

REINFORCEMENT OF STICKLEBACK MATE PREFERENCES: SYMPATRY
BREEDS CONTEMPT

by

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Abstract

Detailed studies of reproductive isolation and how it varies among populations can provide valuable insight into fundamental questions concerning mechanisms of speciation. Here I investigate how the strength of prezygotic isolation varies between sympatric and allopatric populations of threespine sticklebacks to test a prediction of the hypothesis of reinforcement: that female mate preferences should be stronger in sympatry than allopatry. Ecological character displacement between sympatric species may cause significant divergence in phenotypes which might strengthen prezygotic isolation as a by-product of these changes. I control for ecological character displacement by comparing mate preferences of sympatric females (Benthics) with mate preferences of allopatric females that 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 mate preferences of Benthic females. Previous studies have established that hybridization between sympatric species has occurred in the past in the wild and that hybrid offspring have lower fitness than either parental species. This provides the conditions under which natural selection would favour individuals that do not hybridize. The results are therefore consistent with the hypothesis that female mate preferences have evolved as a response to selection against hybridization (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. This suggests that reproductive

character displacement of mate preferences has occurred in Limnetic males as well.

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INTRODUCTION

In the speciation literature, reinforcement is the process whereby prezygotic barriers to gene exchange are strengthened in areas of species overlap as a response to selection against hybridization (Dobzhansky 1940, Blair 1955, Howard 1993). Reinforcement has long been a contentious issue and has been criticized on theoretical grounds (e.g., Moore 1957, Mayr 1963, Paterson 1978, Moore 1979, Barton & Hewitt 1981), for a lack of definitive examples in nature (e.g., Paterson 1978, Butlin 1987, 1989) and for limited experimental support (Rice & Hostert 1993).

However, this model of speciation 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 & Price 1994). However, theoretical proposals and their objections have occupied evolutionary biologists for some time with limited progress towards settling the basic question posed by Dobzhansky over 50 years ago: How frequently and to what extent can isolating barriers between species be regarded as the products of reinforcement (Dobzhansky 1940)? 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 focuses 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 & Wilson 1956, Howard 1993). I retain Brown and Wilson's (1956) definition of character displacement as a pattern of variation rather than a process in order 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 & Baker 1987), however more recent work suggests otherwise. A survey of patterns of reproductive isolation among *Drosophila* species suggests that prezygotic isolation evolves faster than postzygotic isolation in sympatric species pairs but not in allopatric pairs (Coyne and Orr 1989, 1997). A more conservative, phylogenetically based comparative method (comparing mate preferences of sister species, one sympatric with a third species and the other allopatric to it) confirmed the pattern of elevated sexual isolation between closely related sympatric species of *Drosophila* (Noor 1997). Reinforcement of prezygotic isolation could produce such a pattern, although a biased extinction effect in which sympatry is permitted only between all or most populations having sufficient prezygotic isolation could also produce this pattern. After an extensive literature survey, Howard (1993) also concluded that the pattern of reproductive character displacement is much more prevalent than previously thought. Finally, recent studies designed to detect reproductive character displacement have found it in a number of cases (e.g., Albuquerque et al. 1996, Noor 1995, Gerhardt 1994), although not in all (e.g., Doherty & Howard 1996).

Stringent criteria must be met before it can be concluded that reproductive character displacement was caused by the process of reinforcement (Butlin 1989, Howard 1993, Doherty & Howard 1996). These criteria include demonstrating that: 1) heterospecific matings occur or once occurred in nature; 2) hybrids are selected against in the wild; 3) variation in the displaced trait is heritable and capable of responding to selection; 4) 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 a number of recent studies (e.g. Noor 1995, Gerhardt 1994), the interpretation of reinforcement as the cause was strengthened by evidence

that hybrids are produced and selected against (criteria 1 and 2) and that the pattern is repeated among different populations (suggesting chance environmental differences are not responsible; part of criterion 4). 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. One possibility is ecological character displacement, which is the influence of one species on the evolution of resource use in another species as a consequence of resource competition (Schluter and McPhail 1992). Ecological character displacement between sympatric species may cause significant divergence in traits related to feeding (including morphology, behaviour, physiology) and this might enhance prezygotic isolation as a simple by-product. To my knowledge, the effects of ecological character displacement have never been controlled in tests of reproductive character displacement.

