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Assortative mating but no evidence of genetic divergence in a species characterized by a trophic polymorphism

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Abstract

Disruptive selection is a process that can result in multiple sub-groups within a population, referred to as diversification. Foraging related divergence has been described in many taxa, but many questions remain about the contribution of such divergence to reproductive isolation and potentially sympatric speciation. Here we use stable isotope analysis of diet and morphological analysis of body shape to examine phenotypic divergence between littoral and pelagic foraging ecomorphs in a population of pumpkinseed sunfish (*Lepomis gibbosus*). We then examine reproductive isolation between ecomorphs by comparing the isotopic compositions of nesting males to eggs from their nests (a proxy for maternal diet), and use nine microsatellite loci to examine genetic divergence between ecomorphs. Our data support the presence of distinct foraging ecomorphs in this population and indicate that there is significant positive assortative mating based on diet. We did not find evidence of genetic divergence between ecomorphs, however, indicating that isolation is either relatively recent or is not strong enough to result in genetic divergence at the microsatellite loci. Based on our findings, pumpkinseed sunfish represent a system in which to further explore the mechanisms by which natural and sexual selection contribute to divergence, prior to the occurrence of sympatric speciation.

Keywords: Fish, Morphometrics, Natural selection, Sexual selection, Speciation

Introduction

Speciation is the evolutionary process ultimately responsible for the tremendous biological diversity that exists today. Not surprisingly, biologists have placed considerable emphasis on understanding the conditions and mechanisms behind speciation. Traditionally, speciation has been thought to occur almost exclusively in allopatry, i.e. when groups are isolated by geographic barriers (e.g. islands and mountain ranges) resulting in genetic divergence between populations through a combination of natural selection for local environmental conditions and passive genetic drift (Mayr, 1963; Thorpe *et al.*, 2010; Blair *et al.*, 2013); however, speciation can also occur in sympatry, i.e. without geographic isolation (Bolnick, 2011; Thibert-Plante & Hendry, 2011). The process of divergence in sympatry is described as occurring along a "speciation continuum", ranging from a relatively homogeneous population to reproductively isolated sister species, and is generally based on the mechanisms of (1) disruptive natural selection (e.g. negative frequency-dependent) that result in multiple phenotypes, and (2) reproductive isolation between phenotypes, which lead to (3) genetic differentiation between phenotypes (Hendry *et al.*, 2009; Seehausen & Wagner, 2014). Consequently, understanding the ecological and behavioural mechanisms that contribute to phenotypic divergence at different points along the continuum, and the conditions under which this divergence leads to sympatric speciation, is of considerable interest.

Foraging ecology is an important source of phenotypic divergence within many populations, often mediated through both intra- and interspecific resource competition (Schluter, 1996; Siwertsson *et al.*, 2010). There are typically trade-offs such that generalist foragers, which consume a variety of prey items, are at a competitive disadvantage as compared to specialists, which consume a sub-set of the available prey items (Schluter, 1995; Rueffler *et al.*, 2006). When specialists have an advantage in acquiring or processing specific

food items, there can be disruptive selection within species for foraging phenotypes that specialize on different resources (Ackermann & Doebeli, 2004; Bernays *et al.*, 2004; Svanbäck & Eklöv, 2004). The resulting foraging "ecomorphs" contribute to phenotypic divergence within populations, and may thus be an important step towards divergence and potentially sympatric speciation; however, for divergent selection within a continuous environment to result in speciation there must also be a mechanism of reproductive isolation that disrupts gene flow between ecomorphs, such as assortative mating. Positive assortative mating, i.e. an increased likelihood to mate with phenotypically similar individuals, reduces the overall gene flow between ecomorphs and the number of intermediate individuals that will have lower overall fitness (Bank *et al.*, 2012; Jiang *et al.*, 2013). If pre-mating isolation through assortative mating is maintained for a sufficient period of time other reproductive barriers, e.g. post-mating isolation, may develop resulting in the completion of speciation. Assortative mating can be a "passive" process when, for example, disruptive selection changes habitat use or the timing of reproduction between ecomorphs; e.g. apple maggot fly (*Rhagoletis pomonella*; (Feder *et al.*, 1994; Filchak *et al.*, 2000). Alternatively, assortative mating can be an "active" component of sexual selection when individuals show distinct behavioural mate choice preferences; e.g. colour-based mate choice in African Great Lake cichlids (Seehausen & Alphen, 1998; Gray & McKinnon, 2007) and phenotype matching in threespine stickleback (Conte & Schluter, 2013). Regardless of whether the process of assortative mating is passive or active, if gene flow is significantly reduced then it is possible for the combination of divergent natural selection and reproductive isolation to result in genetic divergence and ultimately speciation.

