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Mating behaviour in the endangered Sonoran topminnow: speciation in action

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Two species of the endangered Sonoran topminnow, *Poeciliopsis occidentalis* and *P. sonoriensis*, occur in two isolated drainage systems in southeast Arizona, U.S.A., and are allopatric throughout their range. Although these two taxa are morphologically very similar, and have been previously described as the same species or subspecies, several molecular studies have since indicated that they differ more than their morphology suggests. To determine whether the behaviours of the two species function as premating barriers to reproduction, we investigated their mating preferences and behavioural patterns in a laboratory setting. Results from no-choice mating observations showed that the mating behaviours of the two species differ. Observations conducted during multiple-choice mating trials provided evidence of assortative mating, suggesting an early stage of premating reproductive isolation.

The Sonoran topminnow, a small live-bearing fish, has been the object of extensive research because it is endangered in the U.S.A. and its status in Mexico is uncertain. Initially, Sonoran topminnows from the Gila River, mainly in Arizona, and from the Yaqui River, originating in Arizona and flowing through Sonora, Mexico, into the Gulf of California, were described as different species (Girard 1859). Several authors then either synonymized or retained these classifications (reviewed in Quattro et al. 1996). Based on subtle morphological differences, Minckley (1969, 1971) redescribed the two taxa, Gila and Yaqui topminnow, as different subspecies.

Recent molecular genetic evidence has shown that the two taxa are more different than their morphology suggests. 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 the Gila topminnow samples examined and that the 29 alleles found at a major histocompatibility complex locus comprised nonoverlapping sets in the two taxa. Based on this genetic evidence, which suggested that the two taxa have been long isolated, Minckley (1999) suggested that they should be considered different species, *Poeciliopsis occidentalis* (Gila topminnow) and *P. sonoriensis* (Yaqui topminnow).

Vrijenhoek et al. (1985) estimated that 1.7 million years of separation would be required to explain the amount of observed genetic divergence between these two taxa. This estimate is consistent with geological evidence suggesting that the separation of these drainages occurred during the late Pliocene or early Pleistocene (Melton 1960; Minckley et al. 1986). In a review of over 40 pairs of allopatric fish species, McCune & Lovejoy (1998) estimated that the time required for speciation ranges from 0.8 to 2.3 million years. The estimated time of separation for *P. sonoriensis* and *P. occidentalis* falls in the centre of this range. Their estimated time of divergence, combined with the exceptional ease with which we are able to breed and experimentally examine topminnows in a laboratory setting, makes this system ideal for the study of speciation processes in an incipient species pair.

Here we evaluate the extent of prezygotic isolation in these two taxa by examining the degree of divergence in mating behaviour between these species. More specifically, we tested for the development of ethological barriers, by comparing the mating behaviours of *P. occidentalis* and *P. sonoriensis* in no-choice matings with homospecific mates and with heterospecific mates. Secondly, we conducted multiple-choice mating trials to examine the ability of males from each species to discriminate between conspecific and heterospecific females.

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METHODS

Fish Stocks, Maintenance and Experimental Protocol

Captive stocks of *P. occidentalis* and *P. sonoriensis* were initiated from 20 gravid females in 1994 and 1998, respectively. Since that time, these stocks have been held at the Animal Resource Center (ARC) at Arizona State University in 400-gallon (1514-litre) raceways with about 1000 individuals per stock. Fish were maintained at constant temperature (26 °C) on a 14:10 h light:dark cycle. Fish were fed commercial flake food once daily at around 1300 hours and were given weekly supplements of brine shrimp, *Artemia salina*. Individuals used in behavioural observations were randomly selected as juveniles from each of these stocks and placed in 10-gallon (37.85-litre) aquaria until they were of reproductive age. Fish were separated by sex as soon as males could be differentiated by 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 (Constantz 1989).

No-choice Mating Trials

Observations took place between 1200 and 1400 hours from 1 September to 20 October 2000. We conducted 40 10-min observations, which included 10 observations of each of the four possible mate pairings, two within-species pairs and two reciprocal pairings between species (i.e. *P. sonoriensis* male–*P. occidentalis* female pairs and *P. occidentalis* male–*P. sonoriensis* female pairs). We observed equal numbers of all pair types on each day of observations to control for potential temporal effects. None of the fish were used in more than one observation. One female and two males were placed into a 10-gallon aquarium with 2.54-cm gravel substrate for 24 h before observation to allow them to acclimate to the new environment. Mating trials were videotaped and behaviours were recorded from videotapes into hand-held tape recorders. Taped observations were transferred to 'The Observer' program (Noldus Information Technology 1989) for analysis. A list of scoreable behaviours (see below) was used as a reference during observations. Individual fish in each mating trial were randomly assigned to one observer. We tested for an effect of different observers on scored behaviours by conducting t tests on each scored behaviour, with observer as the explanatory variable. The effect of observer was not significant for any of the scored behaviours ($-0.518 < t_{38} < 0.889$, $P > 0.10$).

