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Morphological plasticity of native and non-native pumpkinseed sunfish in response to habitat type

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ABSTRACT

Background: Aquatic environments contain discrete habitats that vary in structural complexity and resource availability. Non-indigenous organisms that exhibit a high degree of phenotypic plasticity may be more successful at foraging and navigating, in and around the novel environments.

Goal: To investigate differences in morphological plasticity in young-of-year fish from native Canadian and non-native Iberian populations.

Organism: Pumpkinseed sunfish (*Lepomis gibbosus*).

Methods: Fish were reared for 80 days in enclosures that restricted individuals to either the littoral or pelagic zone of an artificial pond, while still permitting the passage of prey. Inter- and intra-population differences were tested using analysis of covariance and discriminant function analysis, while differences in plasticity were tested through a multivariate analysis of variance of discriminant function scores.

Results: Regardless of geographic origin, fish held under pelagic conditions exhibited significantly wider caudal fin bases, and longer dorsal and ventral caudal peduncle lengths. All populations developed longer gill rakers and shorter and narrower jaw structures when held under pelagic conditions. Iberian populations exhibited less phenotypic plasticity than native Canadian populations. All pumpkinseed populations held under pelagic conditions appear to exhibit similar morphological changes geared towards enhancing their ability to swim and forage in open-water habitat.

Keywords: Centrarchidae, foraging, habitat, reaction norm, trophic polymorphism.

INTRODUCTION

Numerous species exhibit divergent foraging strategies and trophic polymorphisms, leading to intraspecific phenotypic differences that are thought to have beneficial fitness consequences under local conditions (Swanson *et al.*, 2003). Divergent phenotypes are especially common in freshwater fishes (Robinson and Schluter, 2000), and can take the form of changes to

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behaviour, life history, and morphology (Smith and Skúlason, 1996). In littoral habitats, for example, Arctic charr (*Salvelinus alpinus*) grow slower and mature earlier than charr from pelagic habitats (Jonsson *et al.*, 1988). The species also exhibits marked morphological divergence, wherein littoral ecomorphs possess fewer gill rakers and shorter jaws than pelagic ecomorphs to enhance feeding and foraging on harder prey items (Malmquist *et al.*, 1992; Snorrason *et al.*, 1994). Environmental characteristics differ across habitats, creating divergent selective pressures that can lead to trophic polymorphisms within individual populations, or multiple populations across isolated systems. While some phenotypic differences within a population are likely genetic in nature (Gíslason *et al.*, 1999; Peichel *et al.*, 2001), a great deal of evidence suggests that many polymorphisms are expressed as a result of environmental differences across habitats (Proulx and Magnan, 2004; Gerry *et al.*, 2012; Woods *et al.*, 2013).

Phenotypic plasticity, which enables a genotype to produce multiple phenotypes under varying environmental conditions (Ghalambor *et al.*, 2007), may be a mechanism that helps perpetuate trophic polymorphism. A single aquatic system can contain several habitats with contrasting structural complexity and prey composition (Crowder and Cooper, 1982; Kovalenko *et al.*, 2012). Fauna experience selective pressures under the disparate conditions (Smith and Skúlason, 1996), and phenotypic plasticity helps individuals to persist by expressing traits that are more optimal, in that they reduce the costs associated with selection (Ghalambor *et al.*, 2007; Fischer-Rousseau *et al.*, 2010; Garduño-Paz *et al.*, 2010). For example, neotropical cichlids possess very little genetic differentiation, but their ability to exhibit phenotypic plasticity in response to habitat type has led to marked morphological differences among sympatric populations (Stauffer and van Snik Gray, 2004; Franchini *et al.*, 2014). A number of empirical and experimental comparisons have also demonstrated that fishes exhibit plastic responses to changes in habitat-specific factors, including narrower gill raker spacing in pelagic habitats and deeper body shapes in littoral areas (e.g. Robinson and Parsons, 2002; Olsson and Eklöv, 2005; Ruehl and DeWitt, 2005; Olsson *et al.*, 2007).

