

REINFORCEMENT OF STICKLEBACK MATE PREFERENCES: SYMPATRY BREEDS CONTEMPT

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Abstract.—Detailed studies of reproductive isolation and how it varies among populations can provide valuable insight into the mechanisms of speciation. Here we investigate how the strength of premating isolation varies between sympatric and allopatric populations of threespine sticklebacks to test a prediction of the hypothesis of reinforcement: that interspecific mate discrimination should be stronger in sympatry than in allopatry. In conducting such tests, it is important to control for ecological character displacement between sympatric species because ecological character divergence may strengthen prezygotic isolation as a by-product. We control for ecological character displacement by comparing mate preferences of females from a sympatric population (benthics) with mate preferences of females from two allopatric populations that most closely resemble the sympatric benthic females in ecology and morphology. No-choice mating trials indicate that sympatric benthic females mate less readily with heterospecific (limnetic) than conspecific (benthic) males, whereas two different populations of allopatric females resembling benthics show no such discrimination. These differences demonstrate reproductive character displacement of benthic female mate choice. Previous studies have established that hybridization between sympatric species occurred in the past in the wild and that hybrid offspring have lower fitness than either parental species, thus providing conditions under which natural selection would favor individuals that do not hybridize. Results are therefore consistent with the hypothesis that female mate preferences have evolved as a response to reduced hybrid fitness (reinforcement), although direct effects of sympatry or a biased extinction process could also produce the pattern. Males of the other sympatric species (limnetics) showed a preference for smaller females, in contrast to the inferred ancestral preference for larger females, suggesting reproductive character displacement of limnetic male mate preferences as well.

Key words.—Prezygotic isolation, reinforcement, reproductive character displacement, speciation, sticklebacks.

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Reinforcement is the process whereby natural selection strengthens prezygotic barriers to gene exchange between populations in response to partially or completely reduced hybrid fitness (Dobzhansky 1940; Blair 1955). Reinforcement is interesting because it can complete the speciation process when postzygotic isolation barriers are incomplete (Dobzhansky 1937, 1940; Butlin 1987, 1989; Rice and Hostert 1993). Reinforcement in speciation has long been a contentious issue and has been criticized on theoretical grounds (e.g., Moore 1957; Mayr 1963; Paterson 1978; Moore 1979; Barton and Hewitt 1981), for a lack of definitive examples in nature (e.g., Paterson 1978; Butlin 1987, 1989) and for limited experimental support (Rice and Hostert 1993).

This model of speciation, however, has received renewed attention due to recent theoretical support. A complex multilocus genetic model that took sexual selection into account demonstrated that, with sufficiently low hybrid fitness, a wide range of genetic and ecological conditions existed under which reinforcement could occur (Liou and Price 1994). However, theoretical proposals and their objections have occupied evolutionary biologists for some time with limited progress toward settling the basic question posed by Dobzhansky (1940) over 50 years ago: How frequently and to what extent can prezygotic isolating barriers between species be regarded as the products of reinforcement? As noted by Howard (1993), the issue will not be settled on theoretical grounds but rather with evidence from populations in nature.

Empirical work on reinforcement has focused on the presence or absence of reproductive character displacement, defined as the pattern of greater divergence of an isolating trait between species in areas of sympatry than allopatry (Brown

and Wilson 1956; Howard 1993). We retain Brown and Wilson's (1956) definition of character displacement as a pattern of variation rather than a process to recognize explicitly that different processes can account for it. One of these processes is reinforcement, but others may also produce the same pattern.

Reproductive character displacement was once considered a rare phenomenon (e.g., Littlejohn 1981; Phelan and Baker 1987), however more recent work suggests otherwise. Extensive literature surveys have revealed that the pattern of reproductive character displacement is much more prevalent than previously thought (e.g., Coyne and Orr 1989, 1997; Howard 1993; Noor 1997). Recent studies designed to detect reproductive character displacement in the wild have found it in a number of cases (e.g., Noor 1995; Albuquerque et al. 1996; Sætre et al. 1997), although not in all (e.g., Doherty and Howard 1996).

Stringent criteria must be met before it can be concluded that speciation was caused by the process of reinforcement (Butlin 1989; Howard 1993; Doherty and Howard 1996). These criteria include demonstrating that heterospecific matings occur or once occurred in nature; hybrids are produced and selected against in the wild (reduced, but nonzero hybrid fitness); variation is heritable and capable of responding to selection; and the displacement has not occurred as a by-product of other processes causing divergence in sympatry (Howard 1993). When reproductive character displacement was detected in two recent studies (Noor 1995; Sætre et al. 1997), the interpretation of reinforcement as the cause was strengthened by further evidence that included demonstrating the production and reduced fitness of hybrids (criteria 1 and

2). However, notably absent from these past studies is any consideration of phenotypic differences among populations. Other causes of phenotypic differentiation between species in sympatry could indirectly lead to exaggerated levels of prezygotic isolation. For example, ecological character displacement between sympatric species may cause significant divergence in traits related to feeding (including morphology, behavior, physiology) and this might enhance prezygotic isolation as a simple by-product. To our knowledge, the effects of ecological character displacement have never been controlled in tests of reinforcement.

