Notes and Comments

Assortative Mating by Diet in a Phenotypically Unimodal but Ecologically Variable Population of Stickleback

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ABSTRACT: Speciation with gene flow may be driven by a combination of positive assortative mating and disruptive selection, particularly if selection and assortative mating act on the same trait, eliminating recombination between ecotype and mating type. Phenotypically unimodal populations of threespine stickleback (*Gasterosteus aculeatus*) are commonly subject to disruptive selection due to competition for alternate prey. Here we present evidence that stickleback also exhibit assortative mating by diet. Among-individual diet variation leads to variation in stable isotopes, which reflect prey use. We find a significant correlation between the isotopes of males and eggs within their nests. Because egg isotopes are derived from females, this correlation reflects assortative mating between males and females by diet. In concert with disruptive selection, this assortative mating should facilitate divergence. However, the stickleback population remains phenotypically unimodal, highlighting the fact that assortative mating and disruptive selection do not guarantee evolutionary divergence and speciation.

Keywords: assortative mating, stable isotopes, individual specialization, speciation, reproductive isolation.

Positive assortative mating takes place when individuals mate with individuals who are like themselves morphologically or behaviorally. Assortative mating has been recognized as an important evolutionary force, creating or maintaining linkage disequilibrium between loci or Hardy-Weinberg disequilibrium within loci. Theory suggests that under certain conditions, assortative mating can establish enough disequilibrium to drive speciation between potentially interbreeding populations (Maynard Smith 1966; Kirkpatrick and Ravigné 2002; Gavrilets 2004). In particular, any disequilibrium arising from ecologically driven disruptive selection may be amplified by assortative mating. Eventually this process may lead to morphologically divergent groups that, due to assortative mating, are reproductively isolated. Sympatric speciation is most likely when a single trait is both under disruptive selection and the basis of assortative mating (Udovic 1980; Dieckmann and Doebeli 1999; Fry 2003). Such a scenario increases the probability of speciation by eliminating recombination between the trait under divergent selection and the trait used in assortative mating (Felsenstein 1981).

Some skeptics of sympatric speciation have questioned whether traits under divergent selection are commonly the basis of assortative mating. Such traits have therefore been dubbed "magic traits" to highlight their uniquely favorable effect on speciation and possible rarity (Gavrilets 2004, 2005). However, there are some clear instances of ecological divergence directly causing assortative mating (Gavrilets 2004). Ecological divergence may lead to allochronic isolation, as in cases where plants grow on different soil types that favor different flowering times (Savolainen et al. 2006) or for insects whose host plants fruit or bud at different times (Feder and Filchak 1999; Groman and Pellmyr 2000). Ecological divergence may also contribute to reproductive isolation when habitat preferences lead to spatial segregation of mating pairs, as occurs when divergent host races of phytophagous insects mate on their host plants (Caillaud and Via 2000; Berlocher and Feder 2002). Adaptive morphological divergence can also serve as a basis of assortative mating. For example, size is an important ecological character in many species, and size differences also contribute to reproductive isolation in sympatric morphs of some fish species (McKinnon et al. 2004). While these examples provide some evidence for the applicability of magic trait models of assortative mating,

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many of these instances of assortative mating are drawn from already divergent host races or incipient species pairs with distinctly bimodal or multimodal trait distributions. In such cases, it is not clear whether the assortative mating preceded, accompanied, or followed the ecotypic divergence. For assortative mating to facilitate divergence, there must be some nonrandom mating within single populations before divergence. Therefore, it would be valuable to look for instances of assortative mating based directly on ecological parameters themselves in a population lacking morphological or behavioral clusters. The combination of disruptive selection and assortative mating on a single trait should quickly lead to divergence under some conditions. However, theoretical models suggest that these forces must be fairly strong to drive polymorphism or speciation; otherwise, they may simply act to maintain genetic variation in a phenotypically unimodal population (Fry 2003; Bolnick 2006; Bürger et al. 2006).

