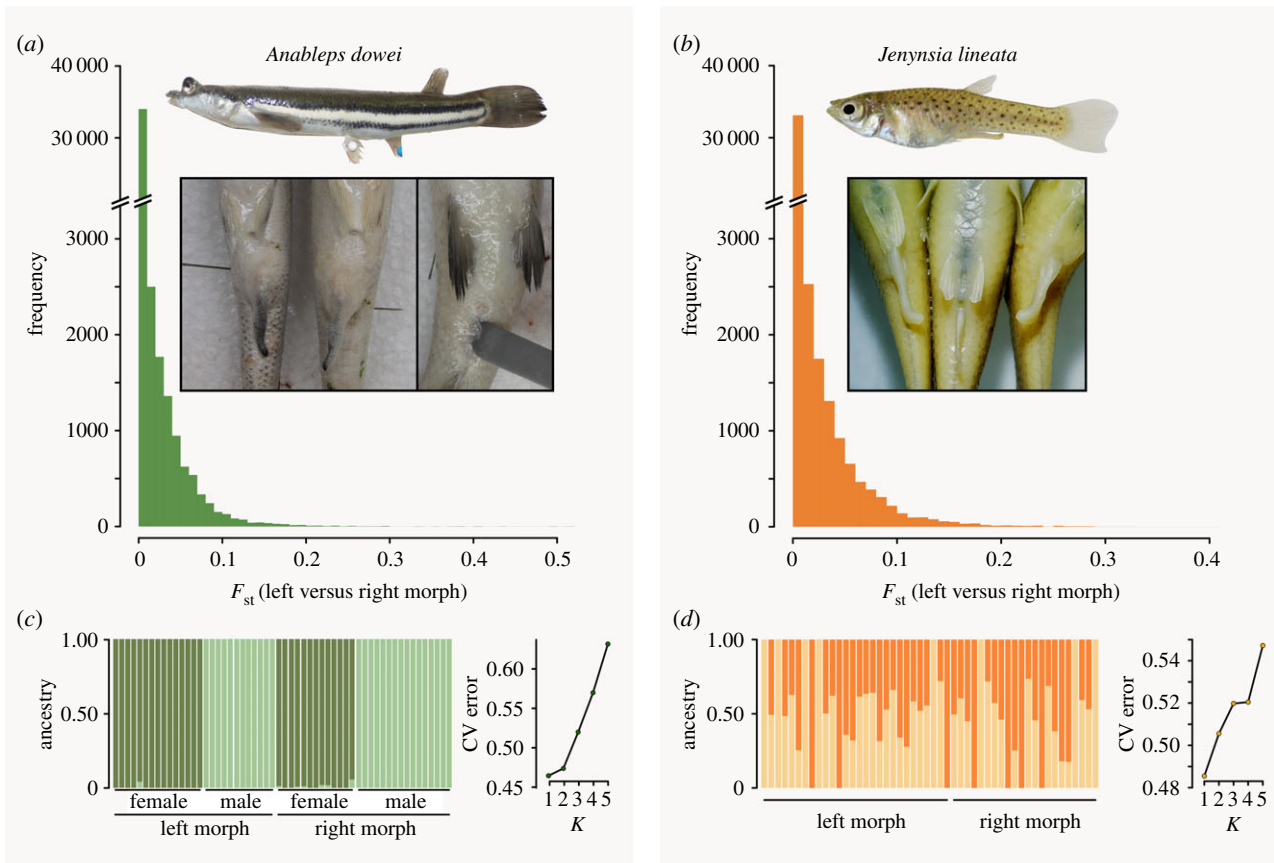


Figure 1. Left and right morphs of *A. dowei* (a) and *J. lineata* (b) show low genetic differentiation, with the vast majority of variants having Weir and Cockerham's F_{st} values equal to zero. Photo insets show two males (a left and a right morph) and a female (a right morph) of *A. dowei* (a), and two *J. lineata* males (a left and a right morph) with a symmetric female in the middle (b). As a consequence of low differentiation between morphs, there is also a lack of population structure where the number of clustering with the lowest cross-validation error was one for *A. dowei* (c) and *J. lineata* (d). Some structure is observed in *A. dowei*, but this is associated with sex and not genital morphology. (Online version in colour.)

and to determine if genetic differences have accumulated between them.

2. Material and methods

(a) Breeding experiment

To determine if variation in the direction of asymmetry has a heritable component, we conducted crosses using *J. lineata*. Females of this species have symmetrical genitalia, whereas males can be either left- or right-sided [31]. A total of 30 couples were placed separately into 9.8 l tanks [11 × 51 × 23 cm] in a recirculating system (12 L : 12D cycle, 25°C ± 1°C) and fed twice daily with recently hatched brine shrimp and commercial flake food. Fifteen pairs were composed of left-sided males and 15 of right-sided males. None of the females used were exposed to mature males before the experiment. Pregnant females (identified by an enlargement of the abdominal cavity) were isolated before parturition and returned to their tank 24 h after giving birth. Offspring were reared in groups of 5–10 individuals and inspected daily for signs of gonopodium development (i.e. elongation of the anal fin [39]), at which point immature males were reared in isolation until their gonopodium was completely formed and genital asymmetry could be determined based on external morphology (i.e. in left-sided males, their gonopodium bends to the left; figure 1).

