BODY SIZE, NATURAL SELECTION, AND SPECIATION IN STICKLEBACKS

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Abstract.—There is little evidence from nature that divergent natural selection is crucial to speciation. However, divergent selection is implicated if traits conferring adaptation to alternative environments also form the basis of reproductive isolation. We tested the importance of body size differences to premating isolation between two sympatric sticklebacks. The species differ greatly in size, and several lines of evidence indicate that this difference is an adaptation to alternative foraging habitats. Strong assortative mating was evident in laboratory trials, but a few hybridization events occurred. Probability of interspecific mating was strongly correlated with body size: interspecific spawning occurred only between the largest individuals of the smaller species and the smallest individuals of the larger species. Probability of spawning between similar-sized individuals from different species was comparable to spawning rates within species. Disruption of mating between individuals from different species can be traced to increased levels of male aggression and decreased levels of male courtship as size differences increased between paired individuals. Interspecific mate preferences in sympatric sticklebacks appears to be dominated by body size, implicating natural selection in the origin of species.

Key words.—Adaptive radiation, body size, natural selection, reproductive isolation, speciation, stickleback.

Divergent natural selection between populations inhabiting different ecological environments has long been thought to be a major cause of speciation (Barton 1989; Rice and Hostert 1993; Schluter 1996a,b). Dobzhansky (1951) and Mayr (1942) felt that reproductive isolation (especially postmating, but also premating) could evolve incidentally between allopatric populations as they accumulate adaptations to alternative environments. Theories of gradual sympatric speciation assume that contrasting abilities of genotypes to exploit two or more discrete resources create disruptive selection that in turn favors the evolution of premating isolation (Maynard Smith 1966; Rosenzweig 1978; Wilson and Turelli 1986; Diehl and Bush 1989). Finally, divergent natural selection on traits used in mate choice may generate divergent sexual selection that brings about the evolution of premating isolation (Lande 1981; Lande and Kirkpatrick 1988; Schluter and Price 1993).

Despite the plausibility of these "ecological" speciation mechanisms, remarkably little evidence exists that divergent selection was crucial to the evolution of reproductive isolation between any species in nature. Most support for the hypothesis derives instead from laboratory simulations in which some reproductive isolation evolved between *Drosophila* lines subjected to opposite patterns of artificial selection (Kilias et al. 1980; Dodd 1989). A shortage of similar evidence from nature stems from our inability to rule out the possibility that reproductive isolation between any two species evolved by nonecological mechanisms instead. These include genetic drift, founder effects, and fixation of alternative advantageous alleles in allopatric populations in response to identical selection pressures (Fisher 1930; Muller 1940; Wright 1940; Mayr 1954). Closely related species frequently differ ecologically, but a compelling connection between this differentiation and the evolution of reproductive

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isolation has rarely been shown. Noor (1995), Sætre et al. (1997), and others have presented evidence that natural selection against hybrids favors reinforcement of premating isolation between sympatric species (see Howard 1993). However, these data do not distinguish the processes that produced lowered hybrid fitness and initial levels of premating isolation, that is, divergent selection or not.

However, strong evidence is gained for selection's role in speciation when the phenotypic traits that diverge between populations, and that improve ecological performance in different environments, form the very basis of reproductive isolation (e.g., Macnair and Christie 1983; Ratcliffe and Grant 1983). In this paper we describe such evidence for premating isolation in an ecologically differentiated pair of sympatric threespine sticklebacks. These species originated very recently from a common ancestor, probably within the past 20,000 years. We show that variation in body size, a trait highly divergent between the sympatric sticklebacks (Fig. 1) and that is linked to exploitation of different resource environments, strongly influences the probability of interspecific hybridization.

Adaptive divergence in body size may lead to premating isolation by one of three routes. First, size-assortative mating within populations may predate the evolution of size differences between populations, in which case premating isolation would necessarily accompany population differentiation in body size. Second, natural selection may directly favor divergent mate preferences based on size as body size diverges—for example, in response to changing size distributions of available mates. Third, divergent size preference may be selected in response to reduced hybrid fitness (reinforcement or reproductive character displacement). We are not able to distinguish among these three scenarios with present data. Nevertheless, adaptation to alternative environments is the ultimate cause of premating isolation in all three cases. Connecting phenotypic evolution to reproductive isolation
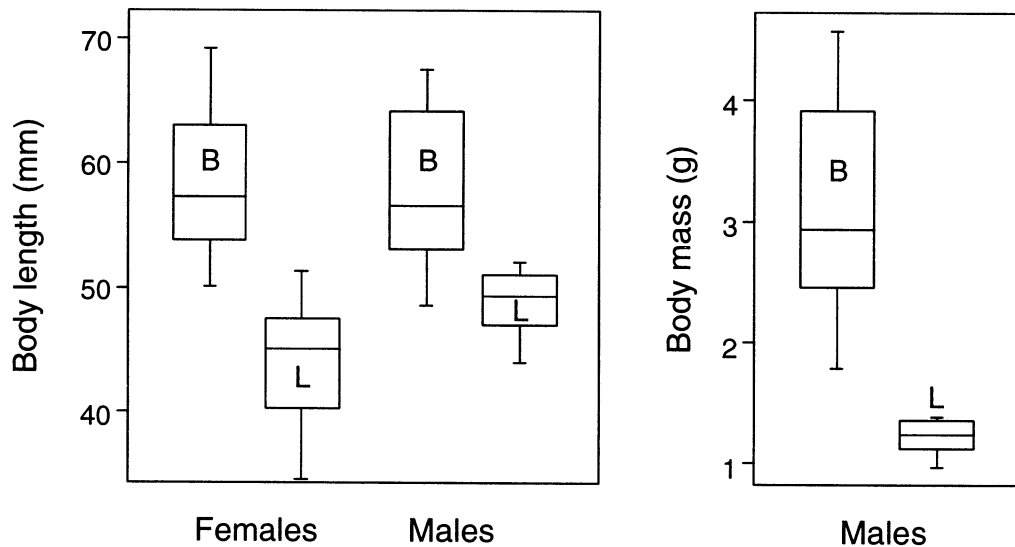
FIG. 1.   Body sizes of benthics (B) and limnetics (L) from Paxton Lake.

would be a major step in understanding speciation by natural selection.

