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Honors Thesis

DOES NEGATIVE FREQUENCY-DEPENDENT SELECTION EXPLAIN
THE MAINTENANCE OF GONOPODIAL ASYMMETRY IN A
LIVEBEARING FISH?

MARY-ELISE JOHNSON

Submitted to Brigham Young University in partial fulfillment of graduation
requirements for University Honors

Biology Department
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Faculty Advisor: Dr. Jerry Johnson

Honors Coordinator: Dr. Steven Peck

ABSTRACT

Mary-Elise Johnson
Department of Biology
Bachelor of Science

How genetic variation is maintained in the face of strong natural selection is an important problem in evolutionary biology. Selection should erode genetic diversity, leading to more and more homogenous populations. Yet in nature, we commonly see high degrees of genetic variation, even for traits that are important to fitness. Negative frequency-dependent selection, a balancing selective force that favors traits when they are rare but not when they are common, is a mechanism proposed to maintain polymorphisms in a population. However, there is little empirical data to demonstrate how negative frequency-dependent selection sustains variation. *Xenophallus umbratilis* is a bilaterally symmetrical species of livebearing fish that exhibits asymmetry in the male gonopodium, the male intromittent organ which terminates with a sinistral or dextral twist. I test the hypothesis that in species such as *Xenophallus umbratilis*, where such asymmetrical morphologies exist, negative frequency-dependent selection maintains variation in the gonopodium within populations.

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INTRODUCTION

The discovery of natural selection has been touted as one of the most important discoveries in modern biology (Ayala, 2007; Lenski, 2017). However, natural selection presents an important paradox. Increased selection decreases genetic diversity, and decreased diversity limits the material upon which natural selection can act (Gillespie, 1998). Consequently, natural selection should be self-limiting if genetic variance is diminished as a consequence. Hence, understanding how genetic diversity is created and maintained is critical to our understanding of natural selection specifically, and how populations evolve in general.

So, if natural selection has a tendency to erode genetic variation, how then does new genetic diversity arise? Several hypotheses have been advanced to explain this. New mutations can increase variation in populations by creating entirely new alleles; however, mutations are rare, and beneficial mutations are even rarer (Fisher, 2009; Kimura, 1991). Recombination in sexual reproduction increases variation in offspring by assorting alleles that are already present. Variation can also be maintained in populations if selection varies over time and space (Kalske, Leimu, Scheepens, & Mutikainen, 2016). Clearly, we know a great deal about factors that can generate phenotypic diversity within populations. However, we still know remarkably little about how this variation is maintained in the face of strong selection. While mutation and recombination are both theoretically and empirically well established, there is an additional mechanism—negative frequency dependent selection (NFDS)—that theoretically can maintain variation within populations (Sato & Kudoh, 2017). Unfortunately, we know much less about this mechanism, and we have very few empirical examples demonstrating evidence for this in

natural populations of organisms [but see (Indermaur, Theis, Egger, & Salzburger, 2018; Weir, 2018; Yenni, Adler, & Ernest, 2012)].

I have identified a species of freshwater fish native to northern Costa Rica, *Xenophallus umbratilis* that may provide evidence for NFDS in the wild. This species is bilaterally symmetrical, with a notable exception. The male intromittent organ—the gonopodium—is asymmetrical. The gonopodium is a modified anal fin used to transfer sperm to females (Evans, Pilastro, & Schlupp, 2011). Males are either left-handed or right-handed for this trait, with the terminus of the gonopodium having either a sinistral or dextral twist (Fig 1.). Preliminary research has shown that ratios of left- to right-handed males vary in populations of *X. umbratilis* over time (Johnson, Not yet published) a pattern consistent with the possibility that this trait is maintained by NFDS.

