Examining the Relative Lifetime Fitnesses for Alternative Mating Phenotypes in *Xiphophorus multilineatus*

A thesis presented to

the faculty of

the College of Arts and Sciences of Ohio University

In partial fulfillment

of the requirements for the degree

Master of Science

Lisa M. Bono

November 2009

This thesis titled

Examining the Relative Lifetime Fitnesses for Alternative Mating Phenotypes in
Xiphophorus multilineatus

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ABSTRACT

BONO, LISA M., M.S., November 2009, Biological Sciences

Examining the Relative Lifetime Fitnesses for Alternative Mating Phenotypes in *Xiphophorus multilineatus* (61 pp.)

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Males of the high-back pygmy swordtail *Xiphophorus multilineatus* display genetically-influenced alternative mating phenotypes. Evolutionary game theory predicts that genetically-influenced alternative phenotypes are maintained at equal fitnesses by negative frequency-dependent selection. I measured age at sexual maturity of the phenotypes and then used this estimate along with an estimate of the relative fertilization success to test the prediction of equal fitnesses. My results demonstrate that the size difference between sneakers and courters is due to differences in the age to sexual maturity. However, the advantages of reaching sexual maturity sooner for sneaker males did not compensate for the higher fertilization success of courter males. Using a model, I demonstrate that the alternative phenotypes did not have equal fitnesses when the frequency of sneakers was high, but preliminary evidence suggests that negative frequency-dependent selection may be maintaining the alternative phenotypes.

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ACKNOWLEDGMENTS

So many people have been instrumental to the completion of this thesis. It is with deep gratitude that I thank my advisors who helped transform my desire to do research into a reality. Dr. Molly Morris graciously welcomed me into her lab and nurtured me with her intellectual and personal insights. As a strong woman in science and life, her passion, energy, fierce loyalty, and joie de vivre have been a source of inspiration to me. Dr. Oscar Rios-Cardenas has provided me with great patience and understanding. He has challenged me to make me to realize my ideas and encouraged my independence.

I would like to thank my committee members, Dr. Matt White for his encouragement and Dr. Don Miles for providing his perspective. I would also like to acknowledge Dr. Janet Duerr, Dr. Soichi Tanda, and Dr. Winfred Just for their technical support as well as Mary Nossek, Dr. Don Holzschu, and Angie McCutcheon. I want to express my gratitude to two members of the Morris Lab in particular, Donelle Robinson who has always been available to lend an ear as well as input, and Scarlett Tudor, who has never restrained her enthusiasm or willingness to help.

I am grateful for my family who helped nurture my love of the natural world through many years of nature walks, books, documentaries, and museums. I am deeply indebted to my husband, Jason Brewer, for his willingness to join me on life’s many adventures. His love and understanding have helped me see many of life’s difficulties as challenges waiting to be overcome.

And, in the words of Douglas Adams, “So long, and thanks for all the fish!”—and, of course, margaritas!

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CHAPTER 1: ALTERNATIVE LIFE HISTORIES IN *XIPHOPHORUS MULTILINEATUS*: MATING PHENOTYPES MATURE AT DIFFERENT AGES BUT GROW AT THE SAME RATE.

Abstract

According to evolutionary game theory, discrete alternative phenotypes as present in many species may have equal lifetime fitnesses realized by maximizing different life history components. The northern swordtail species *Xiphophorus multilineatus* has genetically-influenced alternative male phenotypes, in which the smaller sneaker males have a lower mating success than larger courter males due to sexual selection. I determined if the smaller sneaker males could be maximizing the probability of reaching sexual maturity rather than mating success by determining if sneaker males reach sexual maturity sooner than courters in the wild using otoliths. Males stop growing when they reach sexual maturity, and a previous study showed that otolith counts provide accurate estimates of age at sexual maturity. Because early maturation can be achieved by a faster growth rate, which in turn has implications on the allocation of resources during growth, I also examined growth rates and their potential to influence the degree of fluctuating asymmetries (FA) in the pigment pattern vertical bars. I found that sneakers mature significantly earlier than courters, but there was no difference among the three courter genotypes. The growth rate of one courter genotype, measured as the slope of the relationship between size and age at sexual maturity, was faster than the rest of the genotypes, and this genotype had a higher FA for vertical bar number, suggesting that

males with a faster growth rate may pay the cost of higher FA for a sexually selected trait. My results suggest that the size difference between sneakers and courters is primarily due to differences in the age to sexual maturity and that the faster growth rate of the Y-*II* courter genotype could explain the lack of differences in age to sexual maturity among courters.

Introduction

In many species individuals exhibit distinctly different phenotypes that influence their reproduction and survival. These phenotypes arise by a variety of mechanisms, but evolutionary game theory suggests that phenotypes resulting from a genetic polymorphism should have equal fitnesses in order to be maintained within a population (Maynard Smith 1982). Generally, the balance between these alternative phenotype fitnesses is the result of life history trade-offs. An organism with a lower instantaneous reproductive success may be able to increase its lifetime fitness by maturing earlier and thereby increase its probability of surviving to maturity as well as its lifetime reproductive period (Stearns 1992). Maturing earlier means that an organism either needs to mature at a smaller size or grow faster, both of which may incur certain costs. For animals with determinant growth, maturing at a smaller size means remaining a smaller size for life, and smaller males may not fare well in male-male competition and may be less attractive to females (Luo et al. 2005; Rios-Cardenas et al. 2007; Zimmerer and Kallman 1989). Also, as many predators are limited by mouth gape size, staying small may increase the number of potential predators (Basolo and Wagner 2004; Basolo 2008; Rosenthal 2000). Limited resources early in life (a bad start in life), can lead to compensatory growth which can carry significant costs as well (Metcalfe and Monaghan 2001). One possible outcome of compensatory growth is increased fluctuating asymmetry (FA, “nondirectional deviations from bilateral symmetry”, Bjorksten et al. 2000). Asymmetry in a sexually selected trait may have consequences for mating success (Grammer et al. 2003; Møller 1992; Møller and Thornhill 1998; Morris 1998; Swaddle and Cuthill 1994).

One of the classically cited examples of alternative mating phenotypes based on a genetic polymorphism occurs in the of the northern swordtail species, *X. nigrensis* and *X. multilineatus* (Ryan and Causey 1989; Ryan et al. 1990; Ryan et al. 1992; Zimmerer 1982; Zimmerer and Kallman 1989). These sibling species are found in the Ríos Choy and Coy, respectively, of the Río Pánuco basin on the slopes of eastern Mexico in the state of San Luis Potosí (Rauchenberger et al. 1990). In both species the larger males, called courters, actively court females and defend their territories, while the smaller males, called sneakers, can obtain matings through sneak-chase copulations or courtship depending on the presence of a larger competitor. In *Xiphophorus*, age at sexual maturity is influenced by the pituitary (*P*) locus, which is located on the Y chromosome. Variation in the alleles at this locus produces variation in the activation time of the hypothalamic-pituitary-gonadal (HPG) axis among males. The HPG axis influences the initiation of androgen release, which is associated with the onset of sexual maturity and a drastic reduction in growth (Kallman 1984; Kallman 1989). Due to this drastic reduction in growth, in *X. multilineatus* the sneaker and courter genotypes can be distinguished using a combination of standard length and caudal pigment pattern, although some ambiguity exists in *X. nigrensis*. Crosses using laboratory strains of *X. multilineatus* suggest that there are four different alleles at the *P* locus on the Y chromosome (Zimmerer and Kallman 1989) and the "small" (*s*) allele is fixed on the X chromosome. Consequently, females are homozygous for the small allele (X-*s* X-*s*) and share a similar genetic background as the sneakers (X-*s* Y-*s*) at the *P* locus. The other three alleles produce courters (intermediate-1 X-*s* Y-*I*, intermediate-2 X-*s* Y-*II*, and large X-*s* Y-*L*).

Morris and Ryan (1990) demonstrated that age at sexual maturity can be measured using otolith data and that sneakers mature at an earlier age than courters in *X. nigrensis*. Furthermore, Ryan et al. (1992) showed that this early maturation at a smaller size is able to balance the greater fertilization success of courters in *X. nigrensis*. Age at sexual maturity and its fitness consequences have not been as well studied in *X. multilineatus* as in its sibling species. For this study I estimated age at sexual maturity and growth rates for the different male genotypes of *X. multilineatus.* I compared these results for *X. multilineatus* with the previously published results for *X. nigrensis* (Morris and Ryan 1990). I tested for environmental and genetic effects on age at sexual maturity by comparing maturation age across genotypes for males from three locations. Since males grow before sexual maturity but little afterwards, I expected that males that mature later would be larger. I also tested the effect of age at sexual maturity and genotype on adult male size and compared the growth rates of the genotypes. If the *P*-locus influences the timing of the initiation of sexual maturity but not growth rate, I predicted that the genotypes would grow at the same rate. Alternatively, if maturation age and growth rate are linked in *X. multilineatus* as suggested by Kallman (1989), then the larger male genotypes would grow faster. This would be in contrast to trends in salmonids (Hutchings and Jones 1998) and centrarchids (Gross and Charnov 1980; Neff 2004) where faster growing individuals also mature earlier. Finally, I examined the degree of FA in the vertical bar pigment pattern in relation to the growth rate. Because there may be costs to accelerating growth (Metcalfe and Monaghan 2001), I predicted that genotypes with faster growth rates should have higher FA. A relationship between growth rate and FA would suggest an additional tradeoff to having a larger body size at sexual maturity, as FA of vertical bars has been shown to influence female mate preference in swordtails (Merry and Morris 2001; Morris 1998; Morris and Casey 1998).