Fish found in freshwater lakes provide ideal species to study divergence related to foraging ecology. In general, fish in the shallow littoral habitat of lakes consume a variety of benthic invertebrates and exhibit deep-bodied phenotypes, associated with increased

maneuverability to capture cryptic prey in a structurally complex environment (Robinson *et al.*, 1996; Svanbäck & Eklöv, 2003). In contrast, pelagic fish are more streamlined in body shape, which increases their burst swim speed to catch prey suspended in the water column (Schluter, 1995; Collar & Wainwright, 2009). Due to the strong functional relationships among morphology, swim performance, and foraging efficiency in fish (Webb, 1984; Fisher & Hogan, 2007; Collar & Wainwright, 2009), the development of trophic polymorphisms observed in many fishes are likely related to divergent natural selection on phenotype related to foraging tactic. Indeed, trophic polymorphisms have been linked to foraging tactic within populations of threespine stickleback (Schluter & McPhail, 1992; Svanbäck & Schluter, 2012), Lake Malawi cichlids (Hulsey *et al.*, 2013), lake whitefish (*Coregonus clupeaformis*; (Pigeon *et al.*, 1997; Campbell & Bernatchez, 2004; Rogers & Bernatchez, 2007), and lake trout (*Salvelinus namaycush*; (Chavarie *et al.*, 2013, 2015).

Northern temperate lakes are of particular interest to studies of divergence and sympatric speciation because these lakes represent geologically "young" environments, and were colonized within approximately the last 12,000 years by fish that were displaced during the last ice age (Mandrak & Crossman, 1992; Robinson *et al.*, 2000). The processes of colonization and resource competition has resulted in resource partitioning among species or, when heterospecific competitors are absent, specialization within species (i.e., foraging ecomorphs). For example, in North America, bluegill (*Lepomis macrochirus*) and pumpkinseed sunfish (*L. gibbosus*) co-exist in many lakes across their distribution, with pumpkinseed specializing on benthic invertebrates in the shallow littoral habitat and bluegill specializing on zooplankton in the deeper pelagic habitat (Keast, 1978; Robinson *et al.*, 1993). However, in lakes where only one of the two sunfish species is present, littoral and pelagic foraging ecomorphs may develop within a single species to occupy both resource niches. Indeed, foraging ecomorphs have been identified in populations of bluegill in

Michigan (USA) and Japan where pumpkinseed are absent (Ehlinger & Wilson, 1988; Ehlinger, 1990; Yonekura *et al.*, 2002). For pumpkinseed, almost 30 lakes with foraging ecomorphs have been reported in the Canadian Shield (Ontario, Canada) and Adirondack (New York, USA) regions, consistently in the absence of bluegill (Robinson *et al.*, 2000; Jastrebski & Robinson, 2004; Weese *et al.*, 2012).

In the current study we examine the evidence for disruptive selection, reproductive isolation and genetic divergence between ecomorphs in pumpkinseed. We focus on a pumpkinseed population in Ashby Lake (Ontario, Canada, 45º05'N, 77º21'W), a temperate lake located on the southern portion of the Canadian Shield. Ashby Lake covers an area of approximately 260 ha and consists of a shallow littoral habitat, with a variety of benthic invertebrates, but quickly drops off into the deeper pelagic habitat, with abundant zooplankton surrounding islands and rock shoals in the central part of the lake (Jastrebski & Robinson, 2004). Pumpkinseed colonized this lake after the glacial retreat some 9,000 to 12,000 years ago (Mandrak & Crossman, 1992). The presence of littoral and pelagic foraging ecomorphs in Ashby Lake has been identified based on stomach content analysis of diet and morphological analysis of overall body shape (Jastrebski & Robinson, 2004), and studies of growth rates suggest that differences between these ecomorphs are the result of disruptive selection on morphology (Jastrebski, 2001). However, to our knowledge, there have been no tests of reproductive isolation in this, or any other, polymorphic pumpkinseed population. Based on these prior findings, we identified the Ashby Lake pumpkinseed as a system in which we could further examine the process of diversification and sympatric speciation by using the concept of a speciation continuum to look at (1) the phenotypic differentiation between foraging ecomorphs, (2) the presence of assortative mating, and (3) genetic differentiation.

Materials and methods

Fish collection

In 2011, adult pumpkinseed were collected from Ashby Lake in the spring (May 26 – June 15; n = 49) and summer (August 21 – 22; n = 37). Approximately equal numbers of fish from the littoral (n = 45) and pelagic habitats (n = 41) were collected either by angling with a piece of earthworm as bait or by dip-netting from the water column. The littoral habitat of Ashby Lake was identified as the shallow, relatively macrophyte dense nearshore margins of the lake that rapidly drops off into the deeper open water pelagic habitat of the lake punctuated by rock shoals that provide refuge for fish (Jastrebski, 2001; Jastrebski & Robinson, 2004). Immediately after collection, each fish was euthanized with clove oil and a picture of its left side was taken using an Olympus Stylus Tough-6000 (10 megapixel) digital camera. The wet mass (g) and total length (mm) of each fish was measured prior to removing the stomach contents and liver, which were stored at -20ºC for later analysis of diet. During the dissections, the sex and maturity of each individual was determined by examining the reproductive organs. Only reproductively mature fish were included in the analyses because niche shifts are known to occur between juvenile and adult life stages in pumpkinseed (Osenberg *et al.*, 1988; Arendt & Wilson, 1997).