Here I 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, I evaluate whether reinforcement can account for the existence of this pattern of reproductive character displacement. Threespine stickleback populations inhabit coastal lakes and rivers throughout British Columbia, Canada. Most of these populations are allopatric (solitary 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 reproductive character displacement in this system must take account of the fact that ecological character displacement has also occurred between sympatric sticklebacks in body size and shape (Schluter and McPhail 1992, Schluter 1994, 1996). Body size is known to strongly affect the probability of interspecific mating (Nagel and Schluter ms): 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 ms). Increasing size asymmetry between males and females of the two species resulted in increased levels of male aggression and decreased levels of male courtship (Nagel and Schluter ms).

My test of reproductive character displacement controls for ecological character displacement by comparing mate preferences of sympatric, Benthic females with mate preferences of females from the most Benthic-like allopatric populations. Comparison of mate preferences of Benthic females with those of allopatric Benthic-like females is one of several possible comparisons that might be tested for reproductive character displacement (the others are Limnetic females, Limnetic males, and Benthic males). My 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).

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.

Sympatric Freshwater Sticklebacks

Limnetics and Benthics are part of a complex of threespine sticklebacks species (*Gasterosteus spp.*) inhabiting lakes and rivers of coastal British Columbia, Canada. Sympatric species coexist in several small, low elevation lakes in the Strait of Georgia region of southwestern British Columbia. These species are distinct morphologically and ecologically. Invariably, one of the species (the Limnetic) has a diet consisting largely of plankton taken in the open water (especially during the non-breeding season) and has a relatively slender, small body with long, numerous gill rakers (McPhail 1984, 1992, Schluter & McPhail 1992, Schluter 1993). The other species (the Benthic) feeds primarily in the littoral zone on invertebrates living on or in the sediment or attached to vegetation. It has a larger, deeper body with fewer, shorter gill rakers.

Sympatric species are biological species and not simply morphs of a single polymorphic species. Their morphological differences are partly developmentally plastic but the major part persists for at least two generations when fish are raised in the laboratory (McPhail 1984, 1992; Hatfield 1995, 1997) and are not eliminated when their natural diets are reversed (Day et al. 1994). Limnetics and Benthics differ significantly in allozyme frequencies (Nei's $D = 0.02$; McPhail 1984, 1992) and interspecific hybrids are morphologically intermediate (Hatfield 1995, 1997). F1 hybrids, identified by their intermediate morphology, are encountered only infrequently in the wild (McPhail 1984, 1992).

Analysis of mtDNA suggests that Benthics and Limnetics are more similar genetically to

one another than each species is to its counterpart in other sympatric lakes, which suggests multiple, independent origins of the species pairs (Taylor et al. 1997). These results are also consistent with Benthic and Limnetics arising via sympatric speciation, although mtDNA gene flow following secondary contact would produce the same pattern (Taylor et al. 1997). The alternative to sympatric speciation is a double invasion scenario (see Schluter & McPhail (1992) for details). Under this scenario, the ancestral marine form colonized a lake twice, the second invasion occurring after sufficient isolation had developed between the freshwater form derived from the first invasion and the current marine invader. Molecular data are presently too incomplete to determine which of these hypotheses (sympatric speciation or double invasion) is correct (Taylor et al. 1997). However, whatever their origin, the sympatric species (along with all the freshwater populations) are very young, being descended from numerous freshwater invasions of the marine form (*Gasterosteus aculeatus*) since the retreat of the last ice age (≤ 13000 years ago). These recent origins put an upper limit on the duration of the sympatric species.

Allopatric populations in lakes similar in size to those containing species pairs tend to be intermediate between Benthics and Limnetics in morphology and habitat use (Schluter and McPhail 1992). However, allopatric populations in general exhibit substantial phenotypic variation among lakes, ranging from extremely Benthic-like in small lakes and creeks to more Limnetic-like in large lakes. This range of morphology among allopatric populations made possible the use of populations that controlled for sympatric (Benthic) female morphology in my test of reproductive character displacement.

Evidence of ecological character displacement between Benthics and Limnetics is extensive (Schluter and McPhail 1992, Schluter 1994, 1996). Indirect evidence stems from the observation that the sympatric species tend to be more divergent than randomly paired allopatric

populations in 5 out of 6 traits examined (Schluter and McPhail 1992). Allopatric populations are morphologically intermediate between Benthics and Limnetics in lakes similar in size to those containing sympatric species (Schluter and McPhail 1992). This pattern is repeated over multiple lakes (>10), ruling out chance as an explanation. Morphological differences between individuals closely predict resource use: Limnetics feed mainly on plankton taken in the open water while Benthics feed mainly on benthos taken in the littoral zone. Individuals in allopatric populations, which are intermediate between Benthics and Limnetics in morphology, also tend to be intermediate between Benthics and Limnetics in diet and habitat use (Schluter and McPhail 1992). Although the comparative evidence is compelling, the case is strengthened by a direct experimental test of ecological character displacement caused by resource competition. Addition of Limnetics to experimental ponds containing an intermediate (allopatric) form led to disproportionately severe resource competition against phenotypes most similar to the Limnetics, generating natural selection that favoured allopatric individuals that were most Benthic-like (Schluter 1994, 1996).