One place to look for assortative mating by a trait under disruptive selection is in lacustrine populations of the threespine stickleback (*Gasterosteus aculeatus*). Stickleback are best known for the few lakes where sympatric species pairs exhibit strong ecological and morphological differences and assortative mating (Schluter and McPhail 1992). However, most lake stickleback occur in morphologically unimodal "solitary" populations. In these populations, stickleback may use benthic and/or limnetic prey (Schluter and McPhail 1992), and individuals differ in their propensity to use these alternate resources (Svanbäck and Bolnick 2007; Araújo et al. 2008; L. K. Snowberg, unpublished data). As in the species-pair lakes, fish with larger gapes, deeper bodies, and fewer, shorter gill rakers are more efficient at using benthic prey, whereas the opposite is true for limnetic prey (Robinson 2000). These unimodal morphological traits are commonly subject to disruptive selection (Bolnick and Lau 2008), apparently due to intraspecific competition for alternate prey (Bolnick 2004*a*). This disruptive selection might indirectly promote assortative mating between individuals with similar morphology and resource use (Doebeli et al. 2007).

Assortative mating by diet may take place if stickleback use some cue to evaluate prospective mates or if assortative mating is a passive consequence of another preference, such as habitat choice. Recent laboratory experiments suggest that stickleback can directly assess the diet of other individuals (Ward et al. 2004). The precise mechanism for assessment is unknown, but it appears to be based on olfactory cues (Ward et al. 2004). Regardless of the mechanism, the stickleback's preference for conspecifics with similar diets might lead to assortative mating within an ecologically heterogeneous population, amplifying the effects of disruptive selection arising from competition for alternative prey (Bolnick 2004*a*). We therefore tested for diet-based assortative mating in a wild population of threespine stickleback, using stable isotopes as a measure of diet.

Stable isotope ratios of individuals reflect isotope signatures of their prey over the period that the tissue was synthesized (Hobson and Clark 1992*a*) and are therefore commonly used to infer diet (Tieszen et al. 1983; Newsome et al. 2007). Carbon and nitrogen isotopes provide complementary information on prey. Carbon isotope ratios differ between benthic and limnetic prey (France 1995). Nitrogen isotope ratios display a stepwise enrichment at each trophic level (Hobson and Clark 1992*b*). When individual stickleback vary in their propensity to consume benthic versus limnetic prey, their stable isotope ratios vary accordingly. We present evidence for assortative mating by diet, based on correlations between the isotopes of males and the eggs in their nests. Because egg isotopes are correlated with female isotopes, a correlation between males and the eggs in their nests implies a correlation between the isotope signatures of mated pairs.

Material and Methods

To test for assortative mating based on diet similarity, we collected male stickleback that were guarding nests and the eggs from within those nests. We conducted this study over a 5-day period during June 2007 in Mohun Lake, British Columbia (50°9′49″N, 125°29′17″W). Snorkelers identified nuptial males and their nests by observing male behavior. Males return to their nests regularly and fan the nest with a characteristic head-down position. We collected 41 males and the eggs from their nests using small aquarium nets. We also collected 19 gravid females from the same population using minnow traps. Fish and eggs were frozen in liquid nitrogen for later stomach content analysis, isotopic analysis, and measurement.

We measured the $\delta^{13}C$ and $\delta^{15}N$ isotope ratios of the muscle of males and females and the eggs collected from male nests and females' ovaries. The quantities $\delta^{13}C$ and $\delta^{15}N$ are used to represent the ratios of the uncommon heavy isotopes (^{13}C or ^{15}N) to the more common light isotopes (^{12}C or ^{14}N), adjusted to an international standard and reported in parts per thousand. We used the isotopes of eggs collected from a male's nest as a proxy for the isotopes of the female which he mated with. Egg isotopes have been shown to be correlated with the isotopes of the female fish (Gray 2001), and we confirmed this result by evaluating the correlation between females' isotopes and isotopes of eggs from their ovaries. We used 1–2 eggs from each nest for stable isotope analysis.

