

REPRODUCTIVE CHARACTER DISPLACEMENT OF MALE STICKLEBACK MATE PREFERENCE: REINFORCEMENT OR DIRECT SELECTION?

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Abstract.—Reproductive isolation can evolve between species as a byproduct of adaptation to different niches, through reinforcement, and by direct selection on mating preferences. We investigated the potential role of direct selection in the reproductive isolation between sympatric species of threespine sticklebacks. Each sympatric pair consists of a small-bodied limnetic species and large-bodied benthic species. We compared the mate preferences and courtship behavior of males from one sympatric limnetic population and two allopatric populations. We used limnetic-like allopatric populations to control for the effects of ecological character displacement and adaptation to different niches on mate preferences. The sympatric limnetic males preferred the small limnetic females, whereas the allopatric limnetic-like males preferred the large benthic females, suggesting that adaptation to the limnetic niche does not automatically confer a preference for small limnetic females. This reproductive character displacement of male preference is consistent with the predictions of both reinforcement and direct selection on mate preferences. To test for direct selection, we assessed a prediction of one proposed mechanism: predation by benthic females on eggs guarded by limnetic males. The allopatric males come from populations in which there is no egg predation. Sympatric limnetic males were more aggressive toward benthic females than toward limnetic females, whereas the allopatric limnetic-like males did not treat the two types of females differently. The contrast in male behavior suggests that egg predation has shaped male preferences. Direct selection is potentially more effective than indirect selection via reinforcement, and it is likely that it has been important in building up reproductive isolation between limnetic and benthic sticklebacks.

Key words.—Male preference, mate discrimination, premating isolation, speciation, threespine sticklebacks.

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Under the biological species concept, reproductive isolation from other populations is the defining feature of a species. Any attempt to understand the processes driving speciation must therefore also unravel the processes behind the evolution of reproductive isolation. The buildup of pre- and postmating isolation between allopatric populations is relatively easy to imagine, although the mechanisms in nature are still unclear (Schluter 2001; Turelli et al. 2001). Less well understood is the evolution of reproductive isolation between populations that re-establish secondary contact with gene flow after some divergence in allopatry. There are several proposed mechanisms by which reproductive isolation can evolve between populations that are capable of exchanging genes.

Reproductive isolation can evolve as a byproduct of adaptation to different ecological niches (Kilias et al. 1980; Dodd 1989; Rice and Hostert 1993; Rundle et al. 2000). This adaptation to different niches can result in part from competition and ecological character displacement in sympatry, which may incidentally produce increased prezygotic isolation if traits used in mate choice are those that are diverging through natural selection (Schluter 2000, 2001). For example, if there is ecological character displacement of body size between closely related sympatric species, and size is important in mate choice, there may be an increase in prezygotic isolation without any direct selection on mating preferences.

Alternatively, selection can cause mating preferences to diverge in sympatry by reinforcement if hybrids are less fit than parental types (Dobzhansky 1940; Noor 1995). If individuals that mate with heterospecifics produce less fit offspring, then prezygotic isolating mechanisms might evolve to prevent hybridization (Noor 1995). The reduction in hybrid

fitness can be due to genetic incompatibilities and/or ecological or sexual selection against hybrids (Turelli et al. 2001). Regardless of the nature of selection against hybrids, reinforcement requires that linkage disequilibria are established and maintained between preference genes that reduce interspecific matings and the genes responsible for reduced hybrid fitness (Felsenstein 1981; Kirkpatrick and Servedio 1999; Servedio 2001). Therefore, selection on mating preferences during reinforcement is indirect and potentially weak (Servedio 2001; Kirkpatrick and Ravigné 2002).

Finally, reproductive isolation can evolve between sympatric populations when there is direct selection on mating preferences. Direct selection occurs when individuals lacking mate discrimination face a direct decrease in fecundity or survival (Servedio 2001), which may happen if individuals that hybridize are exposed to novel parasites, or if one of the species is predatory on the other (or its offspring). Interactions between species such as predation could reduce the probability of interspecific matings without the strong selection against hybrids required by some models of reinforcement (Servedio 2001). Direct selection on mate preference is potentially more effective than indirect selection (e.g. Kirkpatrick 1996; Kirkpatrick and Barton 1997), and may consequently be more important for driving the divergence of mating preferences in sympatry (Servedio 2001).

Of these three models (ecological character displacement, reinforcement, and direct selection) most attention has been paid to reinforcement as the major force strengthening prezygotic isolation after secondary contact. Yet, the alternatives are rarely ruled out. One major problem associated with trying to determine which of these three mechanisms is occurring in natural populations is that they all predict similar

outcomes: a pattern of reproductive character displacement in which greater prezygotic isolation develops between sympatric species pairs compared to allopatric species pairs of similar age (Coyne and Orr 1989; Noor 1999). Evidence for reinforcement comes from several empirical examples of reproductive character displacement (for review see Noor 1999; Höbel and Gerhardt 2003) and from theoretical models demonstrating its plausibility (e.g. Liou and Price 1994; Kelly and Noor 1996; Kirkpatrick and Servedio 1999; Kirkpatrick 2001). However, the importance of direct selection relative to reinforcement is completely unknown (Servedio 2001).