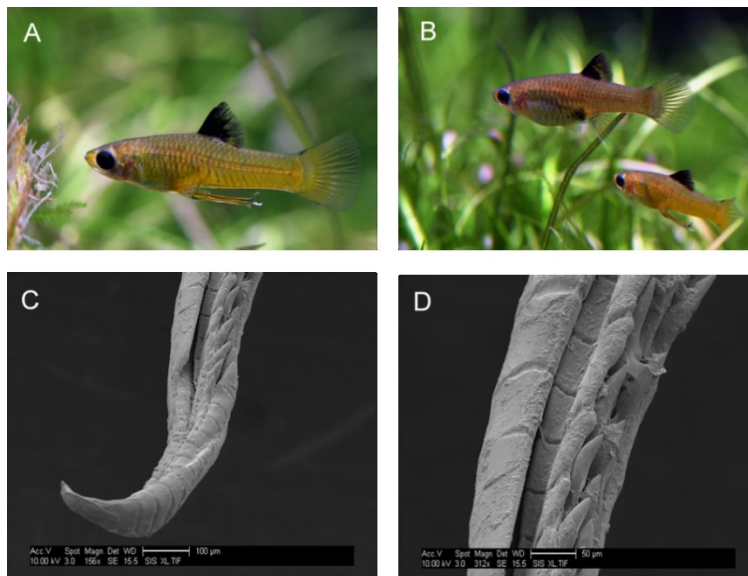


Figure 1. Photographs of male *X. umbratilis* and the male gonopodium. (A) Male with a fully developed gonopodium (dextral morph). (B) Male (bottom) in pursuit of a mature female (top). (C) Electron micrograph of *Xenophallus* gonopodium (sinistral morph) showing the tip of the organ. Note that tissue has been removed from the gonopodium (D) Electron micrograph of *Xenophallus* gonopodium showing barbs along the the shaft of the organ. Again, tissue has been removed from the gonopodium.

Here I test the hypothesis that NFDS maintains a known polymorphism for the male gonopodium within populations of *X. umbratilis* by conducting two behavioral assays to test

this hypothesis. I use these assays, which focus on female avoidance behaviors and male pursuit behaviors, to evaluate the link between male gonopodium morphology and mating behaviors in this species.

METHODS

Study System

Xenophallus umbratilis is unique among livebearing fishes because it exhibits morphological asymmetry in the male gonopodium, a structure used to inseminate females (Bussing, 1998). We scored 14 sites of *X. umbratilis* throughout northern Costa Rica to evaluate what ratios of gonopodium morphologies (Fig.2). For example, at the La Palma River site near the Lake Arenal region of Costa Rica (10.56023333, -84.9407) the population of *X. umbratilis* was predominantly sinistral in February 2018. However, when this site was scored four months later in June 2018, the majority of the population exhibited the dextral morphology. If variation of this trait were maintained by NFDS, this could explain why the ratio of dextral-to-sinistral male morphology appears to oscillate over time.

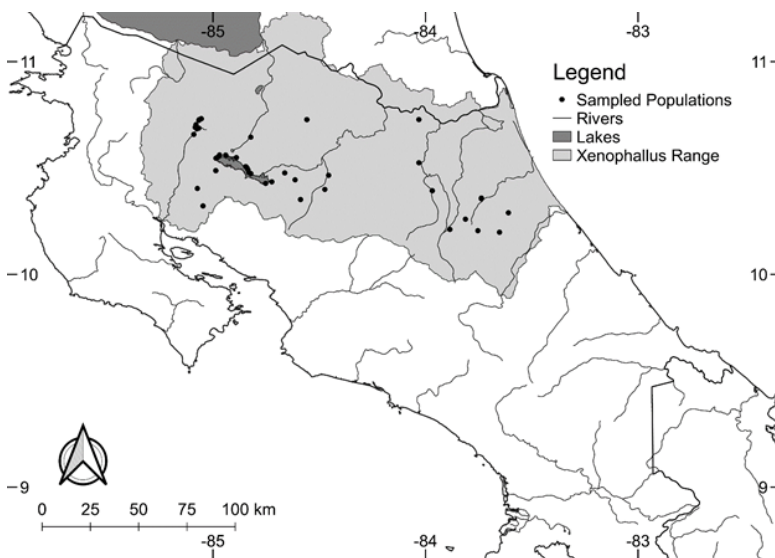


Figure 2. Map depicting the range of *X. umbratilis* in Costa Rica and sampled populations with major river systems. Sampled populations of *X. umbratilis* are depicted by dots, while the range is shown in gray.