Multiple-choice Mating Trials

Observations took place between 1200 and 1400 hours from 6 August to 17 August 2001. We conducted 20 15-min observations, 10 observations for males of each of the two species. Each observation tank contained one male of either *P. sonoriensis* or *P. occidentalis* and one female from each species, selected for similarity in size. We used vernier callipers to measure the standard length of all fish to the nearest millimetre, where standard length is the distance from the tip of the snout to the base of the caudal fin. Because it is difficult to differentiate between *P. occidentalis* and *P. sonoriensis* morphologically, we marked females before observation for ease in identification. Females were marked using injections of red or yellow nontoxic latex paint (Reznick et al. 1996), and colours were alternated between species to control for effects of marking on male preference. Injection of dye was performed at least 1 week before observations to allow females to fully recover from the procedure. Fish were placed in aquaria 24 h before observations to allow them to acclimate to the new environment. Only virgin females were used and no fish were used in more than one observation.

Description of Male Behaviours

The mating behaviours of male *P. occidentalis* and *P. sonoriensis* do not include elaborate courtship displays or the sigmoid curve displays documented in the related guppy, *Poecilia reticulata* (Farr 1989). Topminnow male mating behaviours are described as coercive and females are typically uncooperative during copulation attempts (Constantz 1989). A lack of courtship behaviour is a common trait for live-bearing fish that show superfetation (multiple simultaneously developing broods), which includes most members of the genus *Poeciliopsis* (Reznick & Miles 1989). We scored the following five male mating behaviours during observations.

Mating behaviours

(1) Following. The male swims approximately 1 cm behind or beside the female, possibly in an attempt to gain access to the female.

(2) Posturing. The male assumes a stationary position in close proximity to the female, often positioning himself at a 45° angle, with his head pointed downward towards the female. Posturing may occur with the male facing the female, or more often, with the male's head pointed near the female's caudal fin.

(3) Nibbling. The male places his mouth near the female's genital pore and appears to bite or nibble at the female, often immediately before attempting copulation. Because female topminnows may release oestrogen when they are most fertile (Farr 1989), males may use this behaviour to sense olfactory cues that indicate when a female is receptive (Constantz 1975).

(4) Swing. The male swings his gonopodium forward without directing it towards other fish. This behaviour might be required for transferring of spermatophores to the tip of the gonopodium (Schlossberg et al. 1949; but see Constantz 1989).

(5) Thrust. Thrusting is equivalent to a copulation attempt by the male. The male swings his gonopodium to the left while approaching the female on her right, then rolls to the right while bringing his gonopodium in contact with the female's genital pore. If successful, the male transfers a spermatophore to the female's genital pore. Male *Poeciliopsis* lack the large claws and hook

structures on the tip of the gonopodium, which are used by other groups of Poeciliidae as a holdfast structure during copulation. Males possessing these holdfast structures display extended bouts of thrusting when copulation attempts are successful and are often seen dislodging themselves from the female. Constantz (1989) suggested that *Poeciliopsis* males do not insert the tip of the gonopodium into the female gonopore, but instead place spermatophores on the outside of the female's genitalia. This would explain the extremely short duration of the copulation attempts we observed, most of which lasted for less than 1 s. Because of the short duration of these events, we were not able to distinguish between successful and unsuccessful copulation attempts and therefore we refer to all thrusting bouts simply as copulation attempts.

Description of Female Behaviour

Female topminnows do not appear to actively participate in mating (Clark et al. 1954; Schoenherr 1974; personal observations). This lack of apparent participation in mating by females has also been described in several species of *Xiphophorus* and *Gambusia*, and may be common throughout poeciliids (Farr 1989). However, females may show variation in receptiveness by remaining in the territory of a preferred male or by maintaining a stationary position during male copulation attempts (Clark et al. 1954; Constantz 1975). To assess female receptiveness, we recorded the time that females spent approaching and retreating from males, and the frequency of seven female responses to male copulation attempts during no-choice mating trials. We classified these female behaviours as receptive, neutral or nonreceptive.

Receptive behaviours

(1) Stationary. The female remains stationary.

(2) Approach. The female swims towards the male.

Neutral behaviour

(3) Pace. The female repeatedly swims in a vertical line along the side of the aquarium.

(4) Swim. The female swims normally.

