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Genetic assimilation and the evolution of direction of genital asymmetry in anablepid fishes

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Phylogenetic comparative studies suggest that the direction of deviation from bilateral symmetry (sidedness) might evolve through genetic assimilation; however, the changes in sidedness inheritance remain largely unknown. We investigated the evolution of genital asymmetry in fish of the family Anablepidae, in which males' intromittent organ (the gonopodium, a modified anal fin) bends asymmetrically to the left or the right. In most species, males show a 1 : 1 ratio of left-to-right-sided gonopodia. However, we found that in three species left-sided males are significantly more abundant than right-sided ones. We mapped sidedness onto a new molecular phylogeny, finding that this left-sided bias likely evolved independently three times. Our breeding experiment in a species with an excess of left-sided males showed that sires produced more left-sided offspring independently of their own sidedness. We propose that sidedness might be inherited as a threshold trait, with different thresholds across species. This resolves the apparent paradox that, while there is evidence for the evolution of sidedness, commonly there is a lack of support for its heritability and no response to artificial selection. Focusing on the heritability of the left : right ratio of offspring, rather than on individual sidedness, is key for understanding how the direction of asymmetry becomes genetically assimilated.

Keywords:
Anableps, antisymmetry, gonopodium, *Jenynsia*, time-calibrated phylogeny

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1. Introduction

The role of genetic assimilation, the transition of phenotypic variation from being environmentally or stochastically determined to becoming genetically determined [1–3], has been debated for several decades [4,5]. In the last 20 years, the concept has regained interest owing to the accumulation of theoretical models [6] and empirical examples [7,8] that support genetic assimilation as a plausible evolutionary outcome of natural selection [4] and emphasize its potential role in driving diversification [9,10]. Empirical support for genetic assimilation of phenotypes has come from phylogenetic comparative studies of bilateral asymmetries (e.g. paired structures that differ in size or shape between sides, or medial structures that deflect or rotate to one side) [11,12]. In a number of cases, *antisymmetry* (defined as left- and right-sided morphs being at equal frequencies in a population) was evolutionarily ancestral to *directional asymmetry* (defined as almost all individuals in a population being either

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Figure 1. (*a*) Ventral view of the gonopodia of two *Jenynsia lineata* males (a left- and a right morph) with a symmetric female in the middle. (*b*) Cleared and stained gonopodium of *Jenynsia luxata*, showing that the rays of the modified anal fin are elongated, enlarged and intertwined, rendering the gonopodium asymmetric. In comparison, (*c*) the gonopodium of the fossil *Sachajenynsia pacha* appears to be symmetric (specimen PVL7044 as described in [22]), since all rays are of similar size and show no signs of lateral displacement as seen in other anablepid species (e.g. (*b*)). Photographs by G.A. (*a*,*b*) and Emilia Sferco (*c*). ant., anterior; dors., dorsal. (Online version in colour.)

left- or right-sided) [11]. These evolutionary transitions from antisymmetry to directional asymmetry are considered examples of genetic assimilation [11,13] because the direction of asymmetry is environmentally or stochastically determined in almost all studied antisymmetric species (e.g. [14–16]), but genetically determined in directionally asymmetric species [11,12,17].

Animal genitalia are known to be evolutionarily highly labile [18], and this even includes the evolution of asymmetry in genitalia [19]. In most known cases, directional asymmetry in genitalia evolved from a symmetric ancestral condition, but taxa with antisymmetric genitalia are exceptionally rare [19]. Those few lineages that include species with both antisymmetric and directionally asymmetric genitalia provide the rare opportunity to determine where the evolution of directionality falls on the continuum between a phenotype-precedes-genotype mode, compatible with genetic assimilation, and the more classical genotype-precedes-phenotype evolutionary mode (*sensu* [11]). Fish of the family Anablepidae represent one such lineage [19–21]. This family comprises three extant genera: *Oxyzygonectes* (white-eye fish, 1 species), *Anableps* (four-eyed fishes, 3 species) and *Jenynsia* (one-sided livebearers, 15 species), and three extinct genera: *Tucumanableps*, *Sanjuanableps* and *Sachajenynsia* (each with 1 species) [22–24]. Whereas *Oxyzygonectes* uses external fertilization and lays eggs, all species of the other two extant genera are viviparous and the anal fin of males has been modified into a gonopodium, an intromittent organ for internal fertilization (figure 1) [25,26]. Molecular and fossil data suggest that the gonopodium of anablepid fishes evolved once in the common ancestor of *Anableps* and *Jenynsia* [22,27]. In both genera, the gonopodium is asymmetric and the tip bends either to the left or to the right in approximately equal proportions in the species that have been studied so far (i.e. the three species of *Anableps* and *Jenynsia lineata*) [20,21]. Additionally, *Anableps* females also possess an asymmetric structure that laterally covers the gonopore, called a foriculum [20]. The antisymmetric pattern in males is associated with an apparent lack of heritability for bending sidedness, as the direction of asymmetry was found to be expressed stochastically in anablepid fishes [28]. However, some populations have been reported to be monomorphic in terms of genital sidedness [29,30]. This could indicate possible transitions between antisymmetry and directional asymmetry, which would suggest genetic assimilation of this character because of its lack of heritability [28]. Yet, these reports are anecdotal and not associated with particular populations or species [29,30]. Additionally, the phylogenetics of the family Anablepidae has been in flux [23,31], limiting our ability to understand the evolution of genital asymmetry in these fishes and the corresponding role that genetic assimilation might play in the evolution of this character.

