

Article

Quantifying Shape Variation in an Antisymmetrical Trait in the Tropical Fish *Xenophallus umbratilis*

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Abstract: Antisymmetry is a striking, yet puzzling form of biological asymmetry. The livebearing fish *Xenophallus umbratilis* exhibits antisymmetry in the male intromittent organ and provides a system that is well-suited for studying the nature of variation in antisymmetrical traits. Using geometric morphometrics, we test the hypothesis that because the gonopodium is critical to fitness there will not be significant differences in gonopodium shape between the two gonopodial morphs in this species. Our results are consistent with this prediction, though we found that gonopodium shape differed with gonopodium size.

Keywords: gonopodium; asymmetry; Poeciliidae; geometric morphometrics

1. Introduction

Understanding and explaining morphological variations has long been a topic of interest in evolutionary biology. Some of the most widespread and easily discernable forms of variation are breaks in symmetry, also known as asymmetry. Several types of asymmetries exist. Fluctuating asymmetries are subtle, random deviations from symmetry that are classically associated with parasites, environmental stress, and homozygosity [1–5]. This type of asymmetry typically results from aberrations in development and has been shown to reliably signal genome quality as well [6–8]. Directional asymmetries are those where all individuals share the same direction of asymmetry and the direction of asymmetry usually has a genetic basis [9,10]. Finally, antisymmetry is a type of asymmetry wherein “left-handed” and “right-handed” forms are both present within a population [11]. Antisymmetry is sometimes referred to as random asymmetry because the direction of asymmetry in such traits appears to be random and is sometimes not heritable [12].

Some have suggested that traits that are non-heritable, e.g., many asymmetrical and antisymmetrical traits, are evolutionarily unimportant [13,14]. However, such conclusions are not universally accepted [13,15]. One of the challenges in understanding the evolutionary impact of antisymmetrical traits is that researchers often focus on traits that may not be readily linked to fitness. What is needed is a study that examines antisymmetry in a trait that is clearly and directly linked to fitness, such as those used for reproduction or rearing offspring.

We have identified a species of livebearing freshwater fish, *Xenophallus umbratilis* [16], that fits these criteria. This species exhibits antisymmetry in the male intromittent organ called the gonopodium, a modified anal fin that is used to inseminate females [17,18]. Gonopodia in livebearing fish are often elaborated, featuring barbs, claws, and serrae that presumably help males anchor more securely to the female urogenital pore during copulation [17,19]. The gonopodium in *X. umbratilis* terminates with a hook-like structure that curves to the left (sinistral) or to the right (dextral) (Figure 1).



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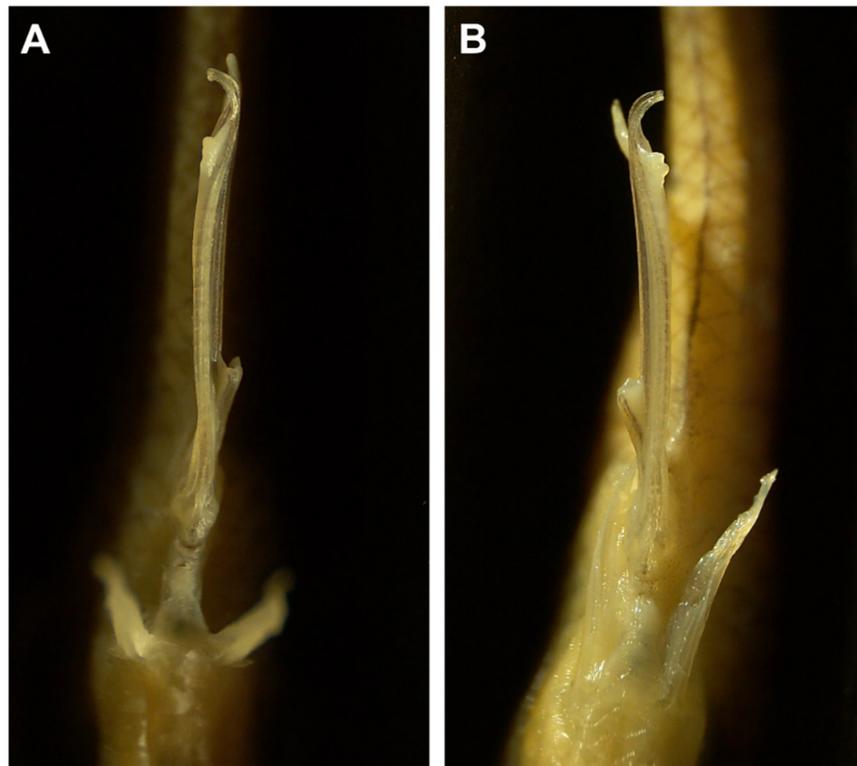