Methods

Otolith removal and reading

Fish lay down tissue layers on their otoliths, and these tissue layers can be counted to determine age much like counting the rings on a tree (Pannella 1971). The interval at which fish lay down layers varies between species, and Morris and Ryan (1990) showed that *X. nigrensis* lays down rings for every day of growth. Since growth ceases or drastically reduces at sexual maturity for males, the number of rings is highly correlated with the age at sexual maturity in males. Since *X. nigrensis* and *X. multilineatus* are sibling species, I assumed that this result for *X. nigrensis* extends to *X. multilineatus*.

Adult males were collected in January 2007 from the Río Coy (*N* = 24) and its two tributaries, Oxitipa (*N* = 18) and Tambaque (*N* = 19). The pigment patterns and standard length (SL) were recorded in the field and used to determine the genotype

(Zimmerer and Kallman 1989). The fish were preserved in 95% ethanol and returned to the lab. I removed the three otoliths and mounted them on a glass microscope slide. Following the methodology outlined in Morris and Ryan (1990), I examined the intermediate-sized otolith (asteriscus), because it is the most translucent of the three. Since the surface of the asteriscus is uneven, I took a series of 2-8 photos at 400X to ensure that all rings were captured clearly in at least one photo. I viewed the series of photos in Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA). I added a layer to the first photo of the series and marked each ring clearly visible marked on the layer using the pencil tool. I transferred the layer was to the next photo in the series, and all visible rings were marked. I then repeated this process for all photos in the series and saved the marked layer separately. I wrote a macro to automatically count the number of marks on the layer in ImageJ 1.37 (Rasband 2008) to avoid manual counting error. See the Appendix for specific directions and macro code.

Since otoliths can be difficult to read (Campana 2001), I counted each sample until the readings were consistent such that all otoliths were read once before proceeding to the second reading and so forth. To test for a learning curve associated with reading an otolith, I used a repeated measures ANOVA to detect an effect of reading (1, 2, 3) on the number of rings ($F_{2, 128} = 20.16$, $P < 0.0001$). Using post-hoc Tukey's HSD test, I detected that the first reading was significantly different from the second and third readings at the 0.05 level of significance. I attributed this to the learning curve associated with reading otoliths (Campana 2001), and therefore, reading 1 was discarded. Since I found no significant difference between the remaining readings, I averaged them for ease of analysis. Because Morris and Ryan (1990) found that an average of 5 rings were laid down before birth in *X. nigrensis*, I subtracted five days from the total count for the measure of age to sexual maturity.

Statistical analysis

The data from 64 males (31 sneakers and 33 courters) conformed to the assumptions of the corresponding tests. I used an ANOVA to test the effects of genotype and population on the response variable age at sexual maturity and used Tukey's HSD test to do pairwise comparisons of the genotypes.

I used an analysis of covariance (ANCOVA) to compare SL by genotype using age at sexual maturity as a covariate. Because the change in SL over the change in maturation age (the slope of the relationship between these two variables) represents the growth rate, I included the interaction between genotype and age at sexual maturity in the model to test if the genotypes are growing at different rates. Post-hoc Tukey's HSD test was used to determine significant differences among genotypes.

I measured FA by comparing the number of bars on the right and left sides of the males. If the number of bars was the same, the male was scored as symmetrical; otherwise, the male was scored as asymmetrical. Due to the tradeoff between growth rate and FA, I grouped genotypes according to significant differences in growth rate and then compared the FA of those groups using a nominal logistic regression. I performed statistical analyses using JMP 7.0.1 (SAS, Cary, NC).

Results

I detected a significant effect of genotype on age at sexual maturity (ANOVA: $F_{3,52} = 18.11$, $P < 0.0001$; Table 1, Figure 1), and using post-hoc Tukey's HSD test, I found that at the 0.05 level of significance sneakers matured significantly earlier than all courter genotypes while courter genotypes did not differ significantly. However, I did not detect an effect of population on the age at sexual maturity (ANOVA: $F_{2,52} = 0.60$, $P = 0.56$) nor was the interaction between population and genotype significant ($F_{6,52} = 0.66$, $P = 0.67$).

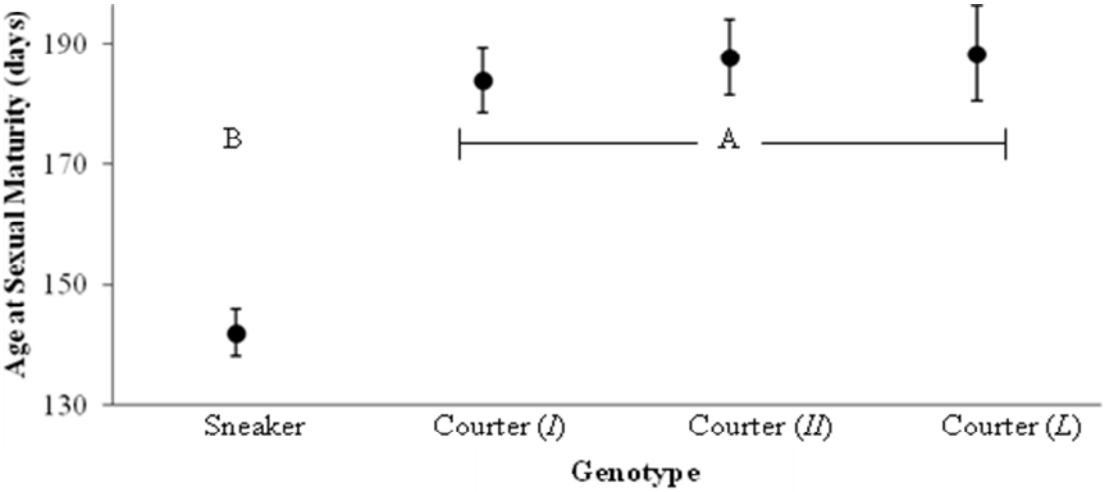

Figure 1. Mean age at sexual maturity (±SE) in days by genotype for *X. multilineatus* males and groupings from Tukey's HSD test.

I detected that age at sexual maturity ($F_{1,56} = 7.28$, $P = 0.0092$) and genotype ($F_{3,56} = 29.58$, $P < 0.0001$) significantly affected the SL of males (ANCOVA: $F_{7,56} = 46.33$, adjusted $R^2 = 0.83$, $P < 0.0001$; Figure 2). When I compared the slopes of the relationship between size and age at sexual maturity across the genotypes, I found a

Table 1. *Summary of results (mean ± SD) for each* P *locus genotype of male* X. multilineatus

Genotype		Sneaker (X-*s* Y-*s*)	Intermediate I (X-*s* Y-*I*)	Intermediate II (X-*s* Y-*II*)	Large (X-*s* Y-*L*)
N		31	11	15	7
Size range (mm)[1]		18-28 mm	22-32	26-39	32-47
SL (mm)		23.43±1.23	29.34±2.05	32.23±3.58	33.27±1.70
Ring count (days)		141.95±21.58	183.93±17.89	187.83±23.70	188.39±21.10
Growth rate (mm/day)		0.17±0.03	0.16±0.02	0.17±0.02	0.17±0.02
Bar number difference	±0	6	5	3	4
	±1	3	3	10	1
	±2	2	3	2	2
	±3	1	0	0	0
Growth rate of symmetrical (mm/day)		0.16±0.03	0.15±0.01	0.17±0.01	0.17±0.02
Growth rate of asymmetrical (mm/day)		0.15±0.02	0.16±0.02	0.17±0.02	0.18±0.02

[1] From several samplings of wild populations (Rios-Cardenas et al. 2007 and unpublished data).

significant interaction of genotype and maturation age ($F_{3,56}$ = 3.29, *P* = 0.027). This significant difference across genotypes suggests that the genotypes are not growing at the same rate; specifically, the Y-*II* genotype had a significantly different slope (*t* = 2.72, *P* = 0.0087) while the growth rates of the other genotypes were not significantly different (*P* > 0.05).