Here we test for reproductive character displacement of female mate preferences between a sympatric and two allopatric populations of sticklebacks in a way that controls for ecological character displacement in sympatry. Second, we evaluate whether reinforcement can account for its existence. Threespine stickleback populations inhabit coastal lakes and rivers throughout British Columbia, Canada. Most of these populations are solitary (allopatric populations). However, a pair of species coexists in a few, small, low-elevation lakes (sympatric species termed "benthics" and "limnetics"; McPhail 1984, 1992; Schluter and McPhail 1992). If natural selection has reinforced divergent mate preferences between these sympatric species, one prediction is that benthic females will mate less readily with limnetic than benthic males, whereas females from allopatric populations will show a lesser difference in their propensity to mate with benthic and limnetic males.

However, any attempt to test for reinforcement in this system must take account of the fact that ecological character displacement has also occurred between sympatric sticklebacks in body size and shape. Although the comparative evidence for ecological character displacement is compelling (Schluter and McPhail 1992), the case is strengthened by a direct experimental test (Schluter 1994, 1996). Ecological character displacement in body size is of concern, as body size is known to strongly affect the probability of interspecific mating (Nagel and Schluter 1998): heterospecific matings occur only between the larger individuals of the smaller species (limnetics) and the smaller individuals of the larger species (benthics; Nagel and Schluter, in press). This opens the possibility that enhanced prezygotic isolation in sympatry could arise as a by-product of ecological character displacement rather than reinforcement.

Our test of reinforcement controls for ecological character displacement by comparing mate preferences of sympatric, benthic females with females from the most benthic-like allopatric populations. Comparison of benthic females with allopatric benthic-like females is one of four possible comparisons that might be tested for reproductive character displacement (the others are limnetic females, limnetic males, and benthic males). Our design was chosen for a number of reasons. First, past work indicated that the combination of benthic females and limnetic males had the lowest mating frequency of all four sympatric combinations (Nagel 1994). This permitted the most powerful test for the presence of reproductive character displacement. Second, lakes with allopatric populations closely resembling limnetics morphologically have not been found. Finally, models suggest that

females should be the more discriminating sex in mating encounters (Wootton et al. 1995).

Conditions for reinforcement may be favorable in the sticklebacks. For reinforcement to occur, heterospecific matings must occur (or have occurred in the past) in nature and selection against hybrids must exist in the wild. Past hybridization between benthics and limnetics is confirmed by the virtually indistinguishable mtDNA of each benthic-limnetic pair (Taylor et al. 1997). Morphological analysis also suggests that hybridization presently occurs at a low rate in the wild (~1%; McPhail 1992). Although no significant hybrid inferiority is detectable in the lab, F_1 hybrids suffer a disadvantage in the wild. Foraging success of hybrids in pelagic and littoral habitats was near or below the average of the parental species (Schluter 1993). Transplant experiments also demonstrated that hybrids grew approximately 25% slower on average than either parental species when raised in either habitat (Hatfield 1995; Schluter 1995).

Small but unique courtship differences often arise between populations in different lakes. These courtship differences can result in a higher probability of spawning of a male and female from the same lake relative to a male and female from different lakes. To ensure that this did not create a bias in the probability of a pair spawning, none of the female populations used coexisted with the male populations used.

MATERIALS AND METHODS

Choice of Study Populations

The experimental design consisted of trials in which a gravid female, from either a benthic species or a benthic-like allopatric population, was introduced into the tank of a sexually receptive benthic or limnetic male. We used benthic and limnetic males from Paxton Lake, a two-species lake on Texada Island, British Columbia (49°43'N, 124°30'W). Benthic females were collected from a different two-species lake: Priest Lake, Texada Island (49°45'N, 124°34'W; Priest and Paxton lakes are in separate drainages on Texada Island). This ensured that none of the female populations coexisted with the male populations used. To choose allopatric populations, we measured five morphological features of females (see Fig. 1; Schluter and McPhail 1992) from approximately 20 allopatric populations. The two chosen, upper Salmon River, lower mainland of British Columbia (49°07'N, 122°37'W), and Beaver Lake, Vancouver Island (48°49'N, 124°04'W), were the most similar to Priest benthic females in body size and form (Fig. 1, Table 1). The allopatric populations fall within the range of morphological variation observed for benthics in the traits that distinguish limnetics and benthics. Although both allopatric populations overlap extensively the size range of Priest benthics, they are smaller on average than benthics (PC1 in Fig. 1). The skewed size (PC1) distribution for Priest benthics resulted from the selection of individuals that most closely resembled females from the allopatric populations.