The conditions required by theoretical models for reinforcement to be possible may be present 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 mtDNA evidence. mtDNA of each Benthic-Limnetic pair in a lake is virtually indistinguishable, indicating the existence of past gene flow between them (Taylor et al. 1997). Morphological analysis also suggests that hybridization between Benthics and Limnetics presently occurs at a low rate in the wild (~1%; McPhail 1992). Although no significant hybrid inferiority is detectable in the lab, F₁ 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).

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 species, was introduced into the tank of a sexually receptive Benthic or Limnetic male. I used Benthic and Limnetic males from Paxton Lake, a two-species lake on Texada Island, British Columbia (49°43'N, 124°30'W). To control for any 'lake effect', 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, I measured 5 morphological features of females (standard length, body depth, gape width, gill raker number and gill raker length; see 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 body size range of Priest Benthics, they are smaller on average than Benthics (average standard lengths: Salmon = 52.1mm, Beaver = 57.1mm, Priest

Benthics = 58.5mm; see also PC1 in Fig. 1).

The phylogenetic relationships of populations and species in the Strait of Georgia region are uncertain, owing to their recent origin (Taylor et al. 1997). However, all populations in this region are closely related and are derived from a Euro-North American clade (Ortí et al. 1994). Paxton Limnetics and Benthics are statistically indistinguishable based upon mtDNA 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 phylogenetic relationships of the Beaver Lake allopatric population.

Maintenance of Fish

First collections of males and females used in the trials were performed in February, 1996. Later collections (March-May) were made as needed. The fish were held in 102 L aquaria in an environment chamber at the University of British Columbia, separated by sex and population. They were fed once daily on a diet of frozen brine shrimp (*Artemia* sp.) and bloodworms (chironomid larvae). The photoperiod was gradually increased from 10L:14D to 16L:8D and the temperature was increased from 8 to 18°C over a two month period to bring the fish into reproductive condition. Reproductive condition of males was signified by the development of nuptial coloration and increasingly aggressive territorial behaviour. Females developed a swollen, distended abdomen when ready to spawn and eggs could be observed through the body cavity in the lower oviduct.

Mating Trials

I conducted 239 separate trials, using 239 females and 122 males, in the laboratory during the 1996 spring breeding season (mid April to late June). I used a 'no-choice' design in which a female was placed in an aquarium with a single nesting male. Allowing female 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 standard in studies of prezygotic isolation (e.g., Noor 1995, Hatfield and Schluter 1996). With sticklebacks, it is a more sensitive and reliable test than another alternative, the 'indirect-choice' test, which consists of placing a single female in an aquarium in view of two males in separate aquaria with which she can interact only visually. Her choice of males is inferred indirectly by her courtship movements because direct interaction is not permitted and thus spawning cannot occur. Previous use of such trials gave similar results to 'no-choice' trials but with weaker interspecific discrimination (Nagel and Schluter ms). Because females terminate courtship at different stages of the sequence (including after nest entry), I felt that the occurrence of spawning itself was the most reliable indicator from which to infer mating probabilities.

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 one half of the aquarium to provide cover, while the other half was left 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', full-spectrum fluorescent lights on a 16L:8D 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 from any population 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 7 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, starting from the first courtship behaviour the male directed toward the female. During each trial the frequency and timing of 4 characteristic behaviours of the female were recorded on an event recorder by a motionless observer at a distance of 1-2m. These behaviours were: 1) following the male to his nest; 2) examining his nest; 3) entering his nest and 4) spawning. These behaviours are sequential, each in turn indicating increasing interest by the female.

Data on the behaviour of males were collected for approximately one-third of the trials. To minimize the influence of female responsiveness on the behaviour of the male, the behavioural data were collected during only the first 5 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 behaviours 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. Zigzags and leads are an integral part of the courtship sequence. Spawning was never observed in a trial in the absence of either. However bites appear aggressive in nature, and are often performed during courtship when the female is not responding to zigzags and leads. Chases are directed towards unresponsive females, and are often very aggressive and involve concomitant bites. Neither chases or bites are necessary for mating to occur (Rundle, pers. obs).