In North America, one teleost that has been well studied for both trophic polymorphism and its ability to exhibit phenotypic plasticity is the pumpkinseed (*Lepomis gibbosus*), a centrarchid that is endemic to rivers and lakes in eastern and central Canada and the United States (Scott and Crossman, 1973). In its larval stage, the species occupies pelagic habitats, where individuals feed on zooplankton (Keast, 1980), but after four weeks, the larvae move to littoral areas where soft-bodied invertebrates are abundant and dense macrophytes provide a refuge from predators (Mittelbach, 1984). When pumpkinseed reach a standard length of approximately 70 mm, they undergo another change in diet: the fish remain in the littoral zone but consume a greater proportion of hard-bodied gastropods (Keast, 1978).

In most aquatic systems, the pumpkinseed is found in sympatry with bluegill (*Lepomis macrochirus*), and their competitive interactions lead to niche segregation; pumpkinseed occupy littoral zones and consume mainly gastropods and other macroinvertebrates, while bluegill inhabit open water areas and consume mainly zooplankton (Werner and Hall, 1979; Robinson *et al.*, 1993). However, pumpkinseed have the capacity to exhibit strong morphological plasticity in response to changes in habitat structure and prey availability (Robinson and Wilson, 1996), and in the absence of bluegill, many pumpkinseed occupy the pelagic zone that, when in sympatry, is occupied by bluegill (Robinson *et al.*, 2000). This pattern of pumpkinseed exploiting an under-utilized pelagic zone has been observed in water bodies throughout eastern North America, and in many of these systems, morphological divergence was detected between ecomorphs that occupy different habitats (e.g. Robinson *et al.*, 1993; Gillespie and Fox, 2003; Weese *et al.*, 2012).

The pumpkinseed was introduced to Europe during the late nineteenth century, and the species is now established in 28 countries (Copp and Fox, 2007). It continues to spread throughout the Iberian Peninsula (Spain and Portugal), but the mechanisms facilitating its range expansion are unclear. The Iberian Peninsula is almost devoid of natural freshwater lakes; rather, the region encompasses a network of over 1200 rivers and artificial reservoirs (Casafont, 2003; Nilsson *et al.*, 2005). Moreover, the Iberian Peninsula is subject to a 'Mediterranean' climate, wherein floods and high flow regimes are common during the spring and autumn, while summers are arid with little or no precipitation (Gasith and Resh, 1999). This cyclical hydrological pattern causes major changes in water levels in most of the artificial systems in the region. For example, the Boadella Reservoir in Spain experienced a 40% reduction in water volume over the course of a single season (Santos *et al.*, 2011).

Pumpkinseed are known to occupy both littoral and pelagic environments (e.g. Gillespie and Fox, 2003; Bhagat *et al.*, 2006), and in the Iberian Peninsula, fluctuating water levels within artificial reservoirs affect the abundance of submerged macrophytes, which limits the availability of littoral habitat and prey. Environmental perturbation favours the evolution or expression of phenotypic plasticity in numerous species (Bradshaw, 1965), and recent evidence suggests that organisms from non-native ranges are capable of expressing stronger phenotypic plasticity than counterparts from native areas (Chown *et al.*, 2007; Chun, 2011). The invasion success of pumpkinseed in this region may be linked to their ability to exhibit strong phenotypic plasticity, facilitating their ability to occupy an alternate habitat as the species continues to expand its non-native range.

In this study, differences in morphological plasticity were tested using pumpkinseed progeny from adults collected in Canada and the Iberian Peninsula. We hypothesized that native and non-native pumpkinseed would exhibit different levels of phenotypic plasticity, with progeny from a non-native region that experiences high environmental perturbations exhibiting greater plasticity than populations from the native range. Yavno *et al.* (2013) identified intercontinental differences in several external morphological traits between populations of Canadian and Iberian pumpkinseed; the authors proposed that the latter group inherited median and paired fins that were longer and more anteriorly-oriented because the morphological changes may have had beneficial fitness consequences in response to water velocity in the non-native region. Those twelve previously examined external traits, together with six internal traits, are functionally significant in numerous fishes for feeding and foraging in different habitats (Malmquist *et al.*, 1992; Snorrason *et al.*, 1994; Hegrenes, 2001; Bhagat *et al.*, 2011). Here, we use a common garden approach (Robinson and Parsons, 2002) to test whether non-native pumpkinseed show greater morphological plasticity than native individuals.