(5) Feed. The female pecks at the floor or the sides of the aquarium.

Nonreceptive behaviours

(6) Avoid. The female swims away from the male, and occasionally bites at the male. Although females rarely made contact with the male during biting, the male usually ended the copulation attempt. Female biting was only observed three times and was therefore not included in the analysis.

(7) Retreat. The female rapidly swims away from the male.

Neutral behaviours were combined into a single category in the following analysis.

Data Analysis

In our analysis of the no-choice behaviour trials we measured the effect of male species and female species on male mating behaviours using a two-factor analysis of variance (ANOVA). We initially included standard lengths of all fish as a covariate in the model. However, standard length did not have a significant effect on male or female behaviours and was therefore removed from the final analysis. We transformed the data sets for behaviours that were not normally distributed using a Box−Cox transformation. Swing behaviour was observed in less than 15% of the individual males and was therefore omitted from our analysis. In the analysis of female behaviours, we measured the effect of male and female species on the proportion of receptive, neutral and nonreceptive responses to male copulation attempts, as well as the time (in seconds) that females spent approaching and retreating from males. Proportions were transformed using a logit transformation. When analysing female responses to male thrusts, we included only those trials for which copulation attempts were observed. Data from the multiple-choice mating observations were analysed using a one-factor ANOVA comparing the proportion of time that males spent performing each mating behaviour and the frequency of copulation attempts directed towards each species of female. These proportions were transformed using the logit or arcsine transformation.

We recorded sequential data using 'The Observer' program. We converted the sequence of mating behaviours from each observation into first-order transition matrices and then summed these for each of the four types of crosses. Repeated behaviours were left out of the matrix (Slater 1975). We identified significant behavioural transitions with a Z test for proportions (Bakeman & Gottman 1986) using the LAG program for behavioural analysis (Roberts 2001). Kinematic diagrams were constructed using all transitions that occurred at a frequency greater than 10%. We compared transition frequencies for homospecific and heterospecific crosses using an expanded Fisher's exact test for $R \times C$ contingency tables (Raymond & Rousset 1995). Tests were performed on a 2×5 contingency matrix, which included the frequency of every possible first-order transition for each of the scored mating behaviours. We assigned total transition frequencies from homospecific and heterospecific crosses to separate columns and assigned each possible lagging behaviour (behaviour occurring immediately after initial behaviour) to a separate row. When a 2×5 matrix differed significantly from random, we tested all possible pairwise transitions within that matrix using a Fisher's exact test. The above analysis was performed on no-choice trials but was not feasible for the multiple-choice trials due to the complicating factor of having both species of females present.

RESULTS AND DISCUSSION

No-choice Mating Trials

Although we identified several differences in the mating behaviours of the two species during no-choice mating trials, there was no significant effect of the interaction of male and female species on any of the scored behaviours, and thus, no evidence of assortative mating in these trials. Species of male had a significant effect on the amount of time that males spent following females (ANOVA: $F_{1,76} = 10.89$, $P = 0.003$) and on the number of attempted copulations ($F_{1,76} = 5.17$, $P = 0.026$). *Poeciliopsis sonoriensis* males spent more time following females of both species and attempted more copulations than did *P. occidentalis* males. Species of female had a significant effect on the amount of time that males spent posturing ($F_{1,76} = 3.97$, $P = 0.050$) and on the number of copulation attempts ($F_{1,76} = 5.02$, $P = 0.012$; Table 1). *Poeciliopsis sonoriensis* males spent less time posturing near *P. occidentalis* females than near *P. sonoriensis* females. Both *P. sonoriensis* and *P. occidentalis* males attempted more copulations with *P. occidentalis* females. We found no effect of male or female species on time spent nibbling. *Poeciliopsis sonoriensis* males tended to be slightly larger than *P. occidentalis* males, with mean standard lengths of 21.4 mm and 24.1 mm, respectively ($F_{1,76} = 9.13$, $P = 0.008$).

The two species of females differed in the time that they spent approaching males ($F_{1,38} = 4.73$, $P = 0.026$; Table 2). *Poeciliopsis sonoriensis* females approached males more often than did female *P. occidentalis*. Females of both species retreated and avoided copulation attempts from *P. sonoriensis* males more often than from *P. occidentalis* males ($F_{1,38} = 3.79$, $P = 0.059$; $F_{1,38} = 8.58$, $P = 0.007$, respectively). Standard length of females did not differ significantly for the two species; mean standard lengths were 26.1 mm for both. As mentioned previously, standard length had no significant effect on mating behaviours in either no-choice or multiple-choice trials.

