

## INITIAL STAGES OF REPRODUCTIVE ISOLATION IN TWO SPECIES OF THE ENDANGERED SONORAN TOPMINNOW

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**Abstract.**—Long-term geographic isolation can result in reproductive incompatibilities due to forces such as mutation, genetic drift, and differential selection. In the Sonoran topminnow, molecular genetic studies of mtDNA, microsatellites, and MHC genes have shown that the endangered Gila and Yaqui topminnows are substantially different, suggesting that divergence took place approximately two million years ago. Here we examined hybrid crosses and backcrosses between these two allopatric taxa to evaluate the accumulation of postmating barriers to reproduction. These results are then compared with results from a previous study where male topminnows were shown to mate assortatively with conspecific females. Despite their preference for conspecific mates, both types of interspecific crosses successfully produced offspring. There was evidence of reduced hybrid fitness, including smaller mean brood size and male-biased sex ratio, for some classes of backcrosses. Brood sizes and interbrood intervals varied significantly when hybrids were subdivided into different cross categories. Our results illustrate the importance of distinctly defining hybrid classes in studies of reproductive isolation. To our knowledge, this is the first such detailed evolutionary analysis in endangered fish taxa.

**Key words.**—Backcross, fecundity, hybrid fitness, postmating barriers, sex ratio.

Received December 18, 2002. Accepted July 18, 2003.

In desert regions of the southwestern United States and adjacent areas in Mexico, vast stretches of arid landscape provide insurmountable barriers to gene flow for aquatic fauna. Consequently, this region contains a high percentage of endemic fishes and fresh-water invertebrates. These factors have resulted in an ideal natural laboratory for the study of allopatric divergence and the evolutionary processes that lead to speciation.

The Sonoran topminnow is a small, livebearing fish native to Arizona, United States, and Sonora, Mexico. Two allopatric species of Sonoran topminnows are found in Arizona. The Gila topminnow (*Poeciliopsis occidentalis*) was once the most common fish found in the Gila River drainage. Destruction of habitat and introduction of the nonnative western mosquitofish (*Gambusia affinis*) have reduced the distribution of the Sonoran topminnow in the United States to four isolated watersheds (Fig. 1). The Yaqui topminnow (*P. sonoriensis*), endemic to the Yaqui River drainage in southeast Arizona, has faced similar threats to its persistence. As a result, the Sonoran topminnow was listed as endangered by the U.S. Fish and Wildlife Service in 1967.

The Sonoran topminnow has undergone several changes to its taxonomy since its initial description as two distinct species, *P. occidentalis* and *P. sonoriensis* (Girard 1859). Several authors either synonymized or retained these taxa (for a review, see Quattro et al. 1996). Based on subtle morphological differences, Minckley (1969, 1971) redescribed the two taxa as different subspecies, *P. o. occidentalis* (Gila topminnow) and *P. o. sonoriensis* (Yaqui topminnow). Since being recognized as endangered, topminnows have been surveyed for a variety of molecular markers in an effort to understand their population structure within and differentiation between taxa.

Collectively these studies suggest that *P. sonoriensis* and *P. occidentalis*, although morphologically very similar, have long been isolated. Quattro et al. (1996) examined mtDNA

variation using six-cutter restriction enzymes and found that the Gila and Yaqui topminnows were fixed for two very diverged haplotypes. Hedrick et al. (2001) found that only two of 25 microsatellite alleles at seven loci found in the Yaqui topminnow were present in any Gila topminnow samples and that the 29 alleles found at a major histocompatibility complex (MHC) locus comprised nonoverlapping sets in the two taxa. Based on this genetic evidence, Minckley (1999) suggested that they should be considered different species, *P. occidentalis* (Gila topminnow) and *P. sonoriensis* (Yaqui topminnow). Here we provide information regarding the reproductive compatibility of these species that is necessary for understanding the biological significance of these genetic differences. Previous attempts at crossing *P. sonoriensis* and *P. occidentalis* were unsuccessful (W. Minckley, pers. comm.). To our knowledge, this is the first time that interspecific hybrids for these species have been produced.

