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Trophic polymorphism in a riverine fish: morphological, dietary, and genetic analysis of mountain whitefish

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Trophic polymorphisms are a prominent form of phenotypic diversification in many animal taxa. Northern temperate lakes have become model systems for the investigation of sympatric speciation due to trophic polymorphisms. Many examples of niche-based phenotypic variation occur in temperate lakes, whereas northern rivers offer few such examples. To further investigate the conditions under which trophic polymorphisms are likely to evolve, the present study examined phenotypic variation related to snout size and shape in the mountain whitefish (Salmonidae: *Prosopium williamsoni*), which has been hypothesized to exhibit a rare example of reproductively isolated trophic morphs in a northern river-dwelling fish species. Variation in snout size and shape increased greatly with body size and, although this variation was continuously distributed, individuals in the largest size class tended to lie at phenotypic extremes. At one extreme were individuals with a large bulbous snout and a sloping forehead ('pinocchio'), and at the other were individuals that lack the bulbous snout and have a concave forehead ('normal'). The pinocchio trait may result from a stage-specific developmental switch that occurs late in ontogeny. Consistent differences were found with respect to diet between individuals with extreme snout morphologies, but no evidence was found for assortative mating within populations at seven microsatellite loci. The explosive mating system of this species may be responsible for this lack of assortative mating. The present study highlights the influence of ecological factors in shaping phenotypic and behavioural diversification due to trophic morphology. *Biological Journal of the Linnean Society*, 2007, **92**, 253–267.

ADDITIONAL KEYWORDS: *Coregonus* – *Gasterosteus* – microsatellite – morphology – *Prosopium* – resource polymorphism – speciation – stage-specific developmental switch – sympatry.

INTRODUCTION

Trophic polymorphisms, namely intraspecific niche-based variation in feeding structures, are hypothesized to reduce intraspecific competition (McLaughlin, Ferguson & Noakes, 1999; Swanson *et al.*, 2003) and play a role in speciation (Wimberger, 1994; Skúlason, Snorrason & Jonsson, 1999; Robinson & Schluter, 2000). Phenotypic variation for trophic, or resource acquisition, structures is often extensive in nature (Robinson & Schluter, 2000) and alternate morphs often have accompanying differences in growth rate, age at maturity, and mating strategies (Skúlason & Smith, 1995). Trophic polymorphisms may be highly genetically influenced or may be the outcome of adaptive phenotypic plasticity (Robinson & Wilson, 1994, 1996; Smith & Girman, 2000). Trophic polymorphisms occur in all classes of vertebrates and may be more common than historically appreciated (Wimberger, 1994; Skúlason & Smith, 1995; Smith & Skúlason, 1996).

Several studies have addressed models for the translation of intraspecific trophic polymorphisms into variation that occurs among species (West-Eberhard, 1986; Wimberger, 1994; Skúlason *et al.*, 1999; Adams & Huntingford, 2004). These models hypothesize that subtle behavioural and/or morphological variation within populations can become increasingly specialized and, under the right conditions, can lead to reproductive isolation and the fixation of alternate traits between species (West-Eberhard, 1986; Wimberger, 1994; Skúlason *et al.*,

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1999). A key factor facilitating divergence in these models is the stability and location of feeding habitats. If pronounced and persistent ecological differences occur among feeding habitats, subsequent behavioural and morphological specialization to these habitats is more likely (Wimberger, 1994; Skúlason *et al.*, 1999). Reproductive isolation may occur as a simple byproduct of ecological specialization if males and females mate within these distinct feeding habitats (Wimberger, 1994; Skúlason *et al.*, 1999; Smith & Girman, 2000). Otherwise, reproductive isolation may also result from positive assortative mating, if animals prefer to mate with individuals of their respective morphological type. In this latter situation, reproductive isolation can arise even in the absence of temporal or spatial segregation.

Fishes in general offer extraordinary examples of trophic polymorphisms. These range from cichlids in the African Rift Lakes (Danley & Kocher, 2001; Bouton, Witte & Van Alphen, 2002; Stauffer & Van Snik Gray, 2004) and lakes in Nicaragua (Meyer, 1987; Wilson, Noack-Kunnmann & Meyer, 2000; Klingenberg, Barluenga & Meyer, 2003) to salmonids, centrarchids, and sticklebacks (*Gasterosteus aculeatus*) in northern temperate postglacial lakes (Robinson & Wilson, 1994; Bernatchez *et al.*, 1996; Schluter, 1996; Skúlason *et al.*, 1999; Robinson & Schluter, 2000). Species-poor northern temperate lakes have become model systems for examining this type of phenotypic variation (Robinson & Schluter, 2000). In these lakes, shallow littoral margins and deeper open waters offer stable and spatially separated habitats in which the whole continuum of divergent biological units, from within-species variation represented by slightly different phenotypes to distinct species, can be found (Robinson & Schluter, 2000). Trophic polymorphisms within these relatively simple and spatially structured environments have improved our general understanding of the ecological causes of phenotypic diversification and adaptive radiation (Robinson & Schluter, 2000; Robinson & Parsons, 2002).

By contrast to lakes, northern temperate rivers offer very few examples of trophic polymorphisms (but see also Kondrashov & Mina, 1986; Osinov, Il'in & Alekseyev, 1990; McLaughlin & Grant, 1994). The paucity of examples of trophic polymorphisms in temperate riverine fishes may be due to the less spatially structured nature of this environment. Adopting alternative foraging tactics may not be an effective means to reduce intraspecific competition in the less structured riverine environment (but, for an example in a spring-fed pool system, see Swanson *et al.*, 2003). It may also be less likely for prezygotic reproductive isolation to arise among specialized trophic morphs, when they occur, if they are not spatially separated by feeding location (i.e. active assortative mating preferences may be required). If trophic variation does occur in riverine fishes, we might generally predict that it will be maintained within populations instead of being spatially partitioned into relatively isolated morphs, as is often observed in temperate lakes.

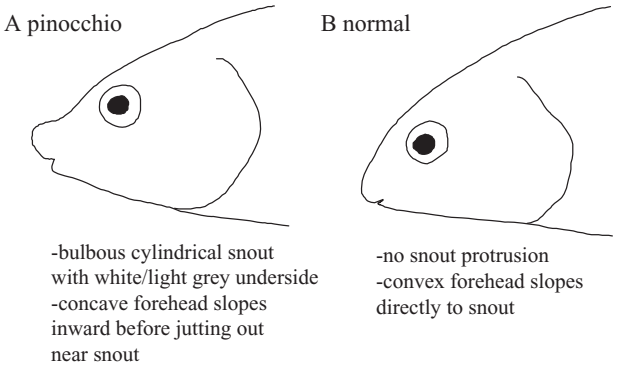

Figure 1. Examples of phenotypically extreme fluvial mountain whitefish. A, pinocchio; B, normal and phenotypic characteristics of extreme individuals.