In this report we test for reproductive character displacement between sympatric threespine stickleback species (*Gasterosteus aculeatus*) using a control for ecological character displacement, and assess the role of direct selection as a cause of the pattern. Threespine sticklebacks are small fish that occur in coastal lakes, streams, and estuaries throughout British Columbia, Canada. Most lakes contain allopatric populations of sticklebacks; however, in each of several low-lying lakes a pair of sympatric species coexists. These species pairs are presumed to have formed after separately evolving allopatric populations came into secondary contact during the retreat of the Pleistocene glaciers (Schluter and McPhail 1992). The "benthic" species is large and deep-bodied and forages on benthic invertebrates, and the "limnetic" species is small and streamlined and forages in the open water on zooplankton (McPhail 1984, 1992; Schluter and McPhail 1992). These species mate assortatively (Ridgway and McPhail 1984; Nagel 1994). However, they seem to produce hybrids at a low rate in the wild (McPhail 1984, 1992) and have a history of mitochondrial DNA introgression (Taylor and McPhail 2000) suggesting that hybridization has been ongoing since secondary contact. Allopatric populations of sticklebacks exhibit a range of phenotypes from limnetic-like to benthic-like (Schluter and McPhail 1992), though less extreme, and display similar differences in feeding and ecology (Lavin and McPhail 1986).

There are reasons for thinking that both reinforcement and direct selection have played a role in the evolution of prezygotic isolation between sympatric stickleback species since secondary contact was established. F_1 hybrids have a lower growth rate than either parental type in their respective habitats (Hatfield and Schluter 1999), and F_1 hybrid males have a lower mating success than parentals (Vamosi and Schluter 1999). Furthermore, a mechanism for direct selection on mating preferences has been identified. Benthic females are known to eat stickleback eggs and will raid the nests of males to eat the eggs inside (Foster 1994, 1995). Males reveal the location of their nest during courtship and will often lead females to nests that contain eggs from previous spawnings (Foster 1994, 1995). This potential for egg predation may lead to selection on limnetic males to avoid courting benthic females and thereby revealing the location of their nests (Rundle and Schluter 1998).

To distinguish between these alternative mechanisms we focus on male mating preferences. Male mate choice is expected in sticklebacks because males are the sole providers of parental care (Whoriskey and FitzGerald 1994). As expected, male sticklebacks tend to prefer larger and therefore more fecund females, and this tendency is probably ancestral

(Sargent et al. 1986; Rowland 1989; Kraak and Bakker 1998). The presence of male mate choice leads to the possibility that males of sympatric species show reproductive character displacement of mate preference.

We tested for reproductive character displacement by comparing the preferences of one population of sympatric limnetic males with two populations of allopatric limnetic-like males. We assessed male preference by allowing males to choose between a limnetic and a benthic female. If there is reproductive character displacement, then limnetic males should prefer the smaller limnetic females, whereas the allopatric limnetic-like males should either show no preference or a preference for the larger benthic females. The use of limnetic-like allopatric populations in our test provides a control for ecological character displacement. If a preference for small limnetic females is a simple by-product of the evolution of a limnetic-like ecotype and morphology, allopatric males should display much the same preference as sympatric males. If allopatric males do not prefer limnetic females, then ecological character displacement is not enough to explain the level of reproductive isolation between sympatric limnetics and benthics.

Reproductive character displacement by itself does not distinguish reinforcement from direct selection on mate preferences. Therefore, we tested for direct selection on male preferences by examining the behavior of males toward their nonpreferred female type. If direct selection on limnetic males to avoid predatory benthic females has been important for the evolution of male preference, then we expect male limnetics to alter their courtship behavior toward benthic females relative to limnetic females. Limnetic males could alter their behavior by increasing the level of aggressive behavior toward benthic females, by hiding from them, or by leading them away from the nest area. Limnetic-like allopatric males would not have been exposed to benthics as nest raiders (Foster 1994, 1995), and should not alter their courtship behavior toward benthic females relative to limnetic females. Instead, we expect that allopatric males will show qualitatively the same type of courtship behavior toward benthic and limnetic females but simply display less overall to their nonpreferred female type. To our knowledge, this is the first attempt explicitly to test the role of direct selection in the reproductive character displacement of mating preferences.

MATERIALS AND METHODS

Collection and Maintenance of Fish

We collected male limnetic sticklebacks from Paxton Lake (a two species lake) on Texada Island, British Columbia (49°43'N, 124°30'W). Allopatric males were collected from Sproat Lake on Vancouver Island, BC (49°14'N, 124°54'W), and Sakinaw Lake on the Sunshine Coast, BC (49°42'N, 123°58'W). We used benthic and limnetic females from Priest Lake on Texada Island, BC (49°45'N, 124°34'W), another two-species lake that is in a separate drainage from Paxton Lake. This ensured that none of the females were from the same lake as any of the males.