McCune and Lovejoy (1998) reviewed studies on reproductive isolation for over 40 pairs of allopatric fishes to estimate the time required for the development of barriers to reproduction in allopatry. They estimated that between 0.8 and 2.3 million years of separation would be required for speciation, as defined by the evolution of reproductive isolation, to be complete. We calculated the divergence time for these two taxa using Kimura two-parameter distance from mitochondrial cytochrome *b* sequence data (P. Hedrick et al., unpubl. ms.). Conservatively, assuming a divergence rate of approximately 1% per million years consistent with what was found for other teleost fish (Bermingham et al. 1997; Garcia et al. 2000), *P. occidentalis* and *P. sonoriensis* have been separated for between one and two million years. This estimate is consistent with the range of time required for speciation. The timing of their divergence is further supported by geological evidence suggesting that the separation of the Gila and Yaqui drainage systems occurred during the late

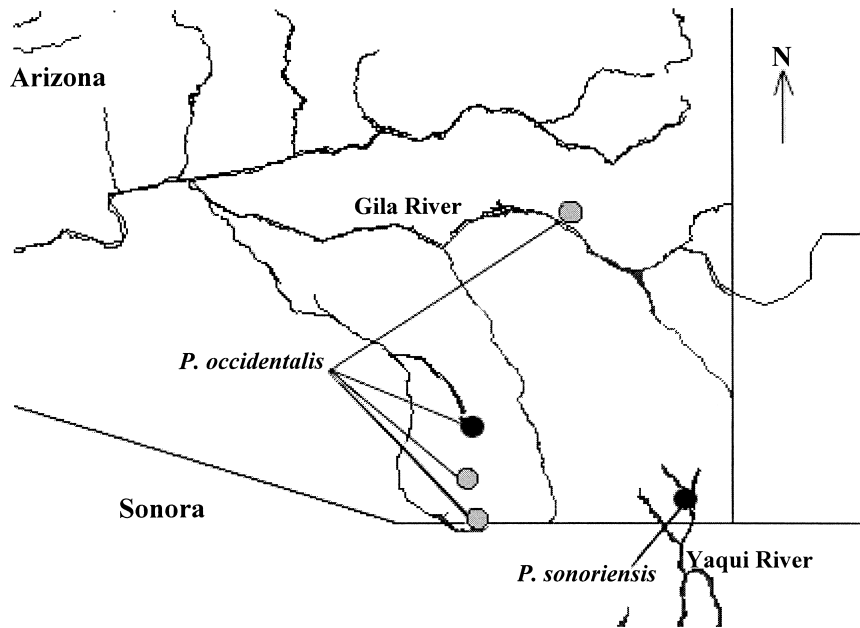


FIG. 1. The distribution of *Poeciliopsis occidentalis* and *P. sonoriensis* in Arizona where solid black circles indicate populations that were used in experimental crosses.

Pliocene or early Pleistocene (Melton 1960; Minckley et al. 1986).

Comparisons of species pairs, which have long been reproductively isolated, may not substantially advance the study of speciation because differences that contributed to speciation can not be distinguished from those that arose after speciation was complete (Coyne and Orr 1998). To gain insights into the process of accumulating barriers to reproduction, it is necessary to study species pairs at an incipient stage. The timing of their divergence makes these two endangered species of Sonoran topminnow ideal for studying the initial development of reproductive isolating barriers.

Previously, we examined mating behavior in crosses between *P. sonoriensis* and *P. occidentalis* to determine the importance of pre-mating barriers to reproduction in incipient allopatric species (Hurt et al. 2004). In multiple-choice trials where males were given a choice between conspecific and heterospecific females, males attempted more copulations with conspecific females. Assortative mating was asymmetric in that *P. occidentalis* males showed a stronger preference for conspecific females than did *P. sonoriensis* males. In contrast, results from observations of no-choice mating trials showed that while the sequence and frequency of certain behaviors differed for *P. occidentalis* and *P. sonoriensis*, there was no difference in the frequency of attempted copulations for interspecific and intraspecific matings. Furthermore, we found no effect of cross type on female response to male copulation attempts. These results suggest that when no choice of mates are given, differences in reproductive output for homospecific and heterospecific matings are likely the result of postmating reproductive barriers.