The phylogenetic relationships of populations and species are uncertain, owing to their recent origin. All populations in this region of the Strait of Georgia are derived from a "southern" clade (Taylor et al. 1997). Paxton limnetics and benthics are statistically indistinguishable based upon mt-

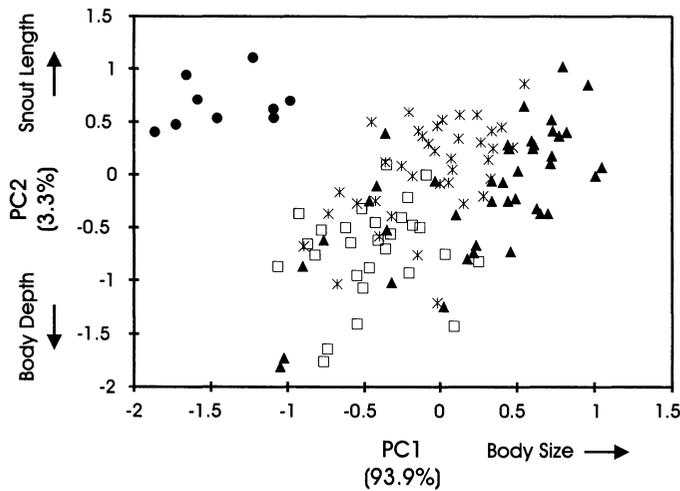


FIG. 1. Principal components analysis of a correlation matrix of eight external morphological traits on a randomly chosen subset of the actual females used in the trials from the three source populations. Priest limnetics are included for reference. The eigen-analysis was performed on the benthic and limnetic females only and then the allopatric (Beaver and Salmon) females were added to the ordination using the original component scores (eigenvectors). Measurements were: standard length, head depth (at the eye), body depth (at the pectoral fin), head length (from tip of snout to end of gill cover), gape width (external), interorbital distance, snout length (from tip of snout to edge of eye), and eye diameter. All measurements were taken on the left side of the fish using an ocular micrometer on a Wild M3C dissecting microscope, except standard length, which was measured using Vernier calipers. All measurements were \ln -transformed before analysis. Percentages indicate amount of variation explained by each factor. Populations are: ● Priest limnetics, ▲ Priest benthics, * Beaver, and □ Salmon River.

DNA sequence divergence (divergence < 0.01%; E. Taylor, pers. comm.). Priest benthic mtDNA sequence divergence from the Paxton limnetic-benthic pair is in the range 0.15–0.19%, while Salmon River females are more distantly related to the Paxton limnetic-benthic pair (0.44–0.49%; Taylor et al. 1997). No information is currently available concerning the relationships of the Beaver Lake allopatric population.

Maintenance of Fish

Males and females used in the trials were first collected in February 1996. Later collections (March–May) were made as needed. The fish were held separately by sex and population in 102-L aquaria in an environment chamber at the University of British Columbia. They were fed to satiation once daily on a diet of frozen brine shrimp (*Artemia* sp.) and bloodworms (chironomid larvae). The photoperiod was gradually increased from 10:14 L:D to 16:8 L:D and the temperature was increased from 8°C to 18°C over a two-month period to bring the fish into reproductive condition.

Mating Trials

Two hundred thirty-nine separate trials, using 239 females and 122 males, were conducted in the laboratory during the 1996 spring breeding season (mid-April to late June). We used a “no-choice” design in which a female was placed in an aquarium with a single nesting male. Allowing female

TABLE 1. Factor loadings (eigenvectors) for the eight morphological traits used in the principal components analysis of female Priest benthic and limnetic sticklebacks.

Trait	Factor scores	
	PC1	PC2
Standard length	0.986	-0.029
Head length	0.990	0.044
Body depth	0.972	-0.188
Head depth	0.983	0.094
Gape width	0.972	-0.164
Interorbital distance	0.974	-0.180
Snout length	0.915	0.376
Eye diameter	0.960	0.141

choice between two males was not possible because benthic males destroy the nests of other males in the same aquarium (Nagel 1994; Hatfield 1995). The no-choice protocol is common in prezygotic isolation studies (e.g., Noor 1995; Hatfield and Schluter 1996) and with sticklebacks, it is a more sensitive and reliable test than alternative designs (Nagel & Schluter 1998). Because females terminate courtship at different stages of the sequence (including after nest entry), we felt that spawning itself was the most reliable indicator of mating probability.

To conduct a mating trial, a single male in reproductive condition, either benthic or limnetic, was placed in a 102-L experimental aquarium with a sandy bottom and supplied with ample Java moss (*Vesicularia dubyana*) and grass clippings as materials for nest construction. Three concrete bricks and three plastic plants were arranged in half of the aquarium to provide cover, whereas the other half was bare. Such a heterogeneous arrangement of open bottom and cover mimics the true lake habitat in which sticklebacks nest and thus allowed the male to choose his preferred site. The aquaria were lit by daylight-simulating fluorescent lights on a 16:8 L:D cycle with a constant 17°C water temperature. All four sides of the tanks were covered with aquarium backing paper to isolate the males from one another and any outside stimulants. Males were encouraged to nest by adding a single, haphazardly chosen, gravid female to his tank for 5–10 minutes a day. A male was determined ready for a trial when he had a complete nest with a well-defined entrance and courted the stimulating female, attempting to lead her to his nest. Males that failed to nest within approximately seven days were replaced.

In each trial a single, haphazardly chosen, gravid female from one of the three female populations was placed into a box suspended from the inside of a male's tank. After approximately 15 minutes a door on the box was opened, allowing the male and female to interact directly. Each trial lasted 30 minutes, timed from the first courtship behavior the male directed toward the female. During each trial the frequency and timing of four characteristic behaviors of the female were recorded on an event recorder by a motionless observer at a distance of one to two months. These behaviors were: (1) following the male to his nest; (2) examining his nest; (3) entering his nest; and (4) spawning. These behaviors are sequential, each in turn indicating increasing interest by the female.