To evaluate whether isotope variance in the wild-caught fish exceeds expectations under a null hypothesis of a similar diet across individuals, we compared isotope variation

in our sample to isotope variation in lab-reared fish fed a shared diet. Lab-reared fish were F1 offspring of wild-caught parents. Eggs were hand-fertilized, and young were raised at 17°C on brine shrimp and then switched to freeze-dried bloodworms after reaching 1-cm standard length. We euthanized 22 9-month-old individuals from different families for isotope analysis.

Benthic and limnetic prey vary in both $\delta^{13}C$ and $\delta^{15}N$ ratios, with the result that benthic/limnetic diet variation in stickleback generates a correlation between these isotopes in fish tissues. We therefore performed a principal components analysis (PCA) on isotope ratios and used the first principal component (PC1) axis as a measure of benthic versus limnetic feeding history. We then tested for assortative mating by evaluating the correlation between isotope PC1 scores for males and the eggs from their nests. Significance of the correlation between male and egg isotopes was evaluated parametrically with Pearson's *r*. To evaluate how robust the parametric result was, we also ran a nonparametric permutation test in R (R Development Core Team 2007) in which we shuffled egg isotopes without replacement 10,000 times, and we determined the distribution of null values for the correlation coefficient and how often null values were more extreme than the observed one.

A correlation between the isotope ratios of males and the eggs in their nests would demonstrate a correlation between male and female isotope ratios (and hence assortative mating). In theory, if the correlation between eggs and females is p_{ef}, and the correlation between females and males is p_{fm}, then the correlation between eggs and males is $p_{em} = p_{ef} \times p_{fm}$. This assumes that the relationships between eggs and females and between females and males are both linear (Sokal and Rohlf 1994) and that male isotopes predict egg isotopes only through their correlation with female isotopes. The correlation between males and females can then be estimated as p_{em}/p_{ef}. An alternative to testing for correlations between principal component axes would be to carry out canonical correlation analysis (CCA). While CCA yields qualitatively similar results, the principal components axis correlations are more intuitive and permit us to estimate the underlying male-female correlation p_{fm} and apply an ANCOVA testing for sex-dependent isotope-morphology correlations.

To test for morphological correlates of isotope variation, we thawed and blotted dry each specimen and recorded mass (to 0.01 g), standard length, and open gape width (using digital calipers accurate to 0.01 mm). We also counted gill raker number under a dissecting microscope and measured the length of the longest gill raker using an ocular micrometer. We log transformed mass, standard length, gape width, and gill raker length. We used these log-transformed variables along with gill raker number to perform a PCA of morphology. We used a linear model to test for a relationship between morphology and isotopes. We used sex and morphological first and second principal components (PC1 and PC2) as independent variables (with sex × PC interactions) and isotope PC1 for fish muscle as the dependent variable.

To test for diet variation directly, we identified the stomach contents of each individual to the lowest feasible taxonomic level. We quantified the degree of among-individual niche variation in the population (E) and the degree to which niche variation reflects dietary clusters using the program DIETA1 (Araújo et al. 2008). Among-individual niche variation (E) ranges from 0 (no individual niche variation) to 1.0 (no overlap in diet between any pairs of individuals; Araújo et al. 2008). The clustering index (C) measures the degree to which the population is organized into discrete groups of individuals sharing a common prey niche and overlapping little with the prey niches of other groups, where a value of 0 represents no clustering, $C = 1$ indicates maximal clustering, and $C = -1$ indicates overdispersed diet variation (Araújo et al. 2008). Both indices were tested against a null hypothesis that individuals sampled randomly from a shared diet distribution, using a Monte Carlo resampling routine implemented in DIETA1.