The purpose of the present study is to evaluate the reproductive potential of *P. occidentalis* and *P. sonoriensis* in no-choice mating trials to gain insight into the accumulation of postmating reproductive barriers in allopatry in relation to

pre-mating barriers and genetic differentiation. In addition, this study is designed to evaluate to what degree different hybrid classes vary in their reproductive fitness. We conducted two sets of laboratory crosses. In the first experiment, we evaluated the reproductive potential of crosses between *P. sonoriensis* males  $\times$  *P. occidentalis* females, the reciprocal interspecific cross, and conspecific crosses for both species. In the second experiment we evaluated the reproductive fitness of  $F_1$  hybrid offspring through a series of backcrosses to parental individuals.

## MATERIALS AND METHODS

### *Experimental Design*

Captive stocks of *P. occidentalis* and *P. sonoriensis* were initiated from 20 gravid females in 1994 and 1998, respectively (the subsequent maintenance and use of these stocks has followed approved animal use protocol from the Arizona State University Animal Care Program). In the following experiments, we used Gila topminnows from the Cienega Creek drainage and Yaqui topminnows from the San Bernardino National Wildlife Refuge (Fig. 1). Since collection, these stocks have been held at the Animal Resource Center (ARC) of Arizona State University in 400-gallon raceways with about 1000 individuals per stock. Fish were maintained at constant temperature (26°C) and under a 14L:10D photoperiod. Fish were fed commercial flake food one time daily at around 13:00 and were given weekly supplements of brine shrimp. Under these conditions, we have been quite successful maintaining these species, although other researchers have experienced substantial difficulty.

Individuals used in parental crosses were randomly selected as juveniles from the two stocks and placed in 10-gallon aquaria until they were of reproductive age. Fish were separated by sex as soon as males could be differentiated by

TABLE 1. For each of the four parental cross types, the percentage of crosses that successfully produced offspring, mean brood size, interparturition period, and interbrood interval (standard error in parentheses).

Male	Female	Successful crosses	% Female offspring	Mean brood	Interparturition period*	Interbrood interval
<i>P. occidentalis</i>	<i>P. occidentalis</i>	85%*		6.19 (1.02)	62.40 (7.27)	17.81 (1.15)
	<i>P. sonoriensis</i>	54%*	8.3*	9.28 (1.27)	87.14 (8.69)*	17.45 (1.31)
<i>P. sonoriensis</i>	<i>P. occidentalis</i>	69%	59.4*	6.92 (0.99)	53.56 (7.66)	17.19 (1.13)
	<i>P. sonoriensis</i>	69%		6.62 (1.00)	63.67 (7.66)	15.48 (1.22)

\*  $P \leq 0.05$ .

development of their gonopodia, to ensure that all fish were virgins at the time of observation. Gonopodial formation, consisting of modification of anterior anal fin rays 3–5, signals the initiation of male sexual development. Upon reaching sexual maturity, the tip of the male gonopodia becomes translucent. Females are considered sexually mature when ova may be visualized through the body wall (Schlossberg et al. 1949).

A total of 52 parental crosses were established, including 13 homospecific crosses for both species, 13 crosses involving *P. occidentalis* females and *P. sonoriensis* males, and 13 crosses involving *P. occidentalis* males and *P. sonoriensis* females. Each cross was initiated between February and September 2000 with one female and two males in 10-gallon aquaria with one-inch gravel substrate. Two males were placed in every tank to ensure mating. Female topminnows can be fertilized by multiple males, even within a single brood, therefore both males may potentially contribute to hybrid progeny (Constantz 1989). Hybrid offspring produced from heterospecific crosses were raised in separate aquaria according to the parental cross type and were separated by sex as soon as males could be identified by gonopodial formation. The sex of all offspring produced by both types of hybrid crosses was recorded.