Figure 1. Photographs of ventral view male specimens with sinistral (A) and dextral (B) gonopodia.

Here, we evaluate the extent of shape variation in the degree of curvature in the gonopodium between sinistral and dextral males in *X. umbratilis* using geometric morphometrics. Because the degree of curvature in the gonopodium likely impacts a male's ability to successfully transfer sperm (too curved or not curved enough may both inhibit copulation), we predict that curvature will be maintained by common selective pressures regardless of chirality and will therefore be identical between the two morphs.

2. Materials and Methods

2.1. Study System and Sampling

Xenophallus umbratilis is a livebearing freshwater fish native to northern Costa Rica. The species is typically found in small streams and is most abundant at the headwaters of river drainages at high elevations [20]. As in all other poeciliid fishes, *X. umbratilis* employs internal fertilization and gives birth to live young. The gonopodium in *X. umbratilis* is antisymmetrical, exhibiting a sinistral (left-handed) or dextral (right-handed) hook at the terminus. Populations of *X. umbratilis* are usually composed of a mixture of sinistral and dextral individuals, though several populations that are fixed for either morph have been observed in the wild [21].

We studied *X. umbratilis* from ten different localities that contained both gonopodium morphs collected from tributaries and streams in Costa Rica between 2005 and 2007 (Figure 2, Table 1). These specimens came from the Brigham Young University Bean Life Science Museum collections. Fish were collected and humanely euthanized in the field with an overdose of the anesthesia tricaine methanesulfonate (MS-222) at a concentration of 250 mg/L [22,23]. Fish were preserved in the field in ethyl alcohol and each specimen was assigned a museum ID number.

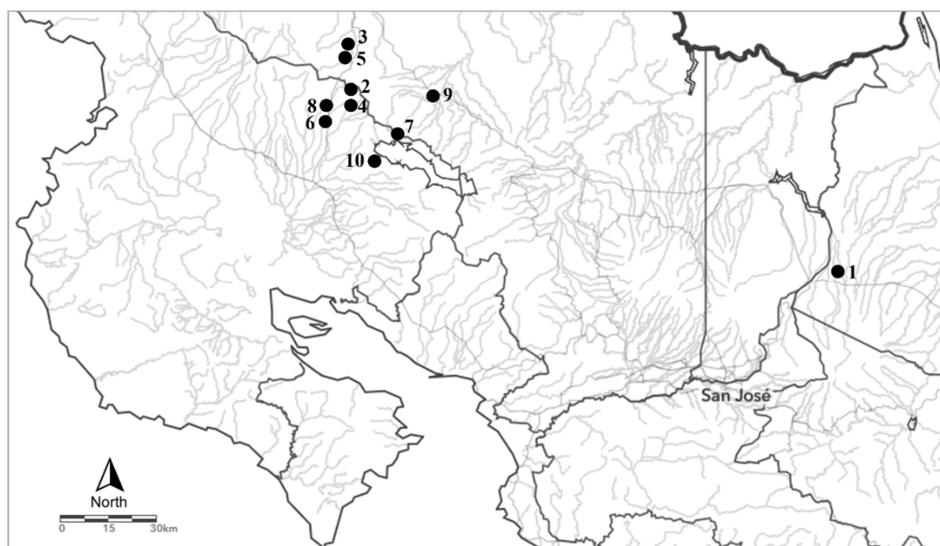


Figure 2. Map of the ten localities sampled in Costa Rica. Rivers and tributaries are shown in gray and localities are denoted by black dots. Localities were sampled between 2005 and 2007. Image generated with ArcGIS.

Table 1. Population identification and location information.