To study the evolution of genital asymmetry in Anablepidae, we first determined the variation in the type of genital asymmetry in anablepids (i.e. antisymmetry, directional asymmetry), by phenotyping almost 2000 individuals (including all but two species with gonopodia) for direction of asymmetry. Second, to map this trait onto a phylogeny, we reconstructed a time-calibrated phylogeny of the family Anablepidae based on genome-wide single nucleotide polymorphism (SNP) data from 114 individuals comprising 17 out of the 19 extant species, as well as calibration times inferred from fossils of extinct species in this family. Finally, we tested if offspring genital asymmetry resembles their parental phenotype by conducting common garden breeding experiments in *Jenynsia tucumana*, a species that shows an excess of left-sided individuals in the wild. Combined, our three-pronged approach highlights the possible role of genetic assimilation in shaping sidedness of male asymmetric genitalia in anablepid fishes.

2. Material and methods

(a) Genital asymmetry phenotyping

To determine the relative abundance of left or right morphs of anablepid fish in natural populations, we combined field samples and examination of museum specimens. Samples of the genera *Anableps* and *Jenynsia* were obtained from Argentina (permit no. 214-9; expte. no. 1757-330-2019 to G.A.), Brazil (ICMBio/SISBIO permit no. 33278-4 to A.C.P. and 14028 to W.J.daG.), Uruguay (permit no. DINARA-002/2014 to G.G.) and Nicaragua (permit no. DGPN/DB-IC-015-2015 to J.T.-D. and A.M.). In total, 1 775 males and 213 females from 57 localities across these countries were examined for genital asymmetry (electronic supplementary material, table S1). The direction of the bending of the distal tip of ray 6 was used to classify males as left- or right-sided (J.T.-D. performed all the phenotyping), a phenotyping method with high repeatability ($R = 0.914 \pm 0.016$; see electronic supplementary material for more detail). Female gonopores were visually inspected to determine the presence of external structures that would render it asymmetric, but not for internal asymmetries, except for one population of *J. lineata*, which showed that internal anatomy of the female gonads is symmetric, at least in this species (J.T.-D. dissected eight females and compared the left and right gonads).

(b) Genome-wide sequencing

To reconstruct a phylogenetic hypothesis for Anablepidae, we included specimens of all three extant genera (*Oxyzygonectes*, 2 samples (monospecific genus); *Anableps*, 20 samples (all three nominal species); *Jenynsia*, 89 samples (13 out of 15 nominal species)), plus two outgroup species from their sister family, the Poeciliidae (*Poecilia wingei*, 3 samples; *Xiphophorus milleri*, 1 sample), resulting in a total of 115 samples (electronic supplementary material, table S2). Raw genomic data were generated following the quaddRAD protocol [32]. All individuals were pooled into three libraries, size-selected for a range of 455–555 bp, and each library was paired-end sequenced (2 × 150 cycles) in one lane on an Illumina HiSeq X10 machine (BGI). Filtering, mapping and variant calling were conducted with the *dDocent* package [33] using a de novo assembly approach. For further details, see electronic supplementary material.

(c) Species tree and divergence time analyses

We estimated the species tree and divergence times among anablepid species using a strict molecular clock method in the SNAPP v. 1.3.0 module [34] of BEAST v. 2.6.2 [35]. Because SNAPP is computationally demanding, we subsampled 34 individuals representing two specimens per species, resulting in a set of 408 bi-allelic SNPs without missing data per species, with an inter-SNP interval of 100. SNAPP was run specifying 1 000 000 Markov-chain Monte Carlo iterations, sampling every 500 iterations. The phylogenetic hypothesis proposed by Aguilera *et al.* [23] was used as the starting tree in the SNAPP molecular clock analysis. Two calibration points were set using a uniform distribution. The first calibration point was set at the last common ancestor of Poeciliinae + Anablepidae following Reznick *et al.* [36]. The minimum age (39.9 million years ago, Ma) was taken from [37,38] based on undescribed poecillid fossils from the Lumbrera Formation in northern Argentina. The maximum age (72 Ma) was taken from [39,40] based on Cyprinodontiformes fossils with uncertain relationships to living taxa in the families of Anablepidae and Poeciliidae from the Upper Cretaceous to Palaeocene of the El Molino Formation, Bolivia. The second calibration point was set at the *Anableps* + *Jenynsia* crown group, with a minimum of 12 Ma and a maximum of 72 Ma. The minimum age was taken from [22] based on *Tucumanableps cionei*, a fossil from the Miocene of Tucumán, Argentina and sister genus to *Anableps*. The maximum age was set as described above. We used the function *contMap* in the package *phytools* [41] in R [42] to map the proportion of left-sided males at the species level, as estimated in §2a (Genital asymmetry phenotyping) above, onto the SNAPP species tree. For further details, see the electronic supplementary material.

