

Natural selection and the evolution of replicated trophic polymorphisms in pumpkinseed sunfish (*Lepomis gibbosus*)

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ABSTRACT

Diversifying selection is expected to operate through all phases of adaptive divergence. If selection is not shown to be diversifying at the earliest stages of divergence when phenotypes are minimally differentiated, then this theory can be challenged. We test for evidence of diversifying selection between lake environments in the nascent divergence of pumpkinseed sunfish (*Lepomis gibbosus*), a divergence that is embedded in the apparent adaptive radiation of North American sunfishes. Pumpkinseed individuals primarily inhabit and appear partially adapted to either inshore littoral or offshore pelagic habitats and resources, the same environmental axes that differentiate various sunfish taxa. We first demonstrate associations between phenotype, lake habitat and diet between ecomorphs in three populations from the Mazinaw region of Ontario. This confirms that the range of pumpkinseed trophic polymorphism extends beyond the Adirondack region of New York State. Second, we show a replicated pattern of divergence between ecomorphs across 26 populations in Ontario and New York, for four of ten external body form traits predicted to influence habitat-specific swimming and foraging performance. Selection is implicated because replicated divergence among populations at this larger regional scale is unlikely to have arisen through random processes. Plastic responses to environmental conditions contribute to body form variation in sunfish, but preliminary evidence from other studies suggests that it is the plastic developmental system that has begun to diverge between ecomorphs.

Keywords: adaptive divergence, Centrarchidae, morphology, phenotypic plasticity, resource polymorphism, thin-plate splines.

INTRODUCTION

Diversifying selection imposed by resources, environments and interactions with other species is thought to be the mechanism that drives adaptive divergence and, in some cases, the formation of new species (Schluter, 2000). Under this theory, natural selection is expected to operate continuously through all phases from the earliest stages of divergence

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to the formation of species and beyond (Simpson, 1953; Dieckmann and Doebeli, 1999; Kondrashov and Kondrashov, 1999). Diversifying selection imposed by resources has been tested in a variety of recently diverged animal taxa, or more obviously divergent subspecies where intermediate forms are already rare and where gene flow is already noticeably restricted (Schluter and Grant, 1984; Smith, 1993; Benkman, 2003; Schluter, 2003; see examples in Schluter, 2000). In contrast, evidence of diversifying selection early on in divergence when phenotypic differentiation is minor has been relatively rare in recent reviews (Schluter, 2000, 2001; but see Via, 2001). Few examples are known among minimally differentiated ecomorphs, particularly where substantial numbers of intermediate forms still occur between more divergent forms (Hori, 1993; McLaughlin *et al.*, 1999). This situation is expected, however, to be the starting condition for adaptive divergence. This paucity of examples at the beginning of divergence challenges our assumption that selection plays a key role early in adaptive divergence.

Here we test for evidence of diversifying selection in a young divergence involving pumpkinseed sunfish (*Lepomis gibbosus*) in post-glacial northern lakes (Robinson *et al.*, 1993, 1996, 2000; Robinson and Wilson, 1996). Trophic polymorphism, an association between divergent phenotypes and habitats, is found in numerous populations of lake fishes, where individuals appear adapted to use resources in either the inshore littoral or offshore pelagic habitat. Polymorphism in sunfish (including that of bluegill sunfish; Ehlinger and Wilson, 1988; Ehlinger, 1990) is interesting because it is embedded in the historical adaptive radiation of North American sunfishes (Wainwright and Lauder, 1992), and occurs along the same resource and habitat axes as those related to the formation of bluegill and pumpkinseed sunfish taxa. Centrarchid sunfishes exhibit many of the hallmarks of adaptive radiation: common ancestry, phenotypic–environment association and the functional utility of traits. Studies of DNA sequences from mitochondria and nuclear genes indicate that the 31 extant taxa form four monophyletic clusters corresponding into genus-level lineages (D. Bolnick, personal communication). Divergence has occurred in almost every major dietary mode known in fishes (Wainwright and Lauder, 1992), including planktivory (e.g. bluegill sunfish), piscivory (e.g. large- and smallmouth bass, crappie) and benthivory (e.g. rock bass, green sunfish). Species are either resource specialists or generalists, and variation in feeding-related traits appears adaptive because it is strongly related to resource use among taxa and influences feeding performance (Lauder, 1983a,b; Mittelbach, 1984; Wainwright and Lauder, 1992). Resource use also responds predictably to competition between closely related taxa (Werner and Hall, 1976, 1979). Our extensive understanding of the nature and consequences of phenotypic variation in this system provides an opportunity to test Simpson's (1953) prediction that selection should cause similar populations to diverge in the same way when exposed to the same set of environmental conditions in different episodes.

During adaptive divergence, populations accumulate and integrate traits that positively affect fitness under different environmental conditions (Mayr, 1963). This results in a strong association between phenotype and resource use among closely related species, such as those seen among many sunfish species. Taxa that exhibit such an association within single phenotypically variable populations are referred to as trophic or resource polymorphisms, and many examples are found in freshwater fishes that inhabit low diversity lakes (Robinson and Wilson, 1994; Wimberger, 1994; Skulason and Smith, 1995; Smith and Skulason, 1996; Robinson and Schluter, 2000). This has led to speculation that trophic polymorphisms may provide insights into early adaptive divergence and perhaps the processes leading to the

formation of new fish species (Lu and Bernatchez, 1999). However, evidence of diversifying selection in trophic polymorphism of fishes is still rare and more evidence is required (Hori, 1993; Robinson *et al.*, 1996; McLaughlin *et al.*, 1999).

Diversifying selection is presumably difficult to test at the beginning of divergence because phenotypic variation is often limited, and the relationship between phenotype and fitness is weak and easily swamped by environmental variation. One approach is to test the prediction that if selection favours divergent phenotypes between alternate environments, then the pattern of phenotypic divergence should be replicated among different populations faced with that same set of environments (Simpson, 1953). Replicated patterns of environmentally related phenotypic divergence among independent populations is strong evidence of selection because a consistent association between particular phenotypes and environments is unlikely to have resulted from other non-ecological mechanisms that can also cause divergence, such as drift or founder effects (Schluter, 1988; Rundle *et al.*, 2000; Robinson and Parsons, 2002). We apply this logic to test for evidence of replicated patterns of divergence among trophically polymorphic populations of pumpkinseed sunfish. We predict that the phenotype–environment association within populations will be replicated with respect to external body form traits primarily expected to influence swimming and feeding performance among populations faced with the same pair of lake environments.

One potential problem is that any environmental gradient common to different lakes could also induce plastic phenotypic responses that are replicated among populations, mimicking an evolutionary response to local diversifying selection. Therefore, some aspects of the divergent phenotypes must reflect heritable differences to infer that divergence represents an evolutionary rather than solely a developmental response to the local environment. We present evidence for heritable variation in body form in our discussion.