Sequence Analysis

Kinematic diagrams (Figs 1 and 2) revealed considerable variation in the complexity and patterns of behavioural sequences. Analysis of single-step transitions revealed two patterns that occurred significantly more often than expected by chance for all crosses examined: the transition from thrust to following (Z test: *P. occidentalis* male × *P. occidentalis* female: $Z = 5.14$, $P < 0.001$; *P. occidentalis* male × *P. sonoriensis* female: $Z = 4.79$, $P < 0.001$; *P. sonoriensis* male × *P. sonoriensis* female: $Z = 8.33$, $P < 0.001$; *P. sonoriensis* male × *P. occidentalis* female: $Z = 7.11$, $P < 0.001$) and the transition from nibbling to thrust (*P. occidentalis* male × *P. occidentalis* female: $Z = 7.34$, $P < 0.001$; *P. occidentalis* male × *P. sonoriensis* female: $Z = 4.38$, $P = 0.003$; *P. sonoriensis* male × *P. sonoriensis* female: $Z = 5.69$, $P < 0.001$; *P. sonoriensis* male × *P. occidentalis* female: $Z = 3.27$, $P = 0.005$). One possible explanation for the frequency with which the transition from thrust to following occurred is that males may resume following because their previous copulation attempt was unsuccessful. Alternatively, males may increase their reproductive success by mating multiple times with the same female. Female topminnows mate with multiple males and more than one male may fertilize individuals within a single brood. By increasing the quantity of sperm transferred to the female, males may ensure that their sperm fertilizes a greater proportion of a female's offspring. The frequency with which the transition from nibbling to thrust occurred suggests that nibbling may be performed by males at a heightened state of sexual arousal; this result is consistent with the suggestion that nibbling enables the male to gain information from the female regarding her reproductive state (Constantz 1975).

Comparisons of behavioural sequences in crosses of male *P. occidentalis* with conspecific versus heterospecific females revealed differences in transition probabilities when following, posturing or nibbling were the preceding behaviour (Table 3). Following led to thrust more often during homospecific crosses and led to nonmating behaviour more often in heterospecific crosses (Fisher's exact test: $P = 0.004$ and $P = 0.016$, respectively). Posturing preceded following and thrust more often in homospecific crosses and led to nonmating behaviour more often in heterospecific crosses ($P = 0.050$, $P = 0.010$ and $P < 0.001$, respectively). Nibbling led to following more often in homospecific crosses and led to posturing and nonmating behaviour more often in heterospecific crosses ($P = 0.017$, $P = 0.021$ and $P = 0.036$, respectively). In

Table 1. Results of no-choice mating trials showing the mean number of seconds (±SE) that males were observed following, hovering and nibbling and the mean number of gonopodial thrusts observed during each 10-min observation period

	Male	*P. occidentalis*		*P. sonoriensis*			
Behaviour	Female	*P. occidentalis*	*P. sonoriensis*	*P. occidentalis*	*P. sonoriensis*	Factor	*P**
Following		86.20 (25.99)	38.30 (22.79)	129.37 (25.37)	120.99 (30.26)	Female	0.213
						Male	0.003
						Interaction	0.372
Posturing		105.29 (34.70)	109.64 (35.27)	18.66 (9.04)	97.09 (33.13)	Female	0.050
						Male	0.126
						Interaction	0.200
Nibbling		9.21 (4.36)	3.68 (2.07)	9.59 (5.22)	5.14 (2.41)	Female	0.512
						Male	0.422
						Interaction	0.225
Thrust		4.30 (1.63)	0.55 (0.26)	7.25 (2.56)	2.75 (0.99)	Female	0.012
						Male	0.026
						Interaction	0.962

*Two-factor ANOVA on the effects of male and female species on male mating behaviours.