Using our full dataset (see §2b (Genome-wide sequencing) above), we also inferred the phylogenetic relationships among anablepid species using four different approaches: a multi-species coalescent approach in PAUP* (v. 4.0a (build 166); [43]), by maximum likelihood implemented in RAxML [44] and IQ-TREE [45], and by Bayesian inference in MrBayes [46]. All these analyses are presented in the electronic supplementary material.

(d) Breeding experiment

To determine if variation in the direction of asymmetry has a heritable component, we conducted crosses with *J. tucumana* following previously described protocols [28]. Females of this species have symmetrical genitalia. Males can be either left- or right-sided, yet there is an excess of left-sided males in natural populations (see Results below). A total of 25 pairs were kept separately in 9.8 l tanks (11 × 51 × 23 cm) in a recirculating aquarium system (12 L : 12 D cycle, 25 ± 1°C) and fed twice daily with brine shrimp nauplii and commercial flake food. Fifteen pairs were composed of left-sided males and 10 of right-sided males. The females used were laboratory reared and not exposed to mature males before the experiment. Pregnant females resulting from the experiment (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 five to ten individuals and inspected daily for signs of gonopodium development (i.e. elongation of the anal fin; [25]), 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 (around 10 to 14 weeks).

We first assessed whether the direction of genital asymmetry in male *J. tucumana* offspring departs from a 1 : 1 ratio and whether this ratio depends on the morphotype of the sire. To test this, we fitted a generalized linear model 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 left- and right-sided offspring in each family. Sire morphotype was initially included as a fixed effect in the model but dropped from the final model owing to the lack of significance.

3. Results

(a) Asymmetry in female genitalia is restricted to the genus *Anableps*

Females of all three species of *Anableps* showed genital asymmetry, with both left- and right-sided morphs observed in proportions not significantly different from a 1 : 1 ratio (table 1). Structurally, this asymmetry is the result of a flap of tissue called a foriculum that is attached to either the left or the right of the gonopore, covering it laterally [20]. In the three species, a few females did not exhibit this tissue overgrowth, rendering them symmetric (table 1). By contrast, none of the *Jenynsia* species showed external asymmetry associated with the gonopore in females.

(b) Directional asymmetry in male genitalia evolved multiple times independently

Males of all species in Anablepidae presented conspicuous genital asymmetry, except for *Oxyzygonectes dovii*, which lacks a gonopodium. Antisymmetry (also referred to as random asymmetry) was the most common pattern observed in natural populations, with right- and left-sided morphs found at approximately equal proportions (i.e. no significant departure from 1 : 1 ratio; figure 2). Three species of *Jenynsia* (*Jenynsia obscura, Jenynsia sanctaecatarinae* and *J. tucumana*) departed from this pattern by exhibiting significant left-biased asymmetry (including both directional asymmetry, i.e. greater than 95% left-sided, and biased antisymmetry, i.e. significantly above 50% left-sided [16]; figure 2). Two other species (*Jenynsia darwini* and *Jenynsia luxata*) exhibited marginally significant ($0.05 < p < 0.1$) left-biased asymmetry (57 and 60%, respectively).

The only completely monomorphic (i.e. directionally asymmetric) species was *J. sanctaecatarinae*, with all 15