The allopatric populations were chosen because they are limnetic-like in morphology (Fig. 1) and therefore in ecology (Lavin and McPhail 1986; Schluter and McPhail 1992). We

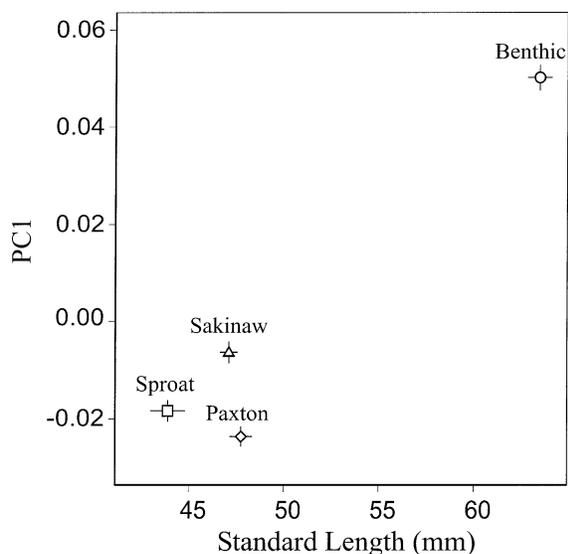


FIG. 1. Population means of the first principal component of body shape (PC1) and body size (standard length) of males from Paxton Lake (diamond), Sakinaw Lake (triangle), and Sproat Lake (square), as well as benthics from Priest Lake (circle). Error bars indicate ± 1 SE.

verified this similarity using a landmark-based analysis of shape (Rohlf and Marcus 1993; Ptacek 2002). We took digital photographs of preserved specimens of limnetics, benthics, and both allopatric populations, and 19 landmarks were placed on each photograph using the image analysis program, tpsDig (Rohlf 2001a). We used the same morphological landmarks as Walker (1997) with an additional five that further outlined the shape of the eye and jaw. The landmarks were analyzed using the program tpsRelw (Rohlf 2001b), which creates a consensus shape that is standardized for geometric size and rotation, and then calculates partial warp scores (Rohlf and Marcus 1993). The partial warp analysis computes the amount of energy required to “bend” the landmark configuration of each fish to the consensus shape. The relative warps produced are the principal components of the bending energy matrix. The first principal component explained 34.9% of the variation between the partial warp scores of fish from the four populations. The majority of the variation was associated with differences between the populations in body depth, eye size, pelvic girdle length, and jaw shape. These morphological traits are associated with differences between limnetic and benthic ecotypes (Schluter and McPhail 1992). The second principal component explained 18.7% of the variation and described the amount of twisting and bowing of the preserved specimens. Since this was not a biologically meaningful measure, we did not use it in further analyses. Similarly, the rest of the principal components were associated with small differences between individual fish rather than differences between populations.

We collected fish using minnow traps and dip nets between March and July 2002 as needed to maintain a stock of reproductive males and gravid females. The fish were held in 102-L aquaria, separated by population, at the University of British Columbia, Vancouver. Males from each population that exhibited reproductive coloration or behavior were

TABLE 1. Number of trials of each type with each male population.

Male type	Choice	No-choice	
		Limnetic	Benthic
Paxton (sympatric)	18	9	8
Sakinaw (allopatric)	13	7	8
Sproat (allopatric)	10	7	0
Total	41	39	

placed individually in 102-L “mating tanks” containing limestone gravel and a 20-cm diameter dish of sand 2–3 cm deep for a nesting substrate. We supplied java moss (*Vesicularia dubyana*) to use as nesting material, and two bladderwort bundles (*Utricularia* sp.) to provide cover. The dish of sand was placed at one end of the aquarium, with a bundle of bladderwort on either side. The sides of the tanks were covered with black plastic to isolate the males from each other visually. All tanks were maintained on a 16:8 light:dark photoperiod at an average temperature of 18°C. Fish were fed a satiation diet of brine shrimp (*Artemia* sp.) and bloodworms (chironomid larvae) daily.

Mating Trials

We conducted 60 choice trials and 54 no-choice trials with 60 males. We used a choice design to evaluate the presence of reproductive character displacement and a no-choice design to further differentiate between the predictions of reinforcement and direct selection. We encouraged males to nest by stimulating them with a gravid female in a jar for 10–15 min each day. The females were selected from limnetic or limnetic-like populations to ensure that the allopatric males had never encountered benthic females before the trials. (Note that this design does not allow us to distinguish between learned and genetic behaviors toward benthic females. However, previous work has found that assortative mating between benthics and limnetics persists in lab-reared fish [Hatfield and Schluter 1996], suggesting a genetic component to behavioral isolation.) Once a male had built a nest and courted the stimulating female, he was considered ready for trials. We used each male in two trials: one choice and one no-choice separated by at least 24 h. The order of the two trials was based on the availability of gravid females. After the two trials, males were anesthetized, measured, weighed, and preserved in 10% formalin. After trials, females were measured, stripped of their eggs to confirm their reproductive condition (eggs are easily released when the females are ready to spawn), and then weighed. Nineteen of the choice trials, and 15 of the no-choice trials were discarded because the male did not see both females (displayed less than five behaviors toward one or both females), or because one (or both) of the females was not yet ready to spawn (see Table 1 for a breakdown of the trials).