In the spring of 2012 (June 11 – 22), nesting parental males that were actively guarding eggs were collected in the littoral (n=13) and pelagic (n=14) habitats. Prior to collection, each nest was visually monitored to confirm that the male was performing guarding and nest care behaviours. The male was then collected using a dip-net and approximately 100 eggs were sampled from the male's nest and stored at -20ºC for stable isotope analysis. Eggs were collected as a proxy for female diet because it has been established for a nearby population of the closely related bluegill (*Lepomis macrochirus*) that the carbon and nitrogen isotopic compositions of eggs and female liver tissue are tightly

correlated, differing by approximately 1 ‰ at any sampling point during egg development (Colborne *et al.*, 2015). Given the physiological and reproductive similarities between bluegill and pumpkinseed we are confident that this relationship is also true for pumpkinseed and, therefore, eggs were used as a proxy for maternal diet. As in 2011, the nesting males were euthanized immediately after collection, photographed, and the liver sampled as outlined above. Stomach contents were not collected in 2012 because nesting males do not actively forage and are therefore unlikely to have stomach contents that are representative of their diet (Gross & MacMillan, 1981).

Samples of potential invertebrate prey were also collected during each of the periods when fish were sampled to establish resource baselines required for stable isotope inferences of diet. Pelagic zooplankton samples were collected from open-water areas adjacent to rock shoals where fish were sampled using a vertical tow net (mesh size 0.5 μm; depth of 3 – 4 m, repeated three times per site). Littoral benthic invertebrates were collected using D-net sweeps of the submerged macrophyte vegetation and the upper 1 – 2 cm of sediment. The D-net samples were then hand sorted through a series of nested sieves to collect littoral benthic invertebrates that were classified to the nearest order. For isotope analysis, snails (Gastropoda) were manually removed from their shells because the shell material remains largely undigested and reflects the inorganic environment at the time of formation (Post, 2002), but all other benthic invertebrate prey were analyzed intact.

Morphological variation

Using tpsDig software (Rohlf, 2008), 15 homologous landmarks were placed on each of the pumpkinseed images (n = 113; see (Jastrebski & Robinson, 2004), for landmark locations). These landmarks were used to calculate partial warp coefficients for each individual, which allow body shape to be examined independently of body size (see Zelditch

et al., 2004). Variation in warp coefficients was further partitioned into axes of major variation using a discriminant function analysis (DFA) comparing four groups based on collection habitat and sex: pelagic males (n = 35), pelagic females (n = 20), littoral males (n = 32), and littoral females (n = 26). Subsequent statistical analyses focused on only those DFA axes that explained at least 20% of the total variation in shape. For each significant DFA axis, two-factor ANOVA models were used to examine variation in DFA score (dependent variable) between sexes and collection habitats (independent factors) and their interaction, with sampling period included as a random effect. Significant differences in body shape identified by these analyses were then visualized using thin-plate splines (Rohlf, 2009).

Diet analysis

The preserved stomach content samples of each fish were thawed to room temperature and sorted using a dissection microscope into one of four prey groups: zooplankton (copepods and cladocerans), molluscs (gastropods, bivalves), benthic prey (ephemeroptera, trichoptera, odonates, and amphipods), and "other" (terrestrial insects, fish eggs, plant material, unidentifiable contents). Prey samples from each fish were then dried at 50ºC for 24 hours and the dry mass (mg) of each prey type was determined. The proportion of each prey type (dry mass of prey type/total dry mass of all stomach contents) from each fish was arcsine transformed to meet the assumptions of normal distribution and homogeneous variance for further statistical analyses (Zar, 1999; Jastrebski & Robinson, 2004). The transformed proportion measures were then used in a two-factor MANOVA test of the four prey groups (dependent variables: proportion of each prey type in diet; independent variables: collection habitat, sex, and their interaction). If an independent factor (habitat or sex) was found to be

significant in the MANOVA, separate t-tests were used for each prey group to compare that independent variable. Only fish with measurable stomach contents were included in the analyses.

Next, stable isotope analysis was conducted in the Laboratory for Stable Isotope Science (LSIS) at The University of Western Ontario (London, Ontario Canada). The liver tissue samples of each fish and eggs (from the nests of parental males collected in Summer 2012), were prepared for stable isotope analysis by freeze drying them at -50 ºC for 24 hours and manually grinding into a fine powder using a mortar and pestle. The isotope ratios of carbon (^{13}C:^{12}C) and nitrogen (^{15}N:^{14}N) were then determined using a Costech elemental analyzer coupled to a Thermo Finnigan Delta^{plus} XL stable isotope ratio mass-spectrometer in continuous flow mode. The ratio of each isotope was calculated as the difference between the measured sample and an international standard reference material:

$$\delta X = (R_{sample}/R_{standard} - 1)$$

where X is the isotope being measured (either ^{13}C or ^{15}N), R is the ratio of ^{13}C:^{12}C or ^{15}N:^{14}N, and δ is a measurement of the heavy to light isotope in a sample expressed as parts per thousand (‰). The international standardization ($R_{standard}$) for $\delta^{13}C$ was Vienna Pee Dee Belemnite (VPDB) and for $\delta^{15}N$ was atmospheric nitrogen (AIR). Two-point curves were used to calibrate $\delta^{13}C$ and $\delta^{15}N$ values to these international standards and internal laboratory standards were used to monitor precision and accuracy (see appendix for details). Additionally, the measured $\delta^{13}C$ values of fish liver were mathematically corrected for the presence of lipids using the mass balance correction for aquatic organisms of (Kiljunen *et al.*, 2006):