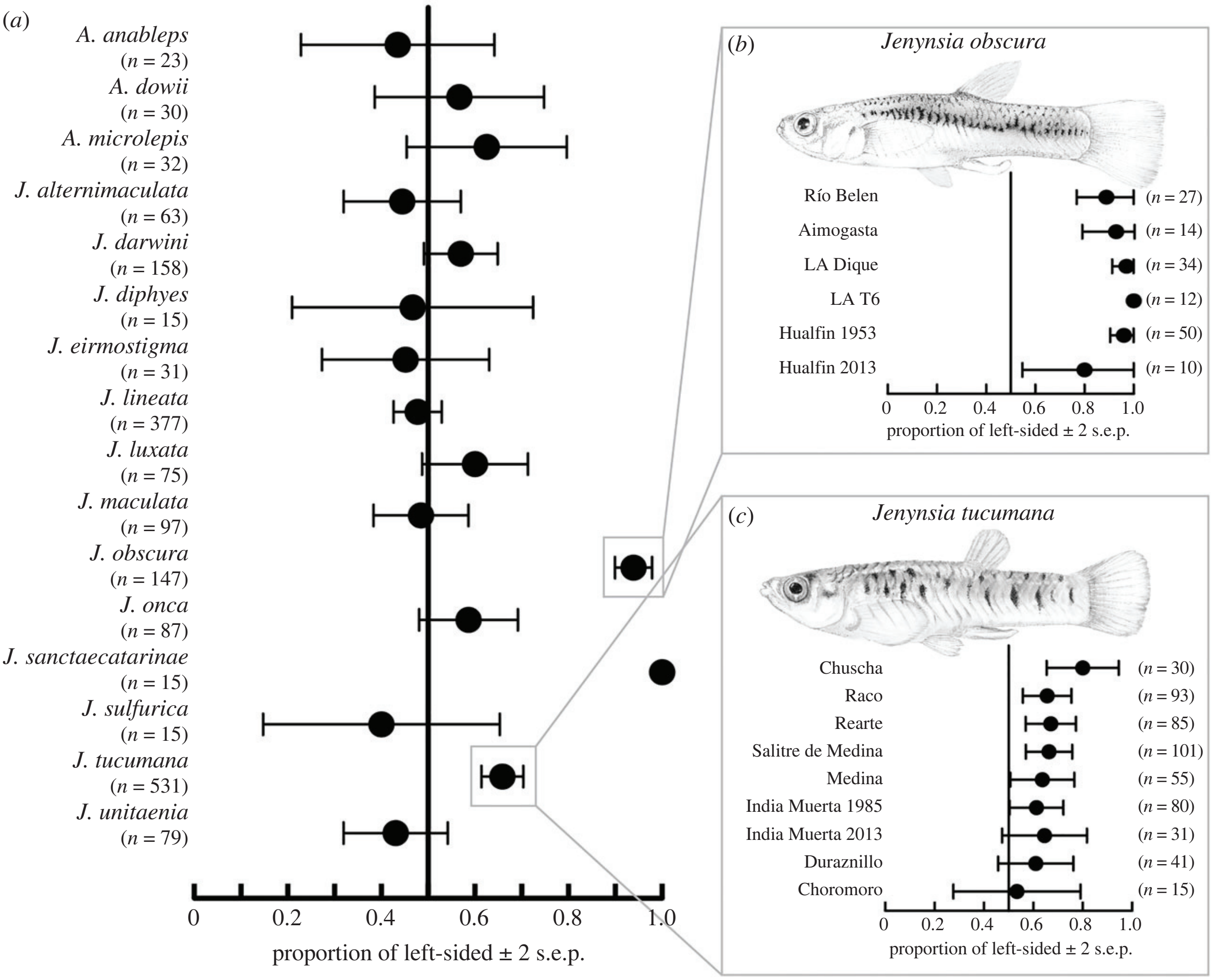

Figure 2. (*a*) Proportion ± 2 standard error of proportion (s.e.p.) of left-sided males among *Anableps* and *Jenynsia* species. Only three species significantly depart from a 1 : 1 ratio, indicating biased antisymmetry or directional asymmetry. For two of those species, *Jenynsia obscura* (*b*) and *Jenynsia tucumana* (*c*), we found that the bias was consistent among populations.

Table 1. Direction of genital asymmetry in female *Anableps*. (All examined females of *Jenynsia* had symmetric genitalia.) n.s, not significant.

species	right-sided	symmetric	left-sided	*z*
Anableps anableps	5	1	9	$1.07^{\text{n.s.}}$
Anableps microlepis	5	3	7	$0.58^{\text{n.s.}}$
Anableps dowii	15	2	16	$0.18^{\text{n.s.}}$

males being left-sided (figure 2*a*). In *J. obscura*, most males had a left-sided gonopodium (93.9% ± 1.9 standard error of the proportion (s.e.p.), $z = 10.64$, $p < 0.0001$). Although there was variation across populations (figure 2*b*), left-sided males were more abundant in all cases, resulting in biased antisymmetry. Finally, *J. tucumana* also showed a pattern of biased antisymmetry, where left-sided males were slightly, yet significantly, more abundant than expected by chance (65.9% ± 2.2 s.e.p., $z = 6.99$, $p < 0.0001$). Considering the relatively large proportion of the right morph in *J. tucumana* (34%), we sampled multiple populations to verify that our results were not biased (figure 2*c*). Indeed, the departure from a 1 : 1 ratio was consistent across nine populations (proportion of left-sided males varied from 53 to 80%; Wilcoxon signed-rank test: $W = 45$, $p < 0.01$).

(c) A novel and well-resolved anablepid phylogeny based on genome-wide SNP data

The SNAPP multi-species coalescent analysis generated a well-supported tree topology (figure 3) that was concordant with those recovered by *SVDQuartets*, maximum likelihood implemented in RAxML and IQ-TREE, and Bayesian inference in MrBayes (electronic supplementary material, figures S3–S7). All nodes received strong support, with only two exceptions: the internal nodes between *Jenynsia maculata*, *Jenynsia alternimaculata* and *Jenynsia sulfurica*, as well as the placement of *J. luxata* and *Jenynsia onca* in relation to the clade composed of *J. lineata* + *J. darwini*. The relationships among genera within Anablepidae in our SNAPP species tree are concordant with previous evolutionary hypotheses for the family that were based on morphology or a few nuclear or mitochondrial loci [22,25,27,36,47]. However, our SNAPP tree is discordant with these previous hypotheses regarding the intrageneric relationships within *Jenynsia* [22,23,47–50]. We recovered *J. obscura* as the earliest split within *Jenynsia*. This novel relationship makes the subgenus *Jenynsia* proposed by Ghedotti [48] paraphyletic. Next, our species tree analysis recovered an early split of a clade consisting of *Jenynsia unitaenia*, *Jenynsia diphyes* and *Jenynsia eirmostigma*, which is congruent with the subgenus *Plesiojenynsia* of Ghedotti [48]. *Jenynsia sanctaecatarinae* was recovered as sister group to a clade composed of all remaining species in the genus. This last clade was