We make three general predictions about how diversifying selection favours the adaptive divergence of phenotypes between shallow littoral and open-water pelagic environments. First, we expect a strong association between phenotype and habitat (or resource use). We test this in three populations of pumpkinseed sunfish that inhabit lakes in the Mazinaw region of Ontario, Canada (Fig. 1). These lakes are ecologically similar to others in New York and Ontario where pumpkinseed trophic polymorphisms have been found (e.g. Robinson *et al.*, 2000; Gillespie and Fox, 2003). Second, if diversifying selection operates frequently between these two lake environments, then we predict that the divergence of body form among ecomorphs will be replicated among polymorphic populations. Third, if the divergence is adaptive, then the replicated patterns of divergence should conform to functional expectations derived from biophysical theories of fish locomotion (see the left-hand side of Table 1). The second and third predictions are tested at a regional scale, using seven populations in Ontario (three from the Mazinaw region described here and four from the Kawartha region identified by Gillespie and Fox, 2003) and 19 populations in the Adirondack region of northern New York State (Robinson *et al.*, 2000). We assume that this larger regional comparison reflects a greater evolutionary independence of populations than is found within any region (an assumption that is being tested in an ongoing population genetic study).

METHODS

We refer to sunfish from pelagic and littoral habitats collectively as a lake's population, and assume that the habitat where pumpkinseeds were captured represents their long-term

Table 1. Description and potential functional utility of 16 linear measurements of external body form used in the meta-analysis of 26 populations of pumpkinseed sunfish from the Mazinaw, Kawartha and Adirondack regions of Ontario and New York State

Trait / Truss	Predicted larger ecomorph	Functional Interpretation	Citations	No. of populations where trait was larger in:		Binomial test p-value	Consistently larger ecomorph
				Pelagic	Littoral		
1) Traits predicted to influence habitat-specific performance:							
Pre-pectoral length	L	Enlarged buccal area allows feeding on larger and harder macrobenthic prey.	Wainwright and Richard 1995, Mittelbach et al 1999	6	18	0.023	L
Pre-pelvic length	L			6	20	<0.01*	L
Pectoral fin insertion width	L	Larger paired fins increase maneuverability, hovering capacity, and median-paired fin locomotion.	Webb 1983, Webb 1984, Weihs and Webb 1984	10	14	0.54	-
Anterior dorsal fin to anterior pelvic fin (mid body height)	L	Greater body depth provides lateral stability, and increases maneuverability. Shallow body depth reduces drag during sustained swimming.	Webb 1983, Webb 1984, Weihs and Webb 1984, Walker 1997	5	19	<0.01*	L
Posterior dorsal fin to anterior anal fin	P	Greater rear body height increases thrust and body/caudal fin acceleration.	Webb 1984, Weihs and Webb 1984, Webb 1986, Walker 1997	8	14	0.29	-
Anterior caudal peduncle depth	P			13	10	0.68	-
Posterior caudal peduncle depth	P	Increased Caudal Peduncle surface area for increased thrust, acceleration, and quick starts.	Webb 1984, Weihs and Webb 1984, Webb 1986, Walker 1997	14	11	0.69	-
Diagonal caudal peduncle depth	P			17	1	<0.0001*	P
Dorsal caudal peduncle length	P	Increased amplitude yields increased thrust, acceleration, and quick starts.	Webb 1984, Weihs and Webb 1984, Webb 1986, Walker 1997	18	6	0.02	P
Ventral caudal peduncle length	P			20	4	<0.001*	P
2) Traits not known to influence habitat-specific performance:							
Pre-Dorsal Length				7	17	0.06	-
Dorsal Fin Base Length				8	13	0.38	-
Anal Fin Base Length				9	15	0.31	-
Anterior Anal to Anterior Pelvic				17	8	0.11	-
Anterior Dorsal to Anterior Anal				5	17	0.02	L
Pectoral Fin Altitude (from dorsal)				7	13	0.26	-

Note: Measurements are grouped into two categories. (1) Traits expected to influence habitat-specific swimming and foraging performance (see embedded references), and the associated prediction of which ecomorph should have the qualitatively larger trait value (L = littoral, P = pelagic). (2) Additional traits for which there is no specific functional prediction. The right-hand side of the table gives the results of separate binomial tests of the null hypothesis that in half of the populations, littoral ecomorphs had the larger mean value for the trait. Two-sided *P*-values are reported and values with an asterisk (*) are significant at a table-wide alpha level of 5% following a sequential Bonferroni adjustment over the 16 traits.

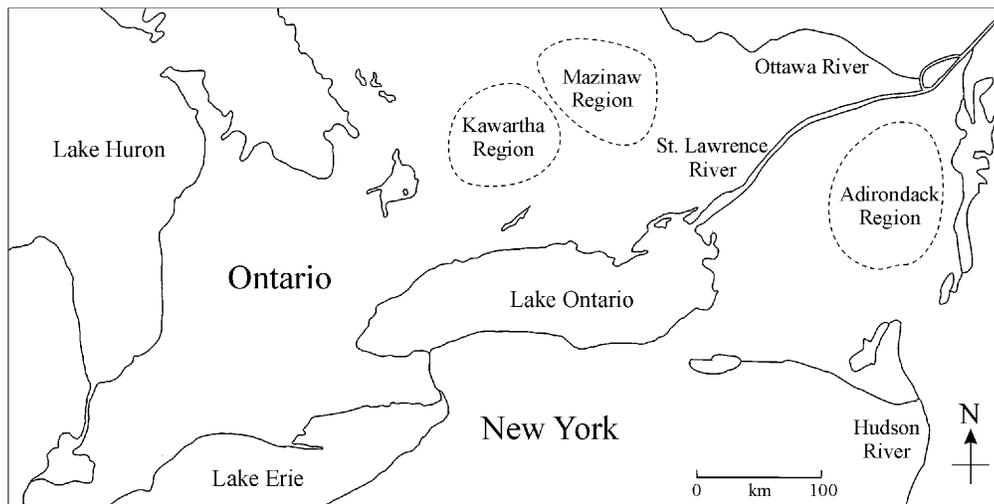


Fig. 1. General locations of lake populations of pumpkinseed sunfish used in this study. We assessed the association between phenotype and lake environment in sunfish sampled from Ashby, Mayo and Salmon Trout Lakes in the Mazinaw region of Ontario (Table 2), all part of the Ottawa River drainage. Variation in body form in Mazinaw region sunfish was then compared with similar data collected by Gillespie and Fox (2003) from five lakes in the Kewartha region (Lake Ontario drainage) and 19 lakes studied by Robinson *et al.* (2000) in the Adirondack region of northern New York State (northern and southern halves are part of the St. Lawrence River and Hudson River drainages, respectively).

feeding habitat (hereafter, the ‘native’ habitat). This is because: (a) tagged pumpkinseeds showed high levels of habitat and site fidelity when transplanted between habitats in a nearby polymorphic population (Gillespie and Fox, 2003); (b) ecomorphs exhibit differences in habitat-specific parasites (Robinson *et al.*, 2000); and (c) our surveys found pumpkinseeds inhabiting both habitats at all times of day between May and October from 1999 to 2001 (see also Robinson *et al.*, 2000).