The mountain whitefish (*Prosopium williamsoni* Girard) appears to exhibit an unusual example of a trophic polymorphism in a northern riverine fish (Taylor, 1999) and, thus, this species provides an opportunity to examine ecological factors that influence the evolution of this type of phenotypic diversification. This species occurs primarily in rivers in western North America (Northcote & Ennis, 1994) and some individuals of this species have an elongated cylindrical snout ('pinocchio'; Fig. 1A), whereas others of similar size do not ('normal'; Fig. 1B), and the variation can be extreme. This trait was originally hypothesized to result from a sexual dimorphism, with elongated snouts present only in males (Evermann, 1893). However, Troffe (2000) and McPhail & Troffe (2001) showed that both males and females have elongated pinocchio snouts. These authors suggested instead that pinocchio mountain whitefish of both sexes use their exaggerated snouts to overturn rocks to feed on benthic invertebrates. Troffe (2000) provided preliminary evidence for differences in foraging behaviour and for reproductive isolation between what he classified as discrete feeding/trophic morphs.

This species is a prime candidate for trophic specialization because populations occur at high densities (Whiteley, Spruell & Allendorf, 2004), which could lead to strong selection for traits that reduce intraspecific competition (i.e. those related to trophic specialization). In addition, other members of this subfamily of Salmonidae (Coregoninae) offer examples of trophic polymorphisms (Lindsey, 1981; Bernatchez, Chouinard & Lu, 1999). However, aspects of mountain

Table 1. Sample locations for mountain whitefish in the Clark Fork River Basin, Montana

Sample location	Date	*N*	Analysis
West Fork Bitterroot River	October 2000	40	G
Bitterroot River, Stevensville, MT	July 2003	117	D, M
Bitterroot River, Stevensville, MT	March 2004	105	D, G, M
Rattlesnake Creek, Missoula, MT	November 2002	46	M
Rattlesnake Creek, Missoula, MT	November 2003	89	M

D, diet; G, genetic; M, morphology.

whitefish mating behaviour (most importantly broadcast spawning in large groups, where multiple males shed sperm on the eggs of each female; Stalnaker, Gresswell & Siefert, 1974; Northcote & Ennis, 1994) suggest that assortative mating by morphotype should be unlikely, if not impossible. Riverine populations of the mountain whitefish thus not only provide a valuable contrast to the better studied trophic polymorphisms of more highly structured northern temperate lakes, but also they may illustrate the critical interplay that must occur between feeding behaviour and reproductive behaviour if morphological variation for trophic behaviours is to lead to the evolution of reproductive isolation and the formation of new species.

The present study aimed to: (1) provide a comprehensive investigation of the distribution of phenotypic variation in snout morphology of the mountain whitefish within natural riverine populations; (2) to test whether this variation is associated with differences in diet (trophic polymorphism); and (3) to test whether phenotypically extreme individuals in this population show genetic signatures of reproductive isolation. Specifically, the following questions were addressed: (1) is there discontinuous variation in snout morphology within populations of mountain whitefish; (2) does diet vary with snout morphology; and (3) is there evidence of genetic subdivision, and thus indirect evidence for assortative mating associated with snout morphology?

MATERIAL AND METHODS

Sample collection

Mountain whitefish were collected from Rattlesnake Creek ($N = 135$) in Missoula, Montana and the Bitterroot River, either from the main-stem near Stevensville, Montana ($N = 222$), or from the West Fork ($N = 41$; Table 1). All sites occur within the Clark Fork River, a tributary of the Columbia River. Fish were collected with a backpack electrofisher or with a boat electrofisher. Specific subsets of these animals where used for morphological, diet, and genetic analyses, as described below.

Morphology

Digital images were captured for all individuals from Rattlesnake Creek and Bitterroot River populations the day of collection with a digital camera mounted on a tripod. The West Fork sample was collected as part of a previous study and used only for genetic analyses (see below). Standard length was used as a measure of overall body size and the sex of all individuals was determined by inspection of internal organs.

Troffe (2000) and McPhail & Troffe (2001) describe variation in snout size and shape in mountain whitefish as being discrete, with two forms (pinocchio and normal). At one extreme of phenotypic variation, individuals have a large bulbous cylindrical snout and an inward curve to the forehead region (pinocchio; Fig. 1A). At the other phenotypic extreme, individuals have a small snout and convex forehead region immediately adjacent to the snout (normal; Fig. 1B). However, in the present study, many individuals with intermediate characteristics could not be readily classified into these two discrete groups. These intermediate individuals tended to have a slightly concave forehead and a slightly cylindrical snout that extended out from where the ventral portion of the snout met the upper maxilla but the snout was not excessively large, bulbous, or cylindrical. Due to the presence of a large number of phenotypically intermediate individuals, variation in snout size and shape was treated as a continuous trait in the present study.

A method to quantify phenotypic variation in snout size and shape (hereafter referred to as the 'snout index') was developed that measured the area of the snout and part of the forehead region. The steps used for this method of quantification were (Fig. 2A): first, landmarks were placed at the tip of the snout and where the operculum meets the ventral lateral margin. Second, these landmark points were connected with a straight line (L1). Third, a line was

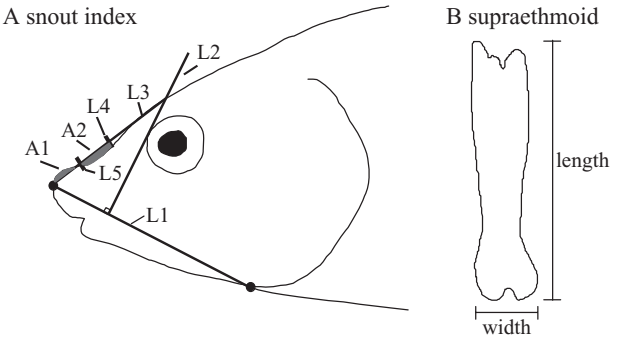

Figure 2. Measurements of snout index (A) and supraethmoid length and width (B). Details of measurements are provided in the text.

drawn perpendicular to L1 and tangential to the anterior orbit of the eye (L2). Fourth, a straight line (L3) was drawn from the landmark at the tip of the snout to the bisection of L2 and the dorsal lateral margin. Fifth, L3 was bisected with a line (L4) drawn perpendicular to L3 and extending to the dorsal surface of the forehead. Finally, the anterior half of L3 was bisected with line L5 drawn perpendicular to L3. Areas A1 and A2 were then measured (Fig. 2A). A1 was the area dorsal to L3 and between the landmark at the tip of the snout and L5 (between L3 and the lateral margin of the fish). A2 was the area dorsal or ventral to L3 and between L5 and L4. Areas dorsal to L3 were positive and those ventral to L3 were negative. The final snout index value was obtained by subtracting A2 from A1. Values tended to be positive for pinocchios and negative for normals. For the example of this method shown in Figure 2A, A1 is dorsal to L3. A2 in Figure 2A lies ventral to L3 and thus would be negative. Subtracting this value from A1 leads to a large positive snout index value. At the other phenotypic extreme, A1 and A2 tended to be positive, leading to small and possibly negative snout index values. All steps for this method were performed with Image J, version 1.23 (http://rsb.info.nih.gov/ij).