We first assessed whether the direction of genital asymmetry in *J. lineata* male offspring departs from a 1 : 1 ratio and whether this ratio depends on the morphotype of the sire. For example, if

the direction of asymmetry was determined by a single autosomal dominant gene, we would expect that, on average, offspring of males expressing the dominant phenotype (i.e. homozygous dominant or heterozygous) will have a 87.5% probability of resembling their sire (assuming a random genotype of females; only heterozygous males that are crossed with heterozygous or homozygous recessive females could produce homozygous recessive males, which overall have probabilities of 4.17% and 8.33%, respectively); whereas, left and right morphs are expected to be equally likely (approx. 50%) among offspring of males expressing the recessive phenotype (again, assuming a random genotype of females). To test this, we fitted a generalized linear model (GLM) in R. The model assumed a binomial distribution with a logit link function and the response variable was specified using the *cbind* function to create a matrix with the number of the left- and right-sided offspring in each family. Sire morphotype was included as a fixed effect in the model.

Additionally, we estimated the narrow-sense heritability following Davies *et al.* [44]. Considering the direction of genital asymmetry as a binary trait (i.e. left or right), we calculated Bayesian R^2 from the fit of a generalized linear mixed model with a logit link function, a binomial error distribution and sire as a random effect [44]. Note that the estimate derived from this procedure refers to the heritability of an assumed latent, continuously distributed variable that underlies the switch between morphs (i.e. a threshold trait; [44]). The statistical significance of the estimate was assessed by comparing it to the distribution obtained by using a permutation-based approach that shuffles the identity of each offspring's sire 1000 times. These analyses were conducted in R using the multiDimBio package [45].

We also bred a species of *Anableps* (*A. anableps*) in captivity to determine if the results were consistent with those obtained with *J. lineata*. However, space limitations necessary for breeding *A. anableps* (mature females are approximately 200 mm and males are 140 mm; J.T.D. 2019, personal observation; also see [46]) affected the number of families that could be raised. We kept *A. anableps* in tanks with a large surface area (200 × 95 cm, and 45 cm depth, 760 l) at 25°C and in brackish water (6 g l⁻¹, Preis Aquaristik Marine Salt). Initial attempts to keep fish in pairs were unsuccessful as they failed to breed, potentially due to stress as *A. anableps* are gregarious fish [32]. Thus, we maintained two group tanks with five females and five males of the same morph each. Thus, results have to be taken with caution as offspring of a female could potentially have been sired by multiple males. Pregnant females were isolated in tanks of the same conditions until they gave birth. Females were allowed to recover for 24 h before being returned to their original tanks. Offspring were maintained in a common tank until they reached sexual maturity, at which point individual fish were phenotyped as described above. In the case of *A. anableps*, individuals were not isolated to avoid stressing them, so the direction of asymmetry might have been also influenced by the social environment. The maintenance of the fish and the experiments described above have been approved by the State of Baden-Württemberg (permit no. 35-9185.81/G-17/110).

(b) RADseq data analyses

(i) Sample collection for genome-wide sequencing

Twenty-eight *A. dowei* males (16:12 right:left-sided) and 28 females (13:15) were collected in Nicaragua in 2015 (permit number DGNP/DB-IC-015-2015). Fifty *J. lineata* males (22:28) were collected in Argentina in 2013 (permit number DFFS-1757/330; electronic supplementary material, table S1). *Jenynsia lineata* males are asymmetric but females are not [31], whereas both males and females show genital asymmetry in *A. dowei* [32]. Phenotyping was based on the external morphology of the genitalia (foricula of the gonopore in females and gonopodium in males; figure 1). We classified each individual as left- or right-sided based on the position of the female in relation to the male during copulation. Both species copulate in a side-to-side position with the head of both sexes pointing in the same direction ([32,41] J.T.D. 2019, personal observations). Accordingly, males and females are classified as left morphs if their genital morphology requires that the female is in the left position during mating (i.e. in lefty males their gonopodium bends to the left and in lefty females of *A. dowei*, the foricula of the gonopore is open to the right). This terminology has the advantage of allowing to refer to compatible mates. Additionally, because laterality in mating behaviour is perfectly associated with genital asymmetry [31], this terminology is in agreement with the one used for the breeding experiment.

(ii) Library preparation and sequencing

Population genomic data were generated following the quaddRAD protocol; a double digest RADseq method that allows for high multiplexing of samples and PCR-duplicate detection [47]. Briefly, all individuals were pooled into one library, size selected for a range of 455–555 bp using a Pippin Prep electrophoresis system (Sage Science, Beverly, MA, USA) and the final libraries were diluted to 10 nM. The library was paired-end sequenced (2 × 150 bp) in one lane on an Illumina HiSeq 2500 machine (TUFTS core facility). Demultiplexing based on Illumina indices produced pools comprising 11–12 samples with unique combinations of inline barcodes. Putative PCR-duplicate reads were identified and removed with the *clone_filter* script prior to demultiplexing based on inline barcodes using the *process_radtags* script in Stacks v. 1.46 [48].