Ontario study populations and sample collection

Pumpkinseeds were observed and collected from three lakes in the Mazinaw region of eastern Ontario (approximately 30 km east of Bancroft), which drain northeast into the Ottawa River (Fig. 1). All three lakes are on the rocky Canadian shield and possess shoals (submerged or slightly emerged outcroppings composed of rock or rubble) used by pumpkinseeds in or near the pelagic environment, as well as littoral habitat along the lake margins (lake characteristics and sampling information are provided in Table 2). Ashby and Mayo Lakes both have relatively extensive pelagic habitat punctuated by many islets and rocky shoals. The littoral habitat of Ashby Lake is relatively small, and limited by a rocky shoreline that drops quickly into the deeper pelagic habitat. Relatively more littoral habitat is present in Mayo Lake, although macrophyte abundance is low and restricted to several shallow bays. Salmon Trout Lake is elongate in shape, with numerous peninsulas tipped with submerged shoals extending into the pelagic habitat. Littoral habitat composed of a

Table 2. Physical characteristics and sampling information for Ashby, Mayo and Salmon Trout Lakes in the Mazinaw region of Ontario

Physical characteristics	Ashby Lake	Mayo Lake	Salmon Trout Lake
Location	45°05'N, 77°21'W	45°02'N, 77°35'W	45°11'N, 77°49'W
Size (ha)	259	182	100
Maximum depth (m)	36.6	38.1	14.0
<i>Sampling information</i>			
Years sampled in this study	1999, 2000	1999	1999
Sample size (littoral, pelagic)	244, 205	43, 58	17, 14
Mean standard length (mm):			
Littoral ± standard error	93.2 ± 1.2	96.0 ± 2.0	94.2 ± 5.4
Pelagic ± standard error	99.4 ± 1.3	104.6 ± 1.6	111.3 ± 8.6
<i>Fish species composition</i> ^b	1, 2, 4, 5, 7, 10, 12	1, 2, 4, 5, 6, 7, 9, 11, 13	2, 3, 5, 7, 8 ^a

Note: Physical characteristics are from unpublished Ontario Ministry of Natural Resources (OMNR) data. Fish species composition represents OMNR data combined with personal observations for each lake.

^a Although bluegill sunfish are recorded by the OMNR as being present in Salmon Trout Lake, they have not been observed or captured there over three summers of research, and are assumed to not be in the lake.

^b Fish species coding: 1 = lake trout (*Salvelinus namaycush*), 2 = common white sucker (*Catostomus commersoni*), 3 = walleye (*Stizostedion vitreum*), 4 = yellow perch (*Perca flavescens*), 5 = smallmouth bass (*Micropterus dolomieu*), 6 = largemouth bass (*Micropterus salmoides*), 7 = pumpkinseed sunfish (*Lepomis gibbosus*), 8 = bluegill sunfish (*Lepomis macrochirus*), 9 = rock bass (*Ambloplites rupestris*), 10 = fallfish (*Semotilus corporalis*), 11 = creek shrub (*Semotilus atromaculatus*), 12 = bluntnose minnow (*Pimephales notatus*), 13 = Iowa darter (*Etheostoma exile*).

sandy or muddy bottom and large beds of macrophytes occurs in the bays at each end of this lake.

Fish were sampled from multiple littoral and pelagic sites in each lake by trapping with wire funnel traps (35 cm in diameter, 90 cm long, 8 cm diameter aperture) and by angling. Littoral samples were taken from vegetated areas along the shoreline, no deeper than the extent of emergent macrophytes (generally < 1.5 m deep and < 5 m from shore). Pelagic samples were collected from the perimeter of shoals adjacent to deeper water, generally at depths of 1–4 m. The Ashby population was sampled repeatedly over two years (1999, 2000), while the Mayo and Salmon Trout populations were sampled only in 1999. Fish were euthanized using 250 ppm clove oil, fixed in 10% buffered formalin for 3 months and rinsed in water before being stored in 70% ethanol.

Phenotype–habitat associations in the Ontario populations

Variation in body and gill raker form

We quantified variation in external body form using a landmark-based geometric technique involving thin-plate splines (Bookstein, 1991). Digital images of the left side of each fish were taken with fins extended, and calibrated against a known size standard. The x and y coordinates of 15 homologous landmarks (Fig. 2) were digitized using the program TPSdig (Rohlf, 2001a). Partial warp scores were then computed for each population using the program TPSrelw, with each population's consensus (average) body form as a standard (Rohlf, 2001b). Partial warp scores represent the changes in multivariate shape space

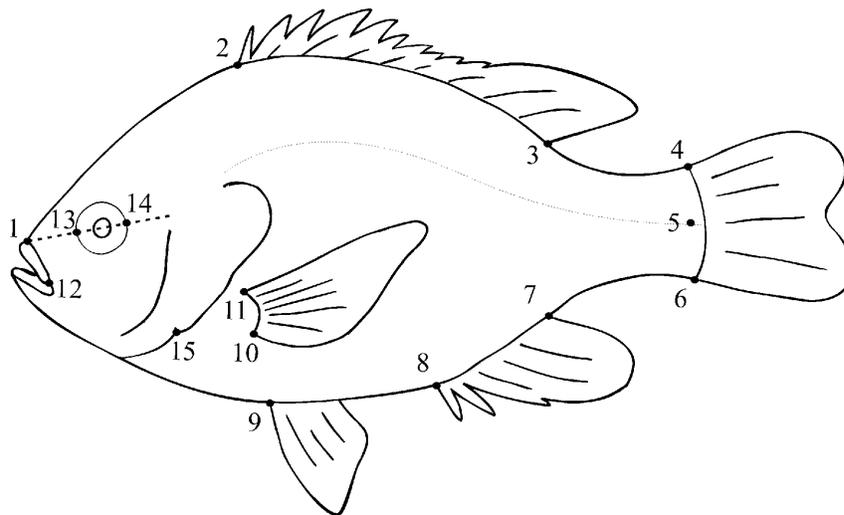


Fig. 2. Location of 15 homologous landmarks used to study variation in body form. Landmarks were recorded from digital images of the left side of each sunfish that included a size reference standard. Both the geometric and linear truss-based morphometric analyses used the same landmarks. Refer to this diagram for clarification of landmark locations shown on the deformation grids provided in Fig. 3.

between each focal individual and the average form for that population independent of body size (one x and one y uniform, and 12 x and 12 y non-uniform, partial warps were estimated for each fish).