Choice trials

To determine the level of male mate discrimination or preference we presented sympatric and allopatric males with a choice of two gravid females: one limnetic and one benthic. We placed the females in a clear Plexiglas box (27.5 cm \times

9.5 cm × 14 cm) suspended at the top of the male's tank, one on either side of an opaque Plexiglas divider. We randomly determined which female was on the left or the right. Opaque dividers extended 5 cm from the front and the bottom of the box so that the males could display to only one female at a time. An observer sitting 1–2 m away watched male and female behavior for 10 min. The timing and frequency of the following male behaviors were recorded: (1) zigzag, courtship display of males that consists of one or more horizontal darts toward and away from the female; (2) bite, the male bites at the female through the Plexiglas. We also recorded several other courtship behaviors commonly used in stickleback behavioral experiments (e.g., see Rowland 1989; Kraak and Bakker 1998; Nagel and Schluter 1998; Rundle and Schluter 1998), but did not analyze them here. We used females for up to two trials with different males, but we never used the same pair of females twice.

No-choice trials

We conducted no-choice trials to evaluate differences in behavior that males showed toward limnetic and benthic females when only one type was present. The female species used for each male was haphazardly selected based on the availability of gravid females of each type and each male was used in only one no-choice trial. Each female was placed in a clear Plexiglas box (17 cm × 9.5 cm × 14 cm) suspended at the top of the male's tank, allowed to rest for approximately 5 min and then released into the tank with the male. An observer sitting 1–2 m away recorded male and female behavior for 20 min or until the female entered the male's nest (spawned), whichever came first. We only analyzed the behavior from the first five minutes of these trials to look at the male's initial response to the female rather than his response to her later behavior (Rundle and Schluter 1998). Females were used only once in no-choice trials, and were not used in any subsequent choice trials.

Analysis

Reproductive character displacement

We tested for reproductive character displacement by calculating a preference score (P), which was the standardized difference in the number of zigzags, N , directed toward the two females during the choice trials:

$$P = \frac{N(\text{zigzags to limnetic}) - N(\text{zigzags to benthic})}{N(\text{zigzags to limnetic}) + N(\text{zigzags to benthic})} \quad (1)$$

A positive preference score indicates a preference for limnetic females, whereas a negative score indicates a preference for benthic females. We then compared the means of these preference scores among the male populations. The number of zigzags directed toward females is widely accepted as a good indicator of male preference (e.g. Bakker and Rowland 1995; Kraak and Bakker 1998), and all males consistently used zigzags as a part of courtship (A. Y. K. Albert, pers. obs.).

We tested for differences between the male populations in mean preference score by fitting a linear model:

$$P_i = c + \beta R_i + e_i \quad (2)$$

where P_i is the courtship score for the i th male, c is a constant, and e_i is the error term. R is an indicator variable that specifies whether male i 's population of origin was sympatric ($R_i = 0$), or allopatric ($R_i = 1$). β is the magnitude of the difference between the male types. A significant β term would suggest that there are differences between sympatric and allopatric populations in preference for female type. Under reproductive character displacement, β should be negative, indicating that sympatric males have a stronger preference for limnetic females than do allopatric males.

We also fitted the data to the full model, which further distinguished between the two allopatric populations:

$$P_i = c + \beta R_i + \gamma Q_i + e_i \quad (3)$$

where Q is an additional indicator variable ($Q_i = 0$ for sympatric males and males from one of the two allopatric populations, and $Q_i = 1$ for males from the remaining allopatric population). A significant γ term indicates that the preferences of the allopatric male populations differ from each other.

We tested for discrimination based on the size difference of the females presented in the choice trials to determine whether males allocate their zigzags differently as females become increasingly different in size. This is expected because in no-choice mating trials between benthics and limnetics, the probability of hybridization increased as the individuals became closer in size (Nagel and Schluter 1998). If there is reproductive character displacement, then we expect the preference of sympatric limnetic males for the small limnetic females to become stronger as the size difference between the females becomes larger. We also expect the preference of allopatric males for the large benthic females to become stronger as the size difference between females becomes larger. We fit the following linear model to test for an effect of female size difference and male population on the courtship score for each trial:

$$P_i = c + \beta_1 z_i + \beta_2 R_i + \beta_3 z_i R_i + e_i \quad (4)$$

z_i is the difference in size between the two females presented to male i (size of benthic female minus size of limnetic female). R is the same indicator variable as in equations (2) and (3). A significant β_2 term would indicate that the relationships for sympatric and allopatric males have different elevations. Under reproductive character displacement, β_3 is predicted to be negative, indicating that the degree of preference of sympatric males for limnetic females becomes stronger with increasing difference in female size, opposite to the pattern in allopatric males. We did not analyze the full model including the Q term (distinguishing the allopatric populations from each other) because it did not improve the fit of the model. Female size was calculated as the first principal component of variation in ln-transformed cube root of size and standard length, all the females combined. The first principal component (body size) explained 98.9% of the variance among females. A positive body size score indicates a long and heavy female (benthic), whereas a negative score indicates a shorter and lighter female (limnetic).