Sympatric Stickleback Species

We studied premating isolation and body size in sympatric species pairs of threespine stickleback (*Gasterosteus* spp.) from small coastal lakes in the Strait of Georgia region of British Columbia, Canada (McPhail 1992, 1994; Schluter and McPhail 1992; Schluter 1996a). Each pair consists of a limnetic species, which is small in size, and a larger benthic species (Fig. 1). Limnetics are mainly planktivorous and forage offshore in open water, especially in the nonbreeding season. Benthics are confined to the littoral zone and deeper areas of open sediment, where they consume invertebrates living on or in the sediment or attached to vegetation (Bentzen and McPhail 1984; Schluter and McPhail 1992; Schluter 1993). Limnetics are smaller, more slender, and have a narrower gape and longer, more numerous gill rakers than the benthic. (Gill rakers are protuberances along the gill arches that assist in prey retention and act either as a physical seive of ingested prey or, when very densely packed, to direct water currents and particle movement within the buccal cavity; Sanderson et al. 1991.) The majority of similar lakes in the same region contain only a single species of threespine stickleback. This solitary form is ecologically and morphologically intermediate between limnetics and benthics when it occurs in lakes similar to those containing two species. This fact, together with experimental tests of competition and selection, suggests that the divergent characteristics of limnetics and benthics are partly the outcome of ecological character displacement (Schluter and McPhail 1992; Schluter 1994). Limnetics and benthics (and solitary species) both breed in the littoral zone, where males build and defend nests on the sediments. Although species differ in microhabitat during the breeding season, they encounter one another regularly (Bentzen et al. 1984; Hatfield 1995).

The precise origin of the species pairs is uncertain, although they are clearly recently derived from the marine threespine stickleback, *G. aculeatus* (Schluter and McPhail 1992; McPhail 1994; Ortí et al. 1994; Taylor et al. 1997). The lakes in which they occur are about 12,000 years old, and were formed when submerged coastal areas uplifted following deglaciation at the end of the Pleistocene (McPhail 1994). This fact places an upper limit on the duration of sympatry between sticklebacks. The lakes were colonized from the sea in the brief period during uplift in which they were accessible. A mtDNA phylogeny suggests that each species pair originated independently (Taylor et al. 1997). However, mtDNA and allozyme evidence conflict over whether species pairs could have evolved within each lake after a single colonization (i.e., via sympatric speciation) or are instead derived from two successive invasions of each lake (Taylor et al. 1997). Resolving the conflict is made difficult by the small genetic differences between populations and species (e.g., sympatric species differ in allozyme frequencies at only two or three of 25 allozyme loci, and no mtDNA markers have yet been discovered) and by a suspected history of gene flow in sympatry (McPhail 1984, 1992, 1994; Schluter 1996b; Taylor et al. 1997).

Sympatric limnetics and benthics are biological species and not mere morphs of a single polymorphic species. They are significantly different in allozyme frequencies (Nei's D = 0.02; McPhail 1984, 1992), and their differences in size and shape persist for at least two generations in the laboratory (McPhail 1984, 1992; Hatfield 1997). Because some morphological traits are developmentally plastic, differences between the species are reduced, but not eliminated, when their natural diets are experimentally reversed (Day et al. 1994). Interspecific hybrids are morphologically intermediate, and comparisons of variation in F_1 and F_2 offspring indicate that some differences between the species are determined by multiple genes (Hatfield 1997). The species mate assortatively in the laboratory (Ridgway and McPhail 1984; Nagel 1994; Hatfield 1995), and F_1 hybrids (identified by their intermediate morphology) are encountered infrequently in the wild (McPhail 1984, 1992, 1994). In the laboratory, F_1 hybrids as

well as F2's and backcrosses are viable and fertile, although growth rate in backcrosses (but not F_2s) is slightly reduced (Hatfield 1995, 1997). In contrast to the laboratory, F_1 hybrid growth is somewhat reduced in the wild, probably because their intermediate morphology renders them less efficient at exploiting the resources on which the parent species are specialized (Schluter 1993, 1995; Hatfield 1995; Vamosi 1996).

Our premise is that size-based assortative mating implicates selection in the evolution of reproductive isolation. This premise rests on the assumption that size differences between limnetics and benthics are the result of divergent natural selection between habitats (littoral zone vs. open water). The following evidence supports this assumption. First, the largest prey are found in the littoral zone; benthics confined to the littoral zone consume prey whose mean size (volume) is 15 times that consumed by benthics confined to open water (Schluter 1993, 1995). Second, the same body size difference between planktivores and benthivores has evolved repeatedly: benthics are always larger than limnetics (Schluter and McPhail 1992; McPhail 1994). Third, solitary species, which tend to exploit both environments in small lakes, are intermediate in size between limnetics and benthics (Schluter and McPhail 1992). Within these solitary populations, use of open-water plankton declines (and use of littoral zone increases) with increasing body size (Schluter and McPhail 1992). This same negative relationship between size and planktivory holds across solitary populations in general (Schluter and McPhail 1992; D. Schluter, pers. obs.). Fourth, large size appears to be a disadvantage when feeding on plankton. Rate of plankton capture in open water (volume/strike) increases steadily with decreasing body size in Paxton Lake benthics, and reaches a maximum at a small body size in limnetics from the same lake (Schluter 1993). In contrast, prey capture success in the littoral zone tends to increase with increasing body size in the benthic species, although this trend was not statistically significant (Schluter 1993).