Results

Morphology was distributed unimodally among the fish sampled. Despite the lack of discrete morphological groups, there was a high degree of among-individual variation in diet. On average, two randomly chosen individuals' stomach contents were 71% different ($E = 0.7101$; $P < .0001$ for the Monte Carlo test of the null hypothesis $E = 0$, that individuals sample randomly from a shared prey frequency distribution). This diet variation is quantitatively very similar to that observed in previous studies of stickleback (Araújo et al. 2008). However, diet variation was not organized into discrete clusters ($C = -0.0294$, $P = 1.0$). Diet variation was reflected in isotope variances: wild-caught fish had significantly more variable isotope signatures than a sample of laboratory-bred stickleback raised on a shared diet (lab-reared fish of unknown sex: $N = 22$, $\mathrm{Var}\,\delta^{13}C = 0.1772$, $\mathrm{Var}\,\delta^{15}N = 0.0520$; wild-caught males: $N = 41$, $\mathrm{Var}\,\delta^{13}C = 2.301$, $F = 12.98$, $\mathrm{df} = 40, 21$, $P < .001$, $\mathrm{Var}\,\delta^{15}N = 0.186$, $F = 3.58$, $\mathrm{df} = 40, 21$, $P = .0013$; wild-caught females: $N = 19$, $\mathrm{Var}\,\delta^{13}C = 3.412$, $F = 19.25$, $\mathrm{df} = 18, 21$, $P < .001$, $\mathrm{Var}\,\delta^{15}N = 0.201$, $F = 3.87$, $\mathrm{df} = 18, 21$, $P = .0019$).

The eggs from males' nests reflect the range of isotope signatures seen in eggs collected from females. There was some evidence of $\delta^{13}C$ and $\delta^{15}N$ depletion when we compared females' isotope ratios with the ratios in eggs from

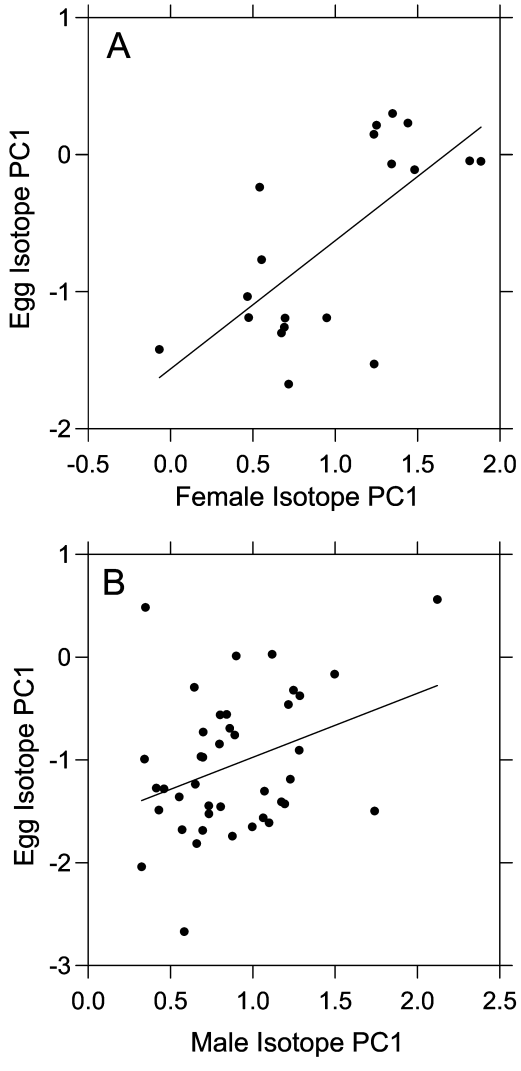

Figure 1: Correlation between the first principal component of isotope variation between females and their eggs (*A*) and males and the eggs collected from their nests (*B*).

their own ovaries. Compared with the isotope ratios in the female from which they were harvested, eggs showed a mean δ^{13}C depletion of 1.79% and a mean δ^{15}N depletion of 1.78% (paired *t*-tests; δ^{13}C: $t = 5.595$, df = 18, $P < .001$; δ^{15}N: $t = 21.627$, df = 18, $P < .001$). The isotopic variances did not differ between females and their eggs (δ^{13}C: $F = 1.761$, df = 18, 18, $P = .24$; δ^{15}N: $F = 0.657$, df = 18, 18, $P = .38$).