TABLE 2. Behavioral data for males from the choice and no-choice trials (means \pm 1 SE).

	Male population		
	Paxton	Sakinaw	Sproat
Choice trials			
Number of zigzags to limnetic	104.6 \pm 17.3	43.2 \pm 10.9	51.8 \pm 20.7
Number of zigzags to benthic	54.8 \pm 10.7	74.1 \pm 13.7	50.9 \pm 11.7
Preference score	0.27 \pm 0.12	-0.30 \pm 0.13	-0.14 \pm 0.15
ln(bite to zigzag ratio) to limnetic	-1.68 \pm 0.23	-1.16 \pm 0.31	-0.98 \pm 0.37
ln(bite to zigzag ratio) to benthic	-0.88 \pm 0.27	-1.17 \pm 0.23	-0.54 \pm 0.29
No-choice trials			
ln(zigzag rate) to limnetic	2.25 \pm 0.26	1.84 \pm 0.56	2.36 \pm 0.25
ln(zigzag rate) to benthic	1.95 \pm 0.19	2.40 \pm 0.17	N/A
ln(bite rate) to limnetic	0.38 \pm 0.32	0.57 \pm 0.46	0.63 \pm 0.31
ln(bite rate) to benthic	1.62 \pm 0.19	1.19 \pm 0.35	N/A

The contribution of direct selection to male preference

To test for direct selection on mating preferences, we compared the behavior of males toward their preferred female type and their nonpreferred female type in the no-choice trials. We used only the data of males from Sakinaw Lake and Paxton Lake in this analysis because of a lack of trials with Sproat Lake males and benthic females (Table 1). We compared both the rate of zigzagging toward females and the rate of biting using two-way ANOVAs. A significant interaction between male type and female type would suggest that sympatric and allopatric males differ in how they behave toward their preferred and nonpreferred females. Rates were calculated as the ln-transformed number of occurrences of each behavior divided by the time elapsed (5 min). We expected sympatric males to behave more aggressively toward the nest-raiding benthic females than the limnetic females, or to adjust their behavior in some other way. In contrast, we expected allopatric males to show no qualitative difference in the type

of behavior directed toward the females, but simply display less overall to the limnetic (nonpreferred) females.

In a second test, we analyzed data from the choice trials to determine whether they supported the patterns found in the no-choice trials. We calculated the ln-transformed ratio of bites to zigzags displayed by males toward preferred and nonpreferred female types. We interpret the ratio of bites to zigzags as reflecting the level of aggression toward each female type. Biting is part of the normal courtship sequence for most stickleback populations, but bites are generally outnumbered by zigzags (Foster 1995). However, in previous trials with limnetic males relative bite frequency rises in displays towards heterospecific females (Nagel and Schluter 1998).

RESULTS

Reproductive Character Displacement

Males exhibited reproductive character displacement of preference. Sympatric limnetic males preferred the smaller limnetic females whereas allopatric limnetic-like males tended to prefer the larger benthic females (Table 2, Fig. 2). The preference scores of the sympatric males were significantly different from the preference scores of the allopatric males ($\beta = -0.461$, $F_{1,40} = 10.537$, $P = 0.001$, one-tailed test). The full model, distinguishing the two allopatric populations, did not fit the data significantly better than the reduced model (F -test, $\gamma = -0.143$, $F_{2,39} = 0.639$, $P = 0.215$), suggesting that there was no discernible difference in preference between the two allopatric populations.

Differences between sympatric and allopatric males in mate preference became more exaggerated the larger the difference in body size between the two presented females (Fig. 3). As the difference in the size of the two females presented increased, there was a trend for sympatric males to increasingly prefer the smaller (limnetic) female. For allopatric males the relationship was completely the reverse ($\beta_3 = -0.601$, $F_{2,38} = 3.263$, $P = 0.04$, one-tailed test). In all three populations, discrimination became weaker as females became similar in size.

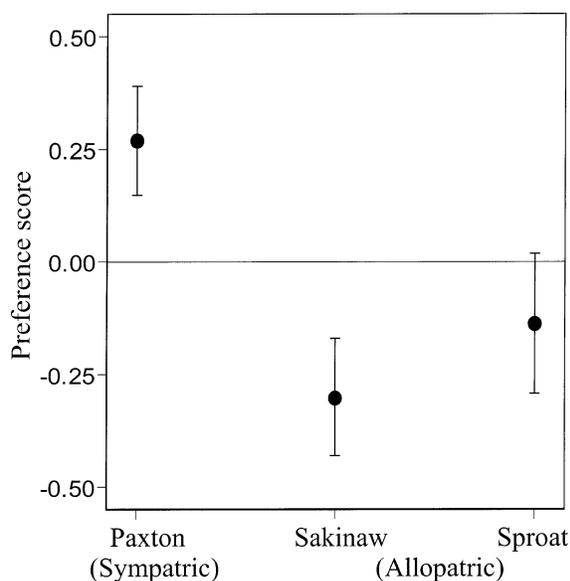


FIG. 2. Mean preference score of each male population from choice trials \pm 1 SE. A positive preference score indicates a preference for limnetic females, whereas a negative preference score indicates a preference for benthic females.

