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MATE CHOICE, SEXUAL IMPRINTING, AND SPECIATION: A TEST OF A ONE-ALLELE ISOLATING MECHANISM IN SYMPATRIC STICKLEBACKS

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Abstract.—One-allele isolating mechanisms should make the evolution of reproductive isolation between potentially hybridizing taxa easier than two-allele mechanisms, but the generality of one-allele mechanisms in nature has yet to be established. A potentially important one-allele mechanism is sexual imprinting, where the mate preferences of individuals are based on the phenotype of their parents. Here I test the possibility that sexual imprinting promotes reproductive isolation using sympatric species of threespine sticklebacks (*Gasterosteus aculeatus*). Sympatric species of sticklebacks consist of large benthic species and small limnetic species that are reproductively isolated and adapted to feeding in different environments. I fostered families of F_1 hybrids between the species to males of both species. Preferences of these fostered females for males of either type revealed little or no effect of sexual imprinting on assortative mating. However, F_1 females showed preferences for males that were similar to themselves in length, suggesting that size-assortative mating may be more important than sexual imprinting for promoting reproductive isolation between species pairs of threespine sticklebacks.

Key words.—Female preference, imprinting, reproductive isolation, size-assortative mating, threespine sticklebacks.

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A major question in speciation research is whether reproductive isolation between populations evolves via one- or two-allele mechanisms (Maynard Smith 1966; Felsenstein 1981; Kirkpatrick and Ravigné 2002; Servedio and Noor 2003). One-allele mechanisms involve the fixation of the same allele at the same locus in diverging populations that increases reproductive isolation as a byproduct. An example is an allele that increases habitat preference when the diverging species inhabit different environments. Two-allele mechanisms involve the fixation of two different alleles at the same or different loci in diverging populations; for example, alleles controlling different male traits and alleles controlling female preferences for different male traits. One-allele mechanisms make speciation theoretically easier because they do not require linkage disequilibria to form between alleles causing adaptation to different environments and alleles for different mating preferences. However, very few empirical studies have demonstrated the existence of one-allele mechanisms promoting the evolution of reproductive isolation in nature (Servedio and Noor 2003).

A type of one-allele mechanism that may be important in speciation is sexual imprinting (Laland 1994; Irwin and Price 1999). Sexual imprinting occurs when the mate preferences of individuals are formed during early development based on the appearance of their parents (Irwin and Price 1999; ten Cate and Vos 1999). This can cause reproductive isolation if an allele causing sexual imprinting (or strengthening it) spreads through two diverging populations (Laland 1994; Irwin and Price 1999; Servedio and Noor 2003). For example, if two populations meet in secondary contact after some period in allopatry, sexual imprinting may promote assortative mating if these populations differ in some trait important for mate choice such as song or body size. This assortative mating can then be strengthened further by reinforcement and other interactions in sympatry (Irwin and Price 1999). The preceding argument illustrates the point that sexual imprinting can only strengthen assortative mating based on traits that already differ between two populations, and these differences are themselves caused by other sources of divergent selection such as competition for shared resources or the fixation of different male display traits (Irwin and Price 1999; Kirkpatrick and Ravigné 2002; Servedio and Noor 2003).

Examples of sexual imprinting within populations are widespread in birds (ten Cate and Vos 1999 and references therein), and have been documented for mammals (Kendrick et al. 1998). However, its importance for promoting assortative mating between diverging populations is less well understood. Interspecific cross-fostering experiments have had mixed results in showing that species recognition is based on sexual imprinting (e.g., Kirchhof-Glazier 1979; Clayton 1990; Kendrick et al. 1998; ten Cate and Vos 1999; Slagsvold et al. 2002). In some cases, sexual imprinting is important for species discrimination, while in other cases the ability of species to distinguish con- from heterospecifics does not appear to be influenced by their rearing environment. This begs the question of how important sexual imprinting is for the evolution of reproductive isolation.