We were most interested in estimating and comparing patterns of variation in body form related to lake habitat, and not in estimating the proportion of total phenotypic variance attributed to habitat. To accomplish this, we analysed variation in body form using multivariate discriminant function analysis (DFA) of the partial warps generated above (uniform and non-uniform) with respect to the two habitats for each population. We chose not to use principal components analysis (PCA) because it is better suited for summarizing variation in complex correlated data sets and confounds within- and among-group components of variation (Manley, 1994).

The DFA was used to test for evidence of phenotypic divergence in each population, and to estimate a canonical factor score for each individual that summarized multivariate information about its external body form. Since individual DFA factor scores did not identify particular traits that differentiated co-existing ecomorphs, we visualized variation in body form by regressing these scores against the partial warp scores above using TPSreg (Rohlf, 2000). The program TPSreg was then used to create deformation grid plots that displayed how the shape of each individual varied relative to the local average form.

Gill raker length was measured on the first left-hand gill arch, which was excised from each fish and stained with alizarin red (Bell, 1984). The length of the second gill raker down from the apical raker (on the distal portion of the arch) was estimated as the mean of two length measurements from the tip of this raker to both its upper and lower insertion points into the arch using an ocular micrometer at $40\times$ magnification. Analysis of covariance was used to compare mean raker lengths (transformed to their natural logarithms) between

littoral and pelagic groups with multivariate centroid body size as the covariate for each population.

Variation in diet

We tested our prediction that variation in body form would be related to differences in resource use by comparing the stomach contents of a random sub-sample of littoral ($n = 56$) and pelagic ($n = 58$) fish collected in June 2000 from Ashby Lake. We were unable to analyse the diets of fish collected from Mayo and Salmon Trout Lakes because ineffective preservation in 1999 resulted in the digestion of stomach contents. However, observations of informal field dissections of at least a dozen stomachs from these two populations were available for comparison with the results from the Ashby Lake population.

For every stomach sub-sampled from the Ashby population, each prey item was identified to Order, then grouped into one of six functional prey categories: benthic prey (larval Trichoptera, Plecoptera, Ephemeroptera, Odonata, Chironomidae; Amphipoda, Decapoda), molluscs (Gastropoda, Bivalvia), cladocerans (*Daphnia*, *Bosmina*), Hydra-carina (occurs in the water column in both habitats), non-aquatic invertebrates (adult insects and adult Odonata) and other (vegetation, seeds, sand). Only fish with four or more prey items per stomach were included in this analysis. The proportion of each prey category (number of prey/total number of prey, in that stomach) was transformed to its arcsin square-root (Zar, 1999), and the transformed mean proportions were then compared between littoral and pelagic samples using Welch's *t*-tests. The diet-wide alpha-level was maintained at 5% across the six prey categories using a sequential Bonferroni adjustment (Rice, 1989).

Comparing patterns of divergence among populations

If diversifying selection consistently favours the evolution of the same pair of divergent phenotypes between the two habitats, then patterns of trait divergence should be replicated among polymorphic populations. We tested for replicated divergence between ecomorphs at the larger regional scale using 26 lake populations from three geographic regions (Fig. 1). We analysed the three Mazinaw populations described here (Ottawa River watershed), four populations from the Kawartha region in the Lake Ontario watershed of Ontario approximately 70 km southwest of the Mazinaw region (Looncall, Monck, Shadow and Wolf Lakes; Gillespie and Fox, 2003), and 19 populations from the mountainous Adirondack region of northern New York State (northern half in the St. Lawrence River watershed, and southern half in the Hudson River watershed; Robinson *et al.*, 2000; and unpublished data). Pumpkinseeds were sampled from littoral and pelagic habitats in all lakes using methods and habitat criteria similar to those outlined above.

Both Gillespie and Fox (2003) and Robinson *et al.* (2000) used conventional linear truss lengths between pairs of landmarks in their analysis of external body form that could not be used in a geometric analysis. For this reason, we converted our geometric data into 16 linear measurements of body form that were common to all studies (Table 1 and Fig. 2). The earlier studies also differed with respect to how body size was assessed, which can influence quantitative differences in truss lengths between ecomorphs when body size is included as a covariate. Thus, our test of consistency in the form of the differences between the mean littoral and pelagic value for each trait was based on their rank size in each population. Following Gillespie and Fox (2003), we analysed the effects of habitat on every trait in each

population using analysis of covariance (ANCOVA) with multivariate centroid body size as covariate (truss lengths and body size were first transformed to their natural logarithm). Adjusted least-squares means were then used to determine the rank size of each ecomorph for that trait when slopes were homogeneous between forms; otherwise, no determination was made. We used ANCOVA solely to estimate the qualitative difference between ecomorphs. Statistical significance was estimated using a binomial test.

Consistency in the qualitative difference between ecomorphs for each trait was tested using a binomial test across those populations where slope homogeneity was found. We tested the null proportion of 0.5 (reporting two sided *P*-values), indicating that both littoral and pelagic ecomorphs would each have the larger value for all traits in 50% of the populations. We applied a sequential Bonferroni correction to each test to maintain an overall alpha level of 5% for the 16 traits. We interpreted statistical significance as evidence of consistent differentiation between ecomorphs among the populations (e.g. significantly more than half of the populations shared the same qualitative difference).

RESULTS

Phenotype–habitat associations in the Ontario populations

Variation in body and gill raker form

Separate DFA analyses of external body form indicated significant differences between pumpkinseeds sampled from littoral and pelagic sites in all three Mazinaw lakes (Table 3). Littoral pumpkinseeds from each of the three Mazinaw populations had enlarged heads (particularly in the eye and buccal areas), wider pectoral fin insertions, and reduced depths of mid-body and tail regions compared with pelagic samples, resulting on average in a more ‘forward-weighted’ phenotype (Fig. 3). Pelagic pumpkinseeds tended to have a more ‘rear-weighted’ body form, characterized by a smaller head region and a deeper mid- and rear-body (particularly in the region of the anterior caudal peduncle). We will return to the functional assessment of this variation after our test of replicated divergence below.

Table 3. Results of discriminant functional analyses performed on sunfish populations from Ashby, Mayo and Salmon Trout Lakes in the Mazinaw region of Ontario

Population	Wilks' λ	<i>F</i> (<i>v</i>)	<i>P</i> -value	Littoral		Pelagic	
				% correct classification	Total # of individuals	% correct classification	Total # of individuals
Ashby Lake	0.442	20.5 (26,442)	<0.0001	85	244	89	205
Mayo Lake	0.443	3.58 (26,74)	<0.0001	84	43	88	58
Salmon Trout	0.018	8.51 (26,4)	0.03	100	17	100	14

Note: Tests for each lake were performed independently of the others, and were based on 13 x,y partial warp scores (12 uniform and one non-uniform) from a geometric morphometric analysis.