Nonlinearity in the relationship between snout index and standard length was tested for using a partial *F*-test (Eberhard & Gutiérrez, 1991). Tests of nonlinearity were conducted for all individuals combined and separately for males and females. The model used was: $Y^* = \alpha_0 + \alpha_1 X^* + a_2 X^{*2} + \varepsilon$, where Y^* is the natural log of body size (standard length, mm); X^* is the natural log of the snout index (mm^2), where 10 was added to each value to make all values positive; α_i is the regression coefficients; and ε is the error with assumed normal distribution, mean zero, and common variance (Eberhard & Gutiérrez, 1991). A significant difference of a_2 from zero would indicate that the relationship between the snout index and body size was significantly nonlinear.

An analysis of covariance (ANCOVA) was used to test for differences in snout index between males and females. Snout index was the dependent variable, natural log-transformed standard length, and standard length^2 were covariates, and sex was the main effect.

To investigate the relationship between external snout morphology and underlying bone structure, the supraethmoid bone of all individuals was measured (Fig. 2B). The supraethmoid lies at the tip of the snout and provides attachment points for cartilage and other tissues within the snout. It was predicted that this bone would be larger in pinocchios relative to nonpinocchios because: (1) it appeared to be larger in X-rays of pinocchio individuals relative to normals (data not shown) and (2) the base width of the supraethmoid is a diagnostic character used to distinguish the sharp-snouted and blunt-snouted morphotypes of another salmonid, *Brachymystax lenok* (Kondrashov & Mina, 1986; Alekseyev, 1995; Alekseyev, Kirillov & Samusenok, 2003). The external morphology of these morphotypes is very similar to that of phenotypically extreme mountain whitefish (Kondrashov & Mina, 1986; Alekseyev, 1995; Alekseyev *et al.*, 2003). Thus, it was predicted that the supraethmoid would be wider and potentially longer in fish with larger snouts. Supraethmoids were dissected from frozen fish and prepared and cleaned using trypsin according to Mayden & Wiley (1984). Image J, version 1.23 was used to measure the length of the supraethmoid as well as its width at its base (Fig. 2B).

A correlation between snout index and supraethmoid base width and supraethmoid length was tested for. To adjust for body size, residuals were used from regressions of each trait on standard length, where variables were natural log-transformed. For snout index, a third order polynomial regression was used because the cubed standard length term was highly significant ($P = 0.001$). For the regression of supraethmoid base width on standard length, natural log-transformed values were used for both variables and a second-order polynomial regression ($P < 0.0001$ for the squared standard length term). For the regression of supraethmoid length on standard length, a second-order polynomial regression was used ($P = 0.04$ for the squared standard length term).

The following equation was used to measure the repeatability (r) of measurements of snout index, supraethmoid length, supraethmoid base width, fin lengths, and standard length: $r = \frac{s_A^2}{s^2 + s_A^2}$, where s^2 is the within-group variance component, $s_A^2 = \frac{MS_{among} - MS_{within}}{n_0}$, and n_0 is the group size

(Lessels & Boag, 1987). For each trait, one individual measured ten individual mountain whitefish three times, blindly, and in a randomized order. All morphological measurements were highly repeatable. The *r*-value was 0.97 for the snout index, 1.0 for supraethmoid length, 1.0 for supraethmoid base width, and 0.99 for standard length.

Diet analysis

Stomach and intestine contents were examined to test for diet differences in relation to snout morphology in the Bitterroot 2003 and 2004 samples. These two samples were each collected from the same location (within approximately 50 m) and, for each sample, all fish were collected at the same time from an electrofishing boat. Fish were kept on ice and stomachs and intestines were dissected as soon as possible after capture and stored in 70% ethanol until analysis. Prey items found in the stomach versus the intestine were not distinguished and for the remainder of this paper stomachs refer to the whole digestive tract.

For the 2003 sample, the diet data were analysed in two ways. First, the entire sample ($N = 117$) was split into three fish size classes: S1, < 180 mm; S2, between 180 mm and 230 mm; and S3, > 230 mm. Second, the fish were divided from the largest size class (S3) into two groups of phenotypically extreme individuals (termed pinocchio and normal for convenience) based on snout index values. Standard lengths of pinocchio and normal groups did not differ (mean standard length ± SD of pinocchio: $N = 14$, 278.6 ± 21.5 mm; normal: $N = 14$, 274.6 ± 15.8 mm; $t_{26} = -0.56$, $P = 0.58$), but these groups varied significantly in snout index values (mean ± SD of pinocchio: 3.02 ± 2.22 mm^2; normal: −1.86 ± 1.14 mm^2; $t_{26} = -7.3$, $P < 0.0001$).

For the 2004 mountain whitefish sample, a pinocchio and a normal group were again formed with 15 individuals of each type with standard length > 230 mm. Mean standard lengths of the pinocchio and normal groups did not differ (mean standard length ± SD of pinocchio: 276.7 ± 17.7 mm; normal: 269.4 ± 12.3 mm; $t_{28} = -1.3$, $P = 0.20$) but, as was the case for the 2003 sample, the groups varied significantly in snout index values (mean ± SD of pinocchio: 3.85 ± 2.66 mm^2; normal: −1.48 ± 1.05 mm^2; $t_{28} = -8.9$, $P < 0.0001$).

Insects in gut samples were sorted to order or family under a dissecting microscope. The total number of each insect taxon per stomach was counted using one reliable body part per taxon (e.g. the head capsule was used for chironomid larvae). Prey item counts were used to calculate the proportion of each food item relative to the total number of food items found in each individual's stomach (proportional contribution by number).