Here I test for the presence of sexual imprinting in species pairs of threespine sticklebacks (*Gasterosteus aculeatus* spp). Threespine sticklebacks are small fish that are found in marine and freshwater habitats around the northern hemisphere. Most lakes contain one type of stickleback, but species pairs co-exist in several lakes in British Columbia, Canada. These pairs consist of a large-bodied invertebrate-feeding benthic species, and a small-bodied zooplankton-feeding limnetic species (Schluter and McPhail 1992). Benthic and limnetic sticklebacks mate assortatively (Ridgway and McPhail 1984; Nagel and Schluter 1998), but the actual basis of assortative mating is still relatively uncertain. Although there is evidence that males display preferences for different sizes of females (Albert and Schluter 2004), and that females choose to mate assortatively by size (Hatfield and Schluter 1996, Nagel and

Schluter 1998; Rundle and Schluter 1998), it is unclear how these preferences are formed within individuals.

Sexual imprinting is plausible in threespine sticklebacks, because males provide parental care. Males build nests and entice female to lay eggs in them. The male then tends the eggs and newly hatched fry until they are able to swim freely (Whoriskey and FitzGerald 1994). During this period of parental care, females may imprint on the phenotype of their father, causing them to prefer the correct species as adults. Sexual imprinting in this case could have facilitated divergence by increasing the probability of assortative mating upon secondary contact.

To test for effects of sexual imprinting on female preferences, I cross-fostered F_1 hybrids of limnetics and benthics to males of both species. I used F_1 hybrids because there is no a priori expectation of what their preferences should be, and it allowed me to examine the effects of imprinting and genetics on female preferences. Another advantage of using F_1 hybrids is that filial imprinting (sexual preference based on the phenotype of siblings) is minimized as a factor influencing mate choice. F_1 females were raised with other F_1 fish and yet never experienced F_1 males during preference trials. The use of F_1 hybrids makes the assumption that the mechanism of imprinting is the same between benthics and limnetics, which is predicted for a one-allele mechanism. Although we have no direct evidence that the mechanism is the same in both species, there is no reason to expect that there has been divergent natural selection on learning via imprinting.

MATERIALS AND METHODS

Imprinting Design

Limnetics and benthics were collected from Paxton Lake on Texada Island between April and July 2003 both for foster fathers and as parents for the crosses. I made F_1 hybrid families in vitro from the gametes of these wild-caught individuals. All F_1 crosses were made between different benthic females and limnetic males (11 families total). This provides a control for any possible species-specific maternal effects, but does not allow a test for the presence of maternal effects. Males that were foster fathers for the crosses were placed singly in 102-L aquaria with a dish of sand and plants for nesting material. I stimulated males to build nests by showing them gravid females in jars for 15–30 min daily. Once males had built nests, they were considered ready to foster an F_1 clutch. Males fostered one clutch each, and were not reused.

In preliminary trials I found that males ate foreign eggs that were added to their nests. I therefore placed the nest of each male containing a family of F_1 eggs into a 250-ml jar covered with mesh to allow water flow. This jar was placed into the male's tank in the location where his nest had been. I added an aerator to the jar to ensure that the eggs were properly oxygenated. Six F_1 families were fostered with limnetic males and five F_1 families were fostered with benthic males. One day prior to hatching, males were put into 1000-ml jars with mesh tops and aerators to prevent them from eating the fry. Once the fry hatched, they were gently shaken from their jars into the male's tank. One month post-hatching, males were removed from the tanks entirely.

Although this setup did not allow for actual physical contact between foster fathers and the eggs and fry, it did allow for both chemical and visual signals to pass between them. Chemical imprinting may occur if females imprint on the odor of the kidney secretions that males use to build their nests, in addition to or instead of visual cues.

All fostered F_1 families were raised in the laboratory until reaching sexual maturity (approximately 10 months) and were fed brine shrimp (*Artemia* sp.) and chironimid larvae to satiation daily.

Testing F_1 Female Preference

I assessed the preferences of individual fostered females using two separate no-choice trials. In one trial, each female was added to an aquarium containing a nesting limnetic male. In the other trial, the female was added to an aquarium containing a nesting benthic male. Wild limnetic and benthic males from Paxton Lake were caught between March and June 2004 and placed singly in 102-L mating tanks. These tanks contained a dish of sand, two plastic plants for cover, and java moss (*Vesicularia dubyana*) for nesting material. I stimulated males to build nests as above. Once a male had built his nest he was used in up to four trials with different females. In total, 12 benthic males and 11 limnetic males were used in the trials.