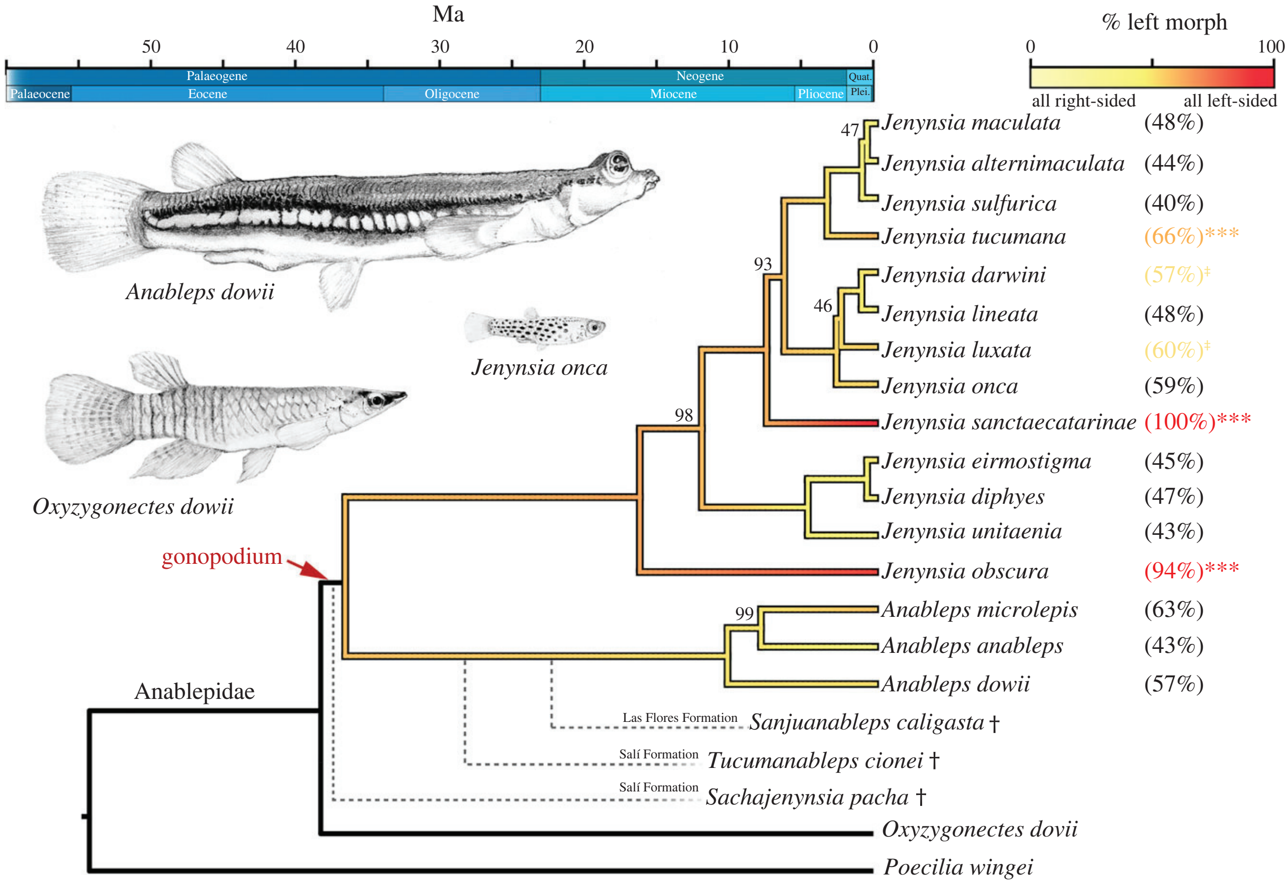

Figure 3. Ancestral state estimation of male genital asymmetry direction mapped as a continuous character in a maximum clade credibility time-calibrated SNAPP tree of Anablepidae (posterior probability of clades is presented only for those not receiving full support; confidence intervals for time estimation of nodes are provided in electronic supplementary material, table S3). On the right, the percentage of left morph individuals per species is tabulated. For the species significantly departing from a 1 : 1 proportion of left-to-right-sided gonopodia (figure 2), the percentage of left morphs is coloured (superscript symbols denote significance: $^{***}p < 0.001$; $^{‡}0.05 < p < 0.1$). Divergence times have been inferred based on calibrations from fossils of the three extinct species included in the tree [22,24] plus unclassified Cyprinodontiformes from Bolivia [40]. Detailed information of the calibration points can be found in electronic supplementary material, table S4. Fish drawings (by J.T.-D.) illustrate male representatives of the three extant genera; notice the lack of gonopodium in *Oxyzygonectes dovii*. *Jenynsia onca* is enlarged to facilitate perceptibility. The red arrow indicates where the gonopodium is assumed to have evolved. †Denotes extinct species that have been manually added to the tree. Their placement in the tree is based on [22] and [24].