(iii) RAD tag assembly, read mapping and variant calling

Mapping and variant calling was conducted with the *dDocent* package [49] using a de novo assembly approach for both species together. *dDocent* was run using the default parameters, internally using *bwa mem* [50] for read mapping and *freebayes* [51] for variant and genotype calling. Variant processing and filtering was done using *VCFtools* v. 0.1.15 [52] following the *dDocent* user guide. Specifically, we set all individual genotype calls with a read depth of less than 3 to missing and then filtered variants using a minimum quality score of 1, a lower minor allele count threshold of 3. We also filtered out loci with more than 60% missing data. Further, variant site filtering was done following the *dDocent_filters* script using the recommended options (<http://ddocent.com/filtering/>). The resulting VCF file for both species contained a total of 38 621 RADtags and 151 275 variants (49 390 polymorphic in *A. dowei* and 46 518 in *J. lineata*) that passed quality filters. Assuming a genome size similar to that seen in Poeciliidae (the closest relatives with sequenced genomes [53,54]) of around 700 Mb, this means that there is around one marker every 18–27 kb for both species, considering the number of RADtags and the final number of variants used in the downstream analyses (see below).

(iv) Association mapping with genome-wide markers

Association mapping with the genome-wide markers generated with quaddRAD was performed using *PLINK* v. 1.90b4.9 [55,56] for each of the species separately, setting direction of genital asymmetry as a binary case/control trait. As *J. lineata* females are symmetric, they were not included in this study. For *A. dowei*, females were considered either left- or right-sided as indicated above (see ‘Sample collection for genome-wide sequencing’). First, we conducted an analysis for both *A. dowei* sexes together considering compatible morphs (as described above). Significant associations in this case will be expected to contribute to genetic differentiation between morphs. Then, we conducted a second analysis grouping incompatible males and females (i.e. males with their gonopodium bending to the left and females with the foricula of the gonopore opening to the left). Significant associations in this case will be expected to promote the maintenance of the left-right polymorphism. For these analyses, we applied some additional variant filters, using only biallelic markers and removing variants with more than 20% missing data or a minor allele frequency of less than 0.05. After this, the dataset for *A. dowei* included 26 568 variants for 56 individuals and the one for *J. lineata* included 25 454 variants for 50 individuals. Significance of the associations was determined using Fisher’s exact tests and adaptive Monte Carlo permutations [57]. *p*-values were then corrected for family-wise error due to multiple testing using the Benjamini–Hochberg false discovery rate.

(v) Population structure

We tested for allelic differentiation in form of Weir & Cockerham’s F_{st} [58] between asymmetric morphs on a per site basis using *VCFtools* [52]. We filtered the dataset in order to include only variants that had data for at least 10 individuals of each morph (i.e. 20 alleles per morph). After this additional filtering step, 43 006 variants remained for *A. dowei* and 40 050 for *J. lineata*. For *A. dowei*, we combined sexes by grouping compatible morphs.

Population structure was examined with *ADMIXTURE* [59], for $K = 1$ to $K = 5$ putative clusters for *A. dowei* and *J. lineata* separately. The optimal numbers of clusters were evaluated based on cross-validation error estimates [60]. Prior to the analysis, variants with more than 20% missing data and a minor allele frequency of less than 0.05 were filtered out, resulting in 32 475 variants for *A. dowei* and of 28 322 variants for *J. lineata*. Admixture results were visualized using the graphics package in R [61].

Table 1. Heritability of the direction of genital asymmetry appears to be low as the proportion of left-sided offspring from a male, independently of its own morph, is on average 50%.

species	pair	parent morph ^a	offspring morph	% (left)				
<i>Anableps anableps</i>	1	L	$n = 7$	♀	LL	RR	50	
				♂	L	RR	33	
	2	L	$n = 23$	♀	LLLLL	RRRRRRR	38	
				♂	LLLLL	RRRR	60	
<i>Jenynsia lineata</i>	1	L	$n = 3$		LL	R	67	
	2	L	$n = 5$		LLL	RR	60	
	3	L	$n = 7$		LLL	RRRR	43	
	4	L	$n = 8$		LLLLL	RRR	63	
	5	L	$n = 9$		LLLLL	RRRRR	44	
	6	L	$n = 14$		LLLLL	RRRRRRRR	36	
	7	L	$n = 19$		LLLLLLLLL	RRRRRRRR	53	
	8	L	$n = 21$		LLLLLLLLL	RRRRRRRRR	48	
	9	L	$n = 25$		LLLLLLLLL	RRRRRRRRRRR	44	
	10	L	$n = 27$		LLLLLLLLL	RRRRRRRRRR	56	
	11	R	$n = 4$			RRRR	0	
	12	R	$n = 4$			LLL	R	75
	13	R	$n = 10$			LLLLL	RRRRR	40
	14	R	$n = 11$			LLLLL	RRRR	50
	15	R	$n = 12$			LLLLLLLLL	RRR	75
	16	R	$n = 13$			LLLLLLLLL	RRRRR	54
	17	R	$n = 16$			LLLLLLLLL	RRRRR	63
	18	R	$n = 16$			LLLLLLLLL	RRRRRRRR	44
	19	R	$n = 18$			LLLLLLLLL	RRRRRRR	56
	20	R	$n = 18$			LLLLLLLLL	RRRRRRR	56
	21	R	$n = 21$			LLLLLLLLL	RRRRRRR	62

^aFor *A. anableps*, we report the morph of the female as the sire was unknown.