F_1 females were tested opportunistically as they came into reproductive condition. I tested 17 females that had been raised with limnetic foster fathers and 19 females that had been raised with benthic foster fathers. The order of trials (limnetic or benthic male first) was alternated between females of each type to ensure that there were equal numbers starting with either type of male. I conducted 20 min no-choice trials following protocol used in previous studies (e.g., Hatfield and Schluter 1996; Rundle and Schluter 1998; Nagel and Schluter 1998; Albert and Schluter 2004). Courtship behaviors of both the male and the female were recorded for each trial by an observer sitting 1 m away. At the end of a trial, I measured the length and weight of the male and female. Females were allowed to rest in a covered container for at least 30 min between the two trials.

I used female nest examination (the penultimate behavior in the stickleback courtship sequence; Hatfield and Schluter 1996) as a measure of female preference. I used examination instead of actual spawning because very few (two) females spawned with males during the experiment, and nest examination has been used in previous experiments as a measure of female preference (e.g., Hatfield and Schluter 1996; McKinnon et al. 2004).

Analysis

I tested for an effect of imprinting on female preference by comparing 2×2 contingency tables counting the number of females of both types (limnetic-reared or benthic-reared) that examined the nest of one, both, or neither males. The contingency tables were compared via a χ^2 test of heterogeneity (Sokal and Rohlf 1995), which tests whether or not two or more tables have the same structure. The χ^2 test of heterogeneity would be significant if the two types of female had different probabilities of examining the nests of the different males. To determine whether the females of each type had an overriding preference for one type of male, I used Fisher exact tests to test for independence of the categories within each table.

As an additional test, I used logistic regression to determine whether there was an effect on nest examination by individual females of foster-father type, male type in the trial, the absolute value of the difference in body length between the male and the female in the trial, and their interactions. Because the two trials with each female were not independent, I only used the first trial for each female in this analysis. In addition, the length measurements were missing for some males, reducing the sample size for this test to 34. An effect of the imprinting treatment on female preference predicts a significant interaction between foster-father type and male type in the logistic regression analysis. I assessed the significance of each fixed effect by comparing the change in the deviance (ΔD) of the model caused by the addition of that factor with a chi-squared distribution (df $= 1$ for continuous variables; df $= k - 1$ for factors, with k number of levels of the factor) (Sokal and Rohlf 1995; Hardy and Field 1998). In addition, the tests of absolute value of size difference are one-tailed because there is an a priori expectation of size-assortative mating in sticklebacks (Nagel and Schluter 1998; McKinnon et al. 2004).

Since individual males were used multiple times (mean = 3), and multiple females were used from each family (max = 8, mean = 4), I conducted four additional tests for effects of male identity and family on the results. In the first two tests, I added male identity or family as fixed effects in logistic regressions. However, as both had nonsignificant effects on nest examination ($P > 0.05$), I did not include them further. In the next tests, I took account of the variance between individual males and families by including them as random factors in mixed-effects logistic regressions. The fit of the mixed-effects logistic regressions was assessed using the penalized quasi likelihood (glmmPQL) function in R (R Core Development Team 2004). Adding male identity and family as random effects changed the results slightly; thus, these analyses were included.

In addition to the tests on occurrences of examination, I looked for differences between the two male types in the amount of time before the first nest examination by females. For this test, I used data only from females who examined at least one of the nests in her two trials. The prediction, if there was an effect of the imprinting treatment, is that limnetic-reared females would examine limnetic nests sooner than benthic nests, and vice versa for benthic-reared females. I used paired samples Wilcoxon signed rank tests on the ln-transformed time to nest examination (in seconds) for each type of female separately. If the female never examined the male's nest, the time was set to 1200s, which was the length of the trial. The time was ln-transformed to achieve a symmetrical distribution of differences. All statistical analyses were carried out in the R language.