Average wet weights of the relevant insect taxa were estimated to determine the proportional contribution of different insect taxa to mountain whitefish diets by weight. Whole insects were collected from the same location in the Bitterroot River and at the same times as the fish used for diet analysis. These insects were collected separately in July 2003 and March 2004 and were stored in 70% ethanol until analysis. Wet weights of five to ten individuals per taxon were used to determine the average proportion by weight for each taxon category within each fish's stomach. To compare total stomach volumes between the 2003 and 2004 Bitterroot River samples, a two-way analysis of variance (ANOVA) was performed using the total weight of the food items in an individual fish's stomach as the dependent variable and sample (2003 or 2004) and phenotype (pinocchio or normal) as the two factors.

The diet data generally appeared to violate assumptions of normality and equality of variance, even after log or arcsine transformation (of proportions). A nonparametric Kruskal–Wallis test was used for the analysis of the three whitefish size classes from the entire 2003 sample along with a procedure that parallels Tukey's test for post-hoc pairwise comparisons following Zar (1984). Mann–Whitney tests were used to test for differences in mean prey proportions (by number or weight) of phenotypically extreme groups. Bonferroni corrected *P*-values for these Mann–Whitney tests would be 0.007 for $\alpha = 0.05$ and 0.014 for $\alpha = 0.10$; however, uncorrected *P*-values are reported because Bonferroni corrections tend to be overly conservative (Nakagawa, 2004).

Genetic analyses

Microsatellite genotypes from seven loci were used to test for genetic subdivision and thus indirectly for assortative mating associated with snout morphology. As with the diet analysis, phenotypically extreme groups were formed. For the Bitterroot 2004 sample, snout index was used to sort individuals into a pinocchio ($N = 20$) and a normal ($N = 20$) group. In this case, pinocchio and normal groups differed in snout index (mean snout index ± SD for pinocchio: 2.85 ± 2.80 mm^2; normal: −1.44 ± 1.00 mm^2; $t_{38} = -6.5$, $P < 0.0001$) and mean standard length (mean standard length ± SD for pinocchio: 271.7 ± 21.0 mm; normal: 222.4 ± 28.0 mm; $t_{38} = -6.3$, $P < 0.0001$).

To replicate genetic analyses, data were used from a sample used in a previous study (Whiteley *et al.*, 2004) and collected in the West Fork of the Bitterroot River in 2000 (Table 1). Conservatively, this sample was not included in the overall morphometric analy-

sis because these individuals were from a different location and photos were taken on specimens after they had been frozen, which appeared to influence snout morphology. A pinocchio ($N = 10$) and a normal group ($N = 10$) were determined by snout index values, although all 41 individuals from this sample were used for some genetic tests (see below). Pinocchio and normal groups differed in snout index (mean snout index ± SD for pinocchio: 2.66 ± 1.38 mm^2; normal: –1.73 ± 0.74 mm^2; $t_{28} = -8.9$, $P < 0.0001$) and standard length (mean standard length ± SD for pinocchio: 285.1 ± 23.8 mm; normal: 247.1 ± 27.3 mm; $t_{28} = -3.3$, $P = 0.004$).

Genotypic data were collected from the following seven microsatellite loci: *COCL4*, *SSA14*, *SSA456*, *ONE8*, *FGT25*, *SFO8-1*, and *SFO8-2* (Whiteley *et al.*, 2004). DNA was extracted from fin clips or liver tissue by standard methods. Polymerase chain reaction (PCR) reagent concentrations and thermal cycler profiles were in accordance with Whiteley *et al.* (2004). The general methods used for visualization of subsequent PCR products followed Spruell *et al.* (1999) and Neraas & Spruell (2001).

Allele frequencies, mean heterozygosities, and mean number of alleles were calculated separately for the pinocchios and normal groups with FSTAT, version 2.9.2.3 (Goudet, 1995; Goudet, 2001). To test for a deficit of heterozygotes (Wahlund effect), for deviations from Hardy–Weinberg proportions were tested for using a one-tailed test with GENEPOP, version 3.4 (Raymond & Rousset, 1995) where the two phenotypic groups were combined. For the West Fork Bitterroot sample, all 41 individuals were used for the test for Hardy–Weinberg proportions. To test for differences in allele frequency distributions between groups of phenotypically extreme individuals, a pseudo-exact test was performed for genic differentiation (Goudet *et al.*, 1996) with GENEPOP, version 3.4. For both tests, Fisher's method was used to combine probabilities following Sokal & Rohlf (1995).

Principal components analysis (PCA) was used to examine the relationship among multilocus genotypes without prior assignment of individuals to phenotypic groups. Pairwise individual-by-individual genetic distances were calculated using the method of Peakall, Smouse & Huff (1995) and with the program GenAlEx, version 6 (Peakall & Smouse, 2006). To statistically test for an effect of snout morphology or size on genetic patterns, correlation analyses were performed with PC scores for each individual from the first two PCs and with snout index and standard length. In addition, an ANCOVA was performed with PC scores (from axis one or two) as the dependent variable, phenotype (pinocchio or normal) as the main effect, and natural log of standard length as the covariate.

RESULTS

Morphology

Little variation was observed in snout index values among individuals below a standard length of approximately 220 mm (Fig. 3). Beyond this standard length, variation in snout morphology increased dramatically and became increasingly bimodal in the largest size classes (Fig. 3). Overall, the relationship between the snout index and standard length was significantly nonlinear. The coefficient α_2 from the equation $Y^* = \alpha_0 + \alpha_1 X^* + \alpha_2 X^{*2} + \varepsilon$ was highly significant ($P < 0.0001$) for all data combined. For males and females analysed separately, the α_2 coefficient was also highly significant ($P < 0.0001$ and $P = 0.001$, respectively). For the ANCOVA used to test for differences in snout size between males and females, the interaction terms between sex and standard length were not significant (standard length, $P = 0.36$; standard length^2, $P = 0.37$). The main effect of sex was not significant ($P = 0.36$). Both covariates were highly significant (ln standard length, $P < 0.0001$; ln standard length^2, $P < 0.0001$).

Length and width of the supraethmoid bone was not significantly correlated with snout index. The correlation between residuals for supraethmoid base width and snout index was positive but not significant ($r = 0.041$, $P = 0.44$). For supraethmoid length, the correlation with snout index residuals was negative but not significant ($r = -0.053$, $P = 0.32$). The results remained nonsignificant if males and females were analysed separately (data not shown).

Diet analysis

For the entire Bitterroot 2003 sample, significant differences were found among age classes in diet (Fig. 4A). For mean proportion of diet by number, there was significant variation among size classes for Chironomidae larvae ($P < 0.0001$), Trichoptera larvae ($P = 0.0003$), Simuliidae larvae ($P = 0.032$), and large Ephemeroptera nymphs ($P = 0.05$; Fig. 3). Significantly more large Ephemeroptera nymphs and Trichoptera larvae occurred in stomachs of the fish in the largest size class (S3). Smaller size classes had significantly greater average proportions (by number) of Chironomidae larvae. Proportion of diet by weight showed similar patterns as proportion by number (data not shown).