We conducted mating trials with benthics and limnetics from two lakes. We tested the role of size in mate discrimination by placing males and females together in aquaria and observing the relative frequency of interspecific and intraspecific matings. If body size is the basis of assortative mating, hybridization should occur disproportionately often between the largest individuals of the smaller species (limnetics) and the smallest individuals of the larger species (benthics). We also recorded behaviors of the mating sequence to determine behavioral changes associated with assortative mating and size discrimination. Pattern and intensity of male nuptial coloration is similar in both species in both lakes: breeding males develop reddish coloration in the throat region as well as blue eye color and a bluish body sheen.

METHODS

Collection and Maintenance of Fish

Sticklebacks were collected from two lakes in separate drainages on Texada Island, British Columbia, Canada: Paxton Lake (49°43'N, 124°31'W) and Priest Lake (49°45'N, 124°34'W). Males were collected with cheese-baited minnow traps prior to the breeding season on February 12 and March 7, 1992, and on February 11, 1993. Benthics and limnetics

TABLE 1. Mean color scores of males and mean rate of two male behaviors (per five minutes) in intraspecific mating trials. Standard deviations are in parentheses.

Population	n	Color score	n	Zig-zags	Bites
Paxton benthic	15	3.7 (0.8)	5	4.4 (4.2)	13.4 (3.6)
Priest benthic	20	2.0 (0.6)	6	3.2 (3.2)	16.8 (11.2)
Paxton limnetic	7	4.3 (0.5)	4	8.0 (7.6)	6.0 (6.9)
Priest limnetic	20	3.1 (1.5)	3	3.0 (0.0)	5.0 (3.5)

were returned to Vancouver where they were held separately in 102-L aquaria in an environment chamber. Photoperiod in the chamber was gradually increased over a two-month period from 10:14 L:D to 16:8 L:D to bring the males into breeding condition. The temperature during the same period was increased from 7°C to 10°C. All fish were fed to satiation once daily with frozen brine shrimp (Artemia) and bloodworms (chironomid larvae). Most males developed some degree of nuptial coloration by early April and were judged ready to use in trials. Males were then returned to Texada Island and housed under similar conditions. No mortality or aberrant behavior was associated with this move. The light regime was maintained at 16:8 L:D. Water temperatures fluctuated between 15°C and 18°C. The experiments were conducted from April 21 until June 17 each year. This covered the peak periods of natural reproductive activity for both species in these lakes.

Gravid females were collected during the breeding season with cheese-baited minnow traps and held in large aquaria (102–180 L). They were fed once daily to satiation with frozen bloodworms and live Artemia nauplii. On the second day after capture, gravid females were used in a single mating trial.

Benthic males from Priest Lake differed from other populations in acclimating to captivity in both years. They were easily frightened even after months in captivity, and they never fully developed the nuptial coloration typical of wild males from this lake (L. Nagel, pers. obs.; cf. Table 1). In mating trials these males had a low probability of spawning even with conspecific females. We take account of this potential problem when analyzing our results. Benthic females from Priest Lake and both sexes of the other species were not affected in this way.

Mating Trials

Mating trials were carried out within and between species from the same lake. We adopted the "no-choice" protocol: a single gravid female was placed in an aquarium with one male and watched to see if spawning occurred. This is the same protocol used by Hatfield and Schluter (1996) in tests of sexual selection on hybrids, and is standard in studies of premating isolation (e.g., Noor 1995). Even though only one male was present, spawning did not appear to be coerced: only 31% of all pairs tested spawned. It was not possible to present females with a choice of two males (one of each species) because benthic males invariably destroyed the nest of limnetic males in pilot studies. However, we also carried out 24 trials using an alternate method in which a single female placed alone in an aquarium could view two males

in separate, adjacent aquaria (e.g., see Milinski and Bakker 1990). Female choice of males was determined by her courtship movements ("head-up"). Results from these trials were similar to those from no-choice trials, although assortative mating was weaker (Nagel 1994, unpubl. data). Because females in the no-choice trials frequently terminated courtship after head-up but prior to spawning, we felt that spawning itself was the most reliable indicator of mating tendencies (Hay and McPhail [1975] reached the same conclusion). We present only the results from no-choice trials here.

Males with typical breeding coloration were chosen haphazardly from the holding aquaria. Each male was placed alone into a 57-L aquarium (60 × 30 × 30 cm) or into one-half of a divided 102-L aquarium (75 × 30 × 45 cm). Each aquarium was covered on three sides by heavy brown paper so that males were visually isolated except from the viewer in front. Each aquarium contained a small box filter and was illuminated from above by a "cool white" fluorescent light. The bottom of each aquarium was covered with coarse gravel. A plastic tray filled with sand was provided as a nesting substrate. Each male was provided with nesting materials obtained from Paxton Lake (primarily *Chara* sp.) and was allowed five days in which to build a nest. Males that did not build a nest within five days were replaced (about 8% of all males used).