Upon reaching sexual maturity, hybrids were backcrossed to one of the two parental species. Forty backcrosses, each initiated with one female and two males, were established from May to September, 2001, and included five replicates of each of the eight possible combinations of hybrid genotype and sex (see results below). No fish were used for more than one cross. All experimental crosses took place in separate, 10-gallon aquaria with one-inch gravel substrate. Females and males were kept in the same aquaria for nine months or until four broods had been produced, during which time the date and size of all broods produced by intraspecific crosses, hybrid crosses, and backcrosses were recorded.

#### Data Analysis

The effect of cross type on reproductive success, as measured by the production of offspring, was tested using a  $\chi^2$  test. Crosses that failed to produce offspring were excluded from further analyses. We tested the effect of cross type on brood size using repeated measures ANCOVA (analysis of covariance) accounting for brood number, female standard length, and multiple broods obtained from the same female. Brood sizes from livebearing fishes may show a maternal effect. Therefore, backcross classes that include  $F_1$  females may produce smaller broods than reciprocal crosses in which the female is not a hybrid. We tested for an effect of the sex

of the  $F_1$  parent in backcrosses on mean brood size using ANCOVA accounting for brood number, female standard length, and cross type nested in cross category (i.e., sex of  $F_1$  parent). Significant results were further analyzed for specific posthoc differences using Scheffe's method of linear contrasts (Zar 1999).

Previous studies have shown that sex ratios of offspring from *P. occidentalis* and *P. sonoriensis* do not significantly differ from 1:1 (Schoenherr 1974; Sheffer et al. 1999). In order to test for the effect of heterospecific crosses on sex ratio a  $\chi^2$  test was used to compare the observed sex ratios of offspring from each of the two heterospecific crosses with a 1:1 ratio. The effect of cross type on the number of days from the initiation of the cross until the production of the first brood (interparturition period) and number of days between broods (interbrood interval) was tested using repeated measures ANCOVA accounting for interval number, female standard length, and multiple intervals obtained from the same female.

#### RESULTS

Offspring were produced from both types of heterospecific and homospecific crosses (Table 1). Crosses between *P. occidentalis* males and *P. sonoriensis* females had the lowest reproductive success with 46% of the crosses failing to produce any offspring. Results from a  $\chi^2$  test showed that the success of heterospecific crosses with *P. occidentalis* males was significantly lower than that of homospecific crosses ( $P = 0.035$ ). However, there was no difference in the reproductive success between heterospecific and homospecific crosses with *P. sonoriensis* males.

Both types of interspecific parental cross had a higher mean brood size than did either homospecific cross; *P. occidentalis* male  $\times$  *P. sonoriensis* female crosses had the largest brood sizes of the four types of crosses, averaging 9.28 offspring per brood. However, the effect of cross type was not significant after accounting for brood number, individual female, and female standard length ( $P = 0.077$ ). Brood size increased significantly across each of the four consecutive broods ( $P < 0.001$ ). Mean brood sizes for broods I through IV were 4.91, 6.45, 8.27, and 8.96, respectively. Brood size was negatively correlated with the length of the interbrood interval ( $r^2 = 0.089$ ,  $P = 0.006$ ). We found no significant effect of female standard length on brood size.

Sex ratios of offspring produced from crosses between *P. occidentalis* males and *P. sonoriensis* females were highly male biased, averaging only 8.3% female offspring ( $P < 0.001$ ). In the reciprocal crosses, sex ratios of  $F_1$  offspring

TABLE 2. For each of the eight classes of backcrosses, the percentage of crosses that successfully produced offspring, mean brood size, interparturition period, and interbrood interval (standard error in parentheses).