For the subset of the S3 size class divided into phenotypically extreme groups (pinocchio and normal) for the Bitterroot 2003 sample, the average proportion by number of large Ephemeroptera nymphs was 0.17 for the pinocchio group and 0.029 for the normal group ($P = 0.014$; Fig. 4B). The average proportion by weight of this prey item was

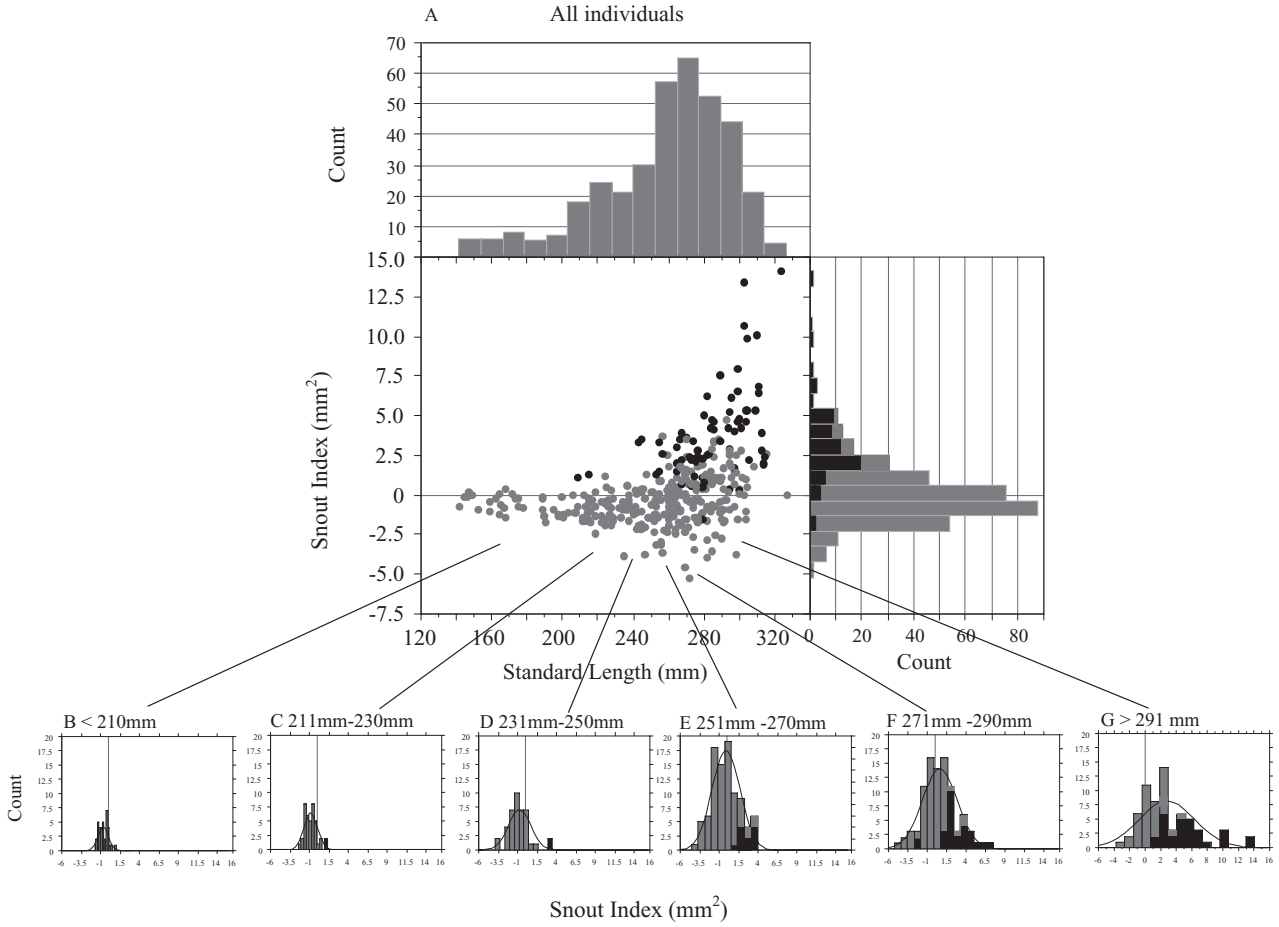

Figure 3. Phenotypic variation in snout morphology and body size for mountain whitefish from the Bitterroot River and Rattlesnake Creek, Montana ($N = 357$). Snout index was determined with the measurement shown in Fig. 2A. Histograms show overall counts for snout index (y-axis) and standard length (x-axis). Bottom panels (B–G) show snout index histograms separately for distinct size classes. Black circles and bars represent individuals with large bulbous snouts and sloping foreheads characteristic of pinocchios and are subjectively shown as such for heuristic purposes only. Grey circles and bars were used for all remaining individuals. Dimorphism in snout morphology appeared to increase with increasing age and was most pronounced in the largest (oldest) size classes.

0.35 and 0.07 for pinocchio and normal groups, respectively ($P = 0.014$). Normal stomachs contained a greater proportion of Simuliidae larvae for both average proportion by number (0.23 for normal and 0.05 for pinocchio; $P = 0.022$; Fig. 4B) and by weight (0.16 for normal and 0.04 for pinocchio; $P = 0.022$).

For the Bitterroot 2004 sample, the average proportion by number of large Ephemeroptera nymphs was greater in the pinocchio group (0.17) than in the normal group (0.06, $P = 0.026$; Fig. 3C). The proportion by weight of large Ephemeroptera was not significantly greater in pinocchios for this sample (0.19 for pinocchio and 0.12 for normal, $P > 0.05$), which was likely due to a masking effect caused by the large proportion by weight of both pinocchio and normal diets that consisted of very large Plecoptera nymphs (data not shown). There was a significantly greater average proportion by number of Chironomidae pupae in normal stomachs (0.05 versus 0.01 for pinocchio, $P = 0.012$; Fig. 3C).

Both phenotypic groups had significantly more food items in their stomachs in the 2004 sample compared with the 2003 sample. There was a significant effect of year for the ANOVA performed on total weight of food items in the stomachs of both pinocchios and normals ($F_{3,53} = 31.5$, $P < 0.0001$). The mean weight of food items did not differ significantly between pinocchios and normals within each sample ($F_{3,53} = 0.56$, $P = 0.46$), nor was the interaction term of this ANOVA (snout phenotype × year) significant ($F_{3,53} = 0.57$, $P = 0.45$).