(1) $$\delta^{13}C' = \delta^{13}C + D \times (I + 3.90 / (1 + 287 / L))$$

and

(2) $$L = 93 / 1 + (0.246 \times C{:}N - 0.775)^{-1}$$

where the estimated lipid content of the sample (L) is based on its measured atomic carbon to nitrogen ratio (C:N), which is used with the measured value (δ^{13}C), the isotopic difference between pure protein and lipid (D; 7.02 ‰, (Kiljunen *et al.*, 2006), and the constant I (0.05, (Kiljunen *et al.*, 2006)) to estimate the lipid-corrected isotope value of a given sample (δ^{13}C').

To create group estimates of the mean resource use between habitats (littoral and pelagic) and sexes in each sampling period we used SIAR (Stable Isotope Analysis in R) two-member mixing models to estimate the contribution of littoral prey and pelagic prey to pumpkinseed diet (Parnell *et al.*, 2010). The SIAR mixing model incorporates both δ^{13}C and δ^{15}N values of each fish collected and the variability both between and within the prey resources of each habitat (Parnell *et al.*, 2010). The 'source' variables of the model were based on the mean (± 1 SD) isotopic composition of snails (littoral habitat) and zooplankton (pelagic habitat). Snails are frequently used as the source value for all littoral invertebrates in these models because snails have similar isotopic compositions to other benthic invertebrates and due to their long-lived nature represent average littoral diet over a period more similar to the fish being sampled than other benthic invertebrates (Post, 2002; Correa *et al.*, 2012). Indeed, comparisons of the isotopic compositions of benthic prey types we collected supported the use of snails as representative of the littoral 'source' values (see appendix for details). Due to the potential for temporal variability in isotopic compositions over our sampling periods, separate mixing models were used for each of the collection periods with unique 'source' values (see appendix for prey isotopic composition details). Mean trophic enrichment factors (TEFs) for δ^{13}C (+0.47 ± 1.23 ‰; (Vander Zanden & Rasmussen, 2001)) and δ^{15}N (+5.00 ± 1.50 ‰; (Caut *et al.*, 2009; Locke *et al.*, 2014)) were estimated based on other studies of temperate freshwater fishes because species-specific TEFs for pumpkinseed are unavailable.

To obtain individual estimates of diet the SIARsolo command for SIAR (see above) was used with the same model components, i.e. sources and TEFs, to generate a % Littoral diet estimate for each pumpkinseed sampled. These % Littoral proportion estimates for each individual were and used in a two-factor ANOVA (dependent variable: % Littoral; independent factors: collection habitat and sex, plus their interaction; random effect: sampling period) to compare this isotopic compositions of individuals across sampling sites and between the sexes.

Morphology, diet, and condition

The relationship between morphology and diet was first tested using a linear model that included the % Littoral estimates for each individual (dependent variable), total body length and DFA 1 scores of shape (independent factors), and sampling period (random effect). Next, we constructed an ecomorph "score" that combined the morphological (DFA 1) and diet (% Littoral) data using principal component analysis (PCA). Given that % Littoral estimates ranged from low values for pelagic consumers to high values for littoral consumers, whereas DFA 1 scores ranged from high values for pelagic body shape to low values for littoral body shape (see results below), the DFA 1 scores for each fish were multiplied by –1 to facilitate interpretation of the axis loadings before use in the PCA. The first principal component (PCA 1) subsequently had positive loadings on both variables such that higher PCA 1 scores, i.e. higher ecomorph scores, were associated with a more littoral shape and diet as compared to lower scores, which were associated with a pelagic shape and diet.

Fulton's condition factor was calculated for each fish using the wet mass (g) and total body length (mm) ($K = \text{mass}/\text{length}^3 \times 10^5$). The condition factor provides an estimate of overall energetic state for each fish (e.g. Neff & Cargnelli, 2004; Magee *et al.*, 2006). Condition factor values (dependent variable) were then compared using ANCOVA models

that included sex (independent factor) and ecomorph score (covariate) and sampling period (random effect). Separate models were run for fish collected from the littoral and pelagic habitats. However, condition factor did not differ between males and females collected in either habitat (both $P \geq 0.11$) and therefore sex was removed from the analysis. Subsequently, ecomorph score and Fulton's condition factor (K) were correlated using separate Pearson's correlation coefficients for littoral and pelagic-caught fish.