MATERIALS AND METHODS

Experimental animals and study sites

Yavno and Fox (2013) describe in detail the biotic and abiotic characteristics of all water bodies where pumpkinseed were collected, and each experimental population has been used in a previous morphological comparison (e.g. Vila-Gispert *et al.*, 2007; Yavno *et al.*, 2013). None of these water bodies are known to contain polymorphic pumpkinseed populations, and none of them contain submerged rocky shoals in open water that are characteristic of the habitat where trophic polymorphism in pumpkinseed occurs (Robinson *et al.*, 1993). Non-native adult

pumpkinseed were collected during the spring of 2007 from one riverine (Ter River: 42°01′N, 3°12′E) and one lacustrine (Susqueda Reservoir: 42°58′N, 2°30′E) water body in Spain, flown to Canada, and held over the winter in an indoor facility at Trent University (44°36′N, 78°28′W). Adult pumpkinseed from the native range were collected from one riverine (Otonabee River: 44°23′N, 78°16′W) and one lacustrine (Rice Lake: 44°14′N, 78°60′W) water body during early June 2008. In late June 2008, all native and non-native adults ($N \geq 20$ for each population) were stocked into their own artificial ponds (9 m long × 3 m wide × 3 m deep; one for each population) in Lindsay, Ontario (44°20′N, 78°43′E). All ponds were isolated from one another, and each contained a mud/silt substrate, an established aquatic macrophyte and invertebrate community that is comparable to that of nearby natural systems, and two common cyprinids that are found within the native range (northern redbelly dace, *Phoxinus eos*; fathead minnow, *Pimephales promelas*). Seining was conducted within the ponds each subsequent year, which confirmed reproduction (i.e. presence of larvae and juveniles <25 mm total length).

Rearing experiment

In late June 2011, F_1 juvenile progeny were collected from each pond using a 9-m seine, and held in individual coolers. Fish were reared within their pond of origin using 16 artificial enclosures (wood structure: 1 m long × 1 m wide × 0.5 m high with 3.2-mm hardware cloth mesh on all sides). Enclosures were assigned to one of the four populations (native: Otonabee River, Rice Lake; non-native: Susqueda Reservoir, Ter River), one of two habitat types (littoral: depressed into the substrate along the shore; pelagic: suspended within the water column), and all treatments were replicated twice (Fig. 1). The mesh siding permitted passage of zooplankton and invertebrates into all enclosures, regardless of their placement within the pond. Juveniles were removed from the coolers and randomly placed into one of the four enclosures assigned to their respective population. This was repeated until each enclosure held an equal number of individuals (18 fish per enclosure; total of 288 individuals). Initial standard length (SL) measurements for all juvenile pumpkinseed placed within the artificial enclosures varied from 21 to 41 mm, with a mean value of 30.9 mm (Table 1). These sizes are smaller than or equal to those used in previous phenotypic

Table 1. Coordinates of source water bodies, and initial standard length (SL) of pumpkinseed placed into the artificial enclosures

Water bodies	Coordinates	SL (mm)
Canada		
Otonabee River	44°23′N, 78°16′W	26.5–33.2 (30.1)
Rice Lake	44°14′N, 78°60′W	34.6–41.7 (37.6)
Iberian Peninsula		
Susqueda Reservoir	42°58′N, 2°30′E	28.3–34.4 (30.7)
Ter River	42°01′N, 3°12′E	21.8–30.8 (25.5)

Note: SL represents variation (minimum–maximum) with mean in parentheses.