Of the males sampled, 45 were barred (18 symmetrical and 27 asymmetrical by ± 1 bar or more, Table 1). All courter males have bars, but the presence of bars is

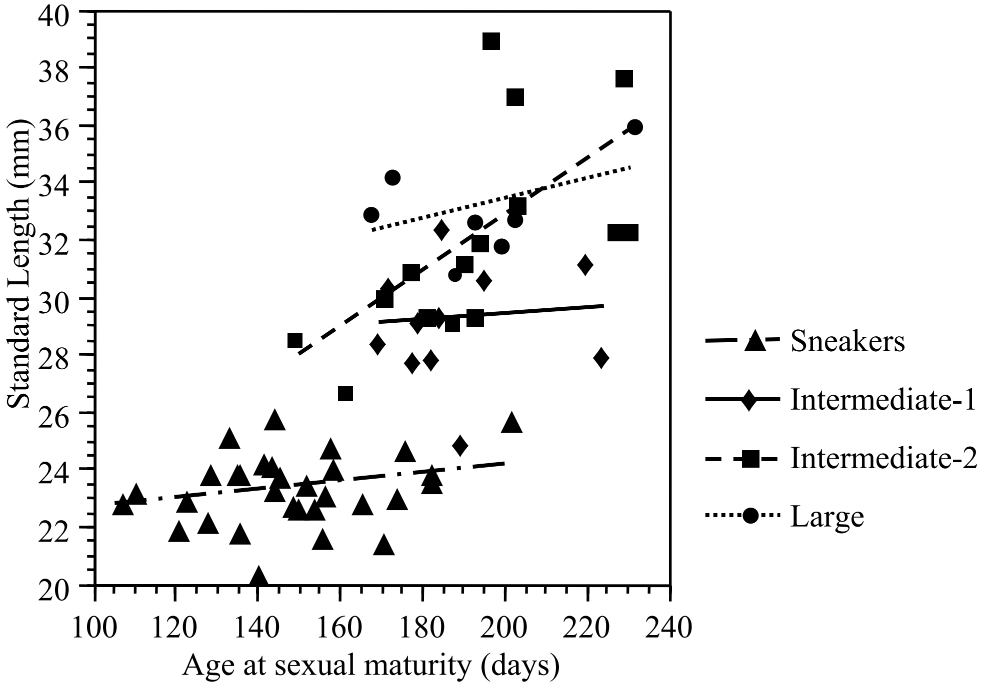

Figure 2. Relationship between body size (standard length) and maturation age in days as counted by otolith rings. Note that size ranges include the range from all rivers sampled and are shown for each genotype (modified from Zimmerer & Kallman 1989).

polymorphic among sneakers in *X. multilineatus* (Zimmerer and Kallman 1988), and 12 of 31 sneakers were barred among my sample. Since the Y-*II* genotype grew significantly faster than the other genotypes, I compared its FA to other three genotypes combined. I found that the Y-*II* genotype was significantly more asymmetrical than the rest of the males (likelihood ratio $\chi^2 = 3.97$, $P = 0.046$).

Discussion

The genetically-influenced courter males have a significant mating advantage due to sexual selection in *X. multilineatus* (Luo et al. 2005; M.R. Morris, O. Rios-Cardenas and J.C. Brewer, unpublished manuscript; Rios-Cardenas et al. 2007). In the current study I demonstrate that in nature *X. multilineatus* sneaker males reach sexual maturity sooner. This early maturation may allow sneakers to balance their fitnesses with courters. The genetically-influenced sneaker males in *X. nigrensis* are able to compensate for their lower fertilization success by maturing earlier, as Ryan et al. (1992) demonstrated equal fitnesses among the sneaker and courters of this closely related swordtail species. By maturing sooner, sneakers have a higher probability of reaching sexual maturity and reproducing, assuming that males of both phenotypes have the same juvenile mortality rate. This is a realistic assumption as phenotype is practically indistinguishable until close to maturity. Maturing earlier may also allow sneakers to have a longer reproductive lifespan assuming that adult mortality rate is constant. Finally, by maturing earlier sneakers have a shorter generation time, allowing the sneaker genotype to increase faster. However, I did not detect a difference in the age of sexual maturity between the wild-caught courter genotypes, suggesting that maturing earlier cannot help explain the maintenance of the three different courter genotypes in this species.

As I found a significant effect of genotype but not of population on age to sexual maturity, the genotype at the *P* locus appears more influential on age at sexual maturity than any environmental differences across populations. This result does not rule out environment as a factor that may affect age at sexual maturity but suggests that environmental differences between the three rivers of the Río Coy drainage I sampled are not sufficient to produce differences in age at sexual maturity. Environmental variation may be responsible for the variation of maturation age within a genotype, but if such environmental variation is present within a population but is consistent across rivers, then

I would be unable to detect it in this analysis. Also, since the interaction between genotype and population is not significant, the river does not affect the age at sexual maturity among the genotypes differently.

The Y-*II* genotype grew faster than the rest of the genotypes in growth rate. This difference was detected when examining the slopes of the relationship between age and size at sexual maturity, not the mean growth rates for the genotypes, which would have been less likely to have detected this variation across genotypes. I did find a correlation between growth rate and symmetry, supporting the hypothesis that growth rate affected FA of vertical bars. Although not a direct test of compensatory growth reducing symmetry, the difference in FA between the Y-*II* genotype and those with lower growth rates suggests that the influence of growth rate on vertical bar FA should be studied further. It should be noted that my method for estimating growth rate does not allow the detection of variation in growth rate within an individual. For instance, males who grew very quickly during a limited period and slowed afterwards and males who grew at a more consistent rate could have very similar growth rates. This type of compensatory growth would not be detected in this study. The relationship between growth rate and FA is interesting for two reasons. First, many organisms have been shown to have a heritable basis for symmetry (Palmer and Strobeck 1986), and these results suggest that there may be a correlation between FA and certain genotypes in *X. multilineatus*. Although both vertical bars and *P* allele are heritable (Zimmer and Kallman 1988; 1989), the heritability of the symmetry of vertical bars is unknown, and future studies of FA in this species may be particularly rewarding. Second, these results suggest that while we may understand the tradeoffs that help to maintain the sneaker and courter genotypes, the maintenance of the three different courter genotypes in this species remains a mystery. While one might assume that each size class is trading early sexual maturity in favor of being a better, larger competitor in terms of sexual selection, it appears that all of the courter male genotypes are optimizing body size over early maturation. The Y-*II* genotype is paying a cost for growing faster (increased FA), but does not use the faster growth to reach sexual maturity sooner but grows as long as the Y-*L* males to the largest size possible. The cost to FA will need to be examined further as well, as vertical bar symmetry and female size affect female preference in *Xiphophorus* (Morris and Casey1998; Morris et al. 2003; Morris et al. 2006), and variation in female preference may affect fertilization success among courters.

X. nigrensis males matured earlier than did *X. multilineatus* males across each of the size classes (Table 2). The size ranges of the courters and sneakers for *X. nigrensis* fall within the range of the size of courters and sneakers for *X. multilineatus*: 22-26 mm for sneakers and 25-40 mm for courters in *X. nigrensis* (Kallman 1989) and 18-28 mm for sneakers and 22-47 mm for courters in *X. multilineatus* (Kallman 1989; Rios-Cardenas et al. 2007; Rios-Cardenas, unpublished data). However, the size range for both phenotypes is greater in *X. multilineatus*, and at least for the courter phenotype, this may be due to the additional courter *P*-allele. Although the size ranges of the males I examined in the current study are comparable to those of *X. nigrensis* examined by Morris and Ryan (1990), the mean age difference between the alternative phenotypes detected in the two

Table 2. *Comparison between* X. nigrensis *(Morris and Ryan 1990) and* X. multilineatus

	X. multilineatus	*X. nigrensis*
Mean courter SL (range)	31.42 mm (25-42 mm)[‡]	33.3 mm (25-40mm)[£]
Mean sneaker SL (range)	23.43 mm (22-28 mm) [‡]	24.1 mm (22-26 mm) [£]
Mean courter age at sexual maturity	191 days	107 days
Mean sneaker age at sexual maturity	147 days	78 days
Mean difference in ages at sexual maturity between courters and sneakers	44 days	29 days

[£] Ranges and means calculated from data reported by Kallman (1989).
[‡] Ranges reported in Zimmerer and Kallman (1989).

studies (sneakers as compared to all courters) was greater in *X. multilineatus* (44 days) than in *X. nigrensis* (29 days). Since size and age at maturity are tightly correlated in both species and *X. nigrensis* of a similar size matures earlier than a *X. multilineatus* male, then both phenotypes in *X. nigrensis* grow faster than in *X. multilineatus*. Because otoliths are notoriously difficult to read, there is a subjective aspect to reading the otolith (Campana 2001). However, while some difference might be attributable to discrepancies among the otolith reading techniques between Morris and Ryan (1990) and those used in the current study, they are unlikely to explain the approximately twofold difference between these two species. The disparity in growth rate could be due to differences in genetic factors other than the *P* locus influencing growth rate in these species, as well as environmental differences between the species' habitats. One of the main differences is that the *X. nigrensis* studied by Morris and Ryan (1990) is found in the headwaters of the Río Choy, which is less subject to seasonal fluctuations in temperature and water flow than the rivers in which *X. multilineatus* were collected. Regardless of which of these two factors are driving the difference, it could have interesting implications for the expected ESSt frequencies of the two morphs in these two species. All else being equal, the advantage to maturing earlier for sneakers would be less in *X. nigrensis* than *X. multilineatus*, and therefore the frequency of courters at ESSt should be greater in *X. nigrensis* than *X. multilineatus*.