Assortative mating and genetic differentiation

To test for assortative mating, the ecomorph scores for the parental males collected in Spring 2012 were used in a Pearson's correlation with the % Littoral estimates based on eggs collected from the male's nest (a proxy for female diet). A positive correlation would indicate assortative mating within the littoral and pelagic foraging ecomorphs. Next, to test for genetic differentiation, DNA from each of the 113 adult fish in this study was extracted using a proteinase K digestion (Neff *et al.*, 2000). Each individual was then genotyped at nine previously described microsatellite loci ((Colbourne *et al.*, 1996): LMA 29, LMA 87; (DeWoody *et al.*, 1998): RB7, RB20; (Neff *et al.*, 1999): LMA 116, LMA 122, LMA 124; (Schable *et al.*, 2002): LMAR 10, LMAR 14). The microsatellite products were visualized using a CEQ 8000 (Beckman Coulter) and manually scored in relation to a known size standard. Micro-checker was used to determine if microsatellite allele frequencies deviated significantly from the expectations of Hardy-Weinberg equilibrium (Van Oosterhout *et al.*, 2004). Only LMAR14 deviated significantly from Hardy-Weinberg equilibrium, showing a homozygote excess consistent with the presence of a null allele. Consequently, this locus was included only in the genetic analyses that accommodate null alleles (Structure, F_{st}), but excluded from analyses that may be biased by null alleles (individual genetic distances).

The microsatellite dataset was first used to test for the presence of discrete genetic groups in Ashby Lake using the Bayesian clustering method implemented by the program Structure v2.3.3 (Pritchard *et al.*, 2000). Specifically, the presence of two genetic clusters was tested using a model with admixture and correlated allele frequencies. To ensure the results converged on a single solution, the model was run using 20 replicate simulations of 100,000 burn-in steps followed by 200,000 resampling steps. The results were then aggregated using Structure harvester and Clumpp (Jakobsson & Rosenberg, 2007; Earl & vonHoldt, 2011).

Next, the global F_{st} (Weir & Cockerham, 1996) was calculated using the null allele correction implemented in FreeNA (Chapuis & Estoup, 2007) to examine genetic differentiation between fish collected from littoral and pelagic habitats. These comparisons were run both for all fish, and for just the subset of nesting males that were collected in 2012. For each test, significance was assessed by resampling over loci to generate 95% confidence intervals from 1000 bootstrap replicates.

Finally, a relationship between the pairwise genetic distance estimates between individuals and the difference in ecomorph score between those individuals was examined. Again, these comparisons were made both for all fish, and for just the nesting males that were collected in 2012. Genalex 6.4.1 was used to calculate the matrix of genetic distances between individuals, and to compare the genetic distance matrix to the ecomorph distance matrix using a Mantel test with 999 permutations to assess significance (Peakall & Smouse, 2006).

Results

Morphological variation between habitats

Body shape differed significantly among the pumpkinseed groups (pelagic males, pelagic females, littoral males, littoral females; DFA: Wilks' $\lambda = 0.17$, $P < 0.001$; Fig. 1), with DFA 1 and DFA 2 accounting for 54% and 34%, respectively, of total variation in

shape. Further examination of the DFA 1 and DFA 2 scores indicated males had higher values than females on both axes (DFA 1: ANOVA, $F_{1, 41.4} = 22.11$, $P < 0.001$; DFA 2: $F_{1, 48.05} = 48.05$, $P < 0.001$). The DFA values also differed between collection habitats with pelagic caught fish having higher DFA 1 scores than littoral fish (ANOVA, $F_{1, 108.7} = 108.19$, $P < 0.001$), but littoral fish having higher DFA 2 values than pelagic fish (ANOVA, $F_{1, 107.1} = 25.98$, $P < 0.001$). There were no interaction effects between sex and collection habitat for either DFA axis (DFA 1: ANOVA, $F_{1, 105.8} = 3.07$, $P = 0.08$; DFA 2: $F_{1, 108.1} = 0.0001$, $P = 0.99$; Fig. 1). Visualization of DFA 1 using thin-plate splines showed that lower values (i.e. littoral females) were associated with decreased body depth in the mid-body and posterior region, whereas higher DFA 2 values (i.e. littoral males) were associated with a larger head region, reduced tail depth, and a more horizontal pectoral fin orientation (Fig. 1).

Diet analysis

Of the fish collected for stomach content analysis, 74% (64 of 86 fish) had measureable contents. Comparisons across prey types indicated that overall there were significant differences in the stomach contents of pumpkinseed based on both the collection habitat and between the sexes (MANOVA; Wilks' $\lambda = 0.58$, df = 12, 151.1, $P = 0.001$). Comparisons of the independent variables indicated that stomach contents of the prey groups differed between collection habitats ($F_{3, 58} = 0.40$, $P < 0.001$; Table 1), but there were non-significant differences between the sexes ($F_{3, 58} = 0.13$, $P = 0.07$) and no interaction between habitat and sex ($F_{3, 58} = 0.05$, $P = 0.40$). Further comparisons of prey types between collection habitats indicated that pelagic caught fish consumed more zooplankton and fewer benthic invertebrates than those caught in the littoral habitat (Table 1).