Data on the behavior of males were collected for approximately one-third of the trials. To minimize the influence of female responsiveness on the behavior of the male, the behavioral data were collected during only the first five minutes of each trial. This is necessary as it is the male's initial reaction to the female's phenotype, and not to her willingness to spawn, that is of interest here. The frequency and timing of each of four behaviors were recorded: (1) zigzag, the characteristic courtship dance consisting of a series of horizontal darting movements by the male as he approaches the female (Rowland 1994); (2) lead, the male successfully leads the female to his nest entrance; (3) bite, the male nips or bites the female; and (4) chase, the male chases the female. Although these behaviors vary in amplitude and conspicuousness, they are sufficiently discreet to permit behavioral analysis and are commonly used in such a manner (e.g., see Rowland 1994). Zigzags and leads are an integral part of the courtship sequence and spawning was never observed in a trial in the absence of either. However bites appear aggressive, often performed during courtship when the female is not responding to the male. Chases are directed toward unresponsive females, often very aggressively, and involve concomitant bites. Neither chases or bites are required in the courtship sequence for mating to occur (Rundle, unpubl. obs.).

These behaviors were then used to calculate a courtship score, defined as the proportion of nonaggressive courtship behaviors:

$$P = \frac{N(\text{zigzags}) + N(\text{leads})}{N(\text{zigzags}) + N(\text{leads}) + N(\text{bites}) + N(\text{chases})}, \quad (1)$$

where $N()$ is the number of occurrences of each behavior.

We discarded five trials in which spawning occurred very rapidly (< approximately two minutes) and data on the behavior of the males was sparse, consisting of only one to three behaviors. The remaining trials with behavior recorded included 25 of male limnetics with allopatric females (19 Beaver and six Salmon) and 23 with benthic (Priest) females, and nine of male benthics with allopatric females (five Beaver and four Salmon) and 11 with benthic (Priest) females.

Each female was used in only one trial, whether or not she spawned. Females that did not spawn were examined afterward to confirm their reproductive condition (when slight pressure is applied to the abdomen, the eggs are easily released). In four cases, the receptivity of the female had been misjudged and the trials were discarded. In eight cases, the female entered the nest and remained for an extended period of time (>10 min) but did not spawn. As it is not known whether this behavior represented a choice by females or a method of avoiding male aggression, these trials were also discarded.

After each trial, the female was anaesthetized, stripped of her eggs if she had not spawned, weighed, and preserved in 10% formalin. Males that spawned were not reused as previous work suggests that having eggs in the nest may influence a subsequent female's decision to spawn with him (Ridley and Rechten 1981; Jamieson and Colgan 1989; Whoriskey and FitzGerald 1994). Males that did not spawn were used again after a minimum three-hour resting period. Males were always retested with a female from a different population

than in his previous trial(s). If the male did not spawn with a female from any of the three populations in three trials, he was not tested again. Males were also anaesthetized, weighed, and preserved in 10% formalin after their trials were completed. Reusing males in such a manner could potentially affect the results. If a correlation between male performance in first, second, and third trials existed, repeated observations on the same male would not be independent. Such a possibility is addressed in the data analysis section.

Because all four possible combinations of sex and species (see Introduction) were not tested, our experimental design did not permit a control for changes in the behavior of the male between trials in which sympatric and allopatric females were presented. For instance, if limnetic males were able to distinguish between benthic and benthic-like allopatric females and not court the former, a low frequency of matings between limnetic males and benthic females could mistakenly be attributed to exaggerated discrimination by benthic females. This was dealt with in two ways. First, behavioral data for the males were used to determine whether any changes could be detected between trials with sympatric and allopatric females. Previous work (Nagel 1994; Nagel and Schluter 1998) has demonstrated that female size must be considered when testing for differences in courtship by the males. Thus, if the pattern of courtship by the male with female body size does not change between sympatric and allopatric females, then a lower rate of spawning with sympatric females is attributed to discrimination by benthic females. Second, we statistically controlled for behavior of the male to determine if the pattern of reproductive character displacement was still observed. This test was restricted to the subset of trials for which behavior of the males is available.

Data Analysis

Increased heterospecific discrimination exists if benthic females mate less readily (have a lower probability of spawning) with limnetic than benthic males, and allopatric females show no such pattern. The presence of such a pattern was tested using logistic regression. The regression model was:

$$P(\text{female spawning}) = \frac{e^x}{1 + e^x}, \quad (2)$$

where $x = \text{constant} + M + F + R$. M and F are main effects of male species (M : limnetic or benthic) and female population (F : Beaver, Salmon, or Priest benthic), and R represents reproductive character displacement and is the main quantity of interest. R is one for the case when the combination of male species (M) is limnetic and the female population (F) is Priest benthic, and zero for all other combinations. Increased prezygotic isolation in sympatry would be indicated by a significant R term in the logistic regression model. This R term represents part of the full interaction term ($M \times F$). Remaining parts of the full interaction are assumed absent and are combined with the error term, reflecting an assumption we tested by comparing the fit of the model having the full interaction term with the model having the partial (R) term only. Significance of R is judged with a one-tailed test,

since we expect the combination of limnetic males with benthic females to have a lower probability of spawning.