Male	Female	Successful crosses	Mean brood*	Interparturition period*	Interbrood interval
<i>P. occidentalis</i>	F <sub>1</sub> ( <i>P.o.</i> ♂ × <i>P.s.</i> ♀)	100%	2.67 (0.94)	112.25 (13.01)	20.75 (5.28)
	F <sub>1</sub> ( <i>P.s.</i> ♂ × <i>P.o.</i> ♀)	80%	2.73 (0.73)	58.25 (13.01)	18.45 (3.18)
<i>P. sonoriensis</i>	F <sub>1</sub> ( <i>P.o.</i> ♂ × <i>P.s.</i> ♀)	100%	4.45 (0.63)	59.40 (11.64)	11.93 (2.72)
	F <sub>1</sub> ( <i>P.s.</i> ♂ × <i>P.o.</i> ♀)	80%	2.47 (0.69)	52.60 (11.64)	21.75 (3.05)
F <sub>1</sub> ( <i>P.o.</i> ♂ × <i>P.s.</i> ♀)	<i>P. occidentalis</i>	100%	7.95 (0.63)	83.20 (11.64)	13.87 (2.72)
	<i>P. sonoriensis</i>	80%	4.00 (0.85)	98.50 (13.01)	14.71 (3.99)
F <sub>1</sub> ( <i>P.s.</i> ♂ × <i>P.o.</i> ♀)	<i>P. occidentalis</i>	80%	5.44 (0.71)	60.00 (15.02)	15.33 (3.05)
	<i>P. sonoriensis</i>	80%	4.57 (0.76)	63.75 (13.01)	16.60 (3.34)

\*  $P \leq 0.05$ .

did not deviate significantly from a 1:1 ratio and actually showed a slight female bias with 59.4% female offspring.

The mean interparturition period differed significantly by cross ( $P = 0.001$ ). Pairwise tests for multiple contrasts showed that crosses between *P. occidentalis* males and *P. sonoriensis* females had a significantly longer interparturition period than any of the other crosses ( $P < 0.05$  for all pairwise contrasts), averaging 87.14 days to the production of the first brood. Interestingly this cross category also had the lowest proportion of successful crosses and the largest average brood size. However, we found no significant effect of interparturition period on brood size in either parental crosses or backcrosses after accounting for female standard length. The reciprocal heterospecific cross had the shortest mean interparturition period at 53.56 days. Interparturition periods for homospecific crosses were nearly equal, with means of 62.40 and 63.67 days for *P. occidentalis* and *P. sonoriensis*, respectively. Female standard length was positively correlated with interparturition period ( $r^2 = 0.226$ ,  $P = 0.002$ ). In the analysis of interbrood intervals there was no effect of either cross type or female standard length. After production of a first brood, subsequent broods were produced regularly at a mean interval of 17.00 days.

### Backcrosses

High reproductive success was observed across the eight categories of backcrosses. At least four of five crosses in each backcross category produced offspring (Table 2); no significant effect of backcross type on reproductive success was detected. As in the first experiment, brood size increased with brood number; the average sizes of broods I through IV were 2.69, 4.03, 5.90, and 6.13, respectively. Again, female standard length had no detectable effect on brood size. Brood size varied greatly by individual cross types ( $P < 0.001$ ). Pairwise contrasts showed that crosses between F<sub>1</sub> males from *P. occidentalis* male × *P. sonoriensis* female crosses and *P. occidentalis* females had a significantly larger mean brood size in all pairwise comparisons ( $P < 0.05$  for all pairwise contrasts), with a mean of 7.95 offspring per brood. Hybrid females from *P. sonoriensis* male × *P. occidentalis* females produced the smallest broods ( $P < 0.05$  for all pairwise contrasts) averaging 2.73 and 2.47 offspring per brood when crossed with *P. occidentalis* males and *P. sonoriensis* males, respectively. In general, crosses between F<sub>1</sub> females and parental type males had a lower average brood size than did the reciprocal cross category with means of 3.21 (0.31) and 5.80 (0.46), respectively ( $P = 0.001$ ) (Fig. 2). Hetero-