Isotopic compositions ($\delta^{13}C$ and $\delta^{15}N$) of pelagic- and littoral-caught pumpkinseed varied among the sampling periods and collection habitats (Appendix Fig. A.1). SIAR mixing model estimates of diet indicated that across all individuals from a given collection habitat the littoral-caught fish consumed 68 - 71% littoral resources as compared to 30 – 55% littoral resources in the diets of pelagic-caught fish (Table 2, Fig. 2). Analysis of variance models based on SIARsolo individual estimates of % Littoral contribution to dies indicate that in addition to differences between collection habitat (ANOVA: $F_{1,\ 100.4} = 16.45$, $P < 0.001$), there was a greater contribution of littoral resources to males (54% littoral) as compared to females (46%) across sampling habitats (ANOVA: $F_{1,\ 98.59} = 5.34$, $P = 0.02$). There was no interaction effect between sex and collection habitat (ANOVA: $F_{1,\ 101.1} = 2.53$, $P = 0.11$). Isotopic compositions of each fish are presented in Appendix Tables A.2 and A.3.

Morphology, diet, and condition

Analysis of covariance found that the % Littoral diet estimates were not related to body length of individual pumpkinseed ($F_{1,\ 101.4} = 1.38$, $P = 0.24$). However, the % Littoral contribution to diet was related to body shape such that lower DFA 1 scores (i.e. increased body depth in the anterior region) were associated with a higher contribution of littoral resources to diet ($F_{1,\ 101.2} = 9.25$, $P = 0.003$). The PCA analysis combining morphology scores (DFA 1; see above) and % Littoral estimates of diet indicated that 61% of the total variation was explained by PCA 1, consequently only these values were used as an overall ecomorph score for each individual. Comparing the ecomorph scores and condition factor of pumpkinseed indicated that there was a significant correlation between these variables in pelagic-caught fish (Pearson's $r = -0.34$, $n = 54$, $P = 0.01$), with more pelagic ecomorph scores being associated with higher condition, although no relationship between these variables was observed in littoral-caught fish (Pearson's $r = 0.01$, $n = 52$, $P = 0.92$; Fig. 3).

Assortative mating and genetic differentiation

There was a significant relationship between a nesting male's ecomorph score and the % Littoral estimates of eggs from his nest: nesting males with a more littoral ecomorph score had eggs with higher % Littoral values (Pearson's r = 0.42, n = 27, P = 0.03; Fig. 4). The microsatellite data, however, did not indicate evidence of neutral genetic differentiation between the ecomorphs. First, the Structure analysis did not identify discrete genetic clusters. When the data were fit to a model of two genetic clusters based on collection habitat, all individuals had intermediate membership in each cluster and the membership coefficients were not related to ecomorph scores (Fig. 5). The F_{st} values also did not indicate significant divergence between littoral and pelagic caught fish when comparing across fish from all sampling periods (F_{st} = 0.0004, n = 113, 95% CI: -0.0029 – 0.0038) or only the nesting males from Spring 2012 (F_{st} = 0.0002, n = 27, 95% CI: -0.0095 – 0.0131). Finally, there was no relationship between genetic distance and the ecomorph score (all fish: Pearson's r = -0.01, n = 113, P = 0.40; nesting males only: Pearson's r = 0.03, n = 27, P = 0.33).

Discussion

In freshwater fish, divergent selection in littoral versus pelagic habitats can result in foraging ecomorphs that have predictable differences in morphology and diet (Skulason & Smith, 1995; Robinson *et al.*, 2000). Here, we found that littoral-caught pumpkinseed had a deeper head region and were less streamlined overall than pelagic fish, consistent with morphological analyses of pumpkinseed foraging ecomorphs across multiple populations (Jastrebski & Robinson, 2004; Weese *et al.*, 2012). Additionally, our stable isotope-based diet analyses indicated that littoral-caught pumpkinseed consumed more benthic invertebrates, such as snails, and fewer zooplankton as compared to pelagic-caught individuals, supporting a previous short-term analysis of diet in Ashby Lake using stomach

contents alone (Jastrebski & Robinson, 2004). There was also a link between body shape and diet independent of habitat associations, indicating that pumpkinseed with deeper bodies consumed more littoral benthic invertebrates than pumpkinseed with shallower body shapes. These data thus support the presence of morphological variation related to foraging tactic, i.e. foraging ecomorphs, in the Ashby Lake pumpkinseed population.

Foraging ecomorphs resulting from disruptive natural selection do not necessarily exist as discrete phenotypes, but may instead represent a phenotypic gradient along which individuals display varying degrees of specialization towards available resource types (Moles *et al.*, 2010; Ellerby & Gerry, 2011). Indeed, morphological variation within polymorphic pumpkinseed populations has been found to range from continuous variation in fish from the littoral and pelagic habitats to nearly bimodal distributions with discrete habitat-related phenotypes (e.g. Robinson *et al.*, 1996). Based on our data, littoral- and pelagic-caught pumpkinseed in Ashby Lake differed significantly in diet and body shape, but there was considerable overlap between the ecomorphs, indicating a gradient of foraging phenotypes within this population. The high frequency of "intermediate" phenotypes in this population may be related to the strength of disruptive selection based on resource use in the different habitats. For example, using Fulton's condition factor, a correlate of energetic condition and fitness in sunfish (e.g. (Neff & Cargnelli, 2004; Magee *et al.*, 2006), we found a relationship between condition and ecomorph score in pelagic-caught pumpkinseed, but not littoral-caught fish. Similar relationships between condition and morphology within the littoral and pelagic habitats were reported for the pumpkinseed of Paradox Lake (New York, USA), another population characterized by foraging ecomorphs with phenotypic overlap the collection habitats (Robinson *et al.*, 1996). Taken together, these data suggest that selection for resource specialization is similar across at least some pumpkinseed populations and that disruptive selection pressure on foraging ecomorphs is likely strongest in the pelagic habitat.