Spawning probability is known from past work to depend on body size (Nagel 1994; Nagel and Schluter 1998), creating the possibility that the differences in spawning probabilities are the result of body size differences among the female populations (see Fig. 1, PC1). For female size to explain differences in spawning probabilities, its effect must differ between trials with benthic and limnetic males. The effect of female size that is of interest is represented by the variable F . F is part of the full interaction between female size and male species: F is female size when male is limnetic and is zero otherwise. To address this possible size effect, the logistic regression testing for reproductive character displacement was repeated, forcing the partial interaction term (F ; female size is \ln -transformed female mass) into the regression model before assessing the significance of the R term. This F term is sufficient because the remaining part of the full interaction (*female size* \times *benthic male*) has a nonsignificant and inconsequential effect on the fit of the model (Rundle, unpubl. obs.).

Reuse of males that did not spawn in previous trials could bias the significance of R if a correlation existed between male performance in first, second, and third trials. This possibility was addressed in two ways. First, logistic regression was used to examine the effect of male trial number (1, 2 or 3) on the probability of spawning. This analysis was performed separately for each female population (Beaver, Salmon, Priest benthic) to control for differing propensities to spawn of the three female populations. Males from the two species (limnetic and benthic) were combined for this analysis, although results were comparable when each male species was examined separately (Rundle, unpubl. obs.). If no effect of male trial number on the probability of spawning is detected, then repeat observations on the same male can be considered independent. Second, the significance of R is reassessed using a subset of the data that includes only first trial males.

No significant effect of male trial number (1, 2, or 3) on the probability of spawning was detected from trials with any of the three female populations. The effect of male trial number was weak in trials with Beaver (logistic regression coefficient: 0.35 ± 0.27 ; $\chi^2_1 = 1.72$, $P = 0.19$) and Priest (-0.49 ± 0.46 ; $\chi^2_1 = 1.23$, $P = 0.27$) females. Although the effect of male trial number approached significance with Salmon females (0.73 ± 0.42 ; $\chi^2_1 = 3.12$, $P = 0.078$), the trend was in the opposite direction to that expected if later trials involved a disproportionate number of inherently unattractive males. Thus we conclude that repeat measurements of males act as independent observations. A reanalysis of the subset of the data that included only first-trial males had little effect on the R term (see Results), indicating that the reuse of males did not bias the significance of R .

As the experimental design did not permit a control for changes in the behavior of the males that could be responsible for a decreased spawning probability in trials with benthic females, this is dealt with in two ways. The first is to determine whether any such behavioral changes existed between trials with sympatric and allopatric females. Behavioral analyses were performed separately for limnetic and benthic

TABLE 2. Number of females performing each of three courtship behaviors during trials with indicated male species and female population. Numbers in parentheses are percentages.

Male	Female	Female population		
		Beaver	Salmon	Priest
Limnetic	<i>N</i>	49	29	44
	Follow male	42 (85.7)	24 (82.7)	24 (54.5)
	Examine nest	42 (85.7)	22 (75.9)	18 (40.9)
	Spawn	17 (34.7)	12 (41.4)	4 (9.0)
Benthic	<i>N</i>	35	30	44
	Follow male	31 (88.6)	25 (83.3)	39 (88.6)
	Examine nest	31 (88.6)	25 (83.3)	39 (88.6)
	Spawn	14 (40.0)	14 (46.7)	15 (34.1)

males using arcsine square-root transformed courtship scores. Linear regression, with female mass as a covariate, was used to test for differences in courtship by the males between trials with allopatric and benthic females. The assumption of no interaction (homogeneity of slopes) was confirmed (interaction term for limnetic males: $F_{1,44} = 0.097$, $P = 0.76$; and for benthic males: $F_{1,16} = 0.003$, $P = 0.96$). Data from the two allopatric populations were pooled for this analysis due to small sample sizes. Results were comparable when allopatric populations were examined separately (Rundle, unpubl. obs.).

The second method to deal with any changes in the behavior of the males between trials with allopatric and sympatric females is to statistically control for such changes in the analysis. To do this, the test for reproductive character displacement was repeated on the subset of the data for which male courtship scores were available. Male courtship scores were forced into the logistic regression model before assessing the significance of the reproductive character displacement term (R).

RESULTS

Heterospecific Discrimination

Priest benthic females strongly discriminated between limnetic and benthic males, whereas females from both allopatric populations did not. Benthic females were less likely to follow a limnetic male to his nest, were less likely to examine it, and were less likely to deposit eggs in it (Table 2, Fig. 2). Although all of the recorded courtship behaviors of the female showed similar patterns of heterospecific discrimination, failure to deposit eggs in the male's nest is the ultimate indicator of premating isolation. Further analysis thus focuses on the proportion of spawnings.