Population	Museum ID	Tributary/River	Coordinates
1	009294c	Rio Corinto	10°12.674' N 83°53.114' W
2	009301b	Rio Esquivetto	10°41.231' N 85°04.002' W
3	009302	Trib. to Rio Bijagua	10°43.887' N 85°03.318' W
4	009310	Rio Tenerio	10°41.285' N 85°04.561' W
5	009320	Trib. to Rio Bijagua	10°43.453' N 85°03.982' W
6	009325	Quebrada Hormiguero	10°41.454' N 85°05.019' W
7	009338c	Quebrada La Palma	10°33.614' N 84°56.442' W
8	009339	Quebrada Hormiguero	10°41.445' N 85°05.036' W
9	009340	Quebrada Isabel	10°38.387' N 84°50.757' W
10	009354	Quebrada Azul	10°29.955' N 84°59.138' W

Populations that were fixed for either the sinistral or dextral morph were excluded from this study. We sorted each of the ten populations to remove females and juveniles, and then sorted the remaining mature males by gonopodium morph. Using an Olympus DP74 camera mounted on an Olympus MVX10 microscope (Tokyo, Japan), we took photographs of males with their ventral side facing up to the camera lens. We placed males in a black, plastic trough for photographing to make their positioning under the lens easier to control and more consistent across images. Following photographing, we returned males to their original museum collection jars.

2.2. Geometric Morphometric Analysis

We employed landmark-based geometric morphometrics to quantify gonopodium shape in *X. umbratilis* [24]. Prior to landmarking, we used Olympus cellSens software [25] to screen images and ensure that specimens were in focus and that the gonopodium was level in the dorso-ventral and anteroposterior axes. We re-photographed specimens to correct any rotation or focus errors and excluded males with damaged or underdeveloped gonopodia from our analysis ($n = 3$). Additionally, for our analysis, we rotated or flipped images so that all gonopodia were oriented such that they appeared to be dextral. This reduced observer bias by making it impossible to visually distinguish sinistral and dextral gonopodia. In total, 246 males (135 sinistral, 111 dextral) were included in this study.

We used the program tpsDig [26] to digitize landmarks on each specimen and to measure variation in sinistral and dextral gonopodia. We used seven landmarks to outline the shape of the gonopodium (Figure 3). Landmarks were placed by a single researcher on each specimen and specimens were processed in random order.