composed of two species groups, one including all species of *Jenynsia* with spots on their bodies (*J. alternimaculata*, *J. maculata*, *J. sulfurica* and *J. tucumana*) and a second group consisting of species that exhibit lines (*J. darwini*, *J. lineata*, *J. luxata* and *J. onca*).

Our SNAPP tree did not recover *J. tucumana*, *J. obscura* and *J. sanctaecatarinae* as closely related, and our ancestral character state estimation suggests that statistically significant left-biased asymmetry evolved independently three times from antisymmetric ancestors (figure 3). This was further supported by the low phylogenetic signal in the evolution of this trait (Pagel's $\lambda = 0.998$, likelihood ratio test$_{(\lambda=0)} = 2.91$; $p = 0.09$). It is also plausible, although less parsimonious, that a significant left-biased departure from a 1 : 1 ratio evolved once in the common ancestor of all *Jenynsia* species and then subsequently was lost three times (figure 3).

(d) Time-calibrated tree suggests early split of extant genera but more recent diversification within genera

Our time calibration analysis suggested that the split between the families Anablepidae and Poeciliidae occurred during the early Eocene, while the three genera of Anablepidae diverged at the end of the Eocene (figure 3). However, the diversification among living species within the genera *Anableps* and *Jenynsia* started only in the Late Miocene and continued until recently (figure 3).

(e) Excess of left-sided offspring in *Jenynsia tucumana* independent of sire sidedness

Fifteen pairs of *J. tucumana* produced more than three male offspring, thereby allowing the ratio of left- to right-sided males to be determined. On average, these 15 breeding pairs had 11.1 male offspring, of which 61.45% were left-sided (electronic supplementary material, table S5). Offspring mortality was negligible. Offspring from left-sided sires had a higher probability of being left-sided than expected if direction of asymmetry was randomly determined (intercept effect estimate = 0.414 ± 0.21 s.e., $p = 0.045$). However, the left : right offspring ratio did not differ between sire morphs (sire$_{\text{(right)}}$ effect estimate = 0.128 ± 0.325 s.e., $p = 0.693$). Thus, we excluded sire morphotype from the model, finding that offspring of all sires departed from a 1 : 1 ratio by producing an excess of left-sided individuals (intercept effect estimate = 0.466 ± 0.195 s.e., $p = 0.003$). Note that some broods were biased towards having more right-sided offspring, including three from a left-sided sire.

4. Discussion

Genital asymmetry appears to be evolutionarily labile (like other morphological characteristics of animal genitalia) [18], with multiple transitions from symmetry to asymmetry across lineages [51–54]. In anablepid fishes, the transition from symmetry to asymmetry appears to have occurred twice: once in males after the evolution of the male gonopodium and again in females of the genus *Anableps*. *Sachajenynsia pacha*, an extinct species from the middle Miocene found in northern Argentina and sister taxa to all *Jenynsia* + *Anableps*, has a simple, symmetric gonopodium (figure 1) [22]. Antisymmetry is likely the ancestral state in the two extant genera with gonopodia (figure 2). Previously, we predicted that the probabilities of observing transitions from antisymmetry to directional asymmetry were low, considering the small additive genetic variation observed for the direction of genital asymmetry [28]. Here, we show that in the family Anablepidae the transition from antisymmetry to directional asymmetry indeed occurred multiple times, suggestive of recurrent genetic assimilation of the direction of asymmetry in this group of fishes. Additionally, our breeding experiment indicates that the sidedness of genital asymmetry cannot be predicted based on the morphotype of the sire, whereas the probability of expressing one morph over the other one might be predictable.