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

$$p = \frac{N(\text{zigzags}) + N(\text{leads})}{N(\text{zigzags}) + N(\text{leads}) + N(\text{bites}) + N(\text{chases})}$$

I discarded 5 trials in which spawning occurred very rapidly (< approximately 2 minutes) and data on the behaviour of the males was sparse, consisting of only 1-3 behaviours. Remaining trials with behaviour recorded included 25 of male Limnetics with allopatric females (19 Beaver and 6 Salmon) and 23 with Benthic (Priest) females, 9 of male Benthics with allopatric females (5 Beaver and 4 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 afterwards to confirm their reproductive condition by determining if eggs were released when slight pressure was applied to the abdomen. In 4 cases I misjudged the receptivity of the female and the trials were discarded. In 8 cases, the female entered the nest and remained for an extended period of time (>10 min.) but did not spawn. These trials were also discarded because I did not know whether this behaviour represented a choice by females or a method of avoiding male aggression.

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 & Rechten 1981, Jamieson & Colgan 1989, Whoriskey & FitzGerald 1994). Males that did not spawn were used again after a minimum 3hr resting period. Males were always retested with a female from a different population than those used 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 statistical analysis.

Because all four possible combinations of sex and species (see Introduction) were not tested, my experimental design does not permit a control for changes in the behaviour of the male between trials in which sympatric and allopatric females are 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 Limnetic male \times Benthic female matings could mistakenly be attributed to exaggerated discrimination by Benthic females. This is dealt with in two ways. First, behavioural data for the males are collected for a subset of the trials. This allows me to determine whether changes in the behaviour of the males occur between trials with sympatric and allopatric females. Previous work (Nagel 1994, Nagel & Schluter ms) has indicated that courtship by Limnetic males is related to female size: large Benthic females are courted less vigorously and elicit aggression more frequently than small Benthic females. Thus, when testing

for differences in courtship by the males among female populations, female size is considered. 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, I statistically control for behaviour of the male (see *Data Analysis*) to determine if the pattern of reproductive character displacement is still observed. This test is restricted to the subset of trials for which behaviour of the males is available.

Data Analysis

Data were analyzed using SYSTAT (version 5.03; SYSTAT Inc. 1993) and SAS (version 6.04; SAS Institute Inc. 1987). 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 in mating frequencies was tested using logistic regression. The regression model was:

$$P(\text{female spawning}) = \frac{e^x}{1 + e^x} \quad \text{where } x = \text{constant} + M + F + R$$

M and F are main effects of male species (Limnetic or Benthic) and female population (Beaver, Salmon or Priest Benthic). R represents reproductive character displacement and is the main quantity of interest. R is 1 when the combination of *male = Limnetic* and *female = Priest Benthic*, and 0 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 I tested by comparing the fit of the model having the full interaction term with the model having the partial (R) term only. The above model provides a more accurate and powerful test of the hypothesis of reproductive character displacement. Significance of R is judged with a one-tailed test, since I expect the combination of Limnetic male \times Benthic female to have a lower probability of spawning.

Spawning success is known from past work to depend on body size (Nagel 1994, Nagel & Schluter ms), which creates the possibility that the differences in spawning frequencies between allopatric and sympatric females are the result of body size differences among these female populations (see Fig. 1, PC1). For female size to explain the observed differences in spawning probabilities, its effect must differ between trials with Benthic and Limnetic males. Thus, the effect of female size that is of interest is part of the full interaction between female size and male species, namely *female size* \times *Limnetic male*. To address this possible size effect, the logistic regression testing for reproductive character displacement was repeated, forcing the partial interaction term (*female size* (ln-transformed female mass) \times *Limnetic male*) into the regression model before assessing the significance of the R term. This partial interaction is sufficient because the remaining part of the full interaction (*female size* \times *Benthic male*) has a non-significant and inconsequential effect on the fit of the model.

Reuse of males which 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. For example, generally unattractive males would tend to be reused. 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 males that did not spawn in the first trial have no lower spawning probability in their next trial. In this case I consider repeat observations on the same male as independent. Second, significance of R is reassessed using a subset of the data that includes only first trial males (i.e., all trials reusing males were eliminated).

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 (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 I 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 reproductive character displacement term (R ; see Results), indicating that the reuse of males did not bias the significance of R .