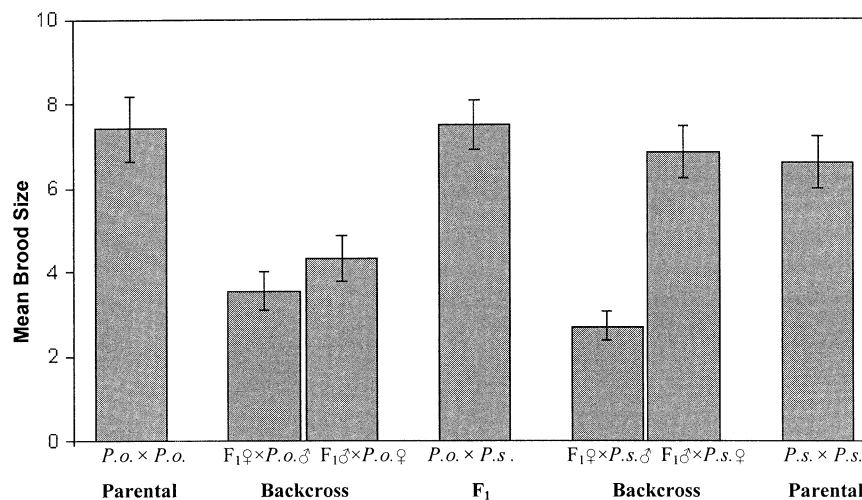


FIG. 2. Mean brood size for all cross classes where *P. o.* and *P. s.* indicate the species *Poeciliopsis occidentalis* and *P. sonoriensis*, respectively. Error bars indicate one standard error.



geneity of brood size was significant among cross types within the category of parental type females  $\times$   $F_1$  males ( $P = 0.034$ ) but not within the category of  $F_1$  females  $\times$  parental type males. Brood sizes from  $F_1$  female  $\times$  parental type male crosses were significantly smaller than those from homo-specific crosses ( $P < 0.001$ ), whereas brood sizes from parental type female  $\times$   $F_1$  male crosses were not. Brood size showed no effect of interbrood interval length.

There was significant heterogeneity of mean interparturition period across the eight types of backcrosses ( $P = 0.014$ ), with means ranging from 52.6 to 112.3 days. A test for multiple contrasts showed that crosses between  $F_1$  offspring resulting from *P. occidentalis* male/*P. sonoriensis* females had significantly longer delays to the production of a first brood than did crosses with the reciprocal category of hybrids ( $P < 0.01$ ). No effect of cross type was found for interbrood intervals. Intervals were similar to those observed in the first experiment, with an overall mean of 16.13 (1.14) days. The effect of female standard length on interparturition period and on interbrood intervals was not significant. Interparturition period and interbrood interval showed no effect of cross type when categorized by sex of the  $F_1$  parent.

#### DISCUSSION

Understanding the process of species formation, the source of biodiversity, remains a primary focus in the study evolutionary biology. In fully isolated species, it is often not possible to differentiate between reproductive barriers contributing to isolation, and those differences that occurred after speciation was complete. To gain insights into the early stages of reproductive isolation, studies should focus on populations that are substantially divergent, yet still capable of gene exchange. Their estimated divergence time and the possibility that we have to use them in laboratory experiments, makes the Sonoran topminnow ideal for addressing questions related to incipient speciation. This study addressed two related questions regarding the onset of barriers to reproduction between the allopatric endangered species, *P. occidentalis* and *P. sonoriensis*. First, to what degree have barriers to reproduction evolved between these two taxa and how does this correspond to their observed amount of genetic divergence. Secondly, are different hybrid classes equivalent in their reproductive fitness.