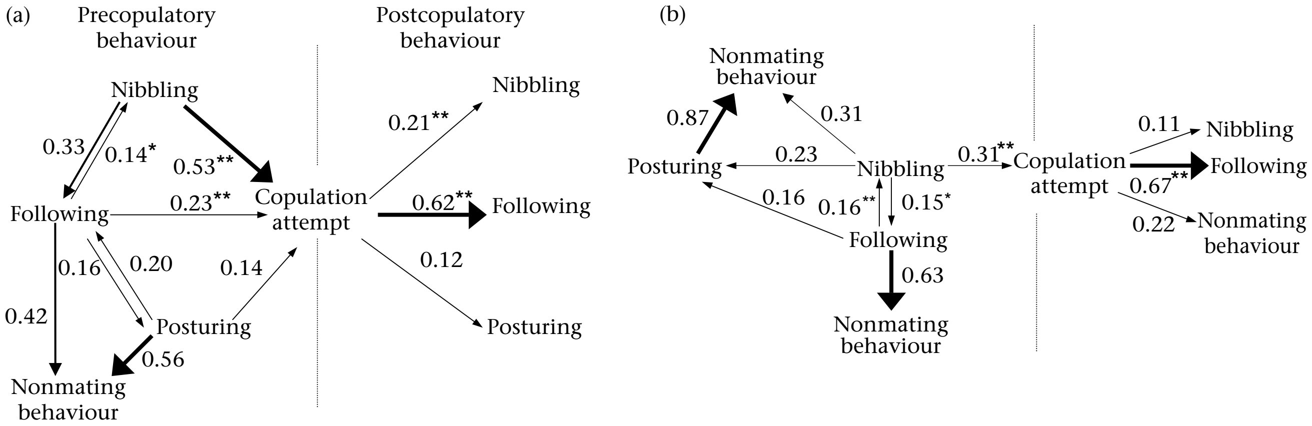

Figure 1. Kinematic flow charts from (a) *P. occidentalis* male × *P. occidentalis* female and (b) *P. occidentalis* male × *P. sonoriensis* female crosses showing all transitions that occurred with a frequency greater than 10%. Numbers and arrow thickness indicate the percentage of occurrence of each transition from one behaviour to the next. Asterisks indicate behavioural transitions that occurred significantly more frequently than expected by chance. *$P \leq 0.05$; **$P \leq 0.01$.

general, the above results indicate that male *P. occidentalis* are more likely to disengage mating activity with heterospecific females prior to copulation, consistent with expectations under assortative mating.

Comparisons of behavioural transitions in crosses with *P. sonoriensis* males did not reveal the same patterns of conspecific mate preference. Our results did show significant effects of homospecific and heterospecific females when following, swing or thrust was the preceding behaviour. Following preceded thrust more often when *P. sonoriensis* males were paired with heterospecific females (Fisher's exact test: $P < 0.001$) and following preceded posturing and swing more often when male *P. sonoriensis* were paired with conspecific females ($P = 0.038$ and $P = 0.012$, respectively). Swing preceded posturing more often in homospecific crosses and preceded nonmating behaviour more often in heterospecific crosses ($P = 0.002$ and $P < 0.001$, respectively). Finally, thrust preceded nibbling more often in heterospecific crosses ($P = 0.033$). The observed differences in transition probabilities for homospecific and heterospecific crosses indicate that male *P. sonoriensis* respond differently to *P. sonoriensis* and *P. occidentalis* females. However, the resulting patterns are not consistent with a preference for conspecific mates as was found for *P. occidentalis* males.

Multiple-choice Mating Trials

Analysis of multiple-choice trials provided stronger evidence of positive assortative mating between the

Table 2. Results of no-choice mating trials showing the mean number of seconds (±SE) that females were observed approaching and retreating from males and the mean number of times females were receptive (stationary), neutral (pace, swim, feed) and nonreceptive (avoid) to male copulation attempts during each 10-min observation period

	Male	*P. occidentalis*		*P. sonoriensis*			
Behaviour	Female	*P. occidentalis*	*P. sonoriensis*	*P. occidentalis*	*P. sonoriensis*	Factor	*P*
Receptive							
Stationary		1.7 (0.80)	0.4 (0.80)	1.6 (0.80)	0.50 (0.80)	Female	0.172
						Male	0.819
						Interaction	0.563
Approach		0.32 (1.63)	5.20 (1.63)	0.94 (1.63)	6.78 (1.63)	Female	0.026
						Male	0.156
						Interaction	0.779
Neutral							
Pace, swim, feed		0.48 (0.18)	0.20 (1.41)	4.20 (1.41)	2.70 (1.41)	Female	0.061
						Male	0.110
						Interaction	0.592
Nonreceptive							
Avoid		0.4 (1.61)	0.7 (1.61)	5.8 (1.61)	2.0 (1.61)	Female	0.310
						Male	0.007
						Interaction	0.387
Retreat		25.87 (39.80)	47.72 (39.80)	135.89 (39.80)	76.00 (39.80)	Female	0.573
						Male	0.059
						Interaction	0.439