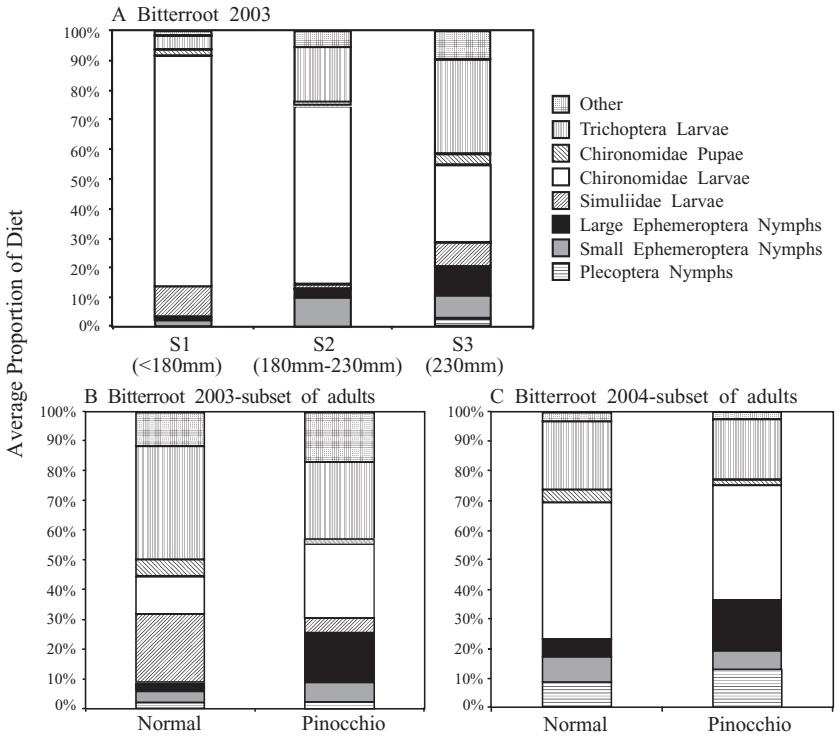

Figure 4. Evidence for trophic specialization in mountain whitefish. Average proportion of eight prey items in the stomachs of mountain whitefish shown for fish separated by size (A) or (for the largest individuals) by snout morphology (B, C). For (B) and (C), phenotypically extreme individuals were grouped by snout index scores (see Material and methods; Fig. 3).

Genetic analysis

General summary statistics were examined and deviation from Hardy–Weinberg proportions was investigated for the Bitterroot 2004 and West Fork Bitterroot samples (Table 2). Allele frequencies were similar for the comparisons of phenotypic groups within each sample, as was the mean expected heterozygosity and average number of alleles (Table 2). For the Bitterroot 2004 sample, *SSA456* deviated from Hardy–Weinberg proportions with a significant deficit of heterozygotes ($P = 0.013$). No significant deviations from Hardy–Weinberg proportions were detected in the West Fork Bitterroot sample. The combined probability for deviations for Hardy–Weinberg proportions based on Fisher's method was not significant for either sample ($P > 0.05$).

Single locus tests for genic differentiation were combined with an analysis of multilocus genotypes using PCA to test for genetic subdivision within each sample. None of the exact tests for genic differentiation was significant for either sample, nor were any of the combined *P*-values based on Fisher's method ($P > 0.05$). In addition, no patterns of genetic differentiation were detected between phenotypic groups within either sample using PCA (Fig. 5). For Bitterroot 2004, PC axes 1–4 explained 19%, 15%, 13%, and 10% of the variation among multilocus genotypes and, again, there was no tendency for individuals to cluster by phenotype in PCA plots (Fig. 5A; axes 3 and 4 not shown). For the West Fork Bitterroot, PC axes 1–4 explained 26%, 16%, 14%, and 11% of the variation among multilocus genotypes. There was no tendency for individuals to cluster by phenotype in PCA plots for this sample (Fig. 5B; axes 3 and 4 not shown). Neither snout index, nor standard length was significantly correlated with scores from either PC 1 or PC 2 in either sample ($P > 0.05$). In addition, none of the effects in any of the ANCOVAs with scores from either PC 1 or PC 2 as dependent variables was significant in either of the two samples ($P > 0.05$).

DISCUSSION

Is there discontinuous variation in snout morphology within populations of mountain whitefish?

Phenotypic variation in snout size and shape was reduced in smaller individuals but increased dramati-

Table 2. Microsatellite allele frequencies for groups of pinocchio and normal mountain whitefish. Sample size (N), mean expected heterozygosity (H_S), and mean number of alleles (A) are shown

	SFO8-1			*SSA456*								
Sample	**158*	**162*	**164*	**138*	**158*	**160*	**162*	**210*	**220*	**222*	**224*	**230*
West Fork Bitterroot River												
Pinocchio	0.300	0.550	0.150	0.450	0.250	0.250	0.000	–	0.050	–	–	–
Normal	0.300	0.450	0.250	0.300	0.100	0.500	0.050	–	0.050	–	–	–
Bitterroot River 2004												
Pinocchio	0.400	0.475	0.125	0.175	0.000	0.625	–	0.025	0.050	0.025	0.075	0.025
Normal	0.300	0.525	0.175	0.350	0.000	0.475	–	0.000	0.100	0.050	–	0.025

	COCL4			*SSA14*					*ONE8*		
Sample	**146*	**150*	**152*	**167*	**169*	**171*	**173*	**175*	**180*	**182*	**184*
West Fork Bitterroot River											
Pinocchio	0.700	0.250	0.050	0.000	0.050	0.550	0.100	0.300	0.100	0.750	0.150
Normal	0.650	0.250	0.100	0.050	0.000	0.600	0.100	0.250	0.100	0.650	0.250
Bitterroot River 2004											
Pinocchio	0.658	0.316	0.026	0.075	0.100	0.450	0.150	0.225	0.025	0.700	0.275
Normal	0.700	0.225	0.075	0.075	0.100	0.650	0.075	0.100	0.075	0.625	0.300

	FGT25			*SFO8-2*				
Sample	**170*	**178*	**180*	**195*	**197*	N	H_S	A
West Fork Bitterroot River								
Pinocchio	0.200	0.050	0.750	0.900	0.100	10	0.494	3.14
Normal	0.000	–	1.000	0.900	0.100	10	0.459	3.00
Bitterroot River 2004								
Pinocchio	0.075	0.050	0.875	0.925	0.075	20	0.461	3.71
Normal	0.100	0.025	0.875	0.875	0.125	20	0.468	3.43

cally with increasing standard length, becoming somewhat bimodal in the largest size classes (Fig. 3). This increase in phenotypic variation after a specific size suggests that the events that occur when individuals are approximately 220–240 mm in length may influence snout morphology. Body length and age are generally highly correlated in fishes and, based on data from nearby populations of mountain whitefish (Wydoski, 2001), this size range would correspond to individuals that are 3 years of age. This size range also corresponds to the large dietary shift that was observed between juveniles and adults (Fig. 4A), a shift that has been observed by others (Pontius & Parker, 1973). Individuals in this size range also undergo an ontogenetic niche shift (*sensu* Werner, 1986) from shallow side-water habitat to deeper, faster flowing sections of rivers (Northcote & Ennis, 1994). Thus, the rapid increase in snout variation observed in the present study coincides with stage-specific behavioural shifts in foraging and with shifts in patterns of habitat use.