3. Results

(a) No evidence for a simple genetic basis of direction of genital asymmetry

For *J. lineata*, 21 families produced more than three male offspring, thereby allowing to determine the ratio of left- to right-sided males (table 1). Offspring from left-sided sires did not significantly depart from 1:1 ratio in the direction of their genitalia (intercept effect estimate = -0.03 ± 0.17 s.e., $p = 0.86$) and the left:right offspring ratio did not differ between sire morphs (sire_(right) effect estimate = 0.24 ± 0.23 s.e., $p = 0.32$; table 1). Then, right-sided sires were set as reference in our model to verify that their offspring did not differ from 1:1 either (intercept effect estimate = -0.21 ± 0.17 s.e., $p = 0.21$). Eliminating four families that had only few offspring (i.e. less than seven male offspring), and, therefore, could have biased the analysis, did not affect these results. Hence, there is no evidence that male offspring of *J. lineata* depart from a 1:1 ratio in the direction of their genitalia. In line with these results, estimated narrow-sense heritability was not significantly different from zero ($h^2 = 2.25 \times 10^{-28}$, $p = 0.48$).

Unfortunately, due to the space required for *A. anableps* to breed, our sample size is small and mostly anecdotal (table 1).

However, given that males and females in this species show genital asymmetry, finding both morphs among offspring of the two broods at approximately equal proportions suggests nonetheless that the direction of asymmetry is not due to a single Mendelian locus.

(b) No evidence for loci associated with the direction of genital asymmetry

In line with the breeding experiment, the whole-genome association analysis identified no genetic variant significantly associated with the direction of genital asymmetry in *A. dowei* or *J. lineata* after family-wise error correction for multiple testing (electronic supplementary material, table S2).

(c) No evidence of population structure between individuals differing in the direction of genital asymmetry

Overall, there was essentially no differentiation between asymmetric morphs within samples of both *A. dowei* and *J. lineata* (Weir & Cockerham's weighted mean $F_{st} < 0.001$ for both species; figure 1a,b). Accordingly, there was no evidence of population

structure, with the highest support for only one genetic cluster in both species (figure 1*c,d*). There was some structure in *A. dowei*; however, this appeared to be associated with sex rather than with the direction of genital asymmetry. These findings suggest that genital asymmetry does not result in the accumulation of genetic differences between incompatible morphs.

4. Discussion

In 1895, Garman [14] described the polymorphic genitalia of fish of the genus *Anableps*, reporting the presence of left and right morphs at equal frequencies within populations and discussed the role of mating incompatibility between morphs as a potential driver of differentiation and speciation into two asymmetric, but monomorphic species. For this to occur, variation in the direction of asymmetry must have a genetic component. Here, a series of experiments, including breeding experiments and genome-wide RAD sequencing, strongly suggest that there is no strong heritable component of direction of genital asymmetry in anablepid fishes. Instead, the breeding experiments suggest that the direction of asymmetry appears to have a large stochastic component (i.e. direction is determined by chance and cannot be experimentally biased towards one particular side; [25]). Moreover, there is no evidence for genome-wide differentiation between left and right morphs neither in *Anableps dowei* nor in *Jenynsia lineata*. These findings provide an explanation for the long-term maintenance of this polymorphism.

The lack of population structure is not necessarily evidence against the direction of genital asymmetry having a genetic component, as there are many examples of genetically determined polymorphisms affecting mating strategy that do not result in genome-wide differentiation (e.g. [62–64]). Stable polymorphisms can result from different selection pressures acting on the population, including disruptive selection and frequency-dependent selection (e.g. [65–67]). In fact, we had previously suggested that the temporal oscillation in the relative abundance of asymmetric morphs in *J. lineata* males might be explained by frequency-dependent selection; the rare morph might have an advantage at forced copulations, given that females are symmetrical in terms of their genitalia [31]. However, for frequency-dependent selection on breeding success to affect the relative abundance of both morphs, there must be a heritable component to the phenotypic variation [68]. Thus, this seems to be a less plausible explanation, given that the heritability of the direction of asymmetry was not different from zero in *J. lineata* (we have no estimate of heritability for *Anableps* due to the low sample size and unknown sires) and that there is no evidence of loci associated with this trait in either of the analysed species. Instead, these results are in line with the general pattern found in other antisymmetric species (i.e. both asymmetric morphs found at equal proportions), where direction of departure from symmetry most commonly lacks a strong genetic basis [20,24] (but see [69]).

A caveat to this conclusion is that determining the genetic component of discrete phenotypic variance is challenging [30,44]. If the genetic component underlying variation is small, modestly sized breeding experiments, such as ours, and even large-scale ones might not have the power to detect it. Additionally, there are clear limitations to association analyses based on RADseq data (sparse markers that do not