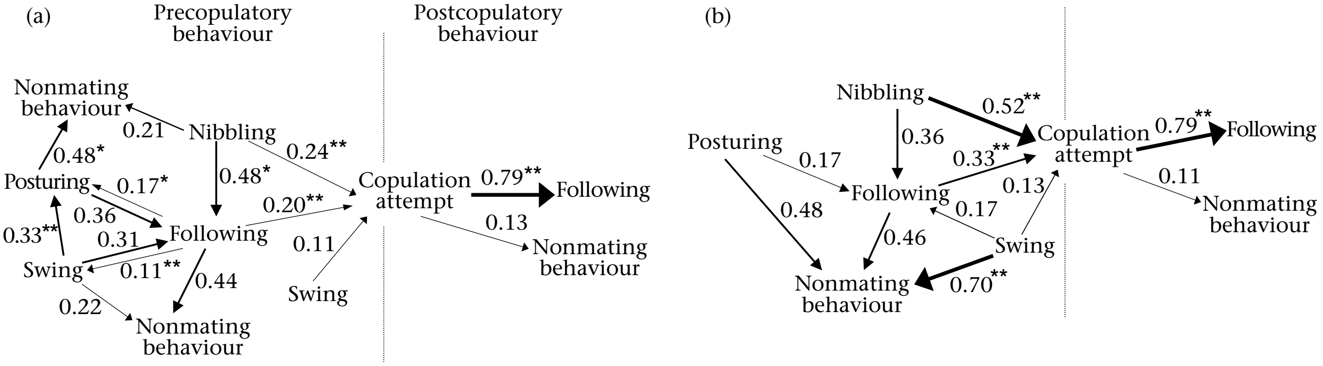

Figure 2. Kinematic flow charts from (a) *P. sonoriensis* male × *P.sonoriensis* female and (b) *P. sonoriensis* male × *P. occidentalis* female crosses showing all transitions that occurred with a frequency greater than 10%. Numbers and arrow thickness indicate the percentage of occurrence of each transition from one behaviour to the next. Asterisks indicate behavioural transitions that occurred significantly more frequently than expected by chance. *$P \leq 0.05$; **$P \leq 0.01$.

P. occidentalis and *P. sonoriensis* than did the no-choice mating trials. We measured the effect of male species on the proportion of time that males spent performing each behaviour towards *P. occidentalis* females (calculated as one minus the proportion of each behaviour directed towards *P. sonoriensis* females; Table 4). We found significant differences between males of the two species for all four measured behaviours (ANOVA: following: $F_{1,18} = 8.680$, $P = 0.009$; posturing: $F_{1,18} = 5.711$, $P = 0.034$; nibbling: $F_{1,18} = 8.28$, $P = 0.010$; proportion of thrusts towards *P. occidentalis* females: $F_{1,18} = 5.882$. $P = 0.036$). Males of both species spent more time performing mating behaviours towards conspecific females than towards heterospecific females. This preference was asymmetric, with

Table 3. Results of sequential behaviour analysis showing the frequencies of transitions from preceding to lagging behaviours

Behaviour		Male	*P. occidentalis*			*P. sonoriensis*		
Preceding	Lagging	Female	*P. occidentalis*	*P. sonoriensis*	*P*	*P. occidentalis*	*P. sonoriensis*	*P*
Following	Total		168	43	0.018	296	219	<0.001
	Posturing		27	7	1.00	21	37	0.038
	Nibbling		24	7	0.811	25	20	0.880
	Thrust		39	2	0.004	99	43	<0.001
	Swing		7	0	0.348	14	23	0.012
	Nonmating		71	27	0.016	137	96	0.748
Posturing	Total		71	69	<0.001	44	102	0.171
	Following		14	5	0.050	—	—	—
	Nibbling		4	3	1.00	—	—	—
	Thrust		10	1	0.010	—	—	—
	Swing		3	0	0.245	—	—	—
	Nonmating		40	60	<0.001	—	—	—
Nibbling	Total		55	13	0.007	44	29	0.065
	Following		18	2	0.017	—	—	—
	Posturing		1	3	0.021	—	—	—
	Thrust		29	4	0.223	—	—	—
	Swing		3	0	1.00	—	—	—
	Nonmating		4	4	0.036	—	—	—
Swing	Total		13	4	0.239	23	36	<0.001
	Following		—	—	—	4	11	0.357
	Posturing		—	—	—	0	12	0.002
	Nibbling		—	—	—	0	1	1.000
	Thrust		—	—	—	3	4	1.000
	Nonmating		—	—	—	16	8	<0.001
Thrust	Total		86	9	0.261	141	61	0.049
	Following		—	—	—	112	48	1.000
	Posturing		—	—	—	4	4	0.240
	Nibbling		—	—	—	10	0	0.033
	Swing		—	—	—	0	1	0.302
	Nonmating		—	—	—	15	8	0.636

Probabilities for individual lagging behaviours were calculated if the total probability for a given preceding behaviour was less than 0.05.