Based on our phylogenetic analyses of genome-wide SNP data, we infer three independent transitions from antisymmetry to biased antisymmetry or directional asymmetry in the genitalia of anablepids. Our phylogenetic hypothesis places *J. obscura* as the earliest lineage to split from the other species in the genus (figure 3), which differs from previous phylogenetic hypotheses that placed this species in the subgenus *Jenynsia* (*sensu* [48]) and as sister species to *J. tucumana* (e.g. [23,47]). These previous hypotheses would indicate only two transitions from antisymmetry to directional asymmetry or biased antisymmetry in *Jenynsia*. Previous phylogenetic hypotheses of the family were based on 71 morphological characters. In these datasets, *J. obscura* often had a large number of autapomorphies (e.g. [47,55]), and its placement was only poorly supported (e.g. [23]). All the synapomorphies for the subgenus *Jenynsia* proposed by Ghedotti [48] and Aguilera *et al*. [56] are recovered in *J. obscura*. However, this species also shows the synapomorphies proposed for the subgenus *Plesiojenynsia* based on additional material examined (G.A. 2021, unpublished data). Phylogenetic analyses of mitochondrial markers again recovered *J. obscura* as sister to all other *Jenynsia* species (electronic supplementary material, figure S8). These results support the placement of *J. obscura* as the sister group to all other *Jenynsia* species and indicate that multiple independent transitions from genital antisymmetry to an excess of left-sided individuals have occurred in the Anablepidae. Alternatively, it is plausible that a left-biased asymmetry might have evolved once in the most recent common ancestor to all *Jenynsia* species and was later lost independently and repeatedly in three lineages (first in *J. unitaenia*, *J. diphyes* and *J. eirmostigma*, then in *J. darwini*, *J. lineata*, *J. luxata* and *J. onca* (but two species in this clade show a marginally significant left-biased asymmetry, figure 3), and third in *J. alternimaculata*, *J. maculata* and *J. sulfurica*). The placement of *J. obscura* (94% left-sided males) as the first split in *Jenynsia* makes it difficult to differentiate these two alternatives. A more thorough evaluation of other species might help resolve this issue, especially given that some of the species without an overall departure from a 1 : 1 ratio might contain particular populations with a significant bias toward one direction [21] (see also electronic supplementary material, table S1). Similarly, a larger sample size for the genus *Anableps* could help, as there are data suggesting that some populations show some bias in female sidedness [20,30].

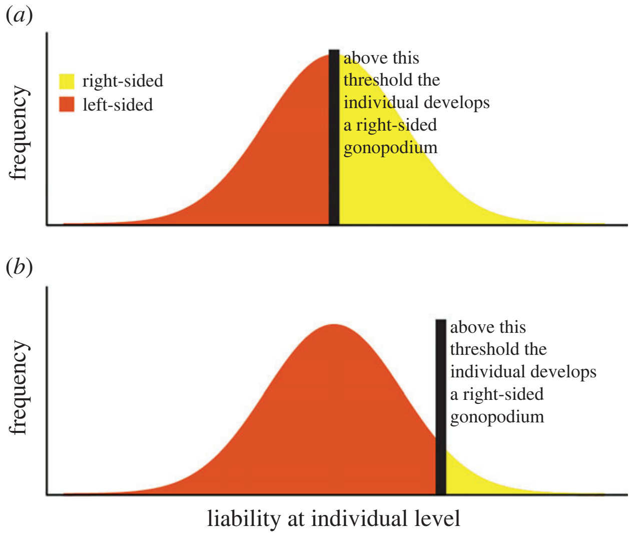

Figure 4. Threshold model for the determination of the direction of asymmetry in anablepid genitalia. Levels of a liability factor below a threshold (black vertical line) result in the expression of left-sided gonopodia and above this threshold in the expression of right-sided gonopodia. (*a*) In antisymmetric species, the threshold is expected to coincide with the mean of the liability factor distribution. (*b*) In species with directional asymmetry or biased antisymmetry, the threshold is displaced from the mean of the distribution. (Online version in colour.)

The inference of multiple transitions in the direction of male genitalia highlights the question of what, if any, is the genetic basis of genital sidedness in anablepid fishes. Previous breeding experiments with two antisymmetric species of anablepids (i.e. *J. lineata* and *A. anableps*) [28] indicated that the direction of the gonopodium is expressed stochastically. The new breeding experiment in *J. tucumana*, a species showing an excess of left-sided males, also suggests a stochastic determination of the direction of the gonopodium, but in this case with a bias towards left-sided offspring. Assuming morphological asymmetries to be threshold traits [12,57,58] allows us to hypothesize how phenotypic variation in the direction of genital asymmetry is assimilated in anablepid fishes (figure 4): under the assumption that variation in a liability factor (e.g. morphogens) is mostly due to developmental stochasticity, individuals with a level of the liability factor above a certain threshold will develop right-sided gonopodia, and below this threshold, left-sided gonopodia. In antisymmetric species, the threshold is expected to coincide with the centre of the liability factor distribution (figure 4*a*). Biased antisymmetry (e.g. *J. obscura* and *J. tucumana*) or directional asymmetry (e.g. *J. sanctaecatarinae*) can evolve if the threshold is displaced from the mean of the distribution (figure 4*b*). This mechanism has been proposed to explain genetic assimilation of other polyphenisms [59] and might also explain the inheritance of sidedness in the polymorphic starry flounder (*Platichthys* *stellatus*) [12,60]. The hypothesis of mostly stochastically determined liability levels and a displaced threshold is also in line with the results of our breeding experiment, which shows an excess of left-sided male offspring, independent of sire morph. However, one caveat to this interpretation is that the contribution of the mother's genotype to the phenotypic variation in gonopodium sidedness of male offspring in unknown. Multiple generation breeding crosses that track the ancestry of the female might be needed to clarify their contribution. This will be less relevant if the loci coding for the phenotype is on sex chromosomes, but unfortunately sex determination mechanisms in anablepids remain unknown. Also, we do not make any assumption about the genetic architecture of the threshold, which could vary from simple to very complex. This would mostly impact the width of the threshold, affecting the variation within broods, but would not conceptually change the model. To sum up, this model might help reconcile the lack of heritability of sidedness (e.g. [28,61]), and the lack of response to artificial selection on the direction of asymmetry (e.g. [14,15,62,63]), with the fact that the direction of asymmetry certainly evolves [11,17]. Thus, future studies might benefit from focusing on the heritability of the threshold, which is manifested in the proportion of offspring of each morph, rather than in the heritability of sidedness *per se*.