Sampling and Housing

I collected approximately 140 live individuals from the La Palma River in Central Costa Rica in February 2018 and transported them to Brigham Young University to establish a breeding colony. Because males from the La Palma population were predominantly sinistral when they were collected in February, I collected approximately 200 live dextral individuals from La Palma and Quebrada Chorros tributary (10.476805, -84.6625319) in May of 2019. This gave us both dextral and sinistral male forms in our breeding population.

Prior to introducing females into my behavioral assays, I isolated 20 females and 15 males from the breeding colony. Isolated females were of similar size (4-5 cm in length) and were housed in individual 2-gallon shoebox tanks (28 cm x 14 cm x 19 cm), each with a unique identification number, for at least 72 hours prior to testing. Males, also of similar size (2.5-3 cm in length) were divided into three groups of five fish. The trial of the assay involved placing one of these groups of males with a single female for the female avoidance trial (see details below). Male groups were rotated out every three trials to avoid male fatigue.

All fish were held in a 12-hour day/12-hour night room and fed twice daily throughout the study. Fish were fed fruit flies in the morning followed by TetraMin flakes or crushed krill in the afternoon. For all other variables, fish used in these assays were held under common environmental conditions (23-24°C room, conditioned water, gravel substrate and plants in tanks).

Female Avoidance Assay

Female livebearing fishes typically actively resist forced copulation mating attempts from males (Plath, Makowicz, Schlupp, & Tobler, 2007). Hence, I hypothesized that X.

umbratilis females would avoid forced copulation attempts from males by moving to the side where the asymmetrical gonopodium is ineffective at transferring sperm. It is not clear whether that would be to the left or right of males, but I did expect to see a non-random bias of the side that females would prefer to orient when males from either dextral or sinistral populations were present. Hence, my first assay focused on female resistance to male pursuit. To test this hypothesis, I used a white, circular tank (Fig. 3) and a camera, all housed within a sound-proof chamber. The circular tank (with no corners to retreat to) eliminated opportunities for females to use the wall to shield against male approaches and the white color made it easier to observe fish as they moved.

I conducted the assay as follows. A group of five males with the same gonopodium morphology (sinistral or dextral) was placed in the tank with a single female and allowed to acclimate for 10 minutes. After acclimation, interactions between the males and the female were video-recorded for 10 minutes. Following recording, the female was removed from the tank and returned to her home shoebox tank, and water in the testing tank was filtered for 10 minutes. A group of males was run through this assay 3 times in a row (each time with different females) before they were replaced by another group of males and placed back in their home tank. The groups were cycled through in this manner until all females (1-20) had run through the assay once. Females remained in their isolated shoebox tanks following this assay, rather than being placed back in a regular 10-gallon tank.

Using the footage of each trial, I recorded how the female positioned her body with respect to the group of males, expecting that females oriented themselves in a manner that prevented most males from successfully mating. Each video was scored by two people using to

an established standard (see details below) and any discrepancies in scoring were noted and resolved. Scoring was recorded in terms of female orientation in 30-second intervals. Female orientation was recorded as “right” (when the female oriented her body to keep males on her right side), “left” (when the female kept males on her left), or “out” (when males were more than a body’s length from a female, or oriented away from females in a way that did not indicate a mating attempt). In most cases, the female was readily distinguished on the video from males based on size, as females are typically larger than males. However, there were two trials (#9 and #12) where the females were indistinguishable from the males. The data from these trials were not considered in our analyses. This assay was completed with our sinistral population, followed by the dextral population.

Male Approach Assay

In our second assay, I focused on whether male gonopodial morph influenced male pursuit of a female. This assay was conducted four months after the first assay. I used the same circular, white tank from the first assay (Fig. 3), as well as the same camera. Prior to the male approach assay, males from the three groups used in the first assay were separated into individual shoebox tanks with unique identification numbers (1-15). An additional ten naïve females were moved from the breeding colony into our experiment. Each were isolated in shoebox tanks with identification numbers (21-30).