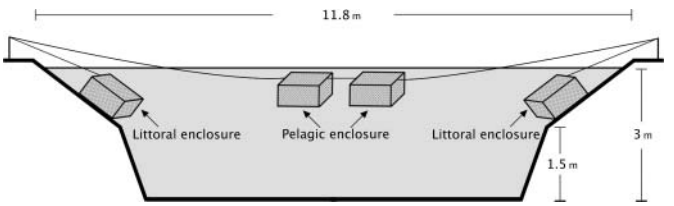

Fig. 1. Arrangement of artificial enclosures in experimental pond of each population. Littoral enclosures were positioned on opposite sides of the pond below the water line, and depressed into the substrate. Pelagic enclosures were paired and suspended within the water column in the centre of each pond.

plasticity studies using pumpkinseed (e.g. Arendt and Wilson, 1999; Yavno and Fox, 2013; Yavno *et al.*, 2014). Fish were reared for 80 days, a sufficient period of time for morphological divergence to occur in pumpkinseed (see Robinson and Wilson, 1996; Yavno *et al.*, 2013). Enclosures were checked every 10–14 days, and cleared of any external algal accumulation to facilitate water exchange and the passage of zooplankton. Because littoral enclosures were depressed into the substrate, submerged aquatic macrophytes (e.g. *Vallisneria americana*, *Zosterella dubia*) grew through the mesh and into the enclosures; these macrophytes were not cleared away in order to reproduce natural littoral conditions.

A total of 121 fish were lost across all populations over the course of the experiment (~42% mortality: Otonabee River, 36%; Rice Lake, 48%; Susqueda Reservoir, 40%; Ter River, 43%). This level of mortality is less than that of previous enclosure studies with young of the same species [e.g. ~66% in Robinson and Wilson (1996)], and may be due to intraspecific competition from outside the enclosures; the presence of stock pumpkinseed within each pond may have led to a decrease in the replenishment rate of zooplankton into the enclosures.

Final morphological traits in juveniles were assessed at the end of the rearing period after euthanizing all remaining fish ($n = 167$) with a lethal dose of MS-222. Fish were photographed on their left sides and dorsal aspects to assess external morphological characteristics. Internal morphological traits were determined by removing the first branchial arch, and both the dorsal and ventral jaws; these structures were stained with alizarin red (Springer *et al.*, 2000), and photographed using a Leica S8AP0 dissection scope equipped with a Lumenera Infinity 1-1C camera. Fins were extended to their maximum, natural position prior to image capture, which reduced any ambiguity with respect to the origin and terminus of morphological characters.

Morphometric analyses

Morphological differences between populations and habitat treatments were identified using a distance-based approach. Photographs were taken under identical lighting conditions and included a scientific ruler to permit standardization of scale. ImageJ analysis software (National Institute of Health: http://rsbweb.nih.gov/ij/) was then used to quantify all internal and external morphometric measurements. A modified box truss design, which consists of homologous points that form inter-landmark distances (Strauss and Bookstein, 1982), was used to analyse pumpkinseed morphology. Centroid size (CS) was used to take the effect of allometry into account during the statistical analysis (Robinson *et al.*, 2000). A residual

analysis performed on a regression of SL against CS identified nine individuals as outliers (>2σ); these fish were subsequently removed from the dataset prior to statistical analysis. Eighteen morphological characters (6 internal, 16 external) identified as being functionally significant for feeding and foraging in pumpkinseed (Gillespie and Fox, 2003; Yavno *et al.*, 2014) were compared among the four populations [internal: Fig. 2; external: see Figure 2 in Yavno and Fox (2013)]; internal and external measurements were tested separately. Because some of the measurements taken on day 80 did not meet the assumption of normality (Kolmogorov-Smirnov test; $P < 0.05$), data were $\log_{10}$-transformed prior to analysis. Transformed data were then regressed against $\log_{10}$-transformed CS in a two-factor analysis of covariance (ANCOVA factors: population and habitat type; covariate: CS). A Bonferroni correction was used to adjust probabilities due to multiple paired comparisons (internal traits: $\alpha \leq 0.0083$; external traits: $\alpha \leq 0.0042$). If no significant interaction term was detected between CS and a main effect (i.e. homogeneity of slopes), then that particular interaction term was