To begin a trial, a single gravid female was placed in an inverted 500-mL clear, plastic jar in a corner of a male's tank away from his nest. Males usually began to court females within two minutes of jar placement into the tank. On rare occasions, a male did not approach the jar within three minutes, in which case the test was cancelled. Usually within about 30 seconds of the male's approach, the female in the jar swam up from the bottom and began to respond to the male's courtship by swimming at the sides of the jar. Three minutes after being placed in the tank, the jar was gently raised above the female using a piece of clear monofilament line attached to its bottom. The line was secured such that the jar was positioned just below the surface of the water to minimize disturbance and to serve as a retreat for females. Courtship in sticklebacks can be aggressive, and in natural situations females who are unwilling to court with a male are able to leave the area. This appeared to be especially important in tests involving limnetic females and benthic males because benthic males were sometimes very aggressive toward the smaller limnetics and chased them until they sought refuge in or near the suspended jar.

We recorded female behavior from approximately one meter in front of the aquarium using an event recorder. Recording began when the male first directed courtship activity to a freed female (usually within one minute of her release). Most trials were also videotaped for later analysis of male behavior. We recorded whether the pair spawned within 30 minutes, a time sufficient to gauge a pair's willingness to mate. Preliminary tests showed that pairs that did not spawn within 30 minutes would not do so even if left for up to two hours.

During the breeding season males of both species develop a red throat that is known to be a target of female choice in intraspecific mating trials (e.g., Milinski and Bakker 1990). We assigned a subjective color score to each male to determine the effect of its intensity on the probability of interspecific spawn-

ing. The score ranged from 1 (almost no color) to 5 (maximum color intensity). Because males usually become more colorful as the time engaged in courtship increased (L. Nagel, pers. obs.) this score was recorded only once: when the male first began courting the female. Color scores were not recorded in the first year and so are missing for 18 trials.

After each trial females that did not spawn were examined to see if their receptivity prior to the test had been properly assessed. When gentle pressure is placed on the abdomen of a female with mature eggs, the eggs slide easily into the lower oviduct and can be seen through the body cavity. If this did not happen, the trial was excluded from the analysis. This occurred only twice in over 150 trials.

A female was used only once in the set of trials, after which her standard length was measured with calipers. Males that did not spawn in their first trial were occasionally used a second time, always with a female from a different population than in the first trial (12% of all males). Such males were left alone for 24 hours after the first trial before beginning the second. Males were then anesthetized, measured, weighed, and preserved in 10% formalin.

Male and Female Behavior

Male and female courtship behaviors were recorded on videotape to determine whether sympatric species differed, and whether the probability of hybridization could be linked to specific behavioral changes. In males these behaviors are: (1) approach: male swims to the female in a straight path; (2) zig-zag: male swims to female in a series of horizontal dashes; (3) bite: male bites female; (4) lead: male leads the female to his nest (both direct and meandering paths are included); (5) show nest: male pokes at nest entrance with his snout; (6) creep-through: male goes through his nest; and (7) nest maintenance: male performs nest-tending behaviors such as fanning, pushing, or gluing nest material. These behaviors tend to be carried out in fixed sequence and hence their frequencies are correlated. For this reason we focus on two behaviors very early in the sequence: frequency of zig-zags and bites. See Rowland (1994) for a more complete description of breeding behaviors. Five video tapes from each test combination (lake, male species, female species) were examined, for a total of 40 trials. Only the first five minutes of each trial were studied. Three of the cases were discarded because spawning occurred within five minutes, leaving 37 trials for study of male behavior.

Female behaviors included: (1) follow: female follows male after he approaches her; (2) head-up: female assumes a posture in which the head is tilted upward while she approaches the male; (3) examine nest: female pokes her snout into the nest entrance; (4) enter nest: female enters nest; (5) leave nest: female leaves the nest after entering it without depositing eggs. We focus on (1) and (3); behavior (2) is not always seen in courtship, and (4) and (5) occur late.

Analysis

We initially treated species pairs in the two lakes separately when analyzing the data because of their presumably independent origins (Taylor et al. 1997). However, limnetics from the two lakes behaved similarly, as did benthics. For example,
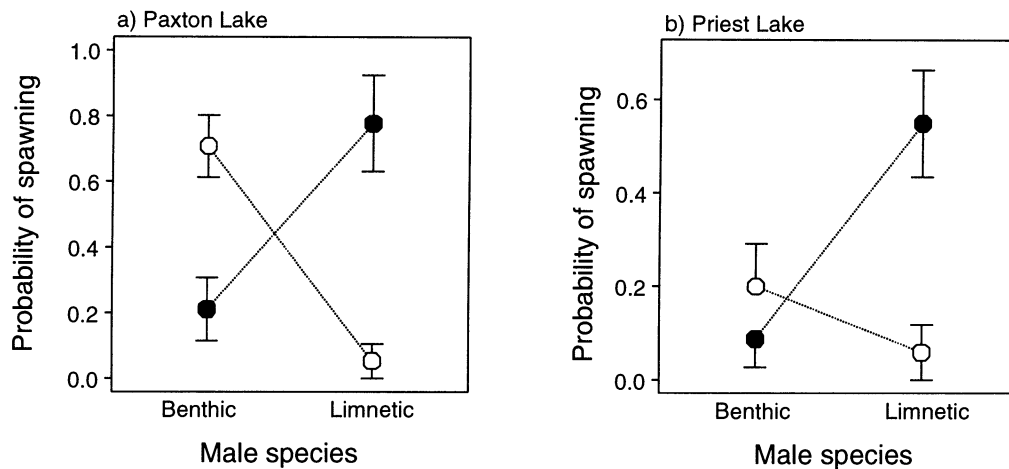
FIG. 2. Assortative mating between sympatric species. Points measure the fraction of trials that resulted in spawning $\pm$ 1 SE. Symbol refers to female species: limnetic (●) and benthic (○). Number of trials are n = 19, 9, 23, and 20 for limnetic females (from left to right) and n = 24, 19, 20, and 17 for benthic females.