A previous ontogenetic laboratory study across *Xiphophorus* compared growth rate and age at sexual maturity (Marcus and McCune 1999). They found that *X. multilineatus* females grew relatively slower (0.17 mm/day) than *X. nigrensis* females (0.21 mm/day), which is a similar pattern to what I detected for males in these two species. *X. multilineatus* sneaker males in the Marcus and McCune (1999) study grew at a rate of 0.15 mm/day, which is comparable to the rate measured in the current study for wild caught sneaker males (Table 1). Male data were not reported for *X. nigrensis*. My results support the findings of Kallman (1989), who suggested that the *P* locus affects "age at cessation of growth" (age at sexual maturity) and growth rate, as I found evidence that the genotypes do not all grow at the same rate in *X. multilineatus*. However, my results indicate that only the Y-*II* genotype grew at a different rate than the rest of the genotypes unlike Kallman's results that growth rate increases with size class of the genotypes. In a laboratory study, Kallman (1989) found for both *X. nigrensis* and *X. multilineatus* that the Y-*s* genotype males grow the slowest, and the Y-*I*, Y-*II* and Y-*L* courter males grew faster in that order. One possible explanation for the differences in these results is that growth rate could be plastic, and the differences are more apparent in a laboratory environment as compared to the field. In combination, these studies suggest that there is a genetic influence of growth rate that is related to allelic state at the *P* locus.

In conclusion, the alternative mating phenotypes among male *X. multilineatus* have life history tradeoffs, and this could have consequences for how the genotypes are maintained within a population. I found a significant difference in the age at sexual maturity between the courters and sneakers in *X. multilineatus*, in concurrence with results found for these two phenotypes in *X. nigrensis* (Morris and Ryan 1990). Sneakers may be using age at sexual maturity as a tradeoff for the increased fertilization success of courters (Luo et al. 1996; M.R. Morris, O. Rios-Cardenas and J.C. Brewer, unpublished manuscript; Rios-Cardenas et al. 2007; Zimmerer and Kallman 1989). This means that sneakers have a higher probability of reaching sexual maturity, assuming equal juvenile mortality, a longer reproductive period if both phenotypes have the same adult mortality rate and a shorter generation time. In addition, both the sneaker and courter phenotypes in *X. multilineatus* took longer to reach sexual maturity than the same phenotypes in *X. nigrensis* (Morris and Ryan 1990). This difference seems to be caused by slower growth rate in *X. multilineatus*, which in turn may be due to genetic differences (at loci other than the *P* locus) between the species or environmental differences between the rivers they inhabit. Since I found a significant effect of genotype but not population, I can conclude that the *P* locus is more influential for age at sexual maturity than environment, at least under the conditions at the sites sampled for this study. I found that the genotype did have an effect on growth rate, indicating that the *P* locus influences age at sexual maturity rather than growth rate. My results did find a relationship between FA in the vertical bar pigment pattern and growth rate with the fastest growing genotype being significantly more asymmetrical than the other genotypes. Overall, my results explain some of the mechanisms that facilitate the maintenance of alternative reproductive strategies in systems with a strong genetic influence.

CHAPTER 2: DO ALTERNATIVE LIFE-HISTORY AND MATING PHENOTYPES IN *XIPHOPHORUS MULTILINEATUS* HAVE EQUAL FITNESSES?

Abstract

Genetically-influenced alternative mating phenotypes are assumed to have equal average fitnesses when at an Evolutionary Stable State (ESSt) and to be maintained at this equilibrium point by negative frequency-dependent selection. I empirically tested this hypothesis for the alternative male mating phenotypes in the swordtail fish, *Xiphophorus multilineatus*. These phenotypes are influenced by a single locus polymorphism on the Y chromosome. Smaller sneakers mate via sneak-chase or courtship behavior depending on social conditions, while larger, ornamented courters use courtship behavior exclusively. A previous study suggested that shifts in female size distribution alter the strength of their preference for the courters, which could change the ESSt to a different frequency of sneakers. I assessed the relative fitnesses of the two phenotypes for *X. multilineatus* at a point in time when preference for the courters was lower and the frequency of sneakers males was higher than previously sampled. I calculated the mortality rate necessary for equal fitnesses given empirical estimates of age at sexual maturity and relative fertilization success of the two phenotypes. I then determined if this mortality rate would be biologically realistic for a stable population using female fecundity data. Separately, I compared the intrinsic growth rates of population growth for each phenotype to determine if they were equal as an additional check. I rejected the hypothesis that this population of *X. multilineatus* is at an ESSt,

although I did find preliminary evidence of negative frequency-dependent selection. I discuss the differences between the two species in relation to potential mechanisms maintaining the alternative mating phenotypes.

Introduction

Alternative phenotypes in mating behaviors and life-history traits were once thought to be difficult to evolve and unstable but are now known to be quite common (Oliviera et al. 2008; Sinervo and Calsbeek 2006). According to evolutionary game theory, alternatives that are produced by genetic polymorphisms are maintained by negative frequency-dependent selection at an ESSt where they have equal fitnesses (Maynard Smith 1982). One way alternatives can arrive at an ESSt is by balancing mating success and survival to reach equal fitnesses. Given that many different factors could influence mating success and survival, which vary both spatially and temporally, one might also expect that different populations have different ESSt's. In such cases the frequency of morphs would not be the same across populations or time. Therefore, variation in the frequency of alternative phenotypes across space and time could reflect shifts in the ESSt due to changes in selection that influence the phenotypes differently. Alternatively, variation in the frequency of phenotypes could suggest that the population is cycling around the ESSt (Hori 1993; Sinervo and Lively 1996; Sinervo et al. 2007), or that mechanisms other than negative frequency dependence, such as temporal and spatial fluctuations, may maintain alternatives without equal fitnesses of the alternatives (Table 3; Austad 1984; Calsbeek et al. 2002; Dominey 1984; Fogel et al. 1997; Fogel et al. 1998; Gross 1996).

Table 3. *Review of mechanisms that maintain alternative mating phenotypes*

Mechanism	Equal Fitnesses required	Frequency Dependence	Variation in predation levels	Variation in female preference	Gene flow
Negative frequency-dependent selection	Required*	Required	Not required	Not required	Not required
Spatially oscillating selection	Not required	Must not exist	Either one must exist		Required
Temporally oscillating selection	Not required	Must not exist	Either one must exist		Must not exist
Disruptive selection/assortative mating	Required	Must not exist	Not required	Required	Not required

*Mean equal fitnesses are required over time but not necessarily at a single time point if the frequencies are cycling

Note: Under ideal conditions these designations would allow for discrimination between the underlying mechanisms. However, most mechanisms are not necessarily mutually exclusive.

Ryan et al. (1992) developed a model to determine if benefits due to sexual selection (greater mating success of larger courting males) were balanced by the benefits of greater survival to reproductive age (smaller sneaker males have a higher probability of reaching sexual maturity) for the alternative mating strategies in the swordtail fish *X. nigrensis*. Using a modified version of the Euler equation and data on relative fecundities (Ryan et al. 1992) and age at first reproduction (Morris and Ryan 1990), they determined the mortality necessary for the two morphs to have equal fitnesses. They then checked if this mortality rate was biologically realistic for a stationary population using female demographic data. Based on these results, they concluded that the two types of males in *X. nigrensis* had equal reproductive successes.

X. nigrensis has a sibling species, *X. multilineatus*, which displays the same alternative phenotypes (Zimmerer and Kallman 1989). These two species were once considered different populations of the same species, but morphological and genetic (allozyme) evidence has since supported the species status of *X. multilineatus* (Rauchenberger et al. 1990). Examining the maintenance of the alternative phenotypes in *X. multilineatus* has several advantages over *X. nigrensis*. First, the phenotypes can unambiguously indicate the genotypes in *X. multilineatus* whereas some ambiguity exists in identifying genotype from phenotypes in *X. nigrensis* (Kallman 1989; Ryan et al. 1990). Second, *X. multilineatus* is more widely distributed than *X. nigrensis*, with several populations identified and studied (Rauchenberger 1990; Rios-Cardenas et al. 2007). In the population of *X. nigrensis* that was previously examined, the frequency of the alternative phenotypes may remain more constant over time than a population of *X. multilineatus* (Figure 3), which vary rather extensively over space and time (Rios-Cardenas et al. 2007). Estimating the relative fitnesses of the genotypes at a point in time when the frequencies are at an extreme can help identify the different mechanisms that may be involved in maintaining these alternative strategies.

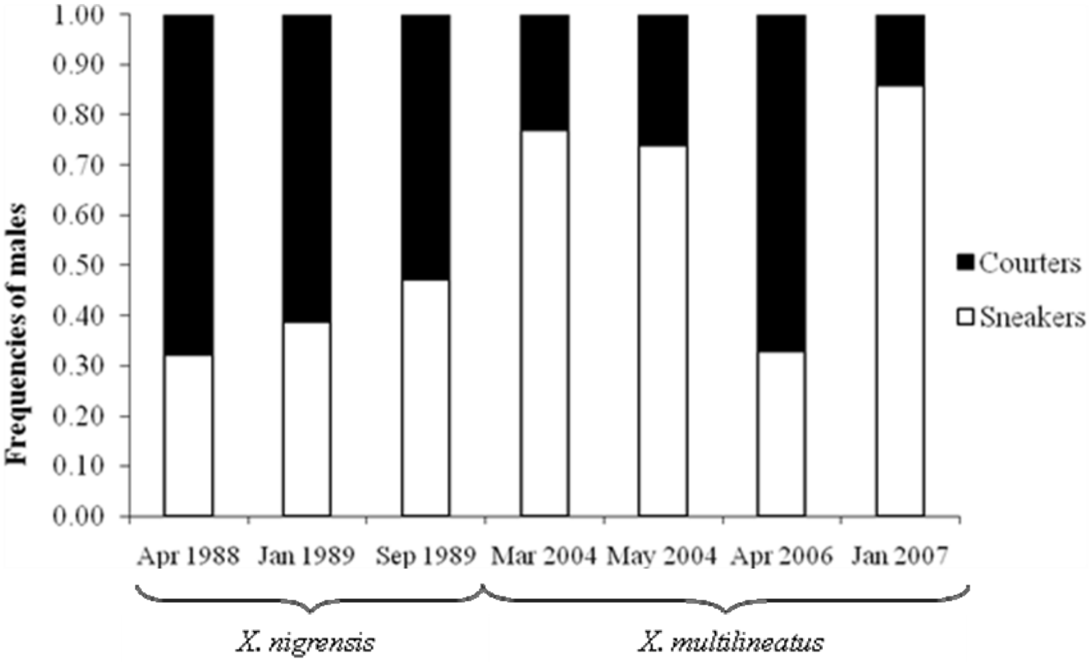

Figure 3. Frequencies of alternative phenotypes in *X. nigrensis* at the *nacimiento* of the Río Choy (M.R. Morris, unpublished data) and *X. multilineatus* at the Río Oxitipa from 2004 to 2007 (Rios-Cardenas et al. 2007).