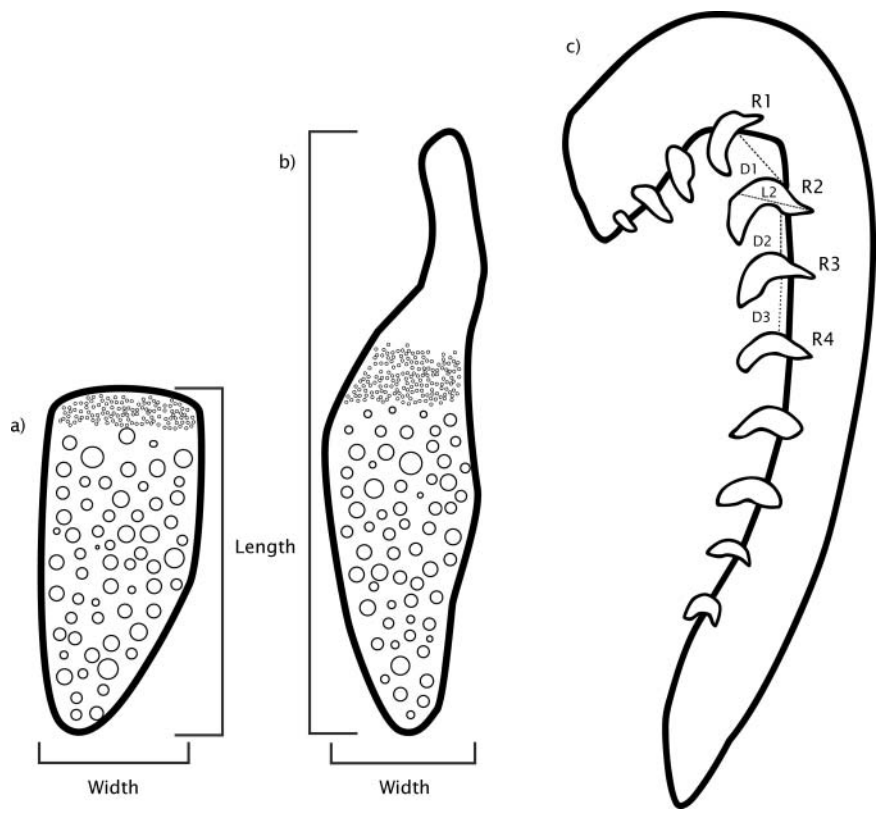

Fig. 2. Illustrations of three internal feeding structures of pumpkinseed sunfish. Length and width measurements were taken for both the (a) pharyngobranchial 3 and (b) ceratobranchial 5 jaw bones. Branchial gill arch (c) measurements were taken on rakers 1–4 (R1–R4), and included the length of the second gill raker (L2) and the average distance between the first four gill rakers [(D1 + D2 + D3)/3)]. Pharyngobranchial 3 and ceratobranchial 5 are presented here in their ventral and dorsal view, respectively; see Figure 1 in Wainwright *et al.* (1991) for schematics depicting the position of these structures within the jaw.

removed from the ANCOVA and the test rerun. Significant main effects were compared only when interactions between both main effects were not significant, and using Tukey's HSD and Student-Neuman-Keuls (SNK) analyses for external and internal traits, respectively. The latter test provides more power than Tukey's HSD, but with an increased chance of committing a Type I error when comparing four or more groups (Zar, 2010). SNK was therefore used for a *post-hoc* comparison between the two habitat types, while Tukey's HSD was used to examine differences between the four populations.

In previous morphological comparisons, replicate effects were examined by comparing differences in multivariate shape among treatments (e.g. Fischer-Rousseau *et al.*, 2010). Here, differences between treatment replicates were tested using the canonical scores for the first axis in the multivariate analysis (discussed below), and replicates were pooled, as no significant differences were detected between replicates in any of the populations (*t*-test; $P > 0.32$ in all cases).