As noted earlier, the experimental design did not permit a control for changes in the behaviour of the males that could potentially 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 behavioural changes existed between trials with sympatric and allopatric females. Courtship scores were arcsine square root transformed before analysis. Behavioural analyses were performed separately for Limnetic and Benthic males. Linear regression was used to

examine the effect of female size on male courtship score. Differences in courtship by the males between trials with allopatric and Benthic females were tested using female mass as a covariate in the regression model:

$$\text{Male courtship} = \text{constant} + \text{female mass} + \text{female population}$$

where *female population* = 0 for Benthic females and 1 for allopatric (Beaver and Salmon) females. The assumption of no interaction (homogeneity of slopes) was confirmed by testing the significance of the interaction term: *female mass* × *female population* (Limnetic males: $F_{1,44} = 0.097$, $P = 0.76$; 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 behaviour 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 behaviours of the female showed similar patterns of heterospecific discrimination, failure to deposit eggs in the male's nest is the ultimate indicator of prezygotic 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 size interaction term (*female size* \times *Limnetic male*) significantly improved the fit of the model ($\chi^2_1 = 21.5$, $P < 0.0001$), but the R term remained significant when tested after first forcing the size term into the regression model ($\chi^2_1 = 3.60$, $P = 0.028$). Thus I 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. In a reanalysis of 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 and thus the reduced power (122 as opposed to 231 trials). This indicates that the pattern observed in the full data set was still present when the repeated measures were eliminated.

Behaviour

As behaviour of the males was not controlled by the experimental design, data on the behaviour of the males were recorded and controlled statistically. Males became increasingly aggressive toward larger females, and this raises the possibility that changes in behaviour 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), non-significantly 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 (linear regression; 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 non-significant *female population* term in the Limnetic male courtship regression model ($F_{1,45} = 0.599$, $P = 0.44$). I conclude that Limnetic males treated Benthic and allopatric females in a similar manner and behavioural differences by the males are therefore not the cause of the significant R term in the logistic regression.

Courtship score of Benthic males showed a weakly positive but non-significant 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 (linear regression; 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 by Benthic males between trials with allopatric and Benthic females, as indicated by the nonsignificant *female population* term in the regression model for Benthic male courtship ($F_{1,17} = 2.16$, $P = 0.16$). Thus I conclude that Benthic males also treated Benthic and allopatric females in a similar manner.

As a final check on the role of the behaviour 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 SE's 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

Evidence based on mtDNA indicates that sympatric species of sticklebacks hybridized in nature in the past (Taylor et al. 1997) and morphological evidence suggests that a low level of hybridization (~1%) persists today (McPhail 1992), 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, a 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 of Benthic females between Limnetic and Benthic compared to that of 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. Furthermore, this pattern of low probability of spawning between Benthic females and Limnetic males is consistent with other data. Nagel (1994) and Nagel and Schluter (ms) demonstrated, under virtually the same experimental conditions, low rates of spawning between Priest Benthic females \times Priest Limnetic males and Paxton Benthic females \times Paxton Benthic males. Ridgway and McPhail (1984), working in another 2-species lake (Enos), demonstrated strong prezygotic isolation between the Benthic females \times Limnetic males. Thus the pattern of reproductive character displacement in sympatric sticklebacks is clear, yet a number of possibilities exist to explain its evolution.

Reproductive character displacement can be produced by reinforcing selection on 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 contrast to the upper river males, preferred smaller females, suggesting reinforcing selection on male mate preferences (Borland 1986).

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 the double invasion scenario is true (see Introduction) and the 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. Evidence based on mtDNA however 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, I conclude that, based upon mtDNA, phylogeny does not 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). 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 have 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 Introduction). As noted earlier, 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 I used, chosen because they were most different morphologically from Limnetics (i.e., most Benthic-like), would exhibit identical and near-zero levels of prezygotic isolation from Limnetics. For this reason, I view the biased extinction hypothesis to be less likely than reinforcement.

Behaviour of the Males

No significant difference in courtship by Limnetic or Benthic males was detected between trials with Benthic and allopatric females. This indicates that males do not treat the allopatric, Benthic-like females any differently than they treat Benthic females.

Positive but non-significant correlations were detected between female mass and courtship by Benthic males. However, courtship scores of Limnetic males decreased as female body size increased (Fig. 3). Courtship by Limnetic males has been shown to depend on female size in a similar manner in past studies (Nagel and Schluter ms) and in this study (Fig. 3), revealing a pattern of interest in itself. 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 in a Long Island tidal marsh pool population (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, demonstrating a shift in male courtship from the expected pattern. As no allopatric 'Limnetic-like' male control was used in the current 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 non-exclusive 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 have a significant parental investment. Males 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 & FitzGerald 1994). Hence males may also experience strong selection against hybridization.