RESULTS

The χ^2 test of heterogeneity found no significant difference in examination frequency between the two types of females ($\chi^2_1 = 1.41$, $P > 0.1$), suggesting that they did not differ in their probability of examining the nests of the two types of males (Fig. 1). There was no significant difference in the proportion of females examining the limnetic male, the benthic male, both, or neither for either type of female (Fisher exact tests: limnetic-reared females $P = 0.10$, benthic-reared females $P = 0.65$). This suggests that there was no effect of the imprinting treatment on female preference. Because of the nonsignificance of the χ^2 test of heterogeneity, I pooled all females to see if there was an overriding preference for one type of male. Again, there was no significant difference in the proportion of females examining one, both, or neither type of male (Fisher exact test: $P = 0.16$), suggesting that F_1 females do not prefer one type of male to the other (Fig. 1). This is especially evident in the high proportion of females (25%) that examined the nests of both types of males.

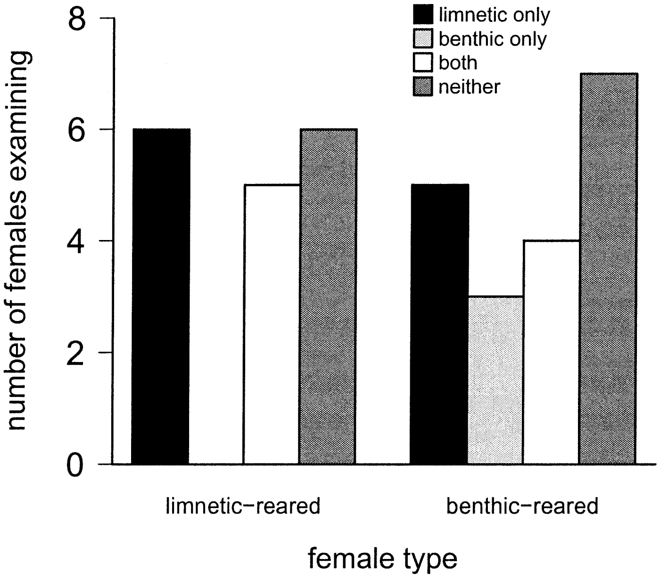

FIG. 1. The number of females of each type that examined the limnetic nest only, the benthic nest only, both types of nests, or neither nest in her two trials.

There was no significant interaction between male type and foster-father type in determining the probability of nest examination (logistic regression $\Delta D_1 = 0.97$, $P = 0.32$); thus, the interaction was removed from subsequent models. Neither male type ($\Delta D_1 = 3.38$, $P = 0.07$) nor foster-father type ($\Delta D_1 = 0.85$, $P = 0.36$) were significant. In contrast, the absolute value of the difference in length (mm) between the female and the male ($|\text{female length} - \text{male length}|$) was a significant predictor of whether the female examined the nest (one-tailed: $\Delta D_1 = 10.05$, $P = 0.01$). The probability of the female examining the nest decreased as the difference in body size increased (Fig. 2).

When male and family identities were included as random effects, the significance of the absolute value of the size difference was weakened (one-tailed: $P = 0.04$ with male id; $P = 0.08$ with family id). This makes sense because variation in body size between individual test males and between female families accounted for some of the effect of size in individual trials. However, the interaction between male type and foster-father type, and either of these factors singly, re-

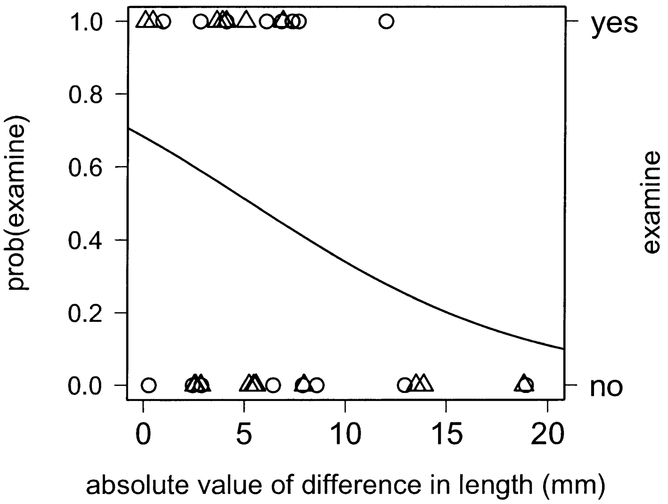

FIG. 2. The relationship between the absolute value of the difference in length between the male and female in a trial (|flength − mlength|), and whether she examined the nest. The female types are shown by different symbols (circles, limnetic-reared; triangles, benthic-reared). The line is the predicted probability of nest examination from a logistic regression of examination on the absolute value of length difference.

mained nonsignificant when either male identity (foster-father type $P = 0.34$, male type $P = 0.13$), or family identity (foster-father type $P = 0.42$, male type $P = 0.09$) was added to the model. This suggests that any imprinting effect that may exist is less important to assortative mating than body size differences.