In interspecific parental crosses, a high degree of reproductive success was observed and brood sizes from interspecific crosses were slightly larger than brood sizes from intraspecific crosses. This is not surprising because theory predicts that reduced fitness from outbreeding due to the breakdown of coadapted gene complexes may not occur until  $F_2$  or backcross generations.  $F_1$  offspring may actually exhibit a higher level of fitness than their parents due to favorable dominance effects (Lynch 1991). Crosses between *P. occidentalis* males  $\times$  *P. sonoriensis* females exhibited the lowest reproductive success, had the longest interparturition period, and had a highly skewed sex ratio in  $F_1$  progeny. This cross category did have the highest average brood size, however, the high percentage of crosses that failed to reproduce, deficiency of female offspring, and the delay in production of the first brood make the overall reproductive success much

lower for this cross category than for any of the others. Interestingly, the reduced reproductive success in this cross category is consistent with the direction of assortative mating observed in multiple choice mating trials where male *P. occidentalis* preferentially mated with conspecific females (Hurt et al. 2004).

Tests for associations between traits such as female size, brood size, and interbrood interval revealed several interesting trends, some of which may reflect life history traits of *Poeciliopsis*. First, both experiments failed to show a correlation between brood size and female standard length. Female *Poeciliopsis* exhibit superfetation, whereby two or more broods at different stages of development occur in the same female (Constantz 1989). Superfetation reduces peak demands on a female from any one brood by distributing maternal investment over time. Also, nearly all superfetators are matrotrophic, which may further reduce maternal investment when ova are yolked (Reznick and Miles 1989). This mode of reproduction may allow smaller females to accommodate greater numbers of offspring because energetic demands during egg yolking and late in gestation are distributed over time. This mode of reproduction could make detecting correlations between female size and fertility difficult. Reznick and Miles (1989) reviewed life-history traits in the family Poeciliidae, and found that in species where superfetation does occur, female size was associated with longer intervals but was independent of brood size. Secondly, Reznick and Miles also found that superfetators tend to produce small number of offspring at regular intervals. This is consistent with our findings that broods were produced regularly at 17-day intervals following the production of the first brood. Finally, the observed increase in brood size with brood number observed in both parental and backcrosses may reflect an increase in female fecundity with age. Female poeciliids continue to grow throughout adulthood, which may also result in an increase in female reproductive potential with age.

Sex-ratio biases in Poeciliids have received much attention since the discovery of the first all-female vertebrate species, *Poecilia formosa* by Hubbs and Hubbs (1932). Since then, the production of all female gynogenetic offspring through interspecific hybridization in Poeciliids has been reported for a number of species pairs (Hubbs 1964; Schultz 1961, 1973), indicating that female-biased sex ratios in hybrid populations are not uncommon. Male-biased sex ratios in hybrid offspring, as we observed in the *P. occidentalis* male  $\times$  *P. sonoriensis* female cross, appears to be a rarer phenomenon, although a deficiency in females has been reported for several hybrid strains (Leslie and Vrijenhoek 1978; Leslie 1982). According to Haldane's rule (1922), a deficiency of female offspring in  $F_1$  hybrids is consistent with females being the heterogametic sex. Sex determination mechanisms for the genus *Poeciliopsis* are not known, although evidence of female heterogamy has been found in certain strains of *Poecilia reticulata* and *Gambusia affinis affinis* (Angus 1989). Alternatively, male biased sex ratios may result from incompatibilities between the paternal X chromosome and the maternal cytoplasm, giving an exception to Haldane's rule in male heterogametic taxa (Turelli and Orr 2000).

There are both disadvantages and advantages to performing reproductive trials in laboratory settings. Evidence of repro-

ductive isolating barriers obtained from laboratory crosses can only be attributed to genetic differences and may not be indicative of the outcome of hybridization in nature. However, laboratory studies are necessary for conducting the large number of replicated crosses necessary to rigorously compare the reproductive output from different categories of hybrid matings and backcrosses. In the present study, replicates of distinct hybrid backcrosses enabled us to identify distinct backcross genotypes that show reduced reproductive success, although other cross categories show no reduction in fitness.