Table 4. Results of multiple-choice mating trials showing the mean number of seconds (±SE) that males were observed following, hovering and nibbling and the mean number (±SE) of male thrusts that were directed towards *P. occidentalis* and *P. sonoriensis* females during a 15-min observation period

	Male	*P. occidentalis*		*P. sonoriensis*		
Behaviour	Female	*P. occidentalis*	*P. sonoriensis*	*P. occidentalis*	*P. sonoriensis*	*P*
Following		166.10 (41.38)	43.69 (57.76)	222.50 (60.53)	283.73 (51.37)	0.009
Posturing		6.17 (3.45)	0.00 (0.00)	21.93 (8.97)	35.86 (14.55)	0.034
Nibbling		66.13 (33.57)	0.56 (0.38)	0.08 (0.08)	1.17 (1.17)	0.010
Thrust		17.6 (7.23)	0.90 (0.41)	8.50 (1.98)	8.80 (2.14)	0.036

P. occidentalis males showing a stronger preference for conspecific mates than did *P. sonoriensis* males.

GENERAL DISCUSSION

In order to increase understanding of speciation processes, it is necessary to examine multiple traits that may reflect species divergence. Several molecular studies have indicated that *P. occidentalis* and *P. sonoriensis* are substantially different genetically, and have suggested that the Sonoran topminnow may be an excellent system for the study of speciation processes in allopatry. The observed degree of genetic divergence between *P. occidentalis* and *P. sonoriensis* lies at the critical threshold of what distinguishes populations from species. This divergence threshold is the ideal stage for examining the accumulation of reproductive isolating mechanisms. Here we have systematically examined the contribution of species-specific mating behaviours to reproductive isolation.

Results obtained from no-choice mating trials provide evidence of divergence in behaviours. Specifically, these trials showed that the two species differ significantly in the proportion of time allotted to the performance of mating behaviours. *Poeciliopsis sonoriensis* males appeared to be the more vigorous of the two species. They spent more time following females and attempted more copulations than did *P. occidentalis* males. Similarly, *P. sonoriensis* females approached males more often than did *P. occidentalis* females. The tendency for females to avoid thrusts and retreat more often from *P. sonoriensis* males may reflect an increase in following and copulation attempts initiated by *P. sonoriensis* males. Species of female did have an effect on male posturing and on copulation attempts, although these differences were not consistent across the studied behaviours.

Comparisons of behavioural sequences for male *P. occidentalis* with conspecific and heterospecific females were consistent with recognition of conspecific mates. Following, nibbling and posturing had a higher probability of leading to copulation attempts or other mating behaviours in homospecific crosses and a higher probability of preceding nonmating behaviours in crosses with heterospecific females. It is surprising that this trend was observed despite the lack of support for conspecific mate preference when comparing mean numbers of copulation attempts. One possible explanation is that males may attempt copulations with heterospecific females if they have not performed other courtship activities. Males that have acquired information about a heterospecific female through her chemosensory or behavioural cues while following, nibbling or posturing may not receive the appropriate signals necessary to proceed with copulations. Sequential analysis of behaviours performed by male *P. sonoriensis* indicated that these males did respond differently to conspecific and heterospecific females. However, they did not show any patterns that would suggest a preference for conspecific females. It appears that formation of premating isolating mechanisms in these groups has been asymmetric, with *P. occidentalis* males showing a greater conspecific mate preference than *P. sonoriensis* males.

Comparisons of duration and frequencies of mating behaviours and copulations in multiple-choice trials provided stronger evidence of conspecific mate preference than did the no-choice mating trials. Both male species spent more time performing mating behaviours and attempted more copulations towards conspecific than towards heterospecific females. Topminnows might opt for a less desirable mate when they are given no opportunity to choose. Mate selectivity is expected to decline when the cost of finding another mate is apparently very high, as it is in a no-choice experiment, where the animal's encounter rate with preferred mates is zero (Wilson & Hedrick 1982; Real 1990, 1991).

The difference in the results for the no-choice and multiple-choice trials may have implications for the design of mate choice studies. No-choice mating trials may circumvent reproductive barriers that are present in natural hybrid zones, where it is likely that a male will be presented with both conspecific and heterospecific females. Failure to detect assortative mating in no-choice mating trials may be due to a lowering of the excitation threshold in the absence of appropriate mates (Schlossberg et al. 1949). The no-choice design may be more useful in testing reproductive compatibility of two forms that show complete isolation in nature or for detecting differences in behaviours when males are presented with conspecific versus heterospecific mates, such as in the sequential analysis performed here. Alternatively, a multiple-choice design may be more informative for the detection of conspecific mate preference in species pairs that show incomplete reproductive isolation.