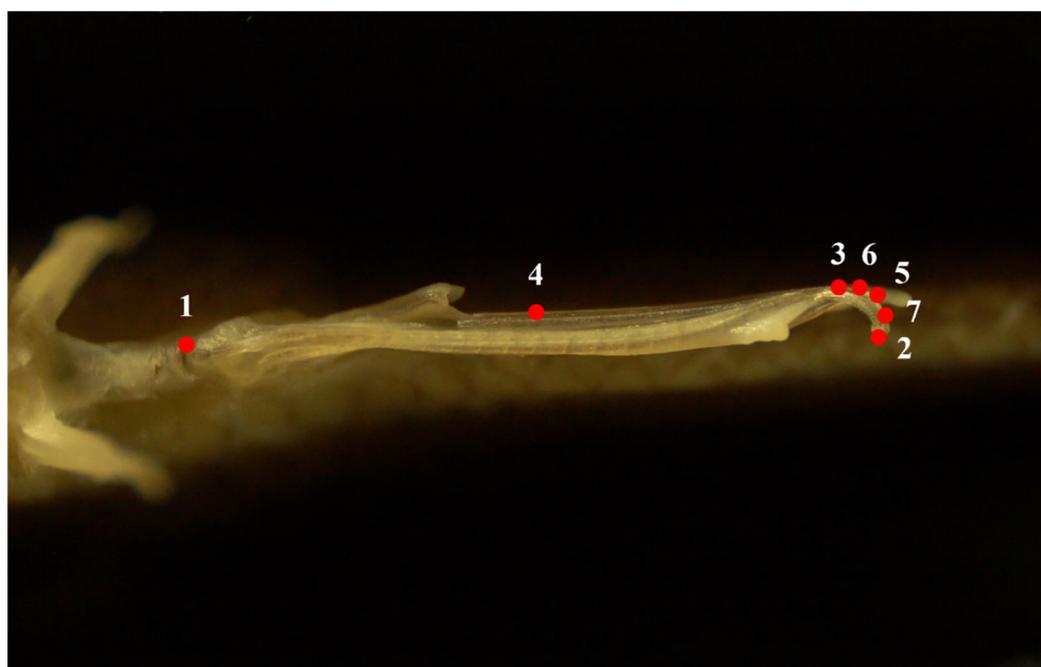


Figure 3. Landmark placement used to analyze gonopodium shape in *X. umbratilis*. Landmarks were placed as follows: (1) origin of the gonopodium; (2) terminus of the gonopodium; (3) point of curvature at the tip of the shaft; (4) midpoint between landmarks one and two; (5) midpoint between landmarks two and three on the terminus; (6) midpoint between landmarks three and five; and (7) midpoint between landmarks five and two. Landmarks one and two are homologous; landmark three is a pseudo-landmark; and landmarks four, five, six, and seven are sliding landmarks.

We generated shape variables from our landmark data in tpsRelw [26]. After producing shape variables using a general Procrustes analysis [27], tpsRelw runs a principal components analysis and calculates relative warps and centroid size. For this analysis, tpsRelw calculated ten relative warps. We used the first six of these relative warps, which accounted for 99.89% of total shape variation, for additional analysis, and excluded four relative warps that accounted for less than 0.5% of variation. Excluding these relative warps allowed us to avoid inflating the degrees of freedom in our shape analysis [28,29].

2.3. Statistical Analysis

We used a multivariate linear mixed model to evaluate the effects of morph (sinistral or dextral), centroid size (a measure of size commonly used in geometric morphometrics) [30], and locality on shape variation [28,29]. In each analysis, we used relative warps (shape

variables) as our response variable. Because relative warps come from a matrix of shape variables, we had to convert the shape variable matrix into columns of vectors to be used in our multivariate linear mixed model. This conversion subsequently required the creation of an index variable that retained individual information for each of the relative warps in our analyses. We included the index variable in our analysis as a predictor variable and it is necessary to meaningfully measure the differences in shape variation between groups. Hence, it is the two-way interactions between morph and the index variable, centroid size and the index variable, locality and the index variable, and the three-way interactions between morph, centroid size and the index variable, and locality, centroid size and the index variable, that allowed us to comprehensively determine what factors significantly influence gonopodium shape variation [31].

To test our hypothesis, we ran three models. We first needed to determine if centroid size and locality were significant predictors of shape variation to inform how we constructed subsequent models that tested for the effect of gonopodial morph on shape. Our first model tested for the impact of centroid size on shape. The second model included centroid size and locality. Our third model tested for the effects of morph and centroid size and included locality as a random effect. Across all three models, relative warps (shape variables) were used as the response variable (see Table 2 for details on each model's components). We used the Akaike Information Criterion (AIC) to determine which of the three models provided the best fit [32].

Table 2. Variables used in each model of the multivariate linear model analysis.

	Response Variable	Random Effect	Predictor Variable
Model 1	Relative Warps	–	Centroid Size Index Centroid Size × Index
Model 2	Relative Warps	–	Locality Centroid Size Centroid Size × Locality Index Locality × Index Centroid Size × Index Locality × Centroid Size × Index
Model 3	Relative Warps	Locality	Morph Centroid Size Centroid Size × Morph Index Morph × Index Centroid Size × Index Morph × Centroid Size × Index

We estimated the degrees of freedom in our analyses using the Kenward-Roger method [33] and ran our multivariate linear mixed models in SAS software, using the Proc MIXED protocol (SAS version 9.4, SAS Institute Inc., Cary, NC, USA).

3. Results

Neither morph nor locality were significant predictors of gonopodium shape. That is, we could not reject the hypothesis that the shape of dextral and sinistral gonopodial morphs are the same (Table 3). Gonopodium shape in *X. umbratilis* did differ significantly by centroid size in both models 1 and 3 (see the two-way interaction between centroid size and the index variable from models 1 and 3 in Table 2). Of the three models, model 2 had the lowest AIC score (−10,768.2) and best fit the data. The terminus of the gonopodium becomes slightly more open moving from the smallest centroid size to the largest (Figure 4). Although a significant predictor, the extent of shape variation that centroid size explains appears to be relatively limited compared to the overall variation in shape captured across

Relative Warps 1 and 2 (Figure 5). In other words, the primary difference in gonopodium as a function of centroid size was a slight change at the tip of the gonopodium (Figure 4).