Rios-Cardenas et al. (2007) demonstrated that the strength of female preference is positively correlated with female size in *X. multilineatus* with larger females having a stronger preference for courters. They assessed female size distribution and frequency of male phenotypes at several sites over time and found that courters are most common in populations with the largest females. These results suggest that changes in the female preference driven by variation in female size distribution may alter the mating success of the male phenotypes over space and time. Therefore, changes in the size distribution of the females could be responsible for the fluctuations in the frequencies of the two phenotypes by changing the equilibrium frequencies of male phenotypes for a population at an ESSt (Figure 4). Alternatively, the varying frequencies of the two phenotypes in *X.*

multilineatus may be due to oscillations around equilibrium via frequency-dependent selection. For example, oscillations due to frequency-dependent selection have been detected in the handedness of the scale-eating cichlid *Perissodus microlepis* (Hori 1993). When frequencies behave in this fashion, the fitnesses are expected to be unequal when the population has fluctuated away from ESSt but balance out over time. Other models suggest that the fluctuations may occur in a less predictable manner unrelated to an equilibrium point without ever requiring fitnesses to be equal (Calsbeek et al. 2002; Ellner and Hairston 1994; Fogel et al. 1997; Fogel et al. 1998; Frank and Slatkin 1990).

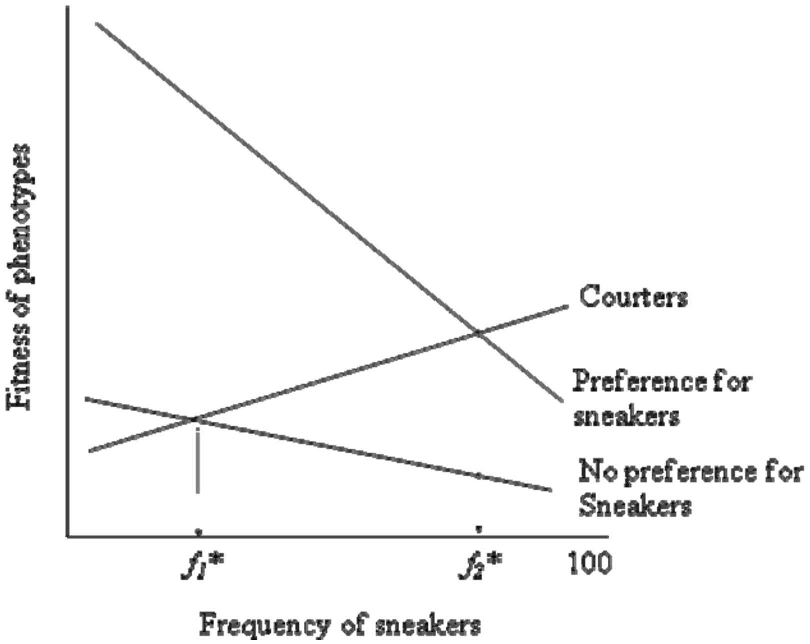

Figure 4. Influence of female preference on the fitness function for sneakers, the preference for sneakers will increase the sneaker' ESSt from f_1* to f_2*.

In the current study, I tested the hypothesis that the two phenotypes have equal fitnesses in a population of *X. multilineatus* when the frequency of sneaker males was at a high point for this population. If the fitnesses of the male phenotypes are equal at an extreme point in the fluctuations (*i.e.* the frequencies are very different from other samples), then I will have evidence to suggest that the ESSt is resilient to variation in female preference. In this case, variation in female preference may just be shifting the equilibrium frequencies, but an ESSt would be maintained. Otherwise, I will be able to eliminate the hypothesis that different ESSt's is the primary reason for the fluctuations in the frequencies of the two morphs.

The System

The high-back pygmy northern swordtail fish (*X. multilineatus*) is found in the Río Pánuco Basin of the Atlantic slopes of San Luis Potosí, Mexico. *X. multilineatus* are livebearing fish that breed continuously (Morris and Ryan 1990) and can reproduce from stored sperm for up to 8.5 months in the laboratory (M.R. Morris, O. Rios-Cardenas and J.C. Brewer, unpublished manuscript). *X. multilineatus* is a commonly cited example of genetically influenced alternative male mating strategies expressed as two distinct phenotypes first described by Zimmerer (1982). These phenotypes differ distinctly in size, morphology, and behavior. The larger males, hereafter called courters, mature later at a larger size, have vertical body bars, and are sexually ornamented with a "sword" extending ventrally from the caudal fin. Males of the other phenotype, referred to as sneakers, mature earlier at a smaller size, do not often have vertical body bars, and rarely have a well-developed sword. This suite of traits is tightly correlated with allelic variation at the pituitary (*P*) locus on the Y chromosome. Androgens are released by the activation of the hypothalamic-pituitary-gonadal axis and their release initiates sexual maturity and the end or drastic reduction of growth. In males, four alleles occurring at the *P* locus (Y-*s*, Y-*I*, Y-*II*, and Y-*L*) influence the onset of this axis and thus the size at sexual maturity (Kallman 1984; Kallman 1989). Since the locus is monomorphic on the X chromosomes (X-*s*), courters (with either Y-*I*, Y-*II*, and Y-*L* alleles) produce only courter sons, and sneakers (Y-*s*) produce only sneaker sons. Growth rate is drastically reduced after sexual maturity, and therefore, courters are larger than sneakers. The genotypes can be identified using a combination of the standard length (SL), which is measured as the length from the tip of the snout to the caudal peduncle, and pigment patterns on their caudal fin (Table 4). One advantage *X. multilineatus* has over *X. nigrensis* in this particular analysis is that the genotypes in *X. multilineatus* can be unambiguously identified from the phenotypes. There are no such secondary phenotypic traits to help distinguish genotypes in *X. nigrensis*, and therefore, some ambiguity existed when Ryan et al. (1992) inferred genotypes based on SL in the range where the size of Y-*I* males overlap with the size of Y-*s* males as well as the overlap between Y-*I* males and Y-*L* males.

The courter and sneaker phenotypes in *X. multilineatus* are associated with different mating behaviors. Courters use only courtship mating behavior, and sneakers are plastic in their mating behavior, using both courtship and sneak-chase mating behavior depending on the presence of competitors (Zimmerer and Kallman 1989). Zimmerer and Kallman (1989) also demonstrated mating behavior of the *X. multilineatus* males was not conditional of body size. Since different *P* alleles produce males that

Table 4. *Genotypes, Y linked pigment patterns, standard length, and presence of sneak-chase behavior of the four male genotypes of* X. multilineatus *(modified from Zimmerer and Kallman 1989)*

Phenotypic size-class	Genotype	Color pattern	Standard length range (mm)			Mating behavior
			Río Coy	Río Oxitipa	Río Tambaque	
Small	X-*s*/Y-*s*	Blue, yellow	18-28	18-28	19-28	Sneak-chase and courting
Intermediate-1	X-*s*/Y-*I*	Margins of caudal fin yellow	22-32	22-32	23-32	Courting
Intermediate-2	X-*s*/Y-*II*	Caudal fin yellow	27-38	26-39	26-38	Courting
Large	X-*s*/Y-*L*	Margins of caudal fin yellow	32-47	32-42	32-42	Courting

overlap in size, they compared the mating behavior of size matched males from the two smallest size classes (Y-*s* and Y-*I*) and found that the Y-*s* males tend to use sneak-chase but could use courtship only in the absence of other males, while the Y-*I* males only use courtship in any competitive situations. They also demonstrated that in competitive mating experiments, the larger courting males produced more than twice as many offspring as the smaller sneaker males. Despite courters having a greater relative reproductive success, both phenotypes have been maintained in the wild at least since their first description (Zimmerer 1982). If courters produce more offspring, what maintains the *s*-allele that produces the small sneaker males?