necessarily cover the entire genome [70]; but see [71]), especially if the extent of linkage disequilibrium is unknown and there is no closely related reference genome available. Finally, whereas offspring in the breeding experiments were raised separately under controlled conditions that limited environmental effects influencing the direction of asymmetry, we cannot completely rule out that environmental factors affect the proportion of left- versus right-sided individuals in natural populations. However, the similarities seen in the near-equal proportion of left and right morphs in natural populations [14,31,33,34] and in the breeding experiment suggest that no other mechanism than stochastic variation is needed to explain this polymorphism in the direction of genital asymmetry. Stochastic developmental variation of direction of asymmetry has been suggested for different systems (reviewed in [25]), including egg formation (e.g. [72]), embryonal development (e.g. [73]), and adult internal [74,75] and external morphology (e.g. [29,76,77]). Thinking of the direction of genital asymmetry in anablepid fish as being determined by stochastic factors allows us to go back and evaluate the predictions originally made by Garman [14] about the evolutionary stability of this peculiar trait. At the population level, the expectation would be a stable polymorphism as most of the phenotypic variation is random. Indeed, no evidence of population structure associated with the phenotype was observed in natural populations of *A. dowei* or *J. lineata* (figure 1). This is in line with previous observations of both asymmetric morphs remaining at approximately equal proportions over time [14,31–33] and space [31]. Similarly, at the phylogenetic level, the expectation that monomorphic, directional asymmetric species will evolve is low, given that the absence of heritability is due to low or no additive genetic variation for this trait (e.g. [78]). Nevertheless, the evolution of directional asymmetry from random asymmetry has occurred a considerable number of times (36–44% of cases; reviewed in [18]), including cases of genital asymmetry (e.g. [13,79,80]). These have been put forward as examples of genetic assimilation of non-heritable phenotypic variation [18]. It is worth noting that other authors have mentioned some populations of *Jenynsia* to be monomorphic in terms of the direction of their genitalia [33,81]. Unfortunately, these reports were not associated with a particular population and the systematics and classification of this family of fishes has been in flux in the last years (e.g. [82,83]), making it difficult to verify these claims. Ongoing studies conducted at our laboratory at the family level will shed light onto the phylogenetic pattern of genital asymmetry in anablepid fishes and inform on the general understanding of the evolution of genital asymmetry.

Ethics. This research was approved by the Regierungspräsidium Freiburg, Baden-Württemberg, Germany (reference number: G-17/110), the Ministerio de Ambiente y Recursos Naturales de Nicaragua (permit number DGP/DB-IC-015-2015) and the Dirección de Flora y Fauna Tucumán, Argentina (permit number DFFS-1757/330).

Data accessibility. The short-read data and associated information have been archived in NCBI SRA database under the Bioproject accession number PRJNA635446 (SAMN15041046-SAMN15041151). The data associated with the breeding experiment are all available in table 1.

Authors' contributions. J.T.-D., S.J.R. and A.M. developed the project. G.A. provided the field-caught populations from Argentina. S.J.R. and J.T.-D. conducted the crosses. A.F.K. and J.T.-D. analysed the RADseq data. J.T.-D. wrote the manuscript with revisions from all authors.

Competing interests. We declare we have no competing interests.