The alternative explanation for a preference for smaller females is direct natural selection in sympatry. Benthic females are known to be nest raiders (Ridgway and McPhail 1988) and a direct selective advantage may be accrued by Limnetic males that do not reveal their nest location by courting them. If a selective advantage is incurred by avoiding nest predation by Benthic females, one would expect adaptations in Benthic males to deal with this same problem. Interestingly, significant differences between Benthic and Limnetic male courtship are seen (Ridgway & McPhail 1984, Rundle, pers. obs.). Benthic males court more aggressively, zigzagging and leading less often, biting more frequently, and physically removing 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 more aggressively than Limnetics and sometimes entering the nest behind her, pushing her out as soon as the eggs are released (Rundle, pers. obs.). These behavioural 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.

CONCLUSION

Reproductive character displacement is demonstrated for Benthic female sticklebacks, which are less likely to mate with a Limnetic than a Benthic male, whereas two populations of allopatric females show no such pattern. Unlike past studies of reproductive character displacement, ecological character displacement, which has resulted in phenotypic divergence in the sympatric species, was controlled for. These results suggest that reinforcement has played a role in the speciation of Benthic and Limnetic sticklebacks, although direct natural selection in sympatry or biased extinctions cannot be ruled out. This study highlights the need for critical, properly controlled tests of reproductive character displacement to evaluate the role of reinforcement in the speciation process.

Table 1: Factor loadings (eigenvectors) for the 8 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

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

Male	Female	Female Population		
		Beaver	Salmon	Priest
	N	49	29	44
Limnetic	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)
	N	35	30	44
Benthic	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)

Figure 1. Principal components analysis of a correlation matrix of 8 external morphological traits on a randomly chosen subset of the females from the three source populations used in the trials. 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.