Heterospecific discrimination by benthic females was confirmed by the significant R term, indicating a reduced probability of spawning between benthic females and limnetic males (logistic regression; $\chi^2_1 = 5.04$, $P = 0.012$). Replacing the R term with the full interaction term in the regression model did not improve the fit over the model having only the specific interaction (R) term ($\chi^2_1 = 0.0015$, $P = 0.97$), indicating that no other interaction between male species and female population was present. The partial female size interaction term (F) significantly improved the fit of the model ($\chi^2_1 = 21.5$, $P < 0.0001$), but the R term remained significant

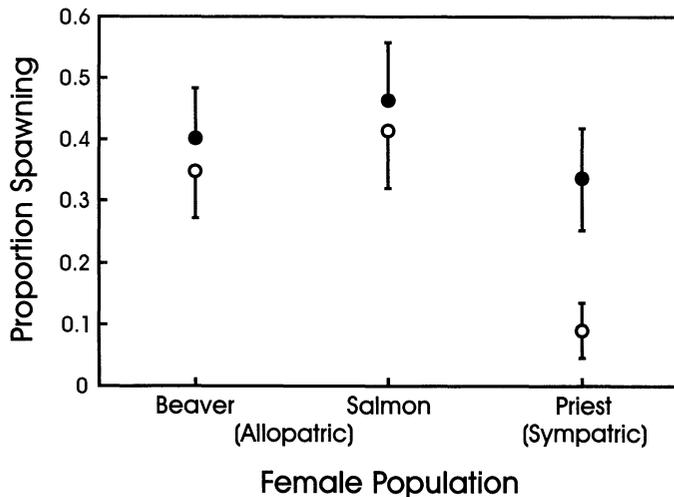


FIG. 2. Spawning probability of each combination of male species and female population. Points represent the proportion of trials \pm 1 SE that ended in females depositing her eggs in the males' nest. Symbols indicate male species: benthics (●) and limnetics (○). Error bars are shown in only one direction in some instances for clarity.

when tested after first forcing the size term into the regression model ($\chi^2_1 = 3.60$, $P = 0.028$). Thus we conclude that the significance of the R term in the original model was not due to the larger mean size of benthic females than allopatric females. Reanalyzing a subset of the original data that included only first-trial males, the R term was the same magnitude as before and approached significance ($\chi^2_1 = 2.15$, $P = 0.071$), despite the smaller sample size (122 as opposed to 231 trials) and thus the reduced power. This indicates that the pattern observed in the full dataset was still present when the repeated measures were eliminated.

Behavior

Males became increasingly aggressive toward larger females, and this raises the possibility that changes in behavior of the males (and not interpopulational differences in female mate preferences) are responsible for the significant R term. Limnetic male courtship score declined with increasing female body size (Fig. 3), nonsignificantly in trials with allopatric females (linear regression; slope coefficient = $-13.9 \pm 17.8\text{SE}$; $F_{1,23} = 0.61$, $P = 0.44$) and significantly in trials with benthic females (slope coefficient = $-19.8 \pm 8.4\text{SE}$; $F_{1,21} = 5.58$, $P = 0.028$). This difference in limnetic male courtship score between allopatric and benthic females was small and inconsequential (Fig. 3), as indicated by a nonsignificant effect of female population in the limnetic male courtship regression model ($F_{1,45} = 0.599$, $P = 0.44$). We conclude that limnetic males treated benthic and allopatric females in a similar manner and therefore are not the cause of the significant R term.

Benthic male courtship score showed a weakly positive but nonsignificant relationship with female size for trials with allopatric females (linear regression; slope coefficient = $6.9 \pm 24.9\text{SE}$; $F_{1,7} = 0.076$, $P = 0.79$) and benthic females (slope coefficient = $5.7 \pm 8.4\text{SE}$; $F_{1,9} = 0.46$, $P = 0.51$). There is no significant difference in the quality of courtship

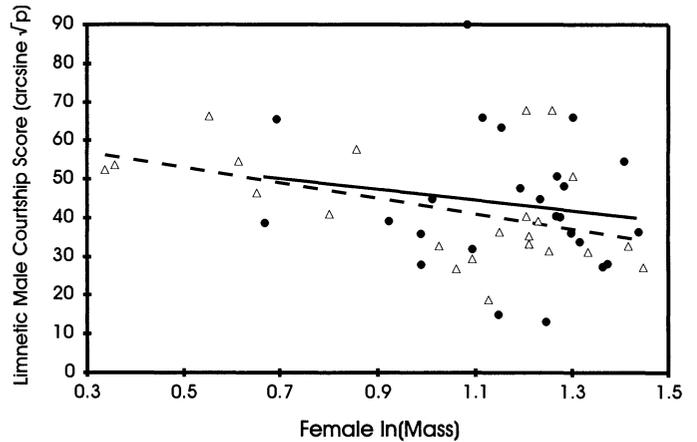


FIG. 3. Quality of limnetic male courtship as a function of female body size. Lines are linear regressions of arcsine square-root transformed male courtship score on \ln -transformed female mass (g). Benthic females are represented by broken line and open triangles, allopatric females are represented by solid line and solid circles). Allopatric female populations (Beaver, Salmon) are combined.

by benthic males between trials with allopatric and benthic females, as indicated by the nonsignificant effect of female population in the regression model for benthic male courtship ($F_{1,17} = 2.16$, $P = 0.16$). Thus we conclude that benthic males also treated benthic and allopatric females in a similar manner.