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References

- Eberhard WG. 1985 *Sexual selection and animal genitalia*. Cambridge, MA: Harvard University Press.
- Arnqvist G. 1997 The evolution of animal genitalia: distinguishing between hypotheses by single species studies. *Biol. J. Linnean Soc.* **60**, 365–379. (doi:10.1111/j.1095-8312.1997.tb01501.x)
- Hosken DJ, Stockley P. 2004 Sexual selection and genital evolution. *Trends Ecol. Evol.* **19**, 87–93. (doi:10.1016/j.tree.2003.11.012)
- Eberhard WG. 2010 Evolution of genitalia: theories, evidence, and new directions. *Genetica* **138**, 5–18. (doi:10.1007/s10709-009-9358-y)
- Schilthuisen M. 2013 Something gone awry: unsolved mysteries in the evolution of asymmetric animal genitalia. *Anim. Biol.* **63**, 1–20. (doi:10.1163/15707563-00002398)
- Abate ME, Eng AG, Kaufman L. 2010 Alarm cue induces an antipredator morphological defense in juvenile Nicaragua cichlids *Hyposiphys nicaraguensis*. *Curr. Zool.* **56**, 36–42. (doi:10.1093/czoolo/56.1.36)
- Huber BA, Sinclair BJ, Schmitt M. 2007 The evolution of asymmetric genitalia in spiders and insects. *Biol. Rev.* **82**, 647–698. (doi:10.1111/j.1469-185X.2007.00029.x)
- Schilthuisen M, de Jong P, van Beek R, Hoogenboom T, Schlochtern MMZ. 2016 The evolution of asymmetric genitalia in Coleoptera. *Phil. Trans. R. Soc. B* **371**, 20150400. (doi:10.1098/rstb.2015.0400)
- Shine R, Olsson M, LeMaster M, Moore I, Mason R. 2000 Are snakes right-handed? Asymmetry in hemipenis size and usage in gartersnakes (*Thamnophis sirtalis*). *Behav. Ecol.* **11**, 411–415. (doi:10.1093/beheco/11.4.411)
- Ludwig W. 1932 *Das rechts-links-Problem im Tierreich und beim Menschen: mit einem Anhang rechts-links-Merkmale der Pflanzen*. Berlin, Germany: Springer.
- Brennan PL, Prum RO, McCracken KG, Sorenson MD, Wilson RE, Birkhead TR. 2007 Coevolution of male and female genital morphology in waterfowl. *PLoS ONE* **2**, e418. (doi:10.1371/journal.pone.0000418)
- Langerhans RB. 2011 Genital evolution. In *Ecology and evolution of poeciliid fishes* (eds J Evans, A Pilastro, I Schlupp), pp. 228–240. Chicago, IL: University of Chicago Press.
- Parenti LR. 1986 Bilateral asymmetry in phallostethed fishes (Atherinomorpha) with description of a new species from Sarawak. *Proc. Calif. Acad. Sci.* **44**, 225–236.
- Garman S. 1895 Sexual rights and lefts. *Am. Nat.* **29**, 1012–1014.
- Ueshima R, Asami T. 2003 Evolution: single-gene speciation by left–right reversal. *Nature* **425**, 679. (doi:10.1038/425679a)
- Schilthuisen M, Davison A. 2005 The convoluted evolution of snail chirality. *Naturwissenschaften* **92**, 504–515. (doi:10.1007/s00114-05-0045-2)
- Richards PM, Morii Y, Kimura K, Hirano T, Chiba S, Davison A. 2017 Single-gene speciation: mating and gene flow between mirror-image snails. *Evol. Lett.* **1**, 282–291. (doi:10.1002/evl3.31)
- Palmer AR. 2004 Symmetry breaking and the evolution of development. *Science* **306**, 828–833. (doi:10.1126/science.1103707)
- Kamimura Y, Iwase R. 2010 Evolutionary genetics of genital size and lateral asymmetry in the earwig *Euborellia plebeja* (Dermaptera: Anisolabididae). *Biol. J. Linnean Soc.* **101**, 103–112. (doi:10.1111/j.1095-8312.2010.01491.x)
- Palmer AR. 2005 Antisymmetry. In *Variation* (eds B Hallgrímsson, BK Hall), pp. 359–397. Amsterdam, The Netherlands: Elsevier.
- Schilthuisen M, Craze P, Cabanban A, Davison A, Stone J, Gittenberger E, Scott BJ. 2007 Sexual selection maintains whole-body chiral dimorphism in snails. *J. Evol. Biol.* **20**, 1941–1949. (doi:10.1111/j.1420-9101.2007.01370.x)
- Hori M. 1993 Frequency-dependent natural selection in the handedness of scale-eating cichlid fish. *Science* **260**, 216–219. (doi:10.1126/science.260.5105.216)
- Lang M, Orgogozo V. 2012 Distinct copulation positions in *Drosophila pachea* males with symmetric or asymmetric external genitalia. *Contr. Zool.* **81**, 87–94.
- Palmer AR. 1996 From symmetry to asymmetry: phylogenetic patterns of asymmetry variation in animals and their evolutionary significance. *Proc. Natl Acad. Sci. USA.* **93**, 14 279–14 286. (doi:10.1073/pnas.93.25.14279)
- Palmer AR. 2016 What determines direction of asymmetry: genes, environment or chance? *Phil. Trans. R. Soc. B* **371**, 20150417. (doi:10.1098/rstb.2015.0417)
- Spéder P, Ádám G, Noselli S. 2006 Type ID unconventional myosin controls left–right asymmetry in *Drosophila*. *Nature* **440**, 803–807. (doi:10.1038/nature04623)
- Policansky D. 1982 Flatfishes and the inheritance of asymmetries. *Behav. Brain Sci.* **5**, 262–265. (doi:10.1017/S0140525X0001181X)
- Govind C, Pearce J. 1992 Mechanoreceptors and minimal reflex activity determining claw laterality in developing lobsters. *J. Exp. Biol.* **171**, 149–162.
- Edelaar P, Postma E, Knops P, Phillips R. 2005 No support for a genetic basis of mandible crossing direction in crossbills (*Loxia* spp.). *Auk* **122**, 1123–1129. (doi:10.1093/auk/122.4.1123)
- Falconer D. 1989 *Introduction to quantitative genetics*, 3rd edn. Harlow, UK: Longman Scientific & Technical.
- Torres-Dowdall J, Rometsch SJ, Aguilera G, Goyenola G, Meyer A. 2020 Asymmetry in genitalia is in sync with lateralized mating behavior but not with the lateralization of other behaviors. *Curr. Zool.* **66**, 71–81. (doi:10.1093/cz/zoz019)
- Miller RR. 1979 Ecology, habits and relationships of the middle american cuatro ojos, *Anableps dowi* (Pisces: Anablepidae). *Copeia* **1979**, 82–91. (doi:10.2307/1443732)
- Hubbs CL, Hubbs LC. 1945 Bilateral asymmetry and bilateral variation in fishes. *Pap. Mich. Acad. Sci. Arts Lett.* **30**, 229–311.
- Neville AC. 1976 *Animal asymmetry*. London, UK: E. Arnold.
- Baughman J. 1947 An early mention of *Anableps*. *Copeia* **1947**, 200. (doi:10.2307/1438859)
- Klinkowstrøm A. 1895 Beitrage zur Kenntnis das Auge von *Anableps tetropthalmus* Skan. *Arch Physiol.* **5**, 67–69.
- Rennison DJ, Owens GL, Taylor JS. 2012 Opsin gene duplication and divergence in ray-finned fish. *Mol. Phylogenet. Evol.* **62**, 986–1008. (doi:10.1016/j.ympev.2011.11.030)
- Turner C. 1938 Adaptations for viviparity in embryos and ovary of *Anableps anableps*. *J. Morphol.* **62**, 323–349. (doi:10.1002/jmor.1050620208)
- Parenti LR. 1981 A phylogenetic and biogeographic analysis of cyprinodontiform fishes (Teleostei, Atherinomorpha). *Bull. Am. Mus. Nat. Hist.* **168**, 335–557.
- Bisazza A, Manfredi S, Pilastro A. 2000 Sexual competition, coercive mating and mate assessment in the one-sided livebearer, *Jenynsia multidentata*: are they predictive of sexual dimorphism? *Ethology* **106**, 961–978. (doi:10.1046/j.1439-0310.2000.00620.x)
- Mai ACG, Garcia AM, Vieira JP, Mai MG. 2007 Reproductive aspects of the one-sided livebearer *Jenynsia multidentata* (Jenyns, 1842) (Cyprinodontiformes) in the Patos Lagoon estuary, Brazil. *Panamjas* **2**, 40–46.
- Bisazza A, Rogers LJ, Vallortigara G. 1998 The origins of cerebral asymmetry: a review of evidence of behavioural and brain lateralization in fishes, reptiles and amphibians. *Neurosci. Biobehav. Rev.* **22**, 411–426. (doi:10.1016/S0149-7634(97)00050-X)