The Contribution of Direct Selection to Male Preference

Sympatric and allopatric males differed qualitatively in the types of aggressive behavior directed toward their preferred

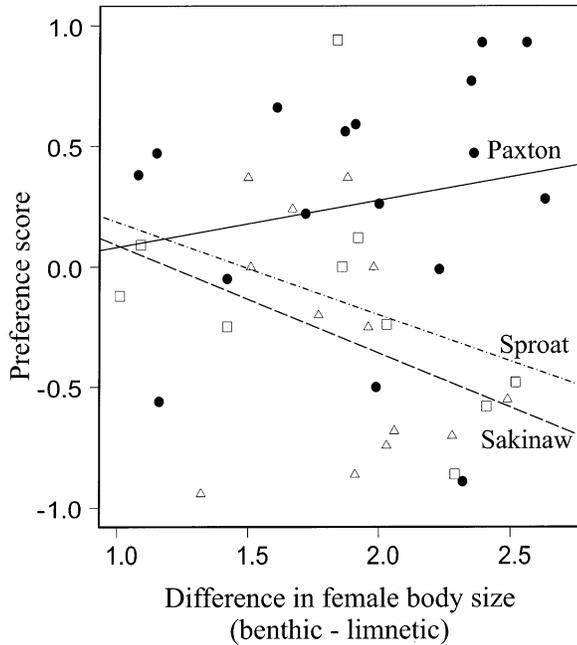


FIG. 3. Relationship between male preference score and the difference in the size of the two females presented during a choice trial. Populations are: Paxton Lake (solid circles and solid line), Sakinaw Lake (open triangles and dashed line), and Sproat Lake (open squares and dotted and dashed line).

and nonpreferred female types in the no-choice trials (Table 2, Fig. 4). Both sympatric and allopatric males displayed a higher rate of zigzagging toward their preferred female type (limnetic females for sympatric males, benthic females for allopatric males) than to their nonpreferred female type. There was therefore no significant interaction between male type and female type (preferred vs. nonpreferred) on the rate of zigzagging ($F_{2,31} = 0.181, P = 0.674$). In contrast, sympatric males elevated their rate of biting toward their nonpreferred benthic females relative to that toward limnetic females, whereas allopatric males displayed a lower rate of biting toward their nonpreferred limnetic females than toward

benthic females. The interaction between male type and female type (preferred vs. nonpreferred) on the rate of biting was significant ($F_{2,31} = 7.622, P = 0.010$). Essentially, allopatric males simply displayed a higher frequency of overall courtship behavior toward their preferred female type with zigzags exceeding bites, whereas sympatric males treated their preferred and nonpreferred females in qualitatively different ways, directing an excessive frequency of bites towards the nonpreferred benthic females. The results from the choice trials show the same pattern of behavior. Sympatric males displayed significantly higher bite-to-zigzag ratios toward their nonpreferred (benthic) female type than toward limnetic females (paired samples t -test, $t_{17} = -3.24, P = 0.005$), whereas allopatric males showed no difference in the bite-to-zigzag ratio displayed toward the different female types ($t_{12} = 0.05, P = 0.962$ for Sakinaw males and $t_9 = -1.35, P = 0.220$ for Sproat males).

DISCUSSION

Reproductive character displacement of male preference was established in this experiment by the observed difference in preference displayed by sympatric and allopatric males. Sympatric limnetic males preferred smaller limnetic females, whereas allopatric limnetic-like males preferred larger benthic females. Preference for large females is probably ancestral as it has been seen in all other allopatric and marine stickleback populations tested to date (Sargent et al. 1986; Rowland 1989; Kraak and Bakker 1998). This suggests that male traits underlying mating that decrease the potential for hybridization between benthic and limnetic sticklebacks have evolved in sympatry. Since the allopatric males were similar in phenotype to the sympatric limnetic males, ecological character displacement is not enough to explain the increased level of reproductive isolation between sympatric species pairs. However, there are at least two other processes besides ecological character displacement that could produce a pattern of reproductive character displacement of male preference: direct selection on mate preferences and reinforcement.