There was a correlation between δ^{13}C and δ^{15}N arising from associations between these signatures in benthic versus limnetic prey. This axis of isotope variation matches that observed between benthic and limnetic species pairs (B. Matthews, personal communication) and between fish in parapatric benthic and limnetic habitats (Bolnick et al. 2008). Therefore, we used the first principal component of isotope variation ($\lambda_1 = 1.19$, percent of total variance explained = 59.43%) to represent benthic versus limnetic feeding history. We did not directly test for correlations between stomach contents (e.g., percent benthic prey) and stable isotope signatures. Our stomach content data was in the form of counts rather than prey mass data, which is required to compare stomach contents with isotope signatures. Previous studies have shown that gut contents are correlated with muscle stable isotopes in solitary lacustrine stickleback populations (Bolnick et al. 2008).

Using the first principal component of isotope variation, we found that there is a significant correlation between isotopes of females and their eggs (fig. 1*A*; $r = 0.687$, $P = .001$). This confirms that eggs may be used as a proxy for females to test for assortative mating (male-female correlations). Consequently, the significant correlation between the isotopes of males and the eggs in their nests (fig. 1*B*; $r = 0.348$, $P = .012$ with the parametric test, and $P = .014$ with the permutation test) can be used to infer that there is a correlation between male and female isotope PC1. This implies that more benthic-feeding males tend to mate with benthic-feeding females (and limnetics with limnetics) more often than expected by chance. Using the observed correlation between male and egg isotopes and between female and egg isotopes, we estimate the male-female correlation to be 0.507.

Log-transformed mass, standard length, and gape width loaded on morphological PC1. Raker number and log-transformed raker length loaded on morphological PC2 (table 1). Both morphological PC1 and PC2 were correlated with isotope PC1 (table 2). Larger fish tended to exhibit a more benthic isotope signature. Fish with fewer and shorter gill rakers also tended to exhibit a more benthic isotope signature. Morphology × sex interaction terms represent a difference in slope but not a difference in trend in the relationship between morphology and isotopes in males and females (fig. A1, available in the online edition of the *American Naturalist*). The effect of morphological PC1 and morphological PC2 on isotope PC1 was stronger for females than males.

Table 1: Principal component analysis of fish morphology

Axis	λ_i	% Variance	SL	Mass	GW	GRL	GRN
PC1	2.669	53.39	.965	.954	.885	.196	.082
PC2	1.385	27.70	−.160	−.190	.127	.790	.826

Note: Principal component analysis results showing the eigenvalues (λ_i), the percentage of total variance explained, and the component loadings for morphology (SL = log-transformed standard length; mass = log-transformed body mass; GW = log-transformed gape width; GRL = log-transformed length of longest gill raker; and GRN = number of gill rakers).

Table 2: Linear model results

Source	SS	df	MS	*F*	*P*
Morphology PC1	2.314	1	2.314	16.501	<.001
Morphology PC2	2.224	1	2.224	15.861	<.001
Sex	1.597	1	1.597	11.389	.001
Morphology PC1 × Sex	1.108	1	1.108	7.9	.007
Morphology PC2 × Sex	.588	1	.588	4.19	.046
Error	7.573	54	.14		

Note: Summary of linear model results showing the source of variation, sum of squares (SS), degrees of freedom, mean square (MS), *F*-ratio, and significance value with isotope PC1 as the dependent variable. The PC1 × PC2 interaction and three-way interactions are not significant ($P > .9$) and for brevity are not included.

Discussion

Stickleback exhibit strong within-population diet variation, or individual specialization (Svanbäck and Bolnick 2007). Individuals vary in their propensity to consume benthic versus limnetic prey, even when held in small (10 m^2) enclosures that ensure that all individuals have access to the same set of prey (Araújo et al. 2008). Because benthic and limnetic prey differ in their stable isotope ratios, diet variation is reflected in isotopic variation among individuals. This isotope variation is consistently correlated with morphology within populations, confirming that among-individual diet differences persist for significant lengths of time. Isotope variance in wild-caught fish was an order of magnitude higher than what we observed when all individuals were reared on the same resource, reflecting prey variation among the wild-caught fish.