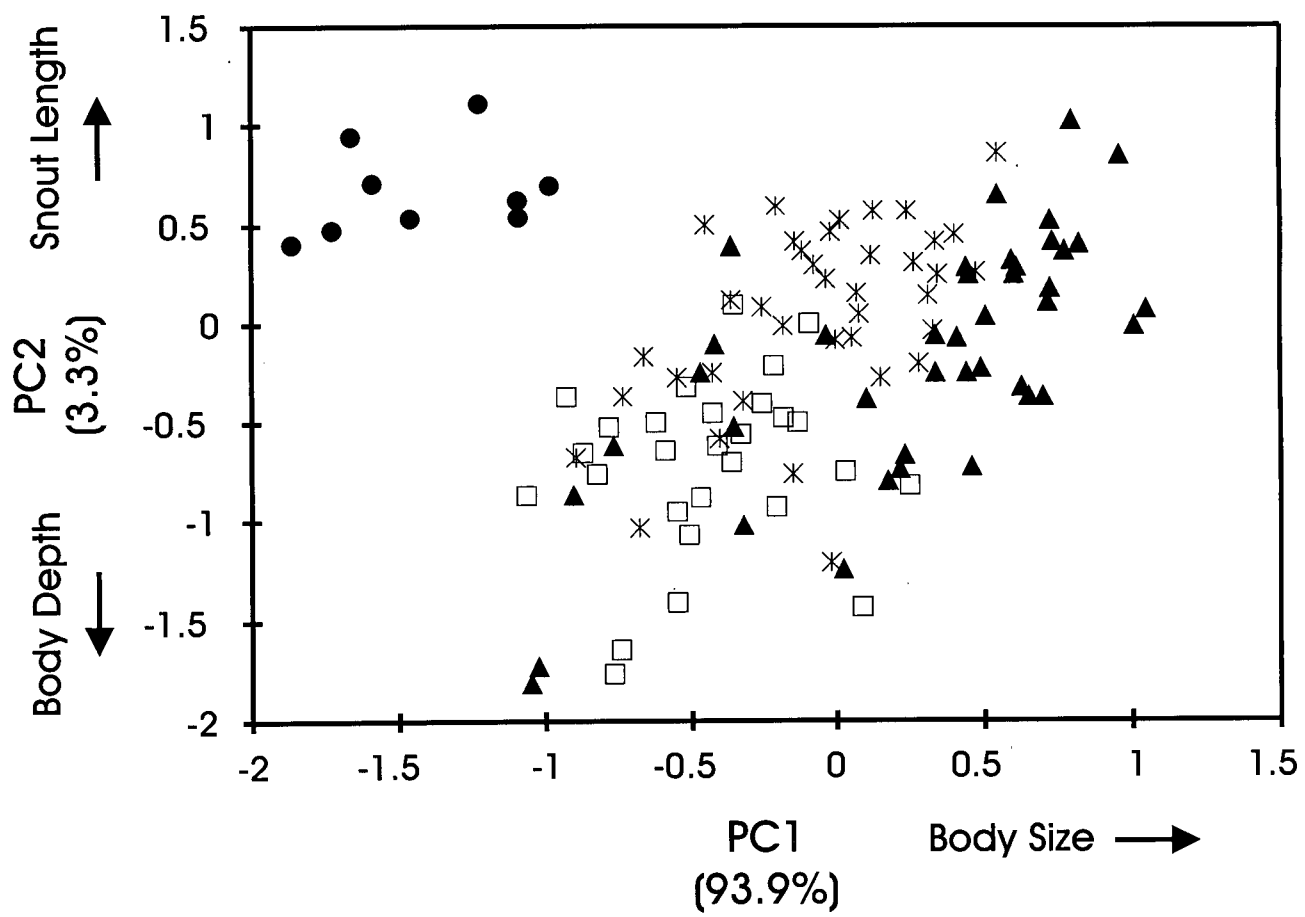


Figure 2. Spawning proportion of each combination of male species and female population. Points represent the proportion of trials \pm 1SE that ended in females depositing eggs in the males' nest. Symbols indicate male species: Benthics (●) and Limnetics (○). Error bars are shown in only one direction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