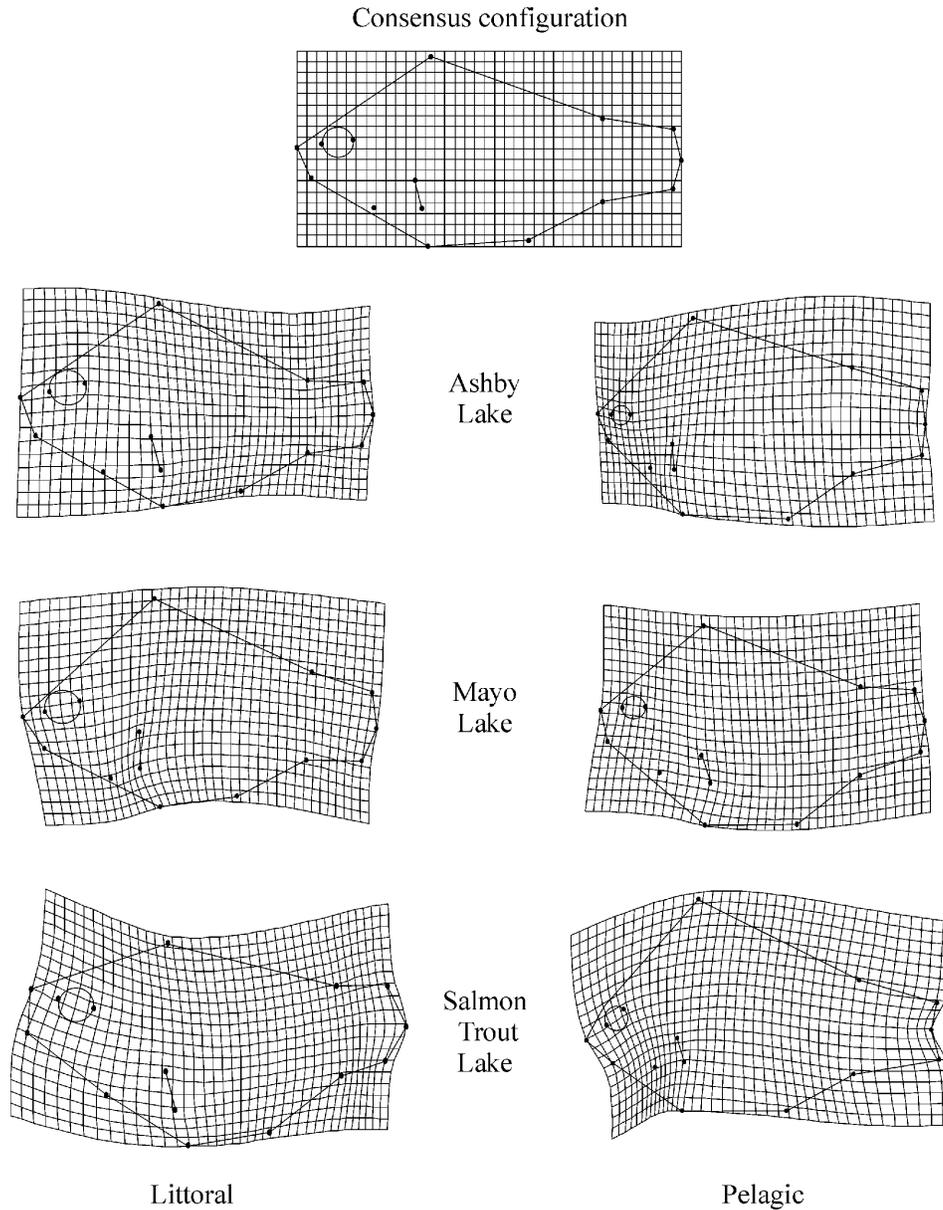


Fig. 3. The variation in external body form (deformation grid plots) between littoral and pelagic ecomorphs in each of the three Mazinaw lakes. The deformation of the gridlines in the outer figures represents the difference in the body form of the most extreme form in each local ecomorph compared with the mean form for all fish combined (consensus configuration shown at top centre for reference). The outlined form is a guide to locate landmarks and does not represent the location of the true body outline (see Fig. 2). For clarity, shape change was magnified 10 times.

Sunfish collected during 1999 and 2000 from Ashby Lake were combined because variation in body form did not vary significantly between years (Welch's *t*-test of DFA scores: littoral samples: $t = -1.10$, $\nu = 122.5$, $P = 0.27$; pelagic samples: $t = 0.49$, $\nu = 97.6$, $P = 0.63$). The average body form of female sunfish was significantly more littoral-like in both habitats of Ashby Lake (Welch's *t*-test of DFA scores: littoral samples: $t = -2.63$, $\nu = 175$, $P = 0.01$; pelagic samples: $t = -2.81$, $\nu = 142$, $P = 0.01$). Sexual dimorphism in body form was ignored in subsequent analyses because the sex of fish could not be reliably determined in the 1999 samples due to deteriorated gonads. Also, sexual dimorphism should not bias habitat-specific variation in body form because sex ratios were similar in both habitats of Ashby Lake in 2000 (females comprised 41% and 40%, respectively, of 178 littoral and 144 pelagic fish sampled).

Pelagic pumpkinseeds from both Ashby and Mayo Lakes had on average significantly longer gill rakers than their littoral counterparts (ANCOVA habitat effect: Ashby, $F_{1,440} = 115.8$, $P < 0.0001$; Mayo, $F_{1,93} = 5.29$, $P = 0.02$). Gill raker lengths were not significantly longer in pelagic pumpkinseeds from Salmon Trout Lake (ANCOVA, $P = 0.15$). The relationships between gill raker length and body size were consistent across the range of body sizes in each population. (There was no evidence of an interaction between gill raker length and body size; all $P > 0.31$.)

Variation in diet

There were large differences in the diets of sunfish sampled from littoral and pelagic sites in Ashby Lake in all six functional prey categories (Table 4). The diets of littoral pumpkinseeds were more generalist, consisting of mostly benthic prey. Prey more commonly found in pelagic lake habitats occurred only at low frequency in the stomachs of littoral pumpkinseeds (Fig. 4). In contrast, the diets of pelagic pumpkinseeds were highly specialized on zooplankton, particularly the larger *Daphnia* (99% of the diet by counts of prey items), and benthic prey were almost completely absent from the diets of pelagic fish. Informal field dissections of pumpkinseeds sampled from Mayo and Salmon Trout Lakes in 1999 also indicated that zooplankton dominated the diet in pelagic fish, while the diets of littoral fish were again dominated by large benthic macroinvertebrates.