Table 3. Results from the multivariate linear mixed model analysis. We ran three different models with different combinations of predictor variables. Predictor variable terms that include an interaction with the index variable are those that evaluate if/how gonopodium shape changes.

	Predictor Variable	Degrees of Freedom	F-Value	p-Value
Model 1	Centroid Size	1,702	6.44	0.0113
	Index	5,657	4.9	0.0002
	Centroid Size \times Index	5,657	4.82	0.0002
Model 2	Locality	9,794	1.02	0.4226
	Centroid Size	1,794	1.25	0.2365
	Centroid Size \times Locality	9,794	1.04	0.4092
	Index	5,659	2.04	0.0718
	Locality \times Index	45,1183	0.98	0.5024
	Centroid Size \times Index	5,659	2.07	0.0671
	Locality \times Centroid Size \times Index	45,1183	0.98	0.5188
Model 3	Morph	1,704	0.01	0.9238
	Centroid Size	1,619	5.09	0.0244
	Centroid Size \times Morph	1,703	0.04	0.8391
	Index	5,653	3.26	0.0065
	Morph \times Index	5,653	1.39	0.2273
	Centroid Size \times Index	5,653	3.07	0.0095
	Morph \times Centroid Size \times Index	5,653	1.23	0.0095

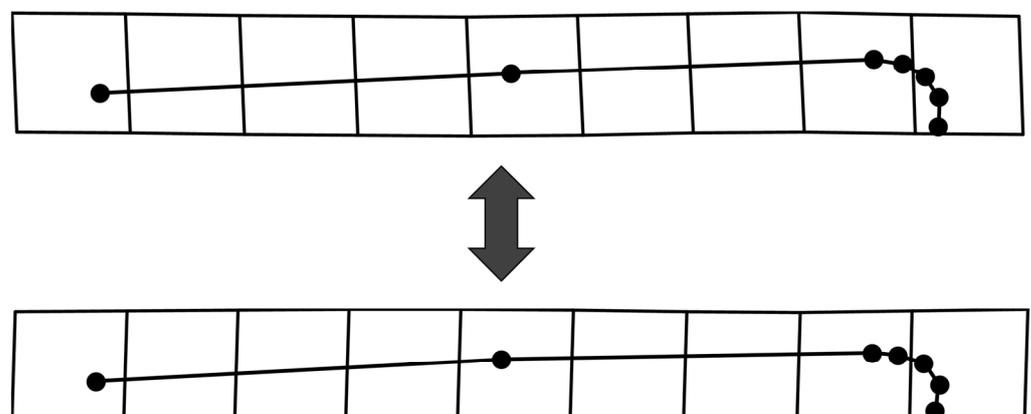


Figure 4. Variation in gonopodium shape explained by centroid size. Shape deformation is represented by thin plate splines. The top thin plate spline visualizes shape at the largest centroid in this dataset and the bottom thin plate spline visualizes the shape at the smallest centroid size.