43. Kasinov VB. 1978 Is there a single basis for biological laterality? *Behav. Brain Sci.* **1**, 301–302. (doi:10.1017/S0140525X00074768)
44. Davies SW, Scarpino SV, Pongwarin T, Scott J, Matz MV. 2015 Estimating trait heritability in highly fecund species. *G3: Genes, Genomes, Genet.* **5**, 2639–2645.
45. Scarpino SV, Gillette R, Crews D. 2014 multiDimBio: an R package for the design, analysis, and visualization of systems biology experiments. arXiv preprint arXiv:14040594.
46. Oliveira VDA, Fontoura NF, Montag LFDA. 2011 Reproductive characteristics and the weight-length relationship in *Anableps anableps* (Linnaeus, 1758) (Cyprinodontiformes: Anablepidae) from the Amazon Estuary. *Neotrop. Ichthyol.* **9**, 757–766. (doi:10.1590/S1679-62252011005000042)
47. Franchini P, Monne Parera D, Kautt AF, Meyer A. 2017 quaddRAD: a new high-multiplexing and PCR duplicate removal ddRAD protocol produces novel evolutionary insights in a nonradiating cichlid lineage. *Mol. Ecol.* **26**, 2783–2795. (doi:10.1111/mec.14077)
48. Catchen J, Hohenlohe PA, Bassham S, Amores A, Cresko WA. 2013 Stacks: an analysis tool set for population genomics. *Mol. Ecol.* **22**, 3124–3140. (doi:10.1111/mec.12354)
49. Puritz JB, Hollenbeck CM, Gold JR. 2014 dDocent: a RADseq, variant-calling pipeline designed for population genomics of non-model organisms. *PeerJ* **2**, e431. (doi:10.7717/peerj.431)
50. Li H, Durbin R. 2009 Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics* **25**, 1754–1760. (doi:10.1093/bioinformatics/btp324)
51. Garrison E, Marth G. 2012 Haplotype-based variant detection from short-read sequencing. arXiv preprint arXiv:12073907.
52. Danecek P *et al.* 2011 The variant call format and VCFtools. *Bioinformatics* **27**, 2156–2158. (doi:10.1093/bioinformatics/btr330)
53. Meyer A, Lydeard C. 1993 The evolution of copulatory organs, internal fertilization, placenta and viviparity in killifishes (Cyprinodontiformes) inferred from a DNA phylogeny of the tyrosine kinase gene *X-src*. *Proc. R. Soc. Lond. B* **254**, 153–162. (doi:10.1098/rspb.1993.0140)
54. Reznick DN, Furness AI, Meredith RW, Springer MS. 2017 The origin and biogeographic diversification of fishes in the family Poeciliidae. *PLoS ONE* **12**, e0172546. (doi:10.1371/journal.pone.0172546)
55. Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, Lee JJ. 2015 Second-generation PLINK: rising to the challenge of larger and richer datasets. *Gigascience* **4**, 7. (doi:10.1186/s13742-015-0047-8)
56. Purcell S *et al.* 2007 PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81**, 559–575. (doi:10.1086/519795)
57. Che R, Jack JR, Motsinger-Reif AA, Brown CC. 2014 An adaptive permutation approach for genome-wide association study: evaluation and recommendations for use. *BioData Mining* **7**, 9. (doi:10.1186/1756-0381-7-9)
58. Weir BS, Cockerham CC. 1984 Estimating F-statistics for the analysis of population structure. *Evolution* **38**, 1358–1370.
59. Alexander DH, Novembre J, Lange K. 2009 Fast model-based estimation of ancestry in unrelated individuals. *Genome Res.* **19**, 1655–1664. (doi:10.1101/gr.094052.109)
60. Alexander DH, Lange K. 2011 Enhancements to the ADMIXTURE algorithm for individual ancestry estimation. *BMC Bioinf.* **12**, 246. (doi:10.1186/1471-2105-12-246)
61. R Core Team. 2019 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
62. Hurtado-Gonzales JL, Uy JAC. 2009 Alternative mating strategies may favour the persistence of a genetically based colour polymorphism in a pentamorphic fish. *Anim. Behav.* **77**, 1187–1194. (doi:10.1016/j.anbehav.2008.12.032)
63. Tuttle EM. 2003 Alternative reproductive strategies in the white-throated sparrow: behavioral and genetic evidence. *Behav. Ecol.* **14**, 425–432. (doi:10.1093/beheco/14.3.425)
64. Machado-Schiaffino G, Kautt AF, Torres-Dowdall J, Baumgarten L, Henning F, Meyer A. 2017 Incipient speciation driven by hypertrophied lips in Midas cichlid fishes? *Mol. Ecol.* **26**, 2348–2362. (doi:10.1111/mec.14029)