Table 4. Comparison of stomach contents of pumpkinseeds sampled from littoral and pelagic habitats in Ashby Lake in 2000

	Mean proportion in diet		Habitat comparison	
	Littoral zone	Pelagic zone	<i>t</i> -value (d.f.)	<i>P</i> -value
Benthic prey	0.56	1.8×10^{-2}	11.7 (73.8)	<0.0001
Molluscs	0.14	3.4×10^{-3}	4.01 (57.2)	0.0002
Cladocera	0.03	0.98	-36.2 (111.6)	<0.0001
Hydracarina	0.05	4.2×10^{-5}	3.05 (55.1)	0.0035
Terrestrial insects	0.17	2.5×10^{-4}	6.46 (55.2)	<0.0001
Other (e.g. vegetation)	0.05	4.1×10^{-4}	2.64 (55.65)	0.01

Note: Mean proportions represent the proportion by item abundance (number) in the stomachs for each prey category averaged in each habitat. Habitat comparisons were performed using Welch's *t*-tests on arcsin square root transformed diet proportions. Each habitat comparison was statistically significant at a table-wide alpha level of 5%, adjusted using a sequential Bonferroni correction. See Fig. 4 for the distribution of prey items in diets.

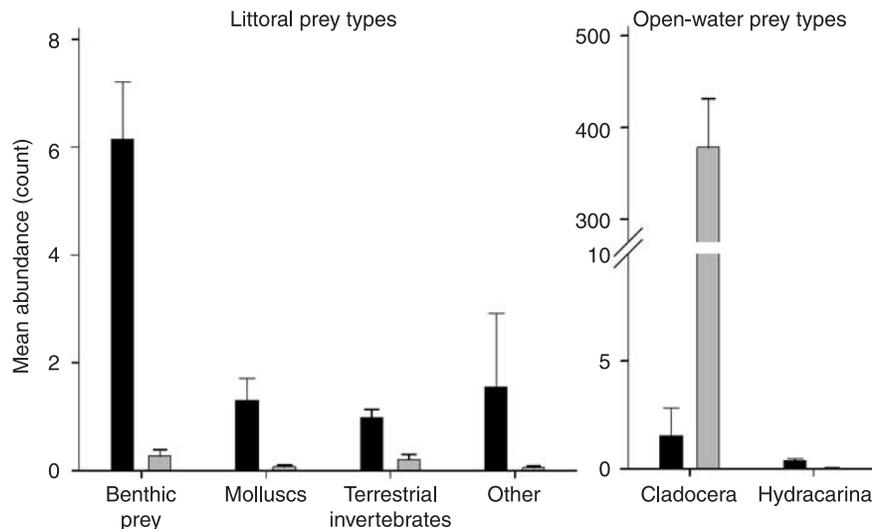


Fig. 4. Variation in the diets of sunfish sampled from littoral (black bars, $n = 56$) and pelagic (grey bars, $n = 58$) habitats in Ashby Lake in June 2000. Bars represent the mean number of prey items per stomach (± 1 standard error) in each of six functional prey categories. Differences between habitats were significant for each prey category (see Table 4).

Testing patterns of divergence among populations in Ontario and New York

We tested for replicated patterns of divergence in external body form among 26 populations from the three geographic regions using the null hypothesis that rank trait order would vary randomly between co-existing ecomorphs. Four of the 16 trusses varied consistently in response to habitat across populations (pre-pelvic length, mid-body height, diagonal caudal peduncle size and ventral caudal peduncle length; Table 1). There was weaker evidence that three additional trusses also varied consistently (individual alpha levels $< 5\%$: pre-pectoral length, the length from the dorsal fin origin to anal fin origin, and dorsal caudal peduncle length). The locations of these additional traits correspond to body regions defined by the significant traits described above. This suggests that these traits are probably correlated with the others, and collectively relate to differences in head size, body depth and caudal peduncle size. Littoral ecomorphs had consistently larger heads and deeper mid-body depths, but generally smaller caudal peduncles, compared with pelagic ecomorphs. Of the seven traits contributing to differences between ecomorphs, six were identified *a priori* as having potential functional utility with respect to swimming and foraging performance, and all of these varied between ecomorphs in expected directions (Table 1).

DISCUSSION

Trophic polymorphism in the Mazinaw sunfish

The association between phenotype, lake habitat and diet in pumpkinseed sunfish from Ashby, Mayo and Salmon Trout Lakes suggests that the trophic polymorphism first described in post-glacial lakes of the Adirondack region of New York (Robinson *et al.*,

1993, 2000) and recently in the Kawartha region of Ontario (Gillespie and Fox, 2003) also occurs in lakes in the Mazinaw region of Ontario. The polymorphism has now been documented in lakes from three major drainages of the St. Lawrence River (north shore of Lake Ontario, west shore of the Ottawa River and south shore of upper St. Lawrence River), some at least 400 km from lakes in the upper Hudson River drainage of New York State where the polymorphism is also found.

Morphology is expected to influence foraging and swimming performance in fishes (Table 1), which are then expected to influence important components of fitness, such as growth rate, fecundity and predator avoidance. For example, larger and more robust jaws influence feeding on macrobenthic prey because mouth gape and pharyngeal jaw size are positively related to maximum prey size and hardness in sunfish (Wainwright *et al.*, 1991a; Wainwright and Richard, 1995). Variation in gill rakers also influences foraging performance in fishes because longer and more closely spaced rakers are consistently and strongly associated with feeding on plankton both within and among taxa (Magnuson and Heitz, 1971; Werner and Hall, 1976; Bodaly, 1979; Lindsey, 1981; Wright *et al.*, 1983; Mittelbach, 1984; Sanderson *et al.*, 1991; Schluter and McPhail, 1992). These foraging-related traits varied consistently among ecomorphs in the Mazinaw populations. The larger heads of littoral fish were related to larger and more robust pharyngeal jaws and shorter gill rakers here as elsewhere (Wainwright *et al.*, 1991b; Robinson *et al.*, 1993; Gillespie and Fox, 2003; unpublished results). The reduced head size of pelagic sunfish was related to less robust pharyngeal jaws and longer or more closely spaced gill rakers (Robinson *et al.*, 1993; Gillespie and Fox, 2003; unpublished results). The variation in this suite of foraging-related traits could explain both the difference in diets and the greater diet diversity of littoral compared with pelagic ecomorphs in Ashby Lake and elsewhere (Keast, 1977; Robinson *et al.*, 1993, 2000; Gillespie and Fox, 2003).