Experimental studies of shoaling behavior, which utilized diet manipulations, have shown that individual stickleback preferred to associate with conspecifics that fed on similar prey, suggesting that diet per se is involved in association behavior (Ward et al. 2004). We posited that shoaling preference might carry over to cause assortative mating by diet in ecologically heterogeneous populations of stickleback. Such assortative mating could be detected as a correlation between the isotope signatures of males and females. Using egg isotopes as a proxy for females, we have demonstrated that such a correlation exists, and we may thus conclude that stickleback in Mohun Lake do exhibit some assortative mating. This population is phenotypically unimodal and diet variation was not in discrete clusters, so this assortative mating occurs within a single population rather than representing reproductive isolation between divergent "morphs."

Mate choice and assortative mating have been studied extensively in stickleback, especially in populations characterized by separate benthic and limnetic groups. Assortative mating between these groups has been demonstrated to be based on size (Nagel and Schluter 1998; Vines and Schluter 2006) and nuptial color (McKinnon 1995; Boughman 2001). However, our results present the first evidence of assortative mating within a phenotypically unimodal population of stickleback. Assortative mating by diet in unimodal populations represents the first example of a potential "magic trait" in stickleback, although more work is necessary to determine whether disruptive selection and assortative mating truly act on the same trait or whether they act on traits that are correlated.

While we found a correlation between male and female isotopes, there are a number of mechanisms that might drive the underlying assortative mating. It is possible that individuals select mates directly based on olfactory cues associated with recently consumed prey, as suggested by studies of shoaling in laboratory aquaria (Ward et al. 2004). Alternatively, stickleback could be selecting mates based on morphological traits that are correlated with diet. We found that isotope PC1 was correlated with size (standard length, mass, and gape width) and gill raker traits (length and number). Assortative mating could be based on size, as is commonly found in fish with different morphs (Foote and Larkin 1988; Nagel and Schluter 1998; McKinnon et al. 2004). Gill raker traits, being internal, are unlikely to be direct targets of mate choice.

Finally, it is possible that the isotope correlations arose from spatial heterogeneity. This spatial effect may be of two types. First, baseline isotope signatures may vary spatially. If stickleback exhibit strong philopatry, there may be spatial gradients in isotope signatures in both males and females, leading to the appearance of assortative mating. However, all our nests were collected along approximately 250 m of homogeneous shoreline. Mark-recapture studies show that stickleback can move that distance within a few days (D. I. Bolnick, unpublished data), so isotopes are unlikely to vary dramatically over such a small distance. Second, spatial effects may arise if individuals that feed on different prey tend to select different microhabitats for mating. In species pairs lakes, benthic and limnetic stickleback differ in their nest location and characteristics (McPhail 1994). Benthic-like and limnetic-like populations from allopatric solitary lakes also differ in their nest location (Vines and Schluter 2006), but differences within solitary populations have not been shown. Hence, nest site selection might be an effective basis for assortative mating that could generate correlations between isotope signatures.

Regardless of whether mate choice is based on diet itself or on morphology or microhabitat that is correlated with diet, the ultimate outcome is assortative mating with respect to diet. Assortative mating based on a trait directly under disruptive selection is the most favorable situation for speciation in the presence of gene flow (Maynard Smith 1966; Felsenstein 1981; Dieckmann and Doebeli 1999; Kirkpatrick and Ravigné 2002; Fry 2003). Disruptive se-

lection is common in solitary lacustrine populations of stickleback (Bolnick and Lau 2008), acting on gill raker traits that are correlated with isotopes in our study. This should be a favorable situation for disruptive selection and assortative mating to lead to speciation. However, both Mohun Lake stickleback and all the populations in surrounding lakes remain phenotypically unimodal and are in Hardy-Weinberg equilibrium (Caldera and Bolnick 2008). This result leaves us with an interesting puzzle: models suggest that sympatric speciation is easiest when disruptive selection and assortative mating act in concert, yet we find no indication of sympatric divergence in stickleback despite the joint action of these processes. We propose that the strength and/or temporal consistency of assortative mating and disruptive selection in these populations may be insufficient for speciation to proceed. Several models have shown that the conditions for sympatric speciation are sensitive to specific parameter values (Bolnick 2004*b*; Bürger et al. 2006), and our results suggest that empirical estimates of these key parameters may be an important step toward understanding when and why sympatric speciation may or may not occur (Bolnick and Fitzpatrick 2007).

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