Regardless of the strength of disruptive natural selection, in order for foraging ecomorphs to drive sympatric speciation there must also be a mechanism of reproductive isolation. We predicted that assortative mating could restrict gene flow between foraging ecomorphs and lead to reproductive isolation in our study population. We found across all the nests sampled, regardless of habitat, that there was positive relationship between the ecomorph scores of nesting male pumpkinseed and the diet, and presumably ecomorph, of the females with whom he mated (inferred from the isotopic composition of the eggs in the nests). Assortative mating may occur passively when ecomorphs forage and breed in different habitats (Feder *et al.*, 1994; Snowberg & Bolnick, 2008, 2012), as is likely in our study population – nesting males were generally separated into the littoral and pelagic habitats during the breeding season. Indeed, a recent review of speciation reported that divergent mate choice was related to habitat use in 54% of the fish studied (Scordato *et al.*, 2014). Assortative mating may also occur through active mate choice, as has been demonstrated in other sympatric populations of fish (e.g. Seehausen & Alphen, 1998; Gray & McKinnon, 2007). Regardless of whether the process of assortative mating between littoral and pelagic pumpkinseed is primarily passive or active, our data provide the first evidence of potential reproductive isolation between pumpkinseed foraging ecomorphs, which could limit gene flow and facilitate increased divergence towards sympatric speciation.

Despite evidence of disruptive natural selection and reproductive isolation, the primary components of the typical sympatric speciation model, multiple analyses of our microsatellite loci provided no evidence of neutral genetic differentiation between littoral and pelagic ecomorphs. This lack of neutral genetic differentiation between ecomorphs could, at least in part, be a reflection of the relatively short amount of time that has passed since the lakes were re-colonized after the last ice age (Weese *et al.*, 2012). Ashby Lake has been populated by pumpkinseed for at most 12,000 years (Mandrak & Crossman, 1992). In

comparison, African rift lake cichlid species flocks have been diverging for between 2.5 to 4.5 million years in Lake Malawi and between 190,000 and 270,000 years in Lake Victoria (Genner *et al.*, 2007). It is possible that genetic divergence between pumpkinseed ecomorphs is present at functional loci, such as those related to body shape, as there is a heritable component to an individual's ecomorph (Parsons & Robinson, 2006). Indeed, littoral-pelagic ecomorphs in a population of Midas cichlids (*Amphilophus* spp.) have been shown to differ at functional loci related to shape and fin placement but not at neutral loci after 22,000 years of divergence (Franchini *et al.*, 2014). Overall, the absence of neutral genetic differentiation in the pumpkinseed ecomorphs examined here does not rule out differentiation at functional loci and the possibility of eventual sympatric speciation. Instead, the absence highlights that this population falls somewhere along the speciation continuum between a homogenous population and separate species (Hendry *et al.*, 2009).

The strength and temporal stability of selection and assortative mating are important factors determining the diversification process and ultimately the likelihood of sympatric speciation (e.g. Bolnick, 2011). For example, northern temperate fishes have been shown to have considerable phenotypic plasticity associated with foraging phenotypes, possibly related to the relative high levels of temporal environmental variability in temperate lakes (e.g. Svanbäck *et al.*, 2009; Bolnick, 2011). Foraging ecomorphs of both pumpkinseed and arctic charr (*Salvelinus alpinus*), have been experimentally shown to arise largely because of phenotypic plasticity during development with a smaller heritable component (Robinson & Wilson, 1996; Adams & Huntingford, 2004; Parsons & Robinson, 2006). Consequently, disruptive selection on "hybrids" (offspring of parents that differed in their ecomorphology) may be weakened if offspring can develop into either ecomorph based on environmental cues during development. Furthermore, reproductive isolation in sunfish may be weakened by the presence of cuckolder male reproductive tactics, e.g. sneaker males, that may be relatively

indiscriminate in their mating preferences (Gross, 1982). Indeed, recent evidence indicates that cuckolders in the littoral habitat do not consistently discriminate among sunfish species (Garner & Neff, 2013), let alone foraging ecomorphs within their own species. Therefore, it is possible that both conditions that favor phenotypic plasticity and high rates of cuckoldry reduce the likelihood of speciation in this system.

In conclusion, we focused on what are likely to be the early stages of divergence by examining phenotypic divergence and assortative mating within a population of pumpkinseed that does not have geographic barriers to gene flow. We found evidence of assortative mating between littoral and pelagic foraging ecomorphs, but no evidence of genetic differentiation. Overall, our data indicate that the Ashby Lake pumpkinseed have not become separate species, but rather represent a population in the early stages of phenotypic divergence along the speciation continuum.