The pinocchio trait may thus provide an example of a stage-specific developmental switch with alternative growth trajectories that are not followed until late in ontogeny. Trophic polymorphisms in other fishes similarly correspond to ontogenetic niche changes, but often occur much earlier in ontogeny (Hindar & Jonsson, 1982; Wainwright, Osenberg & Mittelbach, 1991; Andrews, 1999). Physical constraints on growth and tissue differentiation late in ontogeny may limit how extreme phenotypic variation can become in mountain whitefish.

Many trophic polymorphisms in fishes, amphibians, and reptiles have been shown to be condition sensitive (Pfennig, 1992; Robinson & Wilson, 1996; Queral-Regil & King, 1998; Bouton *et al.*, 2002; Aubret, Shine & Bonnet, 2004). The pinocchio trait in mountain whitefish could either be due to phenotypic plasticity

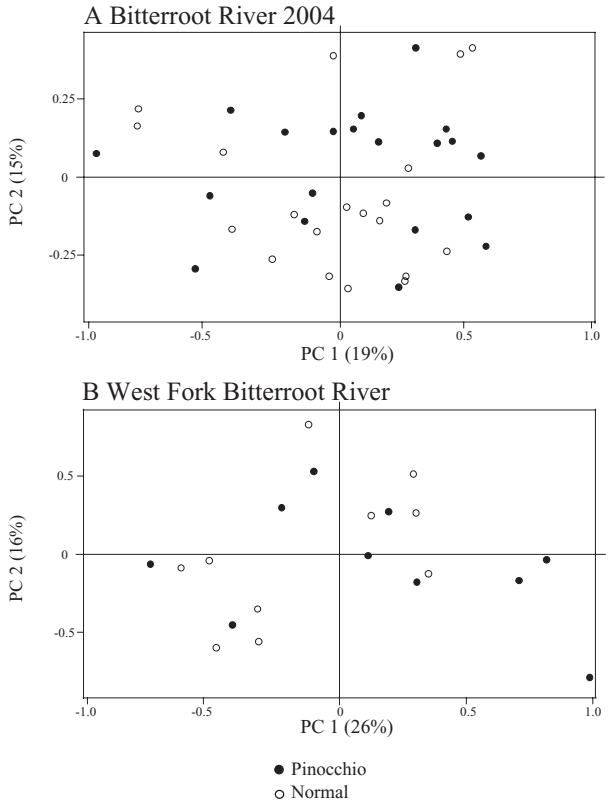

Figure 5. No genetic subdivision associated with snout morphology. Plot of principal component (PC) scores based on multilocus genotypes of individuals from the Bitterroot River 2004 (A) and the West Fork Bitterroot River (B). Individuals at the pinocchio phenotypic extreme are indicated by filled circles, and individuals at the normal extreme are indicated by open circles. Percentages are the proportion of the total variation among genotypes attributable to each axis.

or a genetic polymorphism maintained by frequency-dependent selection. It would be necessary to conduct experimental crosses to determine the degree to which the pinocchio trait is genetically influenced to test these hypotheses.

Snout index was not correlated with supraethmoid base width or supraethmoid length. Dietary differences in fishes can affect bone development due to effects on nutrition (Wimberger, 1993), but these changes likely require dietary differences early in ontogeny. It is possible that the size of the supraethmoid was not correlated with external snout morphology because phenotypic variation increases so late in ontogeny.

Tissue changes beyond underlying bone structure must be responsible for pinocchio snout elongation. Tissues lying between the supraethmoid and outer snout epithelium may have greater ability to change at this late period in development. A preliminary histological analysis of the snout tissue of five pinocchios and five normals was performed to examine snout tissue differences. This preliminary analysis suggested that pinocchios have thicker epithelial tissue layers and large gaps in internal tissue layers due to muscle degeneration that are absent in normals (data not shown). Thus, muscle degeneration might be mechanistically responsible for this morphology. Analysis of more individuals will be required to further explore this hypothesis and to examine this trait's mechanistic basis in greater detail, as has been done for some sexually dimorphic traits (Emerson, 2000).

Finally, variation in snout morphology might be associated with differences in overall body shape. To test this, a preliminary analysis of body shape was conducted for two groups sorted by snout index. A geometric morphometric analysis (Rohlf & Marcus, 1993) was performed using 12 landmarks located along the body of individuals with extreme snout phenotypes according to the methods of Adams, Rohlf & Slice (2004) and Langerhans *et al.* (2003). The preliminary results suggested that body shape differences were correlated with overall shape differences, where pinocchios tend to be less deep-bodied and normal individuals tend to be more deep-bodied with a slight hump along the dorsal margin between the head and dorsal fin (data not shown). However, a more rigorous investigation of this pattern will be necessary, along with other analyses to confirm the presence of alternative growth trajectories.

Does diet vary with snout morphology?

Enlarged snouts of several other fish species represent either sexually dimorphic characters (Fernandes, Lundberg & Riginos, 2002) or are putatively related to differential resource acquisition (Kondrashov & Mina, 1986; Nagelkerke *et al.*, 1994; Pusey, Kennard & Arthington, 2004). The pinocchio snout of mountain whitefish was not sexually dimorphic, but it appears to represent a subtle trophic polymorphism.

Consistent differences in diet were found between adults at opposite extremes of snout morphology. There were significantly more large Ephemeroptera (mayfly) nymphs (in the families Heptageneiidae and Ephemerellidae) in pinocchio stomachs for both of the sample years. For adults, greater consumption of large mayfly nymphs by large-snouted individuals is consistent with the hypothesis that these individuals feed on the bottom more than individuals at the opposite extreme of variation, possibly using their snouts to probe into cracks and crevices and to overturn rocks. Ephemerellidae and Heptageneiidae mayfly nymphs cling to the bottom of the river and feed as scrapers of

organic surfaces on and beneath rocks (Merritt & Cummins, 1996). Mayfly nymphs do also occur suspended in the water column (McIntosh, Peckarsky & Taylor, 2002), and it is possible that pinocchios were feeding on drifting nymphs. However, to feed on mayflies in the benthos would generally require that the fish probe into crevices between rocks.