Priest and Paxton limnetics responded in the same way to size differences in heterospecific trials. Therefore we combined data from the two lakes (limnetics with limnetics and benthics with benthics) in tests of size effects and behavioral differences during courtship, and incorporated "lake" as an additional factor. No significant interactions between lake and other factors were detected in any analysis, and none of the main effects was altered when interactions with lake were dropped. For simplicity we only present results from analyses without interactions involving lake.

Assortative mating between sympatric species was tested using logistic regression. The regression model examined the dependence of spawning probability on male species, female species, and the interaction between male and female species. Assortative mating is indicated by a significant interaction. We also used logistic regression to examine effects of male size and female size on the probability of hybridization between species. Behaviors were analyzed using ANOVA after taking square-root transformations of the raw counts to render homogeneous the variances of residuals.

Trials between males and females were carried out opportunistically as captive males came into breeding colors and according to the availability of wild gravid females. However, seasonal trends in the body sizes of available mates were observed in both lakes. Standard length of benthic males in trials declined through the season (r = -0.54, df = 84, $P <$ 0.001; trends in the two lakes were similar and data were combined) whereas sizes of limnetic females increased (r = 0.60, df = 84, $P <$ 0.001). Weaker negative trends occurred in benthic females (r = -0.19, df = 78, P = 0.08) and limnetic males (r = -0.33, df = 63, P = 0.008). The result is that standard lengths of males and females in trials were correlated, especially in trials involving male benthics and female limnetics. Logistic regressions were unable to disentangle effects of male size on the probability of hybridization from those of female size. We therefore tested the importance of body size to spawning by analyzing measurements of both sexes together. That is, we tested the fit to data of regression models that included both male and female size as variables, with the fit of models lacking both size variables.

Trial date was included in the regression models in addition to lake, to control for possible seasonal changes (unrelated to size trends) in the probability of hybridization. The effect of trial date was not significant in any analysis, and results were unchanged when it was left out. Statistical analyses were carried out using S-Plus (MathSoft 1995).

RESULTS

We first establish the level of assortative mating between the species in our laboratory setting and the associated courtship behaviors. We then compare instances of mating and nonmating between the species to test whether probability of spawning depends on body sizes of paired males and females.

Assortative Mating

Assortative mating between limnetics and benthics was strong and positive (Fig. 2). Of 73 intraspecific trials, 39 resulted in spawning (53%). In contrast, only eight of 78 interspecific trials resulted in spawning (10%). Assortative mating was clearest between the species from Paxton Lake (Fig. 2a), as indicated by a strong interaction between effects of male and female species on the probability of spawning (logistic regression; χ_1^2 = 27.0, $P <$ 0.0001). Assortative mating between the Priest Lake species is clouded by a low spawning rate of benthic males regardless of female species (Fig. 2b; see Methods). Significant assortative mating was nevertheless detected, as indicated by a significant interaction between effects of male and female species (χ_1^2 = 9.2, P = 0.002).

Intraspecific courtship was broadly similar between limnetics and benthics: bites, zig-zags, and bright coloration are common components. Significant differences were nevertheless noted. Benthics were more aggressive than limnetics, biting females two to three times more frequently (Table 1, Fig. 3a). This difference was confirmed with a two-way ANOVA of square root number of bites, with male species and lake as factors (effect of species: $F_{1,15}$ = 8.06, P = 0.012; effect of lake: $F_{1,15}$ = 0.05, P = 0.83). No significant difference between species was detected in the frequency of the zig-zag courtship display in intraspecific trials (Table 1; Fig. 3b) (two-
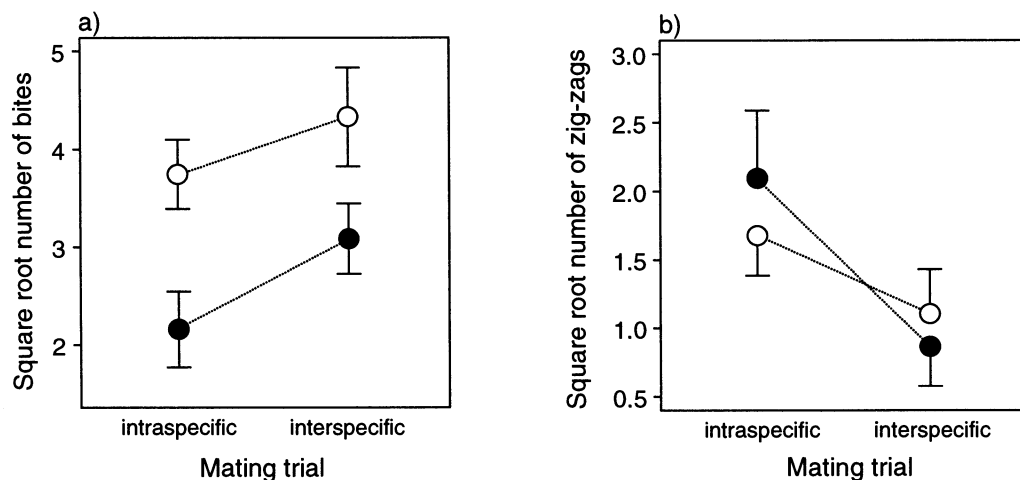
FIG. 3. Two aspects of male courtship in inter- and intraspecific trials: bites (a) and zig-zags (b) (means ± 1 SE). Symbols refer to male species: limnetic (●) and benthic (○). Counts of behaviors are from the first five minutes of the courtship sequence. Data from both lakes were combined.

way ANOVA of square root number of zig-zags; effects of male species and lake: both $F_{1,15} < 0.82$ and $P > 0.38$). Similar analyses of female behaviors in intraspecific trials (Table 2) showed that limnetics examined nests at a higher frequency than did benthics (two-way ANOVA of square root number of nest examinations; effect of female species: $F_{1,40} = 5.32$, $P = 0.026$; effect of lake: $F_{1,40} = 0.26$, $P = 0.61$). Male color scores overlapped broadly between sympatric species (Table 1),' although male limnetics were significantly brighter than male benthics (two-way ANOVA of male color scores on male species and lake; effect of species: $F_{1,60} = 6.36$, $P = 0.014$; effect of lake: $F_{1,60} = 31.3$, $P < 0.0001$).