External body form is expected to influence swimming performance when different modes of propulsion are used in different lake habitats (Table 1). We observed more hovering and orientation by pumpkinseeds foraging in littoral than in pelagic habitats (see also Ehlinger and Wilson, 1988; Ehlinger, 1990). Thus, we expected that paired fin locomotion would be an important mode of propulsion in the more structured littoral habitat where prey are cryptic, while caudal propulsion would be the primary form of locomotion in pelagic habitats when feeding on dispersed planktonic prey. We predicted variation in pectoral fin traits because hovering, rotation and orientation are influenced by the location, size and shape of paired fins that generate the necessary thrust and lift (Blake, 1981). We also predicted variation in caudal traits because pumpkinseed foraging on planktonic prey involves a leisurely caudal swimming motion interrupted by short lunges of one to two body lengths ending in a 'mouth flash' as individual prey are sucked into the buccal cavity (Robinson *et al.*, 1993; personal observations). Rapid acceleration is facilitated by deeper caudal peduncles in many fish (Webb, 1983, 1986; Weihs and Webb, 1984), which may improve foraging success on plankton. Body form differences between ecomorphs in the Mazinaw populations generally conformed to these expectations, with the external form of littoral fish being more robust anteriorly, while that of pelagic fish appeared more robust posteriorly (particularly in the caudal region; Fig. 3). Pectoral fin insertion width was greater in littoral than in pelagic fish, but a more detailed analysis of pectoral fin architecture (e.g. area, shape and rigidity) is required before we can begin to understand the biomechanical consequences of pectoral fin divergence here.

Unexpectedly, pelagic ecomorphs in our Mazinaw populations appeared deeper in their mid-body region and therefore somewhat less fusiform than littoral ecomorphs. Variation in body depth is expected to have several consequences for swimming performance in fish. For example, one cost of a deeper body is increased drag, which should be most pronounced in pelagic taxa that swim long distances (Webb, 1984). This suggests that pumpkinseed movements in the pelagic habitat either do not include long-distance cruising, or that increased body depth confers other functional advantages or is related to other functionally useful traits. We have little evidence that pelagic pumpkinseeds travel extensively in search of planktonic prey and instead observe them feeding within 10–20 m of the rocky shoals in the open-water habitat. Alternatively, the deeper body form may improve acceleration during rapid vertical dives down 3 m that we have observed when pelagic pumpkinseeds were startled in the field (a behaviour not observed in littoral fish). Such diving may be an anti-predator response to loons that we observed frequently hunting adjacent to pelagic shoals in Ashby and other lakes in the region (Barr, 1973). A deeper body form may also be advantageous by allowing fish to more rapidly exceed the size threshold of gape-limited predators (Mittelbach, 1984; Nilsson *et al.*, 1995). The possibility that predation varies between lake habitats and also imposes diversifying selection is only beginning to be studied in this system. For example, Gillespie and Fox (2003) found no variation in life-history traits known to diverge in fishes under differential predation risk between lake habitats. Further tests of predation effects are important because empirical evidence consistently indicates that many fishes simultaneously face the ecological pressures of maximizing foraging return while minimizing predation risk (Werner and Hall, 1988; Grand and Dill, 1997; Walker, 1997; Vamosi and Schluter, 2002).

The association between phenotype and short-term habitat and resource use in pumpkinseeds from the three Mazinaw lakes suggests that selection imposed by habitat-specific resources may be qualitatively similar in these and other lakes, and we focus on testing this prediction next.

Replicated patterns of divergence and inferences about selection

Three groups of external body form traits consistently distinguished littoral and pelagic sunfish from 26 pumpkinseed populations in the Mazinaw and Kawartha regions of Ontario and the Adirondack region of New York. This suggests that diversifying selection has contributed to the divergence of sunfish ecomorphs. That these populations came from distinct geographic regions also increases the likelihood that our comparison includes populations that are more evolutionarily independent than populations from within any one region (Fig. 1). An ongoing population genetic analysis is testing phylogenetic hypotheses about evolutionary relationships among ecomorphs from a subset of these populations.

The results of our larger meta-analysis of external body form shared similarities with, yet also differed somewhat from, our Mazinaw results above. Both analyses found that littoral ecomorphs consistently had larger head regions and smaller caudal peduncles than pelagic ecomorphs (Table 1). However, pectoral fin insertion width was not consistently greater in littoral ecomorphs in our meta-analysis. This casts doubt on the functional role of pectoral fin traits in this divergence, or suggests that insertion width alone does not capture how paired fins are diverging between ecomorphs. A more detailed analysis of fin shape, rigidity and location would be required to distinguish between these two alternatives. Also, the depth of mid-body regions was greater in littoral ecomorphs in our meta-analysis as

predicted, but opposite to the trend observed in the Mazinaw populations. We have no clear suggestion as to why divergence in the Mazinaw populations for mid-body depth should differ from that observed elsewhere.

If selection has favoured the evolution of traits that consistently differentiate ecomorphs, such as variation in head size and rear body depth, then phenotype should be related to fitness in different lake habitats presumably through their effect on swimming and/or foraging performance (Table 1). Robinson *et al.* (1996) and Jastrebski (2001) both found evidence that variation in body form in the pelagic lake environment was related to growth rate, age at maturity and condition factor, all components of fitness in fishes. Laboratory feeding studies have shown that juvenile sunfish with more pelagic-like phenotypes consume zooplankton at a higher rate than other phenotypes, suggesting that body form affects foraging performance at least on pelagic prey (but not on amphipods, a benthic prey; Parsons, 2002). Thus, preliminary evidence suggests that the relationship between phenotype and fitness in the sunfish trophic polymorphism is mediated through foraging performance, at least under pelagic conditions.

Traits that directly influence feeding are likely to have greater effects on fitness than those that influence foraging through their effects on swimming performance. Gillespie and Fox (2003) compared pumpkinseeds from five populations in Ontario and found replicated patterns of divergence with respect to gill raker and pharyngeal jaw traits, but not with respect to external body form traits. Our larger study suggests that selection may act on external body form, but that it can only be detected when many more populations are studied. Qualitative evidence of replicated divergence between littoral and pelagic forms across many fish taxa in lakes is known (Robinson and Wilson, 1994; Robinson and Schluter, 2000), but quantitative comparisons of replicated patterns of divergence within single taxa are rare and additional comparative studies are warranted (Walker, 1997).