I followed the approach of Ryan et al. (1992) to test the prediction of equal fitnesses for *X. multilineatus* courters and sneakers in the Río Oxitipa population for a sample taken in January 2007. By using estimates of reproductive success (M.R. Morris, O. Rios-Cardenas and J.C. Brewer, unpublished manuscript) and age to sexual maturity (Chapter 1), I calculated the mortality rate necessary for the morphs to have equal fitnesses at an ESSt. I conducted this study in the Río Oxitipa at a point in time when this population had significantly smaller females and significantly greater number of sneaker males than the other populations sampled (Rios-Cardenas et al. 2007). Because there is a positive relationship between female size and strength of preference for courters, this population with smaller females would have a decrease preference for larger males (Rios-Cardenas et al. 2007), and this change in female preference may have shifted the ESSt to a higher equilibrium frequency of sneakers (Figure 4). If that is the case, then the alternative phenotypes should have equal fitnesses even at this high frequency of sneakers.

Methods and Results

Modeling morph fitness

I used a modified version of the Euler equation for clonally reproducing populations to determine the mortality rate that would be necessary for the relative fitnesses of the two phenotypes to be equal. Assuming overlapping generations, which is reasonable for *X. multilineatus*, the asymptotic population growth rate of a morph, r_i, is

$$1 = m_i \int_{f_i}^{\infty} e^{-\delta_i x} e^{-r_i x} \, dx \qquad (1)$$

where f_i is the age at sexual maturity, δ_i is the per capita adult mortality rate, and m_i is the fecundity at age x for morph i (1 = sneakers, 2 = courters). This clonal model is appropriate for *X. multilineatus*, because the alleles that encode for the phenotype are located on *P* locus on the Y chromosome and consequently, fathers pass their phenotype directly to their sons. Additional assumptions for ease of calculation include that the age at sexual maturity is equal to the age at first reproduction and the per capita mortality rate is constant across morphs ($\delta_1 = \delta_2$).

Estimate for the relative fertilization success, m_1/m_2

The estimate of the relative fertilization success for the two phenotypes is based on the number of sneakers and courters at the Río Oxitipa in January 2007 compared to the number of sneaker and courter offspring produced by females collected in the field at the same time and location (M.R. Morris, O. Rios-Cardenas and J.C. Brewer, unpublished manuscript). The females were brought back to the laboratory at Ohio University, and each female was isolated in a 18.9 L tank and allowed to drop offspring (up to 5 broods over 10 months) until their stored sperm was exhausted (M.R. Morris, O. Rios-Cardenas and J.C. Brewer, unpublished manuscript). This differs from the method used in Ryan et al. (1992), which only collected fry from the first brood; therefore, I performed my calculations using data from the first brood only and then repeated the analysis using data from the total offspring dropped for each female. The F1 offspring were tracked individually until maturity, recording their sex, standard length (SL), and the pigment patterns correlated with *P*-allele genotype (Zimmerer and Kallman 1989). The frequency of adult sneakers, π_1^A (0.86), and courters, π_2^A (0.14), in the field was then compared to the frequencies of their offspring, π_1^O (0.45 for the first brood and 0.36 for all broods) and π_2^O (0.55 for the first brood and 0.64 for all broods), respectively. The relative fertilization success was then calculated from these frequencies,

$$\frac{m_1}{m_2} = \frac{\pi_1^O \pi_2^A}{\pi_1^A \pi_2^O} \tag{2}$$

such that $m_1/m_2 = 0.130$ for the first brood and 0.090 for all broods (Table 5).

Estimate for the age at sexual maturity, $\mathrm{f_i}$

I used the age to sexual maturity data estimated from otolith ring counts on wild caught *X. multilineatus* males (Chapter 1). Morris and Ryan (1990) demonstrated that the number of otolith rings was an indicator of age (days) at sexual maturity rather than true age in *X. nigrensis*. I followed the methods outlined in Morris and Ryan (1990) for otolith extraction and reading with some modifications. I assumed that the number of rings was an indicator of age at sexual maturity in *X. multilineatus* as well, due to the close genetic relationship between the species (Rauchenberger et al. 1990; Meyer et al. 1994; Morris et al. 2001). Briefly, 64 males (31 sneakers and 33 courters) were collected from the Río Coy (*N* = 27) and its two tributaries, Oxitipa (*N* = 17) and Tambaque (*N* = 20). Once the otoliths were removed and mounted, I took photographs of the intermediate otolith, the asteriscus, at 400X, and I marked and counted all visible rings to estimate the age at sexual maturity. I did not detect a difference across locations.

Table 5. *Comparison of the results from the previous study on* X. nigrensis *(Ryan et al. 1992) and the results from the current study on* X. multilineatus

	Estimates	*X. nigrensis* (Ryan et al. 1992)	*X. multilineatus* (from current study)	
Age at sexual maturity	Sneakers (f_1)	78 d	146.95 d	
	Courters (f_2)	107 d	191.21 d	
	Relative (f_2-f_1)	29 d	44.25 d	
Fertilization success			1st brood	All broods
	Relative (m_1/m_2)	0.44	0.013	0.090
Birth rate	Based on female demographic data	0.25 d^{-1}	0.053 d^{-1}	0.024 d^{-1}
Mortality Rate	δ necessary for ESS	0.028 d^{-1}	0.046 d^{-1}	0.054 d^{-1}
	Bootstrap 95% CI for δ	[0.006 - 0.066 d^{-1}]	[0.019: 0.085 d^{-1}]	[0.030: 0.094 d^{-1}]
	Per capita growth rate (r)	4.62×10^{-5}	-0.038	-0.047
	Bootstrap 95% CI for r	NA	[-0.085: -0.012]	NA
Direct comparison of r	Sneakers (r_1)	NA	6.93×10^{-3}	NA
	Courters (r_2)	NA	5.89×10^{-3}	NA
	$r_2 - r_1$	NA	1.04×10^{-3}	NA
	95% CI for $r_2 - r_1$	NA	[1.21×10^{-4}: $.56\times10^{-3}$]	NA

Therefore, I pooled data across sites and used the mean age to sexual maturity for sneakers (147 d) and for all three genotypes of courters (191 d) as the age to first reproduction (f_1 and f_2) for the two morphs. Thus, the difference in age at sexual maturity (f_2 - f_1) is 44 d (Table 5).

Testing the prediction of equal fitnesses

Estimate of the mortality rate.

To determine the mortality rate necessary for the fitnesses of the alternative phenotypes to be equal, I set the fitness of morph 1 equal to the fitness of morph 2,

$$1 = m_1 \int_{f_1}^{\infty} e^{-(\delta_1 + r_1)x} dx = m_2 \int_{f_2}^{\infty} e^{-(\delta_2 + r_2)x} dx \tag{3}$$

If the per capita growth rate and per capita mortality rate are constant across morphs ($r_1 = r_2 = r$ and $\delta_1 = \delta_2 = \delta$), then

$$\delta + r = \frac{\ln(m_2 / m_1)}{f_2 - f_1} \tag{4}$$

Additionally, I assumed that the per capita growth rate of both morphs is stationary ($r = 0$), I solved for δ that balances the relative age at sexual maturity and relative fertilization success and makes the fitnesses of the two morphs equal in eqn. 5

$$\delta = \frac{\ln(m_2 / m_1)}{f_2 - f_1} \tag{5}$$

I then used my estimates for m_2/m_1 and f_2-f_1 above to solve for δ, which was 0.046 d^{-1} for the first brood and 0.054 d^{-1} for all broods (Table 5).

Confidence interval (CI) for δ

I constructed a bootstrap statistic to quantify the uncertainty of my estimate of δ by creating 10,000 permutations, an increase from the 1,000 iterations used in Ryan et al. (1992), by randomizing the data with replacement used to calculate δ. For each iteration, I used the raw data (number of rings averaged across readings for each otolith) used to estimate f_i. I randomly drew a sample from the sneaker data of the same size as the original sample ($N = 31$) and repeated this process for the courters ($N = 34$). To estimate the relative reproductive success, I created a binomial distribution based on the sneaker frequency (~0.86) among adults in the field ($N = 22$) and another based on the sneaker frequency (~0.45 for the first brood and ~0.36 for all broods) among all male offspring ($N = 113$). Since the number of courters in my field sample was so small, it was possible to randomly draw a sample that did not contain courters, which I found affected ~5% of samples in practice. Such a sample would be biologically unrealistic as females produced courter offspring, and both phenotypes have always been found in natural populations. In addition, such a sample does not exist mathematically as ln(0) (as calculated in eqn. 5) is undefined. Therefore, to avoid a draw of zero, I doubled the sample size while keeping the frequencies (π_1^A and π_2^A) constant; since the relative reproductive success is based only on frequencies, this change had no effect on the estimate of m_1/m_2 as in eqn. 2. These estimates of f_1, f_2, and m_1/m_2 were used to calculate δ using eqn. 5, and a 95% confidence interval ([0.019: 0.085 d^{-1}] for the first brood and [0.030: 0.094 d^{-1}] for all broods) was constructed (Table 5).

The 95% confidence interval assumes that each offspring is statistically independent, meaning that each is sired by a different male. Offspring from the same brood are likely sired by the same male (Luo et al. 2005), and such violations effectively inflate the true sample size. Therefore, the large confidence interval is conservative and should be accepted with caution. Also, without evidence indicating the contrary, I assumed that the mortality rates for the males and females were the same.

Biologically realistic mortality rate?