Direct selection on mating preferences occurs when indi-

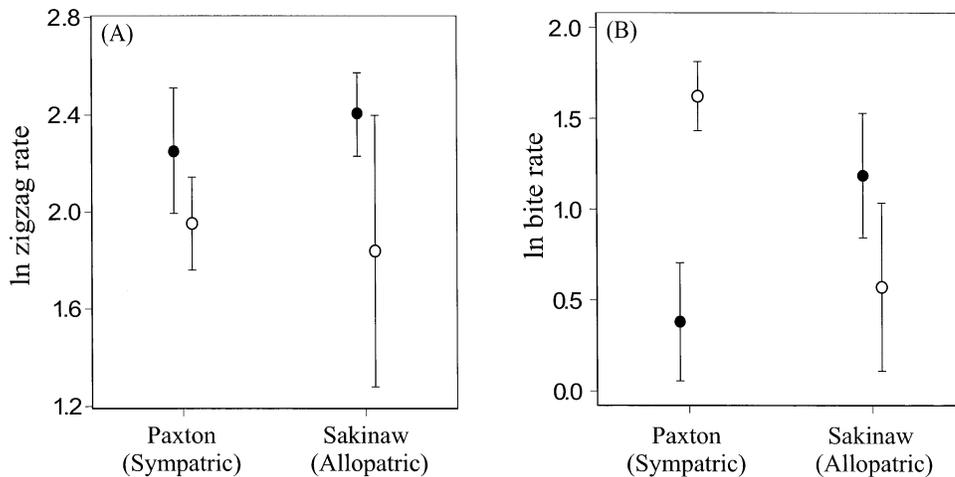


FIG. 4. Ln-transformed rates of (A) zigzagging and (B) biting displayed by males during no-choice trials toward their preferred (solid circles) and nonpreferred (open circles) female type. Points are means \pm 1 SE.

viduals displaying a particular mate preference face a direct increase or reduction in fitness or fecundity (Servedio 2001). Benthic females eat eggs in nests guarded by males (Foster 1994, 1995) and this could lead to selection on limnetic males to avoid courting large benthic females and revealing the location of their nest. In contrast, allopatric limnetic-like males have not evolved with benthic egg predation and are therefore free from selection to avoid large females. Direct selection on mate preference due to nest predation by benthics predicts that sympatric limnetic males should be more evasive or behave more aggressively toward benthic females than toward limnetic females. However, allopatric limnetic-like males should not direct more aggression or evasive behavior toward one type of female and should just display less overall toward their nonpreferred female type. In agreement with this prediction, we observed differences in the level of aggressive behavior displayed by sympatric and allopatric males toward benthic and limnetic females. Sympatric males decreased their rate of zigzagging and increased their rate of biting toward benthic females compared to their behavior toward their preferred limnetic females. In contrast, allopatric males consistently displayed less overall toward their nonpreferred limnetic females but did not increase their relative rate of biting.

These results suggest some influence of direct selection on male preferences in sympatry. However, processes other than direct selection can potentially explain this difference in aggressive behavior seen between males from different populations. Perhaps the increased aggression of limnetic males toward benthic females is the sum of two tendencies characteristic of all populations: an increase in the rate of biting toward benthic females and an increase in the rate of biting toward their nonpreferred female type. These two effects would cancel each other in allopatric males causing them to treat benthic and limnetic females similarly. Contrary to this hypothesis, allopatric males did not bite limnetic females more than did sympatric males (Fig. 4), suggesting that they were not elevating bite rates toward their nonpreferred type. Although this observation lends support to the idea that an increased rate of biting by sympatric males toward benthic females is an indication of direct selection on courtship behavior, the fact remains that male behavior provides only an indirect test of selection on traits involved in assortative mating and other explanations for differences in behavior are conceivable.

The threat of egg predation by benthic females is not restricted to limnetic males. Benthic males also run the risk of having their eggs eaten by benthic females (Foster 1994). This begs the question: why do benthic males continue to mate preferentially with benthic females when nonpredatory limnetic females are available? One possibility is that egg predation by benthic females imposes different costs on benthic and limnetic males. Benthic males are larger than limnetic males and are probably better able to defend their eggs against large benthic females and to control female behavior around the nest. If so, limnetic males may have more to lose than benthic males by courting benthic females. Interestingly, benthic males and limnetic females are slightly more likely to hybridize in the laboratory than limnetic males and benthic females (Nagel and Schluter 1998). This is concordant with

the idea that there is weaker direct selection on benthic males than on limnetic males resulting from mating with the wrong female type.

Alternatively, male preference may experience additional, indirect selection via the cost of producing less fit offspring (reinforcement). F_1 hybrids are less fit in the wild than the parental species (Hatfield and Schluter 1999; Vamosi and Schluter 1999), providing a possible source of indirect selection on mate preferences through reinforcement in addition to direct selection via egg predation. Reinforcement is potentially less effective than direct selection because it requires linkage disequilibria between alleles for mate preferences and alleles that cause reduction in hybrid fitness (Servedio 2001). However, the effectiveness of reinforcement can be increased when there is physical linkage between these types of alleles (Noor et al. 2001). We have no evidence at present to suggest such linkage is present in sticklebacks but it remains an interesting possibility for study. Reinforcement of male mate preferences, to be effective, also requires that those males preferring to mate with heterospecific females raise fewer conspecific offspring than males preferring conspecific females. This is expected if the behaviors are negatively genetically correlated (males preferring one type of female discriminate against the other type), if sperm limitation constrains the number of clutches that can be fertilized, or if nest size or oxygen requirements of eggs inhibit the number that can be raised successfully to hatching. Spermatogenesis in male stickleback is inhibited during the breeding season but males appear to manufacture enough sperm ahead of time to fertilize an excessive number of clutches (Zbinden et al. 2001). Evidence for negative genetic correlations between behaviors and for inhibitory effects of nest crowding are lacking. However, the important point is that our evidence for direct selection on male mate preferences in sympatry does not rule out an additional contribution of reinforcement to the pattern of reproductive character displacement. It is likely that both reinforcement and direct selection have been important in shaping male and female mate preferences, but teasing apart their relative contributions remains an important challenge.