As a final check on the role of the behavior of the males, the subset of the data for which male courtship scores was available was retested, forcing male courtship into the model before testing the significance of the R term. Adding male courtship to this model had only a slight effect on the coefficient for the reproductive character displacement term ($R_{\text{without male courtship}} = 0.84 \pm 1.28\text{SE}$; $R_{\text{with male courtship}} = 0.75 \pm 1.29\text{SE}$), suggesting that the significant R term in the full model was not a result of changes in male courtship. It must be noted however that this last test is not strong because of the large SEs of the R term. In this subset of the data, the R term was not significant before the addition of the male courtship term ($\chi^2_1 = 0.431$, $P = 0.26$).

DISCUSSION

Reinforcement

Mitochondrial DNA evidence indicates that sympatric species of sticklebacks have hybridized in nature in the past (Taylor et al. 1997) and morphological evidence suggests that a low level of hybridization ($\sim 1\%$) persists today (McPhail 1992), thus satisfying the first precondition for reinforcement. Although there is no detectable reduction in hybrid fitness in the lab, selection against hybrids has been demonstrated in the wild (Hatfield 1995). Foraging success of hybrids was found to fall near or below the average of the parental species (Schluter 1993), and transplant experiments demonstrated that hybrids raised in either parental habitat had approximately 25% lower mean growth rate than either parental species (Hatfield 1995; Schluter 1995). Although the intensity of natural selection against hybrids appears less than the theoretical models indicate is required for reinforcement (e.g.,

Liou and Price 1994), two possibilities exist. First, 25% reduction in growth rate of subadults, in combination with other selection pressures (e.g., sexual selection; Hatfield and Schluter 1996; Vamosi 1996), may create strong selection against hybrids. Second, the models may be wrong and reinforcement may be possible with even weak selection against hybrids. Thus, the conditions under which selection would reinforce prezygotic isolation barriers may exist for sympatric species of sticklebacks.

The expected pattern produced by reinforcement is reproductive character displacement, although such a pattern is consistent but not sufficient to prove reinforcement as the driving mechanism (Butlin 1989). The current study demonstrates the existence of reproductive character displacement for benthic female sticklebacks and provides evidence consistent with reinforcement as the mechanism generating it.

Reproductive character displacement was demonstrated by the greater discrimination between limnetic and benthic males shown by the benthic females as opposed to the two populations of allopatric females (Fig. 2). Benthic (Priest) females had a lower probability of mating with limnetic males than expected, indicating the pattern of reproductive character displacement in female mate preference. Although this pattern is inferred using only a single type of female from a two-species population (Priest benthics), its existence is strongly supported by other data. First, the pattern of low probability of spawning between benthic females and limnetic males is consistent with past work. Nagel (1994) and Nagel and Schluter (1998) demonstrated, under virtually the same experimental conditions, low rates of spawning between Priest benthic females and Priest limnetic males, and between Paxton benthic females and Paxton limnetic males. Ridgway and McPhail (1984), working in another two-species lake (Enos), demonstrated strong premating isolation between benthic females and limnetic males. Second, the lack of discrimination by Priest benthic females against Paxton benthic males is also consistent with other data. Nagel (1994) demonstrated a lack of discrimination between Paxton benthic females and Enos benthic males. Thus the pattern of reproductive character displacement in sympatric sticklebacks is clearly demonstrated in this study and is consistent with past work, yet a number of possibilities exist to explain its evolution.

Reproductive character displacement can be produced by reinforcement of benthic female mate preferences. As noted previously, the conditions under which reinforcement could occur are present. Female sticklebacks are also known to choose mates, discriminating among conspecific males using criteria such as body size (Rowland 1989a) and color (Rowland 1994; McKinnon 1995).

These results are not the first suggesting a role for reinforcement in stickleback speciation. Borland (1986) studied mate preferences of a stream-resident stickleback population. In the lower reaches of the stream, the freshwater population was sympatric (during the breeding season) with an anadromous population. In the upper river, where the freshwater population was allopatric, males preferred larger females, likely due to the positive female body size–fecundity relationship. However in the lower river where the populations

are sympatric, the anadromous females are consistently the largest females encountered by the freshwater males. The lower-river freshwater males, in complete contrast to the upper-river males, preferred smaller females, suggesting reinforcement of male mate preferences.

Alternatives to Reinforcement

If phylogeny and not phenotype determines the probability of spawning, the observed spawning probabilities could be the result of patterns of relatedness. If Paxton limnetic and allopatric populations were more recently derived from the marine ancestor than the Priest benthics, the lack of prezygotic isolation between Paxton limnetics and the allopatric females could be due to their more recent common ancestry. However, mtDNA evidence suggests that this is not the case. Salmon River females are more distantly related to Paxton limnetics (0.44%; Taylor et al. 1997) than are Priest benthics (0.15%; Taylor et al. 1997), suggesting the lack of prezygotic isolation between limnetics and the allopatric populations is not due to a more recent shared ancestry. Although the stickleback phylogeny remains uncertain due to the recent origin of the populations and potential past hybridization between populations, based upon mtDNA, we conclude that phylogeny does not appear to explain the observed pattern of discrimination.