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Table 1. Summary of the stomach contents of pumpkinseed (*Lepomis gibbosus*) collected from the littoral and pelagic habitats. The mass and proportion of total stomach mass estimates for each other the four prey groups are presented as the mean ± 1 standard error. Test statistics (t-stat, df, and p-value) comparing the proportion of stomach content mass between collection habitats are also shown.

	Mean mass (mg)				Mean proportion of diet		Habitat comparison		
	Littoral	n	Pelagic	n	Littoral	Pelagic	*t*-stat	df	*P*-value
Zooplankton	0.65 ± 0.31	32	14.04 ± 5.91	32	0.09 ± 0.04	0.50 ± 0.08	4.44	62	< 0.001
Molluscs	3.23 ± 1.04	32	5.31 ± 4.54	32	0.20 ± 0.06	0.10 ± 0.30	-1.01	62	0.31
Benthic prey	21.08 ± 4.24	32	29.98 ± 25.70	32	0.39 ± 0.07	0.13 ± 0.06	-2.52	62	0.01
Other	7.24 ± 2.46	32	21.36 ± 13.15	32	0.32 ± 0.07	0.27 ± 0.06	-0.70	62	0.49

Table 2. Summary of the isotopic compositions and mixing model diet estimates of pumpkinseed (*Lepomis gibbosus*) collected from the littoral and pelagic habitats over three sampling periods. Isotopic compositions of liver tissue are presented as the mean (± 1 SD). SIAR mixing model estimates of the proportion of diet from littoral resources are presented for each sex (and combined) for each sampling period; estimates are presented as the mean and 95% Bayesian credibility interval values.

				SIAR – Proportion Littoral Estimates		
Sampling Period	Collection Habitat	$\delta^{13}C$	$\delta^{15}N$	Males	Females	Sexes Combined
Spring 2011	Littoral	–25.5 ± 2.0	+6.4 ± 1.1	0.82 (0.67 – 0.98)	0.61 (0.46 – 0.76)	0.71 (0.59 – 0.82)
	Pelagic	–26.6 ± 1.5	+7.5 ± 0.7	0.55 (0.42 – 0.67)	0.51 (0.32 – 0.65)	0.53 (0.44 – 0.63)
Summer 2011	Littoral	–23.1 ± 1.9	+6.1 ± 0.7	0.75 (0.49 – 1.00)	0.59 (0.22 – 0.96)	0.68 (0.54 – 0.82)
	Pelagic	–24.0± 1.7	+6.8 ± 0.5	0.55 (0.21 – 0.87)	0.48 (0.20 – 0.75)	0.54 (0.34 – 0.73)
Spring 2012*	Littoral	–23.0 ± 1.3	+6.7 ± 0.5	0.68 (0.52 – 0.84)	--	--
	Pelagic	–25.7 ± 0.7	+7.5 ± 0.3	0.30 (0.19 – 0.42)	--	--

*Only nesting parental males were collected in Spring 2012 (see methods for details)

Figure Legends

Figure 1. Discriminant function analysis (DFA) of body shape variation among four groups of pumpkinseed (*Lepomis gibbosus*): littoral males, littoral females, pelagic males, pelagic females. The plot depicts the mean (± 1 SD) values of each pumpkinseed group for the first two discriminant function axes (DFA 1 and DFA 2). Thin-plate splines below the scatterplot depict the maximum and minimum observed values for each DFA axis at 3× magnification.

Figure 2. Boxplots of SIAR isotope-mixing model estimates of the littoral prey resource contribution to the diets of male and female pumpkinseed (*Lepomis gibbosus*) collected from the littoral and pelagic habitats. Stable isotopic compositions of liver tissues were used in independent mixing models for each sex and sampling period. The boxplots represent the inner 50% of observations, with the mean value indicated by the line within each box. The whiskers represent the 90^{th} and 10^{th} percentiles and dots are the 95^{th} and 5^{th} percentiles.

Figure 3. Relationship between body condition and ecomorph scores of male and female pumpkinseed (*Lepomis gibbosus*) collected from the (a) littoral and (b) pelagic habitats. Condition was estimated using Fulton's condition factor. Ecomorph scores were generated for each individual based on DFA 1 scores and % Littoral diet values (see methods for details).

Figure 4. Relationship between nesting male ecomorph scores and egg isotopic composition of pumpkinseed (*Lepomis gibbosus*) collected from nests in the pelagic (○) and littoral (●) habitats. Ecomorph scores for each nesting male were generated based on DFA 1 morphology scores and % Littoral resource use estimates (see methods for details). The % Littoral

estimates of eggs for each nest (SIARsolo mixing models; see methods) were used as a proxy for maternal diet and ecomorph.

Figure 5. Genetic clustering of individual pumpkinseed (*Lepomis gibbosus*) collected from the littoral and pelagic habitats. Each vertical bar represents one individual, presented in rank order based on ecomorph scores, and indicating proportional membership coefficients in the two genetic clusters modelled by Structure.