Prey items found more often in the stomachs of individuals at the normal extreme were consistent with these individuals feeding in the water column more often than individuals at the large-snouted phenotypic extreme. These prey items included Simuliidae larvae, which occur attached to the outer surface of rocks (Merritt & Cummins, 1996) and Chironomidae pupae, which occur almost exclusively suspended in the water column or at the water surface (Merritt & Cummins, 1996).

The diet results reported in the present study might be under-representative of true diet differences for two primary reasons. First, by collecting each diet sample at the same location and at the same time, potential diet differences associated with variation in habitat preference or diel foraging, both of which could vary with snout morphology, were minimized. Second, the 2004 sample was intentionally collected at a time of year when morphology related diet differences are less likely, under the rationale that the results obtained would more likely be biologically meaningful if they could be replicated under unlikely circumstances. Densities of aquatic macroinvertebrates are generally highest in spring (Boulton *et al.*, 1992; Wipfli & Gregovich, 2002), which was supported by the observation that fish in the 2004 sample had more food in their stomachs than those from the summer-collected 2003 sample. Due to greater prey abundance, Spring should be the time of year when mountain whitefish are least selective with respect to prey choice and, therefore, replication of the Ephemeroptera results, in particular, appears to represent a meaningful diet difference. Additional sampling at times of low prey densities (e.g. in the Autumn) might reveal even stronger differences in diet between phenotypically extreme individuals.

Diet results were consistent with behavioural observations of Troffe (2000), who observed that pinocchios directed feeding attempts towards the substrate significantly more than normal individuals for two sites within a tributary to the Fraser River, BC, Canada. Overall, the evidence presented in the present study suggests that the observed phenotypic variation in snout size and shape of mountain whitefish corresponds to differences in diet and foraging strategies for individuals with extreme phenotypes. The differences in diet observed are not as great as have been observed in northern lacustrine fishes with trophic polymorphisms (Skúlason, Noakes & Snorrason, 1989; Snorrason *et al.*, 1994). Instead, the results are more similar to the more subtle differences observed in riverine populations of another northern temperate fish species, the brook trout, *Salvelinus fontinalis* (McLaughlin & Grant, 1994; McLaughlin *et al.*, 1999; McLaughlin, 2001). Whether the differences observed for mountain whitefish have fitness consequences remains to be determined. For example, it will be important to determine whether these differences in diet reduce intraspecific competition between individuals with extreme phenotypes (Swanson *et al.*, 2003).

Is there evidence of assortative mating associated with snout morphology?

The genetic results presented here provided no evidence for genetic subdivision associated with snout morphology. A strong signature of genetic subdivision would have provided indirect evidence for assortative mating by snout phenotype. Together, the lack of any loci with significant differences in allele frequencies, only one locus with a significant deficit of heterozygotes, and a lack of significant clustering of multilocus genotypes by phenotype in the PCA (Fig. 5), strongly suggest that assortative mating by phenotype does not occur.

The genetic results found in the present study are not consistent with those of Troffe (2000). These authors proposed secondary contact among distinct evolutionary groups in the Fraser River as a possible mechanism to explain their genetic observations. It is possible that secondary contact among distinct evolutionary groups occurred in the Fraser River but not the Clark Fork River. However, this scenario appears unlikely based on genetic analysis of populations from both river systems (Whiteley, Spruell & Allendorf, 2006). An alternative explanation is that the results of Troffe (2000) reflect drift, potentially among cohorts, at the mtDNA locus, especially because mtDNA has a small effective population size (N_e).

Implications for the evolution of trophic polymorphisms

The combination of riverine environment and mating system may serve as a constraint that prevents the translation of intraspecific trophic variation into among-species variation in the mountain whitefish. Comparisons with species that have a similar mating system as mountain whitefish but occur in lakes, as well as comparisons with species that have different mating systems but occur in rivers, provide information about the ecological conditions that favour the evolution of trophic polymorphisms.

In several cases where trophic morphs have arisen sympatrically within lakes, it appears that trophic

morphs have become highly specialized and reproductively isolated despite mating systems in which assortative mating is unlikely. For example, two reproductively isolated trophic morphs of lake whitefish (*Coregonus clupeaformis*) occur within northern lakes (Bernatchez *et al.*, 1999). These morphs have arisen sympatrically in at least some lakes (Bernatchez *et al.*, 1996). The pygmy whitefish (*Prosopium coulteri*) potentially has two or three morphs that may have evolved within several Alaskan lakes (McCart, 1970). Information on the mating system of these two species is limited but both are group broadcast spawners (Fabricius & Lindroth, 1954; Stearley, 1992). If multiple males simultaneously fertilize the eggs of females, as is likely in these species, reproductive isolation will be unlikely to occur without spatial or temporal segregation of morphs at the time of spawning. This suggests that the distinct and spatially structured foraging habitats found in lakes, and their subsequent correlation with spawning location, may be critical for superceding the homogenizing effect of a broadcast spawning mating system in these species. Furthermore, if extant morphological differences originated in isolation, the spatially structured lacustrine environment would be more conducive to the maintenance of these differences upon secondary contact.

The present study provides a valuable contrast to those lake systems because the riverine environment of mountain whitefish is not as spatially structured as that of lakes. The mountain whitefish shares the same basic mating system (broadcast spawning) as other whitefishes and shows a pattern of morphological variation consistent with trophic specialization, but it lacks the critical factor of a highly segregated feeding environment. Also, as predicted, the extent of morphological dimorphism is less extreme and genetic divergence between forms is absent. Thus, by providing an example from a less stable environment (rivers), the present study highlights the importance of stable environments (lakes) and physical/geographical separation for promoting phenotypic diversification. This raises the hypothesis that, in rivers, because spatial segregation is less likely than in lakes, temporal segregation of reproduction may be a more important factor for promoting phenotypic diversification.

The present study also highlights the potential importance of mating systems for the origin and maintenance of trophic polymorphisms in rivers. In riverine lenok populations, where two reproductively isolated morphs occur (Osinov *et al.*, 1990), small groups spawn in nests called redds (Baimukanov, 1996). Spawning in redds provides an opportunity for effective mate choice in salmonids (Stearley, 1992). It is possible that this mating system has allowed assortative mating by trophic phenotype to occur in this species, and thus has permitted these putative trophicaly polymorphic forms to become reproductively isolated from each other despite the reduced spatial segregation of resources in the riverine environment. If morphological differences in this species arose in isolation, the more derived mating system may have provided conditions that allowed these differences to be maintained upon secondary contact. This example highlights the inference that the less derived mating system of the mountain whitefish may serve as a constraint that prevents reproductive isolation by trophic morphology from occurring in this species.

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