One interesting question that arises from this experiment is why sympatric limnetic males still choose to court the large benthic females at all. The answer may lie in the potential benefits that could be gained from mating with a benthic female when no other options are available. Sexual selection theory suggests that males stand to lose less than females by mating with the wrong type (Andersson 1994), and may therefore be less choosy even when the stakes are high. In this case, a limnetic male might increase his fitness by mating with a large benthic female and producing lots of offspring even if those offspring are less fit hybrids. This is likely to be especially true if a male has the choice between mating with a benthic and not mating at all. A similar situation occurs between sympatric species of flycatchers where late in the breeding season females of one species face the choice between hybridization and not breeding at all. In this case females generally choose to mate with males of the other species although their offspring face a reduction in fitness relative to parental types (Veen et al. 2001). Alternatively, because the limnetic species are derived from the marine

ancestral type only recently (Taylor and McPhail 2000), it is possible that courtship displays toward larger females have not yet been fully eliminated by selection.

This is not the first experiment to implicate reinforcement and/or direct selection as causes of reproductive character displacement of mate preferences in threespine sticklebacks. Similar evidence for the potential role of direct selection on male preferences comes from a previous study on marine and stream sticklebacks. In the Salmon River in British Columbia, one population of stream resident sticklebacks is sympatric with marines during the breeding season, but a population that is further upstream is not (Borland 1986). Marine sticklebacks are larger than the stream residents and are known egg predators (Foster 1995). In the downstream (sympatric) population, males prefer to mate with females that are smaller than themselves. Conversely, the upstream (allopatric) males prefer to mate with females that are larger than themselves, indicating that there has been reproductive character displacement of male preference. Furthermore, consistent with the predictions of direct selection, sympatric males are more aggressive toward females that are larger than themselves, whereas allopatric males show no difference in the level of aggression displayed toward females of different sizes (Borland 1986). This leads to the interesting possibility that direct selection on males to avoid nest predation may also have contributed to the evolution of reproductive isolation between stream resident and marine sticklebacks.

In the limnetic/benthic species pair system, Rundle and Schluter (1998) documented reproductive character displacement of the mating preferences of benthic females. Their test compared the spawning probabilities of sympatric benthic females, presented with either benthic or limnetic males, to spawning probabilities of benthic-like allopatric females. They determined that the sympatric benthic females were less likely to spawn with limnetic males than with benthic males, whereas allopatric benthic-like females responded similarly to both types of males (Rundle and Schluter 1998). Since their experiment also controlled for the effects of ecological character displacement on preferences by using benthic-like allopatric females, the results implicate some process in sympatry that strengthened premating isolation over and above differences due to adaptation to different niches. They viewed reinforcement as the most likely explanation for reproductive character displacement of female preference. However, there is still the possibility that benthic females are under direct selection to avoid mating with small limnetic males that are less able than large benthic males to defend their nests against predatory raids (Rundle and Schluter 1998).

One final explanation for reproductive character displacement we have not yet considered is biased extinction. Biased extinction occurs when allopatric populations vary in their degree of reproductive isolation from each other (Butlin 1987). When some pairs of these populations come into secondary contact, only pairs that are strongly reproductively isolated remain as separate species, whereas pairs with insufficient reproductive isolation collapse into hybrid swarms (Butlin 1987; Noor 1999). This produces the pattern of greater isolation between sympatric species pairs than between allopatric species pairs. Biased extinction predicts that there should be a range of reproductive isolation between randomly

chosen allopatric populations (Rundle and Schluter 1998). Although we have now only tested four allopatric populations (two from Rundle and Schluter [1998] and two here), none of them seem to show anywhere near the level of discrimination displayed by both males and females from species pair lakes (Nagel and Schluter 1998). Therefore, current evidence indicates that biased extinction is unlikely to explain the pattern of reproductive character displacement seen between benthic and limnetic sticklebacks.

Although the results of these experiments support a role for direct selection in the evolution of reproductive isolation between limnetic and benthic sticklebacks, further tests are possible. This experiment provides only indirect evidence of direct selection on male preference. We require some measure of the direct and indirect fitness costs associated with hybridization to fully understand how direct selection and reinforcement interact to strengthen reproductive isolation in sympatry. However, these results provide some preliminary evidence that direct selection on mate preferences may have been important for speciation in this system. To our knowledge this is one of the first studies to provide empirical evidence that direct selection on mating preferences in sympatry may contribute to reproductive character displacement.

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