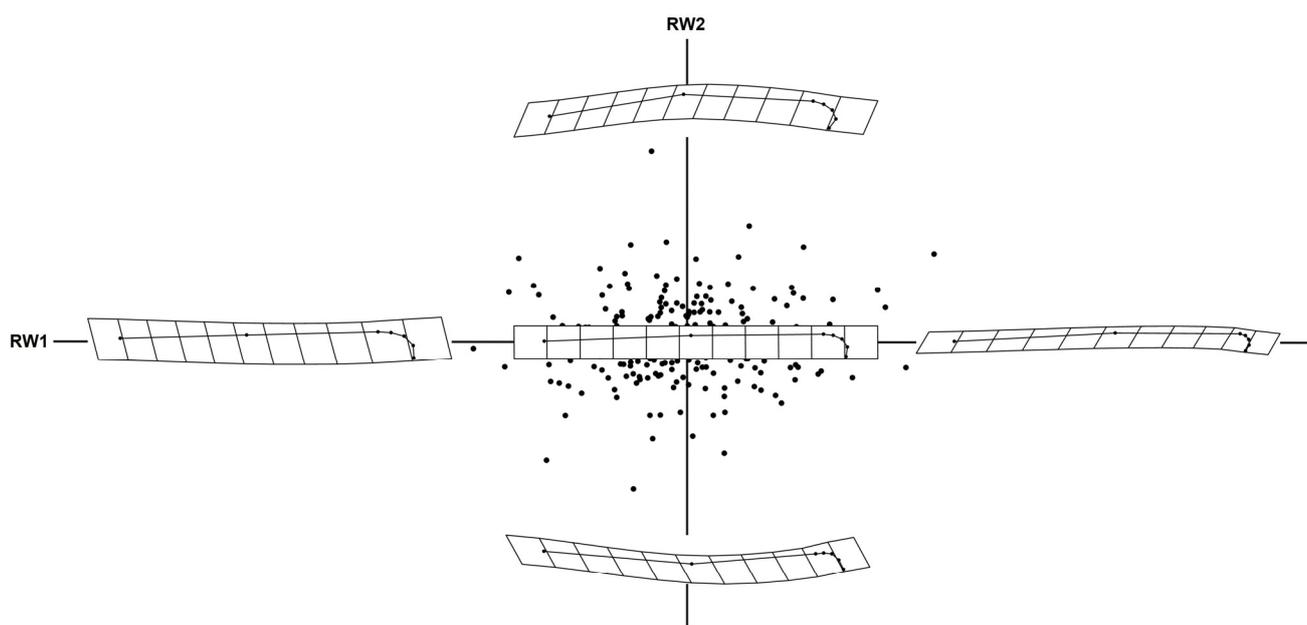


Figure 5. Visualization of gonopodium shape variation along Relative Warp 1 (RW1) and Relative Warp 2 (RW2). Thin plate splines along the x-axis represent shape deformation across Relative Warp 1. Moving from left to right, these splines show the terminus of the gonopodium becoming more tightly curved. Thin plate splines along the y-axis represent shape deformation across Relative Warp 2. These splines show some bending in the midpoint of the gonopodium shaft and some changes in the curvature of the gonopodium tip.

4. Discussion

This study provides insight on variation in antisymmetrical traits. We predicted that the shape of gonopodial curvature would not differ between sinistral and dextral individuals in *X. umbratilis* and our results support this prediction. We also found that centroid size is a significant predictor of gonopodium shape. Males of many poeciliid species exhibit determinate growth, so this finding suggests that the size at which males mature may affect gonopodium shape [34,35]. However, it seems unlikely that centroid size impacts gonopodium shape in a biologically meaningful way, as the variation due to centroid size is minimal when considered with the overall shape variation in the gonopodium we observed.

Asymmetry is typically considered in a morphological context, though asymmetry can also be demonstrated behaviorally [36]. Previous research in *X. umbratilis* from Johnson et al. [21] found that gonopodial morphology reliably predicted detour behaviors and eye-bias for potential mates and predators. In that study, dextral (right morph) males detoured to the right to view potential mate and predator stimuli. Sinistral (left morph) males' behaviors were completely opposite, with males detouring to the left for the same set of stimuli. Our work aligns with these observed patterns. We found that sinistral and dextral gonopodial morphs were essentially mirror images of each other, which is consistent with a specific morph type predicting detour behavior in this species. Another study found that individuals from a fixed sinistral population of *X. umbratilis* preferentially positioned themselves wherein males were primarily on the left side of a female during mating interactions [37]. However, individuals from fixed dextral populations did not display side-biased positioning behavior. Our results provide some context for the strong side-bias in the sinistral population and the lack of side-bias in the dextral population by ruling out the possibility that unequal degrees of curvature in the gonopodium influenced mating positioning behaviors.

A critical component of studying morphology and its evolutionary implications is tying morphology to function and fitness. Though the gonopodium certainly is important to

fitness in *X. umbratilis*, very little is understood about the actual mechanisms that facilitate insemination in this species and livebearing fish in general. Given that dextral and sinistral gonopodia are essentially mirror images of each other, we might expect that functional behavior associated with fertilization in this species will also reflect antisymmetry in the gonopodium. Future work should focus on understanding the mechanisms involved in copulation and how this compares between gonopodial morphs.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/sym15020489/s1>.

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