Because this assay focused on how male morphology influenced male approach to females, one male and one female (from tanks 21-30) were placed in the testing arena. Fish were acclimated in the tank for 10 minutes and then recorded for 10 minutes. Following recording, I

removed the male, and filtered the water for 10 minutes. Each of the fifteen males was evaluated three times with three different females. To avoid male fatigue, no males were used in trials more than once in a 24-hour period.

I analyzed recordings in a similar manner to that described above in the female avoidance assay. All recordings were scored by the same two people, again using an established rubric (see details below) and resolved any discrepancies. Videos were scored in 30-second intervals and scoring was recorded in terms of the orientation of the male with respect to the female. The three possible scores were “right” (when the male approached from the female’s right side), “left” (when the male approached from the female’s left side) or “out” (when the male was either not close enough to the female or not oriented towards her in a way that indicated a mating attempt). This assay was completed with our sinistral population, followed by the dextral population.

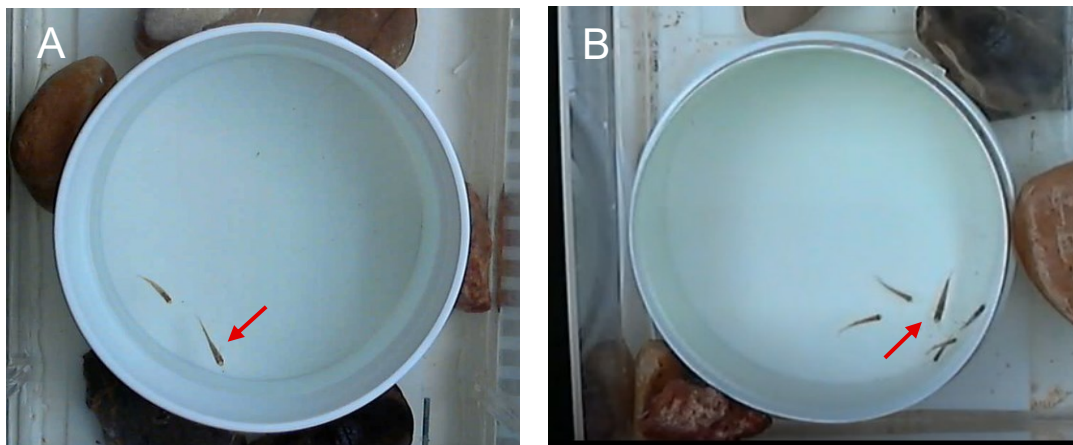


Figure 3. Screenshots of video recordings from both the male approach and female avoidance assays. (A) Experimental arena for male approach assay. Female is indicated by the arrow. Other individual is a male. (B) Experimental arena for female avoidance assay. Female is indicated by the arrow. Other individuals are males.

Data Analysis

To evaluate whether females showed a distinct side bias to avoid males, and to determine whether males showed a lateralized approach preference, we analyzed these data with a paired t-test. Scores from each assay corresponding to a left- or right-handed population were evaluated with a t-test to test for significant differences in avoidance or approach behavior.

RESULTS

Female Avoidance

Females from our sinistral population showed a significant left-handed avoidance bias (p-value 0.001635) when avoiding sinistral males, positioning themselves to keep sinistral males on the left. Females from our dextral populations showed no significant side bias. Sinistral males preferred to approach a female from her left side, while dextral males did not demonstrate a clear lateralized approach bias (Fig. 4).