Reduced probabilities of spawning in interspecific trials are associated with changes in male courtship patterns (Fig. 3). The zig-zag display was performed one-half to one-third as frequently in interspecific trials as in intraspecific trials. This difference was significant, as indicated by a sizeable interaction between male and female species ($F_{1,32} = 8.11$, $P = 0.008$) in a three-way ANOVA of square root number of zig-zags on male species, female species, male × female species, and lake (the effect of lake was also significant: $F_{1,32} = 5.47$, $P = 0.026$). The frequency of bites also rose in interspecific trials (Fig. 3) but not significantly (three-way ANOVA, effect of interaction between male and female species: $F_{1,32} = 3.32$, $P = 0.078$). Similar analyses showed that the frequencies of two male behaviors associated with nest display decreased in interspecific trials: number of leads to the nest ($F_{1,32} = 5.52$, $P = 0.025$) and number of nest showings ($F_{1,32} = 4.44$, $P = 0.043$). None of the female behaviors showed a significant

TABLE 2. Mean rate of female courtship behaviors (per five minutes) in intraspecific mating trials. Standard deviations are in parentheses.

Population	n	Follow	Examine nest
Paxton benthic	8	3.0 (2.0)	0.4 (0.5)
Priest benthic	12	4.5 (1.8)	0.5 (0.5)
Paxton limnetic	9	2.8 (1.6)	0.9 (0.6)
Priest limnetic	14	4.0 (2.3)	1.3 (1.2)

change from interspecific to intraspecific trials. This does not mean that changes in female behavior were absent. It is possible that changes in male behavior are a response to changes in female behavior that we did not record.

Body Size and Hybridization

Results in the previous section show that assortative mating is strong, and that changes in courtship are associated with a lower probability of spawning in interspecific trials. We now determine whether the assortative mating and associated behaviors are influenced by body size differences between the species (Fig. 1).

The probability of spawning between individuals from different species was strongly associated with their body sizes (Fig. 4). Spawning occurred only between the largest limnetic females and the smallest benthic males (Fig. 4a; $\chi_2^2 = 34.45$, $P < 0.0001$), and between the largest limnetic males and the smallest benthic females (Fig. 4b; $\chi_2^2 = 11.97$, $P = 0.003$) (these analyses include lake and trial date as factors; see Methods). In both crosses hybridization was restricted to males and females of approximately the same length (Fig. 4). Probability of interspecific mating was also positively related to the intensity of male coloration, but coloration was not significantly correlated with body sizes of males and females; adding it to the regression model did not alter the results for size.

The mean probability of spawning between a limnetic female and a benthic male of approximately the same size was 0.7 (estimated from those females whose lengths were greater than 45 mm and those males whose lengths were less than 55 mm; cf. Fig. 4a), which is similar to spawning probabilities within species (Fig. 2). The mean probability of spawning between a benthic female and a limnetic male of about the same size was near 0.3 (estimated from those males whose lengths were greater than 50 mm and those females whose lengths were less than 55 mm; cf. Fig. 4b), which is lower than that within species, with the exception of Priest benthics (Fig. 2).
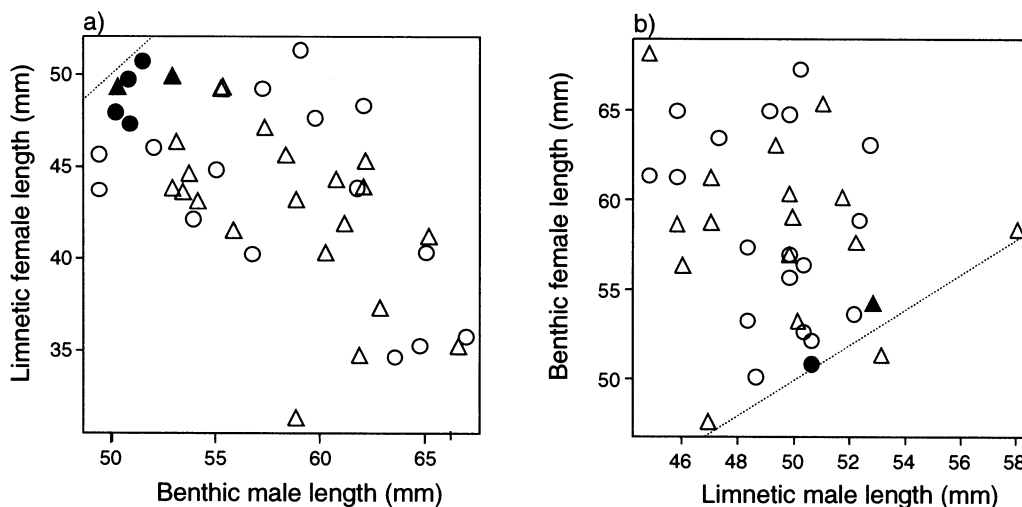
FIG. 4. Interspecific spawning in relation to body lengths of males and females. Circles are pairs from Paxton Lake; triangles are from Priest Lake. A filled symbol indicates that the pair spawned. Dashed lines indicate $Y = X$. (a) Limnetic females with benthic males. (b) Benthic females with limnetic males.