Benthic females that ignore limnetic male courtship could potentially gain a direct fitness advantage (instead of indirectly via the fitness of their offspring as required by our definition of reinforcement; see Introduction). Such direct natural selection in sympatry could also be responsible for increased discrimination by benthic females. If for instance, small limnetic males are poor defenders of eggs compared to large benthic males, a direct fitness advantage could be gained by benthic females that ignore limnetic male courtship and thus do not deposit their eggs in a nest that has a high probability of being raided. No evidence exists suggesting such a direct benefit to females, although the necessary data has not been collected. Thus a direct benefit to benthic females who ignore limnetic male courtship could potentially produce the observed pattern of reproductive character displacement.

Reproductive character displacement can also be produced by a biased extinction effect. When similar species come together multiple times, two species are maintained only in cases in which the populations already have sufficient reproductive isolation. Those cases in which one or both species have insufficient levels of isolation result in fusion of the genomes or the extinction of one (Butlin 1987, 1989). The outcome of such a process is greater isolation in surviving sympatric populations than randomly chosen allopatric populations. In sticklebacks, this possibility requires that species pairs are the result of two successive invasions of the lakes (double invasion scenario; see Schluter and McPhail 1992). The molecular data are presently too incomplete to decide whether this is the case (Taylor et al. 1997).

Biased extinction predicts that a range of levels of prezygotic isolation should exist among randomly paired allopatric populations, with the most extreme allopatric pairs exhibiting virtually complete assortative mating such as

found in existing sympatric species pairs. However, if such a range exists, it is unlikely that the two allopatric populations we used, chosen because they were most different morphologically from limnetics (i.e., most benthic-like), would exhibit identical and near-zero levels of premating isolation from limnetics. For this reason, we view the biased extinction hypothesis to be less likely than reinforcement.

Behavior of the Males

No significant difference in courtship by the limnetic or benthic males was detected between trials with benthic and allopatric females. This indicates that males do not view the allopatric, benthic-like females as any more or less attractive than benthic females.

Positive but nonsignificant correlations were detected between female mass and courtship by benthic males. However, limnetic male courtship scores decreased as female body size increased (Fig. 3). Courtship by limnetic males has been shown to depend on female size in past studies (Nagel and Schluter 1998) and in this study (Fig. 3), revealing an interesting pattern. Positive body size–fecundity relationships are well documented in sticklebacks in general (Baker 1994) and a positive body size–fecundity relationship has been demonstrated in Paxton limnetic and benthic females (Schluter 1995). Thus, a preference by males for larger females is the expectation. Such a preference by males has been demonstrated using models of females (Rowland 1989b). Borland (1986), as previously noted, described a similar change in courtship by the male for a stream-resident population. Upstream, allopatric males preferentially courted larger females, as expected. However downstream, where the freshwater population was sympatric with a larger anadromous marine population, males preferentially courted smaller females. As no allopatric “limnetic-like” male control was used our study to confirm a preference for larger females, the discussion of the evolution of a preference of limnetics for smaller females must remain speculative in nature. However, a limnetic male’s preference for smaller females is contrary to the pattern expected, and elsewhere seen (except Borland’s [1986] downstream males) and it is interesting to consider how this may have arisen.

Two nonexclusive possibilities exist for the evolution of a preference of limnetic males for smaller females. One is reinforcement of male mate preferences in sympatry. It has been argued that because mating mistakes are more costly to females than males in terms of lost reproductive success, reproductive character displacement should be observed more commonly in females than males (Gerhardt 1994). However, male sticklebacks raise the young and can spend three or more weeks from fertilization defending and caring for the eggs before they are ready to spawn again (Whoriskey and FitzGerald 1994). Hence males may also experience strong reinforcing selection.

The alternative explanation for a preference for smaller females is direct natural selection in sympatry. Benthic females are known nest raiders (Ridgway and McPhail 1988), creating a direct selective advantage for limnetic males that do not reveal their nest location by courting them. If nest predation by benthic females is a strong selection force, one

would also expect adaptations in benthic males to deal with this. Interestingly, significant differences between benthic and limnetic male courtship are seen (Ridgway and McPhail 1984; Rundle, unpubl. obs.). Benthic males court much more aggressively and physically remove conspecific females from their nest area if they do not enter the nest immediately to spawn. When a female is in the nest, benthic males appear agitated, nipping her protruding caudal fin aggressively and sometimes entering the nest behind her, pushing her out as soon as the eggs are released (Rundle, unpubl. obs.). These behavioral differences between benthic and limnetic males could represent different adaptations to nest raiding by benthic females, perhaps due to different costs and benefits to the males. The hypothesis that selection imposed by cannibalism could contribute to reproductive isolation between benthics and limnetics was originally proposed by Foster (1994) and the possibility that limnetic male’s preference for smaller females is a direct result of selection to avoid nest raiding by benthic females remains an interesting possibility to be explored.

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