65. Olendorf R, Rodd FH, Punzalan D, Houde AE, Hurt C, Reznick DN, Hughes KA. 2006 Frequency-dependent survival in natural guppy populations. *Nature* **441**, 633–636. (doi:10.1038/nature04646)
66. Rosenblum EB. 2006 Convergent evolution and divergent selection: lizards at the white sands ecotone. *Am. Nat.* **167**, 1–15. (doi:10.1086/498397)
67. Torres-Dowdall J, Machado-Schiaffino G, Kautt AF, Kusche H, Meyer A. 2014 Differential predation on the two colour morphs of Nicaraguan Crater lake Midas cichlid fish—implications for the maintenance of its gold-dark polymorphism. *Biol. J. Linn. Soc.* **112**, 123–131. (doi:10.1111/bij.12271)
68. Ayala FJ, Campbell CA. 1974 Frequency-dependent selection. *Annu. Rev. Ecol. Syst.* **5**, 115–138. (doi:10.1146/annurev.es.05.110174.000555)
69. Raffini F, Fruciano C, Franchini P, Meyer A. 2017 Towards understanding the genetic basis of mouth asymmetry in the scale-eating cichlid *Perissodus microlepis*. *Mol. Ecol.* **26**, 77–91. (doi:10.1111/mec.13699)
70. Lowry DB, Hoban S, Kelley JL, Lotterhos KE, Reed LK, Antolin MF, Storfer A. 2017 Breaking RAD: an evaluation of the utility of restriction site-associated DNA sequencing for genome scans of adaptation. *Mol. Ecol. Resour.* **17**, 142–152. (doi:10.1111/1755-0998.12635)
71. Catchen JM, Hohenlohe PA, Bernatchez L, Funk WC, Andrews KR, Allendorf FW. 2017 Unbroken: RADseq remains a powerful tool for understanding the genetics of adaptation in natural populations. *Mol. Ecol. Resour.* **17**, 362–365. (doi:10.1111/1755-0998.12669)
72. Haigo SL, Bilder D. 2011 Global tissue revolutions in a morphogenetic movement controlling elongation. *Science* **331**, 1071–1074. (doi:10.1126/science.1199424)
73. Delattre M, Félix M-A. 2001 Development and evolution of a variable left–right asymmetry in nematodes: the handedness of P11/P12 migration. *Dev. Biol.* **232**, 362–371. (doi:10.1006/dbio.2001.0175)
74. Kurnit DM, Layton WM, Matthyse S. 1987 Genetics, chance, and morphogenesis. *Am. J. Hum. Genet.* **41**, 979.
75. Alcorn MR, Callander DC, López-Santos A, Torres Cleuren YN, Birsoy B, Joshi PM, Santure AW, Rothman JH. 2016 Heterotaxy in *Caenorhabditis*: widespread natural variation in left–right arrangement of the major organs. *Phil. Trans. R. Soc. B.* **371**, 20150404. (doi:10.1098/rstb.2015.0404)
76. Socha R, Nedvéd O, Zrzavý J. 1993 Unstable forewing polymorphism in a strain of *Pyrrhocoris apterus* (Hemiptera: Pyrrhocoridae). *Ann. Entomol. Soc. Am.* **86**, 484–489. (doi:10.1093/aesa/86.4.484)
77. Daugeron C, Plant A, Winkler I, Stark A, Baylac M. 2011 Extreme male leg polymorphic asymmetry in a new empidine dance fly (Diptera: Empididae). *Biol. Lett.* **7**, 11–14. (doi:10.1098/rsbl.2010.0726)
78. Maynard SJ, Sondhi K. 1960 The genetics of a pattern. *Genetics* **45**, 1039.
79. Kamimura Y. 2006 Right-handed penises of the earwig *Labidura riparia* (Insecta, Dermaptera, Labiduridae): evolutionary relationships between structural and behavioral asymmetries. *J. Morphol.* **267**, 1381–1389.
80. Parenti LR. 1996 Phylogenetic systematics and biogeography of phallostethid fishes (Atherinomorpha, Phallostethidae) of northwestern Borneo, with description of a new species. *Copeia* **1996**, 703–712. (doi:10.2307/1447535)
81. Langer WF. 1913 Beiträge zur Morphologie der viviparen Cyprinodontiden. *Gegenbaver's Morph. Jahrb.* **47**, 193–307.
82. Aguilera G *et al.* 2019 Molecular and morphological convergence to sulfide-tolerant fishes in a new species of *Jenynsia* (Cyprinodontiformes: Anablepidae), the first extremophile member of the family. *PLoS ONE* **14**, e0218810. (doi:10.1371/journal.pone.0218810)
83. Amorim PF. 2018 *Jenynsia lineata* species complex, revision and new species description (Cyprinodontiformes: Anablepidae). *J. Fish Biol.* **92**, 1312–1332. (doi:10.1111/jfb.13587)