Measurements of male courtship showed that behaviors in interspecific trials changed with body sizes of tested individuals, suggesting that these behaviors may have played a part in establishing size effects on spawning probability. Aggression by limnetic males (bites) tended to increase in frequency with increasing size of female benthics (Fig. 5) and decreasing size of Limnetic males, whereas the frequency of zig-zags showed a decline (Fig. 5). Neither behavior trend is significant on its own, but the difference between the trends (i.e., number of bites minus number of zig-zags) is significantly related to the body sizes of paired individuals ($F_{2,5} = 7.80$, $P = 0.029$). As with analyses of spawning behavior, the contributions of male and female size could not be separated and so both are analyzed together. The frequency of bites and zig-zags by limnetic males toward the smallest ben-
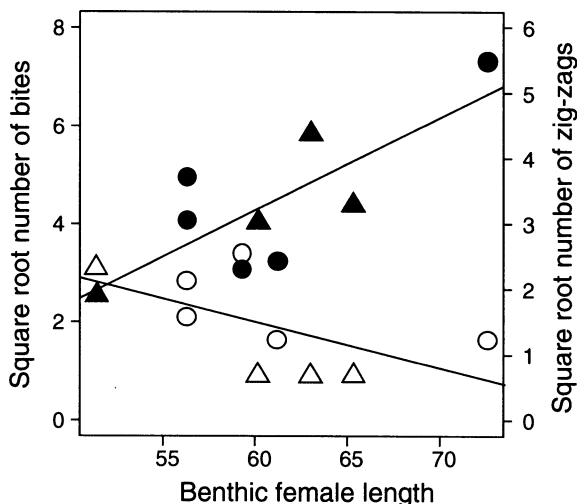

FIG. 5. Courtship behaviors of male limnetics with female benthics of varying size. Counts of zig-zags (open symbols, lower line) and bites (filled symbols, upper line) are from the first five minutes of a trial. Circles and triangles indicate observations from Paxton and Priest Lakes, respectively.

thic females was nearly the same as that toward conspecific (limnetic) females (Table 1). Analogous trends were seen between male benthics and female limnetics, although none was significant.

DISCUSSION

Body Size and Premating Isolation

Natural selection is implicated in speciation if adaptive phenotypic differences between species also form the basis of their reproductive isolation. We summarized evidence that body size has diverged between two species of threespine stickleback because of contrasting selection pressures between the two environments they principally exploit. Limnetics are smaller than benthics, with only slight overlap in size. We showed that these two species are reproductively isolated, and that the probability of hybridization between them is strongly associated with differences in body size between paired members of the two species. Courtship behavior of male limnetics toward the smallest benthic females was similar to that toward females of their own species, and spawning probability was equally high. Limnetic male behavior became more aggressive toward benthic females as the size difference between them increased, and spawning probability dropped to zero. Analogous trends were seen between male benthics and female limnetics, although they were weaker and spawning probability between them remained lower than that within species. These results suggest that body size is a major determinant of reproductive isolation between sympatric stickleback species. Hence they support the hypothesis that natural selection stemming from foraging habitat differences was a cause of speciation in these sticklebacks.

The results extend earlier work showing that limnetics and benthics in Paxton and Enos Lakes, and other sympatric forms of sticklebacks, mate assortatively (Hagen 1967; Moodie 1972; Hay and McPhail 1975; Ridgway and McPhail 1984; Borland 1986; Blouw and Hagen 1990; Hatfield 1995;

Hatfield and Schluter 1996; reviewed by McPhail 1994). Borland's (1986) study of premating isolation between marine anadromous sticklebacks (*G. aculeatus*) and stream resident threespine sticklebacks also indicated that size was a cue in mating decisions. Size differences evolving in the context of ecological differentiation are wholly or partly responsible for premating isolation between other closely related sympatric species including sockeye and kokanee salmon (Foote and Larkin 1988) and several species of Darwin's finches (Ratcliffe and Grant 1983). Intraspecific mate choice in sticklebacks is known to be influenced by male coloration (e.g., McLennan and McPhail 1990; Milinski and Bakker 1990), territory size (e.g., Goldschmidt and Bakker 1990), territory quality (Sargent 1982), presence of eggs in the males' nest (e.g., Jamieson and Colgan 1989) and male courtship behavior (e.g., Hay and McPhail 1975; Jamieson and Colgan 1989). We found that the probability of interspecific spawning was also positively correlated with intensity of male color, but accounting for this association did not diminish the effects of body size.

Alternative Explanations

Until size can be manipulated independently of the rest of the phenotype, we cannot be completely certain that size differences alone caused the patterns of premating isolation documented here, or that size itself is the direct cue used in mating. We considered date of spawning and courtship as additional factors affecting the relationship between size and probability of interspecific mating. A nonsignificant effect of trial date was seen in both combinations of interspecific crosses: hybridization occurred more often later in the breeding season than earlier (logistic regressions: limnetic female × benthic males, $\chi_1^2 = 3.45$, $P = 0.06$; benthic female × limnetic males, $\chi_1^2 = 3.18$, $P = 0.07$). The trends, if real, might reflect a reduced level of discrimination in males and/or females as the season progresses. However, our regression models tested the effects of male and female size after effects of date (and lake) were already incorporated; therefore date alone cannot explain the relationship between size and hybridization.