While this model may explain the evolution of the direction of asymmetry, the question remains why there is a consistent bias towards left-sided individuals: all species of anablepids that departed from a 1 : 1 left–right ratio have an excess of left-sided males (figure 3). The low number of species with directional asymmetry or biased antisymmetry impedes strong conclusions, as the probability of three species evolving in the same direction by chance is not negligible (e.g. $p = 0.125$, although this probability would be reduced if we consider marginally significant species like *J. darwini* and *J. luxata*). However, this pattern is predicted by some models proposed to explain the molecular mechanisms that determine asymmetry sidedness of morphological structures [12,64,65]. The direction of asymmetry might result from intracellular or cytoplasmic chirality that biases the production of one asymmetric morph over the other one by influencing the side of expression of certain morphogens (e.g. [66–70]). Under this scenario, subcellular asymmetries might favour the shift of thresholds consistently towards one direction, resulting in one morph having higher probability of fixation (e.g. more than 90% of snail taxa are sinistral) [71]. This is further supported by the observation that some of the species that show a non-significant trend (i.e. *J. darwini* and *J. luxata*) are also left-biased (electronic supplementary material, table S1). Alternatively, there could be fitness advantages of one morph over the other due to correlated traits or constraints [72]. This topic warrants further investigation.

How often do we see evidence for genetic assimilation in the evolution of sidedness of genital asymmetry? As previously reviewed [19], genital asymmetry is rare in nature, albeit phylogenetically widespread, and most asymmetric species are directionally asymmetric. Antisymmetry occurs in only few taxa, and lineages that might be informative for the study of genetic assimilation (i.e. those with both antisymmetric and directional asymmetric species) are rare [19]. In a few of these groups directional asymmetry is ancestral to antisymmetry [54,73], a seldom-seen trend in the evolution of asymmetry sidedness [11,17]. However, as in anablepid fishes, antisymmetry is ancestral to directional asymmetry in other taxa ([11,61,74], this study), suggesting that genetic assimilation of direction of genital asymmetry occurred independently multiple times. In anablepid fishes, sidedness evolved consistently in one direction (i.e. left-sided males became more abundant or fixed), and the same was observed for earwigs [72,75,76]. However, in phallostethid fishes, entirely right-sided as well as entirely left-sided species evolved [74,77]. In summary, our study shows that asymmetric traits could evolve in a manner compatible with the postulants of genetic assimilation (reviewed in [11]), and this occurred repeatedly in different lineages. It also suggests that future studies should focus on the heritability of the proportion of offspring of each morph, rather on the heritability of sidedness *per se*, to better understand the evolution of directional asymmetry by genetic assimilation.

Ethics. This research was approved by the Regierungspräsidium Freiburg, Baden Württemberg, Germany (reference no.: G-17/110), the Ministerio de Ambiente y Recursos Naturales de Nicaragua (permit no. DGPN/DB-IC-015-2015), the Dirección de Flora y Fauna, Tucumán, Argentina (permit no. DFFS-1757/330-214-9), the Sistema de Autorização e Informação em Biodiversidade, Brazil (SISBIO permit no. 33278-4 and 14028) and the Dirección Nacional de Recursos Acuáticos, Uruguay (permit no. DINARA-002/2014).

Data accessibility. The short-read data and associated information have been archived in the NCBI SRA database under Bioproject accession no. PRJNA822935 (SAMN27284034-SAMN27284138). *COX I* sequences were deposit in Genbank (accession nos ON156456–ON156467). The data associated with the phenotyping of individuals and the breeding experiment are available in the electronic supplementary material, tables S1 and S5, respectively. The nexus and xml files used in the phylogenetic analyses are archived in the Dryad Digital Repository [78].

The data are provided in electronic supplementary material [79].

Authors' contributions. J.T.-D.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, visualization, writing—original draft, and writing—review and editing; S.J.R.: data curation, formal analysis, and writing—review and editing; G.A.: data curation and writing—original draft; A.F.K.: data curation, and writing—review and editing; J.R.V.: formal analysis, and writing—review and editing; G.G.: data curation, resources, and writing—review and editing; A.C.P.: data curation, resources, and writing—review and editing; G.C.D.: data curation, and writing—review and editing; W.J.daG.: data curation, resources, and writing—review and editing; A.M.: conceptualization, data curation, funding acquisition, resources, and writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed herein.

Conflict of interest declaration. We declare we have no competing interests.

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