As in the no-choice mating trials, the preference for conspecific females was stronger for male *P. occidentalis* than for male *P. sonoriensis*, providing further evidence of asymmetric assortative mating. It is not unusual to find asymmetry in premating isolation barriers. When two males differ in sexual vigour, asymmetries in assortative mating may occur, where the more vigorous male is the less discriminating species (Markow & Hocutt 1998). Asymmetry in reproductive isolation may also be a common intermediate stage of speciation between two closely related taxa (Panhuis et al. 2001).

Although not explored in the present study, mate choice by females may also play an important role in the reproductive isolation of these two taxa. Females are commonly thought to be the more discriminating sex, but in the majority of poeciliids studied, females appear to be passive with regard to mate choice (Farr 1989). Within the genus *Poeciliopsis*, the primary determinant of male reproductive success appears to be competition among males (Cox & LeBoeuf 1977), with the level of intermale aggression depending on the attractiveness of the female at stake (Farr 1989). Our preliminary observations suggested that females might retreat more frequently from ‘unattractive’ males. However, to avoid inaccurate interpretation of ambiguous female behaviours, we focused only on the role of male choice for detecting behavioural barriers to interspecific matings. During our observations, males appeared to maintain longer thrusting bouts when females remained motionless. Clark et al. (1954) found a positive correlation between successful transfer of spermatophores and duration of copulation attempt using a smear technique to examine the female reproductive tract for the presence of sperm.

Mechanisms for species recognition in the family Poeciliidae are not known; however, several possibilities have been suggested in the literature. Males may recognize conspecific females through chemosensory cues released by females to ‘advertise’ their fertility (Constantz 1989). Alternatively, visual stimuli have been recognized as important in mate choice throughout the family Poeciliidae. For example, female guppies base mate choice, at least in part, on rare or novel colour patterns in males (Houde 1987).

Poeciliopsis males may similarly respond to the pigmentation spot surrounding the female gonopore, which darkens when females are most fertile or pregnant, and which is larger in *P. sonoriensis* females than in *P. occidentalis* females. In the genus *Gambusia*, female pigmentation spots act as visual cues for guiding copulation attempts (Schoenherr 1974; Constantz 1989).

The theory of reinforcement predicts that selection drives the evolution of premating 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, behavioural reproductive barriers may evolve more slowly than when secondary contact is established (Ritchie & Stephen 1998). Coyne & Orr (1989) observed that in sympatric pairs of *Drosophila* species, prezygotic isolation evolves more rapidly than postzygotic isolation, possibly as a result of reinforcement. However, in allopatric populations they found that both forms of isolation contributed equally to speciation and occurred at approximately the same stage of divergence. Additional studies of the patterns of speciation in a diversity of organisms are needed to assess the robustness of these findings. Our results suggest that in the two geographically isolated populations of the Sonoran topminnow, premating and postmating barriers to reproduction evolved concurrently.

Although crosses between *P. occidentalis* and *P. sonoriensis* in our laboratory produced viable offspring and showed no evidence of reduced reproductive success compared with broods from homospecific crosses, one hybrid cross had a male-biased sex ratio and some hybrid backcrosses had reduced brood sizes, suggesting an early stage of postmating reproductive isolation (C. R. Hurt & P. W. Hedrick, unpublished data). A delay in the onset of postmating barriers to reproduction may be characteristic of the family Poeciliidae. There are numerous reports of successful hybridization throughout this group, both in the laboratory and in nature. Hubbs (1971) discusses a highly successful hybrid swarm in Texas resulting from crosses between *Gambusia affinis* and *G. heterochir*. *Xiphophorous maculatus* and *X. helleri* have been shown to produce viable, fertile offspring in the laboratory (Clark et al. 1954). Offspring from crosses between *Mollienisia latipiinna* and *M. sphenops*, species so different that they were long identified as distinct genera, have established successful hybrid populations in areas of Texas and Mexico (Hubbs & Hubbs 1983). Finally, species of the genus *Poeciliopsis* that occur throughout Mexico hybridize extensively, sometimes with unusual consequence. Crosses between female *Poeciliopsis monacha* and *P. occidentalis* form the all-female hybrid swarm *P. monacha–occidentalis* in the Rio Fuerte in Sonora, Mexico (Schultz 1989).

Given the differences in mating behaviours in no-choice mating trials and the asymmetric conspecific mate preference detected in multiple-choice trials, we conclude that Sonoran topminnows from the Gila and Yaqui drainage systems are at an intermediate stage of reproductive isolation. These findings are consistent with the estimates of speciation times suggested by McCune & Lovejoy (1998). This study demonstrates that species-specific mating behaviours contribute to reproductive isolation even when secondary contact and subsequent reinforcement is not a contributing force.

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