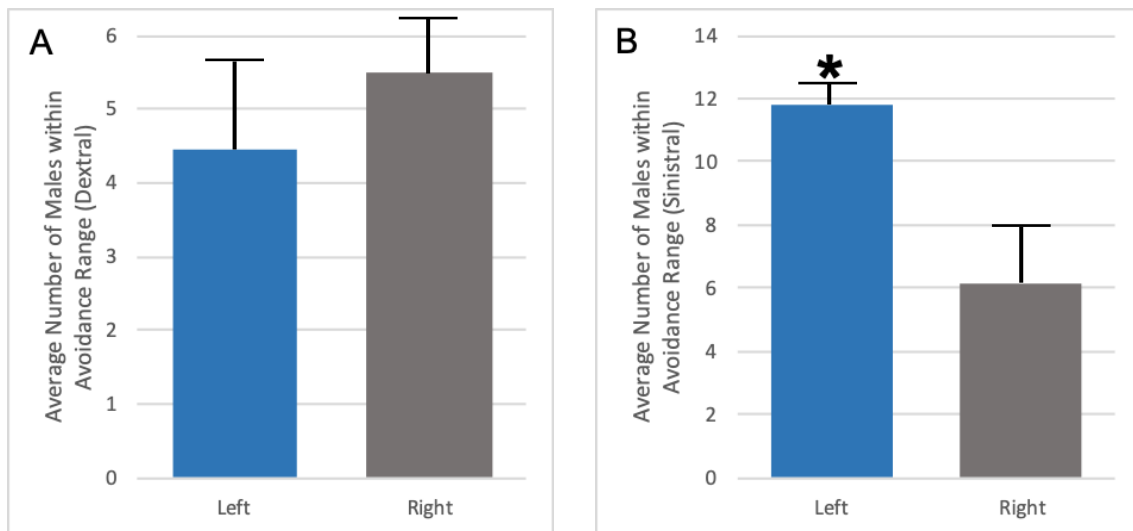


Figure 4. Results from the female avoidance assays. (A) Graph showing the average number of times a dextral male approached a female and the side she kept him on. Results were not significant (p-value 0.2465). (B) Graph showing the average number of times a sinistral male approached a female and the side she kept him on. Results were significant (p-value 0.001635).

Male Approach

Sinistral males show a significant approach bias (p -value < 0.001), preferring to approach a female on her right side. Dextral males did not exhibit a clear approach bias (Fig. 5).

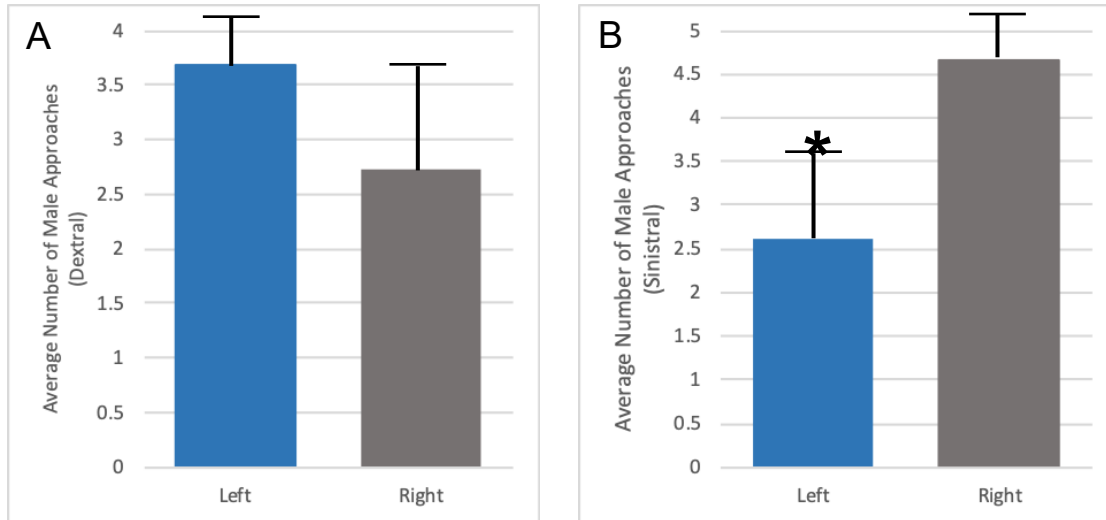


Figure 5. Results from the male approach assays. (A) Graph showing the average number of times a dextral male approached one side of a female. Results were not significant (p -value 0.1742). (B) Graph showing the average number of times a sinistral male approached one side of a female. Results were significant (p -value < 0.001).