The evidence also suggests that selection on body form may be stronger under pelagic than littoral conditions. Individual phenotype is related to components of fitness, such as growth and condition factor in the pelagic but not in the littoral lake environment (Robinson *et al.*, 1996; Jastrebski, 2001), and is also related to foraging performance on pelagic zooplankton but not benthic type prey (DeWitt *et al.*, 2000; Parsons, 2002). If the response to selection varies proportionally with the intensity of selection between lake environments, then we may expect less phenotypic variation and/or a more tightly integrated set of traits in pelagic than in littoral ecomorphs. Preliminary evidence exists in support of both of these predictions. Our DFA results indicate that variation in the canonical body form scores of pelagic fish was less than that of littoral fish in Ashby Lake (standard deviation of littorals = 1.1, pelagics = 0.92; Levene's equality of variance test, $F_{1,447} = 6.0$, $P = 0.015$; variation also appears reduced for pelagic fish in both Mayo and Salmon Trout Lakes but were not significantly different). With respect to testing trait integration, K.J. Parsons and B.W. Robinson (unpublished data) have found that the plastic responses of individual body form traits to littoral and pelagic conditions are more coordinated in pelagic than in littoral ecomorphs. The available evidence strongly suggests that the littoral ecomorph is ancestral because the pelagic ecomorph is only found in young post-glacial lakes at the northern extreme of the pumpkinseed distribution, and because studies of habitat use (Werner and Hall, 1976; Mittelbach, 1984) and ecomorphology (Lauder, 1983a; Wainwright and Lauder, 1992) all indicate that pumpkinseed sunfish are adapted to littoral conditions. If this is correct, then our results suggest that the invasion of

the pelagic habitat has placed pumpkinseed sunfish under selection for a new phenotypic optimum.

Our conclusion that diversifying selection is operating in the pumpkinseed trophic polymorphism is preliminary and perhaps simplistic for two reasons. First, it does not explicitly recognize that the fitness landscape between littoral and pelagic habitats may vary among lakes. Second, it does not reflect the role played by phenotypic plasticity in generating phenotypic variation in this system. It is likely that fitness landscapes vary considerably between lakes, depending on the relative size of littoral and pelagic habitats and prey abundance. Nevertheless, trophic polymorphisms in lake fishes are widely argued to have evolved under divergent selection imposed by a fitness landscape composed of two adaptive peaks separated by a valley (Smith and Todd, 1984; Robinson *et al.*, 1996; Schluter, 1996; Smith and Skulason, 1996). Such a landscape could result in the repeated evolution of the association between phenotype and resource use or habitat as found here, but other fitness landscapes can also generate such an association, and so ideally should be ruled out.

One such fitness landscape is an adaptive ridge that can favour the evolution of a correlation between phenotype and fitness across habitats but without any reduction in the fitness of intermediate phenotypes (Schluter, 2000). Trade-offs in the performance of divergent phenotypes between different environments or resources can evolve on an adaptive ridge, and if such a ridge is a common feature of lakes, then replicated patterns of divergence would result. Consequently, neither the existence of trade-offs nor replicated divergence alone are sufficient evidence to conclude that diversifying selection is in operation. The key evidence is that intermediate fitness is reduced relative to more divergent forms (Schluter, 2000). It is also possible for an apparent trophic polymorphism to exist for a period of time on certain fitness landscapes before a single optimal phenotype becomes fixed. For example, if directional selection favoured a pelagic ecomorph in a population initially composed of littoral forms, there could be an intermediate phase where both forms persist before fixation of the pelagic ecomorph. Such an apparent polymorphism is also possible in a population colonized separately by divergent littoral and pelagic ecomorphs (derived, for example, in allopatry) that have begun to hybridize under weak stabilizing selection for an intermediate form. Such alternatives are plausible because pumpkinseeds colonized post-glacial lakes in Ontario relatively recently some 9000 years ago (Mandrak and Crossman, 1992) and their glacial history is as yet unknown. Many trophic polymorphisms in the fishes of post-glacial lakes are equally recent, or were derived from ancestors that survived glaciation in multiple refugia, or were influenced by multiple colonizations, and so may have evolved under circumstances other than local diversifying selection.

Furthermore, it is also possible that the replicated pattern of divergence found in the pumpkinseed system represents a plastic ancestral genotype responding developmentally to the same environmental gradient in each lake rather than independent evolutionary responses to diversifying selection in each population. Replicated patterns of phenotypic divergence between co-existing littoral and pelagic ecomorphs are known in many taxonomic families of lake fishes (Robinson and Wilson, 1994; Robinson and Schluter, 2000) and are unlikely to represent an inherited plastic response at this larger taxonomic scale because so many distantly related groups are involved. However, the same cannot be said of replicated divergence among isolated populations from one taxa. Sunfish body form responds strongly to environmental conditions during development (Robinson and Wilson, 1996; Hegrenes, 1999; Parsons, 2002), and so the extent to which the divergence in body form represents an evolved response must be tested. The best evidence we have to date

comes from common-garden experiments where juveniles of both ecomorphs have been split and reared under common littoral or pelagic conditions. Robinson and Wilson (1996) performed such a test with sunfish from Paradox Lake in the Adirondack region of New York State and found that 14% of the variation in external body form was attributable to presumed heritable ecomorph differences, while phenotypic responses induced by rearing environment accounted for 54% of the variation. Parsons (2002) continued this work by testing whether the plastic developmental systems that generate body form have diverged between littoral and pelagic ecomorphs, and made two interesting observations. First, considerable variation in plasticity exists between ecomorphs within and among three Ontario sunfish populations in response to littoral and pelagic type diets, indicating that plasticity can evolve in the system. Second, he found a greater degree of correlation among the phenotypic reaction norms of multiple body form traits in pelagic than in littoral fish split and reared between environments. In other words, the coordination of plastic responses was more similar among the same ecomorphs from three different populations than between ecomorphs that co-existed in any single population. This indicates that ecomorphs have consistently diverged with respect to how their developmental systems respond to the environment.

Trophic polymorphisms are widely believed to be systems that can provide insights into the early adaptive divergence and perhaps later formation of species, particularly in fishes. However, this will only come about if we can distinguish among polymorphic populations that evolve on different adaptive landscapes, and perhaps understand better the role of phenotypic plasticity during adaptive divergence. For example, further adaptive divergence and perhaps the initiation of species formation should be more likely on fitness landscapes composed of two adaptive peaks and less likely on other types of landscapes. More definitive tests are required that assess the fitness of intermediate phenotypes relative to more divergent ecomorphs (e.g. Schluter, 1995; Robinson *et al.*, 1996; McLaughlin *et al.*, 1999), test for selection against intermediate phenotypes within habitats (e.g. Schluter, 1994), or quantify adaptive landscapes based on extrinsic ecological conditions such as the availability of resources (e.g. Schluter and Grant, 1984; Hori, 1993). A better understanding of how adaptive landscapes drive contemporary taxonomic divergences may also provide insight into those believed to have driven historic divergences that subsequently resulted in the evolution of new species. For example, the ecological factors that promote polymorphism within pumpkinseeds may also operate among different sunfish species, and thus trait divergence may be similar, as predicted by Simpson (1953), despite being from different episodes. The extensive knowledge already available about Centrarchid sunfishes will make this task easier. Lastly, our study of the sunfish trophic polymorphism suggests that some of the very first morphological traits to diverge are those that are largely developmentally induced by environmental conditions. Thus, ecological conditions appear to influence divergence in this system in at least two important ways: with respect to how natural selection filters the variation present within a population, and how habitat induces phenotypic patterns during development.

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