Consistent with the results for occurrence of nest examination, there was no difference in the length of time to the first nest examination between benthic and limnetic males for either type of female (Wilcoxon signed rank tests, limnetic-reared females $P = 0.08$, benthic-reared females $P = 0.91$).

DISCUSSION

The results of this experiment suggest that there is little or no effect of sexual imprinting on the development of female preferences in the species pairs of threespine sticklebacks. Females that were raised by benthic males were not more likely to examine the nests of benthic males than females raised by limnetic males (and vice versa). This lack of an imprinting effect has important implications for the evolution of reproductive isolation between these species.

The primary conclusion is that the ability of females to distinguish con- from heterospecifics must be genetically controlled as opposed to learned. Comparisons can be made between the behavior of the F_1 females in this experiment, and females of the parental types in another experiment (Hatfield and Schluter 1996), to estimate the genetic basis of female preferences. Parental females displayed preferences for their own type of male, whereas the F_1 females in this experiment did not prefer one type of male to the other. Although the details of the genetic architecture are impossible to determine with this design, the lack of preference in the F_1 females suggests that there is little or no dominance of one type of preference. Admittedly, this experiment was not designed to extract quantitative genetic data about female preferences, and this remains an interesting avenue for further research.

The lack of evidence for sexual imprinting in this experiment does not necessarily rule out its existence. First, if there is a difference in the mechanism of imprinting in limnetics and benthics, an examination of cross-fostered individuals of the pure species would provide better evidence for or against a role of imprinting. However, it seems unlikely that the ability to imprint would be under divergent natural selection between the species pairs.

Second, the crossing design did not allow for an assessment of the role of maternal effects on imprinting. Although we found no evidence for sexual imprinting in one cross direction (benthic female by limnetic male), it is possible that the reciprocal cross would have exhibited imprinting. Previous experiments have failed to find any evidence for maternal effects on morphology, growth rate (McPhail 1992; Hatfield 1997), and male attractiveness to females (Hatfield and Schluter 1996), suggesting that the identity of the maternal species does not influence these traits.

Third, divergent female preferences that were originally shaped by imprinting may have become genetically fixed in sympatry (Irwin and Price 1999). One way to determine whether this is true is to look for evidence of sexual imprinting in allopatric populations of sticklebacks that have not been under selection for greater mate discrimination due to hybridization with a sympatric species.

Fourth, females were not allowed to choose between males simultaneously in this experiment, possibly masking their preferences (Wagner 1998). However, a pilot experiment allowing for female choice in a seminatural pond setting resulted in the same outcome as the no-choice setting. When given a choice, the fostered F_1 females did not prefer their foster-father type (six of 10 females made the ''correct'' choice), and they did not prefer one type of male to the other (six of 10 females mated with a benthic male).

Remarkably, the absolute value of the difference in length between the male and female was more important than the test male species and the female's foster father in determining female preference. This reconfirms the importance of size-assortative mating in the evolution of reproductive isolation between differentially adapted forms of sticklebacks (Nagel and Schluter 1998; McKinnon et al. 2004). The fact that F_1 hybrids between parental types still exhibit a tendency for size-assortative mating suggests that it is controlled by the same genetic mechanism in benthics and limnetics. Furthermore, McKinnon et al. (2004) documented size-assortative mating in marine and stream pairs of sticklebacks, suggesting that it may be an ancestral feature of stickleback mating behavior.

Ultimately, it seems clear that imprinting is not presently important for explaining assortative mating between benthic and limnetic sticklebacks, and that divergent selection on size and a mechanism for size-assortative mating are more important for the evolution of reproductive isolation.

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