DISCUSSION

I predicted that negative frequency-dependent selection maintains gonopodial polymorphism in *X. umbratilis*. If this were the case, I would observe females consistently positioning themselves in a way that prevents the asymmetrical male gonopodium from transferring sperm. I also predicted that males would prefer to approach females on the side opposite to the side she actively avoids males from. Our results neither demonstrate nor disprove that negative frequency-dependent selection is the mechanism that maintains polymorphism in *X. umbratilis*. While results from our sinistral populations alone align with what negative frequency-dependent selection could predict in terms of behavior (females avoided males by keeping them on her left side, males preferred to approach a female on her right side), behavior in the dextral population was mixed and did not lend itself to the same interpretation. What could explain this ambiguous result?

One possible explanation for the differences between the sinistral population and the dextral population may lie in our sampling. Though our sample size was sufficient, I recognize that our single level of replication makes it difficult to draw definitive and meaningful conclusions from the data. With only one point of reference, I am unable to accurately account for variation between individuals or establish observed trends between the two morphologies in this species. Collections from additional localities could potentially remedy this problem.

Beyond sampling, our ambiguous results may stem from our approach to testing for negative frequency-dependent selection. Behavioral work is easily confounded by a variety of factors, especially when specimens are studied outside of their natural habitat. Though care was taken to house fish in semi-natural conditions and provide long enough acclimation periods during trials, the assays themselves were conducted in non-natural conditions. These non-natural conditions were intentional, as the rocky substrate, water flow, and foliage that would be present in *X. umbratilis*' native environment would make mating behaviors difficult to observe and accurately quantify. Though morphology-influenced mating behaviors would explain how negative frequency-dependent selection acts on populations of *X. umbratilis*, there are other methods like paternity testing and mesocosm experiments that can more definitively test if negative frequency-dependent selection is acting on populations in the first place.

It is also possible, though not supported directly by our data, that the lack of significant side-biases in the dextral populations is because the dextral gonopodium morph is more recently evolved and therefore more novel than the sinistral morph. Under this explanation, the mixed results from the dextral population may demonstrate a scenario in which avoidance and approach behaviors in females and males, respectively, have not “caught up” with this newer trait. I find this explanation to be unsatisfactory given that, while theoretically plausible, it is not supported by our data and isn't particularly parsimonious.

I plan to continue this work on evaluating whether and how negative frequency-dependent selection acts on *X. umbratilis*. One of the main theoretical components of NFDS is that rare individuals experience greater fitness when they are rare relative to common individuals. Because our system highlights negative frequency-dependent selection in the context of mating, I would expect to see males with a rare morph siring a greater number of

offspring than common males. I plan to test this aspect of negative frequency-dependent selection by conducting a series of mesocosm assays in which I artificially bias populations of *X. umbratilis* to be left- or right-skewed and determine how ratios of left- to right-handed individuals change over generations. This, coupled with simple paternity tests, should provide some of the necessary information to understand how morphology is inherited and how the relative frequency of these morphs within populations influences individual reproductive fitness. Future work would ideally include consistently scoring a variety of populations of *X. umbratilis* in the wild. Doing this would require continuous field work on-site in Costa Rica for several months, and consequently would require additional funding and labor.

In summary, the mechanism that maintains polymorphism in the male gonopodium ultimately remains unknown. Our current data do not clearly confirm or disprove my hypothesis that negative frequency-dependent selection acts on populations of *X. umbratilis*. While this work did not yield entirely conclusive analyses, until more definitive conclusions can be drawn regarding this question, the question itself merits further investigation. I am confident that additional testing, including the paternity assays, mesocosm experiments, and population monitoring mentioned above, could further clarify our understanding of how negative frequency-dependent selection works—or doesn't work—within polymorphic populations. This work presents a foundation for contextualizing the resulting data from this future work and should bring us closer to identifying and describing how negative frequency-dependent selection can act on wild populations.

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