Ryan et al. (1992) reasoned that the predicted mortality rate needed to maintain the two phenotypes is biologically realistic if it indicates a stable population, suggesting that the fitnesses of the phenotypes are equal at an ESSt. Conversely, if the fitnesses of the two morphs are not equal, the predicted mortality rate would be consistent with a population that is rapidly growing or shrinking. I determined if my estimated mortality rate would be biologically realistic for a stationary population ($r = 0$) using female demographic data from the first brood (M.R. Morris, O. Rios-Cardenas and J.C. Brewer, unpublished manuscript) and my estimate of δ to determine r. Since the female demographic data were not used in estimating δ, this is an independent check of my assumption that $r = 0$. Because I do not have an estimate of time between broods in the wild, I assumed an interbrood interval of 30 days (following the methodology in Ryan et al. 1992) and a sex ratio of 1:1. Therefore, the average fecundity is 1.58 females born every month or 0.053 per day. Given that the sneakers and females both have two copies of the *s* allele, I assumed that the females mature at the same time as the sneakers ($f = 147$ d). I substituted these values into eqn. 1 and solved for r numerically ($r = -0.035$) using Newton-Raphson method (Burden-Faires 2004).

Because r did not equal zero, I calculated the 95% confidence interval for r using bootstrap to determine if its approximated distribution included zero. I used brood size data (M.R. Morris, O. Rios-Cardenas and J.C. Brewer, unpublished manuscript) to bootstrap the data and calculate a 95% confidence interval for r. I used the permuted ages of the sneakers (Chapter 1) and permuted the number of females born per day from the data collected simultaneously but independently as the data used to calculate the male relative reproductive success (M.R. Morris, O. Rios-Cardenas and J.C. Brewer, unpublished manuscript). I only used data from the first brood and again assumed that broods were born every 30 days. I solved for r numerically and repeated this process for 10,000 bootstrap samples. The 95% confidence interval for r did not include zero [-0.085: -0.012].

Even though my estimate of r is not zero, is r small enough for me to consider the death rate biologically realistic? If a population decreases at $r = -0.035$, it would half in 21 days; given the range of r from the bootstrap, a population would halve in 8.15 - 577.62 days. While the range of r from my bootstrap analysis is rather large, it always predicts that the population would be decreasing in size and in some cases rather rapidly. Therefore, my results suggest that the two phenotypes did not have equal fitnesses at the time they were sampled. There are at least two caveats to consider in my analysis of r. As described earlier for the confidence interval calculated for δ, the confidence interval is conservative because the offspring are not statistically independent. Although some of these predictions seem dire, halving times can be crude estimates and assume that both the population is not affected by its age distribution and that r does not vary over time, which is unlikely over long periods of time (Rockwood 2006).

Since my r estimate is so influential to the conclusions drawn about the existence of an ESSt, I assessed the accuracy of my estimate by testing how other measures of female demographic data affected my *r* for *X. multilineatus* (first brood only) and also for *X. nigrensis* using data from Ryan et al. (1992). The parameters brood size and time between broods used to calculate *r* are subject to environmental influences in *X. multilineatus* and *X. nigrensis*, and therefore, I substituted other reasonable estimates to test their effect on *r* (Table 4). Mean brood size in *X. multilineatus* has been measured as low as 3.16 in the lab (M.R. Morris, O. Rios-Cardenas and J.C. Brewer, unpublished manuscript) to as high as 10.1 in the field (Morris and Ryan 1992). Similar ranges in brood size have been documented for *X. nigrensis* (3.6 in the field, Morris and Ryan 1992 to 15 in the lab. Ryan et al. 1992). The interval between broods is likely subject to environmental influences as well and ranges in the lab from 30 days for *X. nigrensis* (Ryan et al. 1992) and 60 days in *X. multilineatus* (M.R. Morris, O. Rios-Cardenas and J.C. Brewer, unpublished manuscript) for the two species. Brood interval has yet to be estimated in the field. Although using field data for mean brood size would have been preferable to estimate the female reproductive success to a lab estimate, I found that the difference between field and lab data had little influence on my estimate of *r*. Despite the differences in the estimates of brood size and brood interval used, the calculations for *r* changed very little (from -0.038 to -0.028) in *X. multilineatus*. Note that the calculation presented in Ryan et al. (1992) for *X. nigrensis* has the *r* value closest to zero.

Comparing intrinsic growth rates of phenotypes

I performed an additional and more direct comparison of the fitnesses of the phenotypes not discussed in Ryan et al. (1992). Since the same data used in my estimate of mortality rate is reanalyzed, it is not a completely independent check of the previous method. My additional test of equal fitnesses estimates the *r* for each phenotype population and then determines the difference. If the difference between the estimates is zero, then the two phenotype populations are growing at an equal rate, and I can conclude that their fitnesses are equal. An advantage of this method is that it compares *r* for the two morphs rather than assuming that *r* is constant across morphs. It does assume, however, that δ is equal for sneakers and courters as does the previous method. The population assumptions regarding the Euler equation are also the same as in the previous method.

Table 6. *Comparison of the* r *calculations using differing female demographic data*

Species	Brood interval (in days)	Brood size	Birth rate based on female demographic data	*r*
X. multilineatus	30	5.6§	0.093333	-0.033
	30	3.16†	0.052667	-0.035
	30	15◊	0.25	-0.028
	60	5.6§	0.046667	-0.036
	60	3.16†	0.026333	-0.038
X. nigrensis	30	7.3¥	0.121667	-0.0062
	30	3.6§	0.04625	-0.011
	30	15◊	0.25	0.000046

[§] Brood size data collected from females in January (Morris and Ryan 1992)

[†] Brood size from the first brood born in lab from M.R. Morris, O. Rios-Cardenas and J.C. Brewer (unpublished manuscript)

[◊] Brood size used in Ryan et al. (1992) for *X. nigrensis* from Kallman (1989)

[¥] Brood size from Ryan et al. (1990)

Using the mean number of each phenotype born per brood (0.21 sneakers and 0.43 courters, M.R. Morris, O. Rios-Cardenas and J.C. Brewer, unpublished manuscript), I divided that by the mean number of days between broods, which was assumed to be 30 days, to provide an estimate of the mean number of each phenotype born each day (0.0069 sneakers and 0.014 courters per day). This indicates that almost twice as many courters were born per day as the sneakers despite the imbalance of male morphs sampled in the field. Returning to eqn. 1, I combined δ and r into a single term n ($\delta + r = n$) as follows

$$1 = m_i \int_{f_i}^{\infty} e^{-n_i x}\, dx \tag{7}$$

Therefore, n is the sum of the mortality rate and the intrinsic growth rate. I used the male demographic data from M.R. Morris, O. Rios-Cardenas and J.C. Brewer (unpublished manuscript) and solved for n numerically in the same manner that I solved for r using the female demographic data. I used the Newton-Raphson method (Burden-Faires 2004) to calculate the value of n for each phenotype ($n_1 = 3.89\times10\text{-}^{3}$ and $n_2 = 5.19\times10^{-3}$). To assess what this means in terms of differences in fitnesses ($n_2 - n_1$) for the different morphs, I again assumed that δ was constant across both phenotypes. Since $\delta_1 = \delta_2 = \delta$, I can then say

$$n_2 - n_1 = (\delta + r_2) - (\delta + r_1) = r_2 - r_1 \tag{8}$$

From this I estimated that the fitnesses differ by 1.30×10^{-3}. Using the methodology outlined previously for r, I generated a 95% confidence interval using 10,000 bootstrap samples for each morph. I found the difference between the morphs for each permutation, and the 95% confidence interval was [1.21×10^{-4}:3.56×10^{-3}]. Since the confidence interval does not include zero, r_2 is significantly larger than r_1, and the courters have significantly higher fitness than the sneakers. All calculations and analyses were performed using SAS 9.1 (Cary, NC) and MATLAB 7.1 (Natick, MA).

Discussion

I determined that the mortality rate necessary for the sneakers and courters to have equal fitnesses in *X. multilineatus* was not biologically realistic, and therefore the two morphs did not have equal fitnesses at the time point sampled. If reproduction had balanced the mortality rate need for equal fitnesses, *r* would have been zero, indicating a stable population. Instead, the *r* from the model suggests a population decreasing in size by half every 21 days. Because a decrease at this rate would drive the population to extinction in a very short time, the mortality rate necessary to provide the sneakers with equal fitnesses was determined not to be biologically realistic. Therefore, my data does not support the hypothesis that the Río Oxitipa population of *X. multilineatus* was at an ESSt when sampled. The results of my second analysis based on the comparison of the population growth rates of each strategy also suggest unequal fitnesses and support this conclusion. However, under the assumptions of this study the courters had higher fitness than the sneakers but were less frequent in the January 2007 sampling than other samples for this population; therefore, I suggest negative frequency-dependent selection may be acting on the system. A frequency-dependent experiment is currently underway to provide more conclusive evidence of negative frequency-dependent selection.

The unequal fitnesses I detected are in contrast to the previous study on the sibling species *X. nigrensis*, where the two phenotypes were shown to have equal fitnesses (Ryan et al. 1992). Differences in the habitats for the two species may be partially responsible for these contrasting findings, if *X. multilineatus* is more likely to be perturbed away from its ESSt than *X. nigrensis*. The environmental conditions for the *X. multilineatus* populations found in the Ríos Coy and its tributaries are more subject to fluctuations. The population of *X. nigrensis* examined is located in a spring (*nacimiento*) that originates the Río Choy, the only river in which this species is found. By living at the origin of the river, the *X. nigrensis* population may not be subject to upstream effects, such as migration of predators and competitors. Also, the water temperature and quality at the spring should be more consistent than for the rivers in which *X. multilineatus* is found. The Río Oxitipa population of *X. multilineatus* examined in the current study is subject to upstream effects and the associated varying environmental conditions. The consistent conditions at the Río Choy may aid in keeping the frequencies of the sneakers and courters more constant over time (see Figure 3), and at an ESSt, as compared to the Río Oxitipa where conditions may be constantly perturbing the frequencies of the sneakers and courters away from equilibrium.