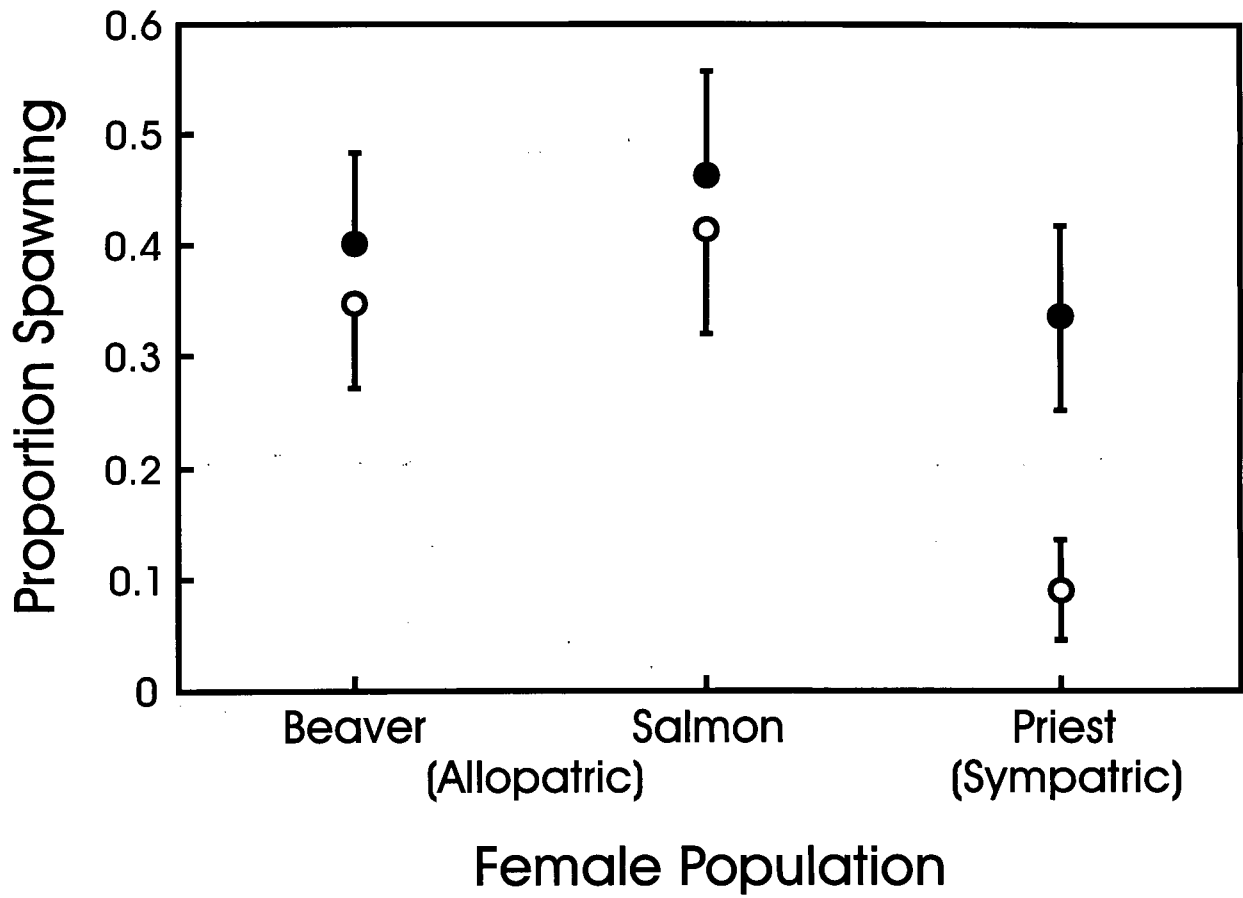
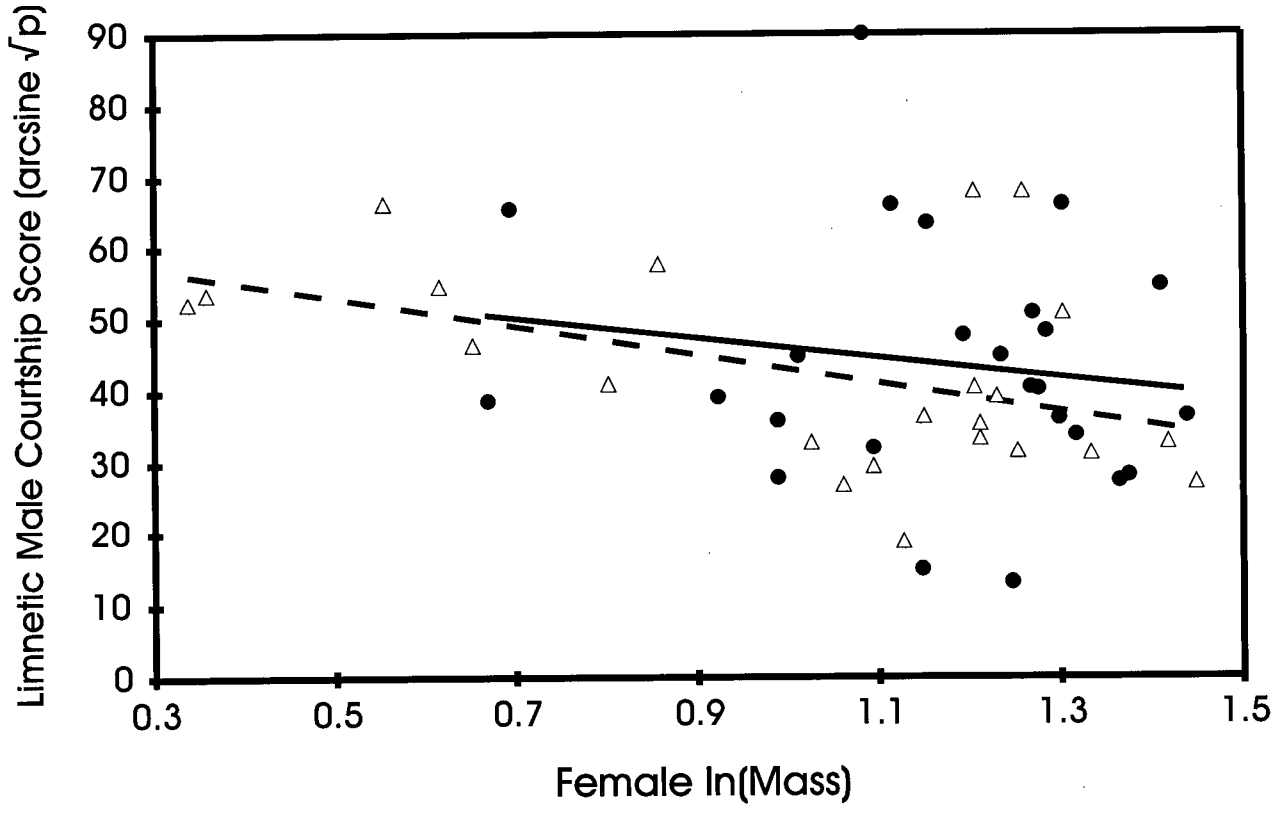


Figure 3. Courtship score of Limnetic males as a function of female body size. Lines are linear regressions of arcsine square root transformed male courtship score on ln-transformed female mass (g). Benthic females (broken line, open triangles), allopatric female (solid line, solid circles). Allopatric female populations (Beaver, Salmon) combined.



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