Fixed differences between the species in male courtship may have contributed to assortative mating. For example, the high baseline levels of aggression by male benthics toward females in general may explain the somewhat (but not significantly) lower frequency of hybridization between male benthics and female limnetics (19% of 42 trials; Fig. 4a) than between male limnetics and female benthics (6% of 36 trials; Fig. 4b; Fisher exact test, $P = 0.28$). Nevertheless, behavior was flexible and appeared to respond to size differences between mates: differences were greatest in interspecific trials when size asymmetries were largest (e.g., Fig. 5). This suggests that size differences between benthics and limnetics influence mating probabilities through context-specific changes in courtship. This latter hypothesis cannot be proved without experimental manipulation of behavior; nevertheless the correlation between size and behavior in interspecific trials is striking (Fig. 5). We cannot say whether changes in male behavior are the sole proximate determinant of these size-dependent patterns; for example, changes in male be-

havior may have been initiated by subtle changes in female behavior that we did not record.

Body size may influence premating isolation between sticklebacks because it is an obvious trait that can be easily assessed. Males and females may additionally react to a size asymmetry for reasons quite apart from mating (see below), with the result that courtship is disrupted. Other aspects of morphology such as shape may also contribute, as in the Darwin's finches (e.g., Ratcliffe and Grant 1983). Limnetics and benthics are different in shape (e.g., Schluter and McPhail 1992), and it is possible that shape changes correlated with size also underly interspecific mating frequencies. Some indication of the contribution of factors other than size might be gained by asking whether the relationship between size and spawning probability recorded between species is just a continuation of trends existing within species. A problem with this idea is that body size distributions of sympatric species overlap very little (Fig. 1), making extrapolations difficult. Relationships between size and spawning within limnetics and benthics are weak, as judged by logistic regressions of spawning probability on the difference between male and female length (lakes combined; limnetics: $n = 27$, $\chi_1^2 = 0.8$, $P = 0.39$; benthics: $n = 36$, $\chi_1^2 = 0.0$, $P = 0.96$). Results were similar when the absolute value of the difference between males and females was used instead. Both small and large limnetic females mated with the largest males, and these large males had higher mating success overall (logistic regression, $n = 27$, $\chi_1^2 = 4.5$, $P = 0.03$). No size trends were apparent in benthics. The dissimilarity of relationships between size and spawning frequency with and among species suggests that size plays a different role in mate discrimination within species than between species. If so, then factors other than size may contribute to hybridization (e.g., body shape). Alternatively, the effect of size may differ depending on the magnitude of the difference, which is greater between species than within species (Fig. 1).

Evolutionary Mechanisms

Three general mechanisms for the evolution of premating isolation may account for its dependence on an adaptive trait such as body size. First, size-based preferences may be an old feature of the mate recognition system, and cross-matings diminish as a correlated response to size divergence without further selection on the preferences. This is the "by-product" mechanism envisioned by Slatkin (1982) and Rice (1984, 1985, 1987; Rice and Hostert 1993). The finding that spawning probability is not related to the difference between male and female sizes within stickleback species is evidence against this hypothesis, but these findings are tentative given the low overlap in body sizes between the species.

Second, selection may directly alter mating preferences during and after divergence. For example, size evolution in a population may result in secondary selection on mate preferences to facilitate detection of novel properties (e.g., size) of available mates (a "chain of selection"; Schluter and Nagel 1995); mate preferences may then diverge between populations upon divergence in body size. Antagonistic interactions between species very different in size could also favor the evolution of behavioral responses that incidentally change

patterns of size-dependent mating. For example, female benthics raid nests of male limnetics, eating the eggs and destroying the structure (Ridgway and McPhail 1988; reviewed by Foster 1994). Such interactions could lead to aggressive defence of nests in male limnetics against large benthic females, and this would be manifested as a reduced probability of hybridization when size differences are large. This second mechanism is also a by-product in that reduced mating between forms is never directly favored. By-product speciation has been shown experimentally to be a feasible mechanism in lab studies of flies (Hurd and Eisenberg 1975; Kilias et al. 1980; Dodd 1989; Rice 1985; Rice and Salt 1988, 1990), but it is not yet clear whether mate preferences diverged purely as a correlated response to natural selection on other aspects of the phenotype or by direct selection on mate preferences.

The third mechanism is reproductive character displacement (Brown and Wilson 1956) or reinforcement (Dobzhansky 1951), whereby a low fitness of hybrids indirectly favors further divergence in mate preferences. Theoretical models of the process require hybrid fitness to be considerably lower than that of the parent species (e.g., Liou and Price 1994). F_1 hybrids between limnetics and benthics have reduced feeding success and growth rate in the wild (Hatfield 1995; Vamosi 1996), though the strength of the fitness effect remains unclear. Rundle and Schluter (1998) present evidence that size-based assortative mating is stronger in sympatry than allopatry, lending support to the reinforcement hypothesis.

Adaptive Radiation in Sticklebacks

The mating patterns described herein have implications for our understanding of adaptive radiation in threespine sticklebacks. Population divergence in sticklebacks is almost certainly driven by natural selection (Schluter 1993, 1994, 1995, 1996a; McPhail 1994), and opportunities for reproductive isolation to evolve via one or more of the three above mechanisms seem high. Marine threespine sticklebacks that invaded freshwater at the end of the Pleistocene responded quickly to the local selection regimes, and reproductive isolation quickly followed. These results may help explain a common feature of adaptive radiations in general: an association between phenotypic (ecological and morphological) diversification and elevated speciation rates (Huxley 1942; Simpson 1944, 1953).

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