What are the consequences of the sneakers and courters having unequal fitnesses for the presence of an ESSt? Stability can either be achieved at an equilibrium point or a limit cycle, and negative frequency-dependent selection can push the frequencies back toward equilibrium after a perturbation or oscillate around the equilibrium in a limit cycle. For instance, negative frequency-dependent selection has been found to give a fitness advantage to the rarest of the three throat-color morphs among male side-blotched lizards (*Uta stansburiana*) and allows the morphs to coexist without equal fitnesses in a cyclical rock-paper-scissors game (Sinervo and Lively 1996). Since the fitnesses are unequal, my data suggest that if there is an ESSt, the Río Oxitipa population was not at equilibrium frequencies when sampled in January 2007. As the frequencies could be cycling around an ESSt, my data cannot confirm or reject the presence of an ESSt. However, I do have some preliminary evidence for negative frequency-dependent selection acting on the population. First, I rejected the hypothesis that sneakers and courters have equal fitnesses, so the population was not at ESSt. Second, the sampled courter frequency was likely below the equilibrium frequency for the phenotype, since the courters were at their lowest sample frequency. While courters ranged from 23% - 67% of males in previous samples (Figure 3, Rios-Cardenas *et al*. 2007), the frequencies were at an extreme low with courters comprising ~14% of males during January 2007. Despite this low frequency, ~55% of all male offspring in the first brood were courters (M.R. Morris, O. Rios-Cardenas and J.C. Brewer, unpublished manuscript). Third, because the mortality rate necessary to balance the courters' fertilization success and the sneakers' early maturation was so high that it would drive the population to extinction, the sneakers' lifetime fitness was likely lower than the courters' at the time of sampling. In combination, these three factors suggest negative frequency-dependent selection is acting on *X. multilineatus*.

Variation in female preference due to changes in the female size distribution was proposed to shift the equilibrium frequencies (Rios-Cardenas et al. 2007). However, as the fitnesses were not equal when the frequencies were at an extreme point for this population (see Rios-Cardenas et al. 2007), my data did not support the hypothesis that variation in female preference had moved the population to a different ESSt where sneakers were more frequent than courters. Rather than assuming that variation in female preference does not affect the frequencies of the alternative strategies in *X. multilineatus* populations, I suggest that my results could indicate that it may be strong enough to shift the frequencies in the population. Subsequent negative frequency-dependent selection may tend to return the population to its ESSt. Indeed it is unlikely that only one mechanism is involved in the maintenance of alternative strategies. Right- and left-handedness is maintained in scale-eating cichlids (*P. microlepis*) by both natural selection due to prey alertness (Hori 1993) and sexual selection due to disassortative mating (Takahashi and Hori 2008). Systems where frequency-dependence is known to exist can exhibit a complex interplay between multiple mechanisms, and ecological interactions both within and between species have consequences for the maintenance of alternative phenotypes (Sinervo and Calsbeek 2006).

Equal average fitnesses among alternative mating phenotypes has rarely been demonstrated in nature (Gross 1996). In addition to the study of *X. nigrensis* (Ryan et al. 1992), the three genetically-influenced alternative male phenotypes in the marine isopod (*Paracerceis sculpta*) have been shown to have equal mating success over time (Shuster and Wade 1991). However, equal average fitnesses are not required for some of the mechanisms proposed to explain the coexistence of alternative phenotypes. These mechanisms can result in fluctuating frequencies without the presence of an equilibrium point or a limit cycle around one. For instance, neither temporal nor spatial oscillating selection requires equal fitnesses. Models have shown that temporally fluctuating environments can maintain genetic variation in organisms where generations overlap and age- or stage-specific selection is present (Ellner and Hairston 1994). Similarly, fitness variations created by spatial heterogeneity can maintain genetic variation (Frank and Slatkin 1990) given gene flow between populations. Calsbeek et al. (2002) found that environmental variation can drive fluctuating frequencies of alternative phenotypes in small populations and that the alternative phenotypes can be maintained without equal fitnesses. In *U. stansburiana*, ‘usurper’ males have a higher mean fitness but also a higher variability in mating success, which can lead to intergenerational variation in fitness, and thus allow for the coexistence of ‘defender’ males. Other models have shown that the fluctuations in the frequencies of alternative phenotypes can converge on a limit cycle that is unrelated to the ESSt despite having started the trials at ESSt frequencies, particularly in small populations (Fogel and Fogel 1995, Fogel et al. 1998). Further understanding of population size, gene flow, and environmental variation are needed to help tease apart the potential roles of temporally and spatially oscillating selection acting on *X. multilineatus*.

In conclusion, unlike the previous study on *X. nigrensis*, I did not find equal fitnesses for the courters and sneakers in the Río Oxitipa population of *X. multilineatus* sampled in January 2007. Instead, even though the courters were at the lowest frequency detected, they had a higher fitness than the sneakers. While the results for these two species differ, equal fitnesses may not be necessarily expected even for the classic ESSt and negative-frequency dependent theory given the more environmentally variable habitat of *X. multilineatus*. In addition, since the courters were at their lowest sampled frequency, the hypothesis of equal fitnesses was rejected, and sneakers likely have lower lifetime fitness, I have evidence for negative frequency-dependent selection, albeit limited. Since the male phenotypes were not at an ESSt, I can reject the hypothesis that variation in female preference driven by variation in the female size distribution has shifted the stable equilibrium point where the fitnesses are equal, explaining the fluctuating frequencies of the phenotypes (Rios-Cardenas et al. 2007). However, this does not eliminate the possibility that female preference may be influencing the frequencies of the alternative male mating morphs; on the contrary, I suggest female preference may have a stronger effect than expected and thus may be playing an important role in driving variation in frequency of the mating morphs in this system. My results reflect a single snapshot in time of the Río Oxitipa population, and as I know that the frequencies of these phenotypes vary over time in this population as well as across populations, further testing over space and time will help elucidate the mechanisms involved in maintaining the alternative male phenotypes in *X. multilineatus*.

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APPENDIX. COUNTING THE NUMBER OF RINGS FROM OTOLITH PHOTOGRAPHS.

This methodology is not limited to counting the number of rings on an otolith and could be extended to counting a variety of types of objects from photographs. This method can be used to help minimize counting errors and allows a user to record exactly what was counted for later viewing.

Part A. Marking the rings in Adobe Photoshop.

I used Adobe Photoshop 7.0, an image editing software, to add transparent layers to digital photographs and mark rings on that layer.

1. Open Photoshop.
2. Open the series of photographs taken of an otolith, and click on the first photo in the series to activate it.
3. Click **Layer→New→Layer** (alternately **Shft+Ctrl+N**) to add a new layer to the photo. When the **New Layer** window appears, click **OK**.
4. Select the **Pencil Tool**. Adjust the **Color** to R: 0, G: 175, and B: 231. I found that this color stood out against the otolith photos, and the ImageJ macro below is adjusted to this color.
5. Ensure the **Layer** is selected rather than the **Background**. Click on each ring to make a mark, being careful the markings do not touch each other as the ImageJ macro will count only distinct markings.

6. When the rings can no longer be clearly distinguished, right click on the photo and select **Duplicate Layer…** to transfer the layer to the next photo in the series. In the **Duplicate Layer** window, click on the **Document** dropdown menu in the **Destination** box and select the next photo in the series. Click **OK**.
7. Activate the next photo in the series and repeat step 6 until the rings are no longer distinguishable at the edge of the otolith. Again, right click on the marked layer and select **Duplicate Layer…** Under the **Document** dropdown menu in the **Destination** box, select **New** and type in a name for the marked layer.
8. Click **File→Save** and save the marked layer as a TIFF file. Click **OK** in the **TIFF Options** window.

Part B. Counting the markings in ImageJ.

I used ImageJ 1.37 (Rasband 1997-2008), a free image analysis software developed by NIH and can be downloaded from http://rsbweb.nih.gov/ij/download.html.

1. Save the macro as count_rings.txt, the code for which can be found at the bottom of the Appendix, in the file in the ImageJ macro (C:\Program Files\ImageJ\macros) directory.
2. Open ImageJ.
3. Open the photo that has only the marked layer in ImageJ.
4. Click **Plugins→Run…** and the **Run Macro…** window will open. Select the **macros** folder and then the file count_rings.txt. Click **Open**. Several windows will open and close automatically. The number of markings will be listed in the **Summary** window under the **Count** column heading.

Part C. ImageJ macro code.

Copy the code below in a text editor and save it as a TXT file, such as Notepad.

```
run("8-bit");
setAutoThreshold();
//run("Threshold...");
setThreshold(129, 146);
run("Convert to Mask");
run("Analyze Particles...", "size=0-Infinity
circularity=0.00-1.00 show=Nothing clear summarize");
close();
```