Brood sizes resulting from backcrosses varied greatly depending on cross type. Most of this variation was explained when crosses were combined into categories according to sex of the  $F_1$  parent. Brood sizes resulting from crosses with  $F_1$  females were significantly lower than those obtained from the reciprocal class of backcrosses and brood sizes from homospecific crosses. This result is consistent with brood size being more dependent on maternal than paternal gene interactions and with females being the heterogametic sex. Reduced fecundity is usually expressed in the heterogametic sex due to incompatibilities between loci on autosomal and hemizygous sex chromosomes (Turelli and Orr 2000). High variation in brood size across backcross classes within the category of parental type females  $\times$   $F_1$  males suggests that hybrids are not uniformly unfit and variation in brood size is not completely explained by sex of the  $F_1$  parent.

Interparturition period also showed a large effect of cross type for both experiments. Hybrid crosses had the longest and the shortest periods while interparturition periods from homospecific crosses were nearly equal. Interparturition periods from backcrosses also showed a large effect of hybrid class. Cross classes that include hybrids from *P. sonoriensis* male  $\times$  *P. occidentalis* females had longer intervals than cross classes with reciprocal type hybrids. The large and unpredictable nature of variation observed across hybrid classes for brood size and interparturition period may have implications for designing studies on the accumulation of reproductive barriers. Combining hybrid classes may result in inaccurate conclusions regarding overall hybrid fitness (Arnold and Hodges 1995). For example in the present study, if all backcross categories are combined, ANCOVA shows no significant difference in brood size obtained from backcrosses versus those obtained from intraspecific parental crosses ( $P = 0.1370$ ). In the combined analysis, the larger brood sizes obtained from backcrossed  $F_1$  males masks the reduction of brood sizes from backcrossed  $F_1$  females.

Results from these reproductive trials as well as from behavioral observations (Hurt et al. 2004) indicate that *P. occidentalis* and *P. sonoriensis* are at an early stage of speciation. The timing of their divergence, based on both molecular and geological evidence, is consistent with McCune and Lovejoy's (1998) estimates of time required for allopatric speciation lending further supports for their findings. However, our study is distinct from those reviewed by the former study in that there are multiple lines of evidence regarding the amount of differentiation between *P. occidentalis* and *P. sonoriensis*, including differences in mating behavior, divergence for nuclear and mitochondrial markers, and characterization of morphological differences. Species pairs reviewed in the former study were recognized on the basis of mor-

phological characters and mitochondrial divergence and may not have been reproductively isolated.

The theory of reinforcement predicts that selection drives the evolution of pre mating isolating barriers when hybridization is detrimental to fitness (Dobzhansky 1951). Species that do not come into contact due to geographical or temporal separation cannot be subject to selection pressures against hybridization. Therefore, it follows that in cases where populations remain separated, assortative mating may evolve more slowly than when secondary contact is established (Ritchie and Stephen 1998). Combined with our previous study, our results indicate that both pre mating and post mating isolating barriers are evolving concurrently between *P. occidentalis* and *P. sonoriensis*. Evidence of reduced hybrid fitness includes a strong male biased sex ratio in crosses between *P. occidentalis* males and *P. sonoriensis* females and a reduced brood size for  $F_1$  females when crossed with parental type males. However, given that all cross types were able to produce offspring, it is unlikely that post mating barriers alone would be sufficient to stop hybridization were these two species to come into contact. In the Sonoran topminnow, pre mating isolating mechanisms may be as critical for early stages of speciation in allopatry as are post mating barriers to reproduction.

#### ACKNOWLEDGMENTS

This project was partially funded by a grant from the National Science Foundation and the Ullman Professorship. We thank J. Badman and S. Stears-Ellis for help with maintaining laboratory topminnow populations and setting up experiments. We are greatly indebted to the late W. L. Minckley for his insight into the husbandry of Sonoran topminnows and his many suggestions that focused our research efforts. This research was carried out under approved animal use protocol from the Arizona State University Animal Care Program. Comments were provided by K. Hughes and several anonymous reviewers.

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