Multivariate discriminant function analysis (DFA) was used to help identify differentiation patterns among populations and between habitat treatments. Analyses were performed using measurements that were statistically adjusted to a common body size using the CS of all pumpkinseed populations (e.g. Bhagat *et al.*, 2006). Adjusted values were first analysed in multivariate analysis of variance (MANOVA) and then in DFA; differences among populations were evaluated using Wilk's λ and the associated *P* statistic.

Analysis of phenotypic plasticity

Previous studies testing for differences in phenotypic plasticity between Canadian and Iberian populations used external traits (Yavno and Fox, 2013; Yavno *et al.*, 2014). To compare the level of morphological differentiation due to observed habitat type with the level of environmentally induced plasticity due to water velocity and competitive pressures in the aforementioned studies, we also used external traits as a means to identify differences in phenotypic plasticity. The canonical scores from the multivariate DFA were used in an independent analysis to provide a representation of the relative contribution of population (genetic) and environmental (habitat type) factors to morphological differentiation. Following Robinson and Wilson (1996) and Yavno *et al.* (2014), the size-adjusted measurements were separated by geographic origin, and two new sets of canonical scores generated by performing multivariate DFAs for native and non-native populations. To assess plasticity differences attributed to population origin, an analysis was then performed independently on each of the new sets of canonical scores. The mean canonical scores for each factor were first run in ANOVA to provide an estimate of the variance components for each factor. This variance was then adjusted by the canonical correlations of each axis, and the resulting values were then summed and expressed as a percentage of the total variation. This percentage represents the contribution of population factors (genetic) and habitat type (phenotypic plasticity) to the morphological differentiation observed in the study.

RESULTS

Morphological trait differentiation

Three fin traits and three body and caudal peduncle traits showed significant among-population differentiation (Table 2; Figs. 3 and 4). Tukey's HSD test revealed that, relative

Table 2. Results of *post-hoc* analyses of morphological traits that exhibited significant differentiation under Bonferroni-corrected ANCOVA (a and b, $P < 0.0042$; c, $P < 0.0083$)

Morphological trait	Population				Habitat type		Expected optimal state		Population exhibiting optimal pattern of change
	OR	RL	TR	SQ	Littoral	Pelagic	Littoral	Pelagic	
(a) Body and peduncle dimensions									
Body depth	A	AB	A	B	A	A	Deeper	Narrower	RL, SQ
Body width	A	A	A	A	A	A	Narrower	Deeper	OR, TR
Anterior caudal peduncle depth	A	A	A	A	A	A	Deeper	Narrower	RL
Posterior caudal peduncle depth	A	B	B	B	A	A	Deeper	Narrower	
Dorsal caudal peduncle length	A	A	B	B	A	A	Shorter	Longer	RL, SQ, TR
Ventral caudal peduncle length	A	A	A	A	A	A	Shorter	Longer	OR, RL, SQ, TR
(b) Position and size of median/paired fins									
Anal fin base length	B	A	AB	A	A	A	Longer	Shorter	OR, RL, SQ, TR
Dorsal fin base length	B	A	A	AB	A	A	Longer	Shorter	RL
Maximum pectoral fin length	A	A	A	A	A	A	Longer	Shorter	OR, RL, TR
Pre-dorsal length	A	C	BC	AB	A	A	Shorter	Longer	
Pre-pectoral length	A	A	A	A	A	A	Shorter	Longer	
Pre-pelvic length	A	A	A	A	A	A	Shorter	Longer	
(c) Jaw bone and gill rakers									
Second gill raker length	A	A	A	A	A	A	Shorter	Longer	RL, SQ, TR
Distance between gill rakers	B	A	BC	C	A	A	Wider	Narrower	
Ceratobranchial 5 width	BC	A	C	B	A	B	Wider	Narrower	RL, SQ
Ceratobranchial 5 length	B	A	B	B	A	B	Longer	Shorter	OR
Pharyngobranchial 3 width	B	A	B	B	A	A	Wider	Narrower	OR, RL, SQ
Pharyngobranchial 3 length	B	A	B	B	A	A	Longer	Shorter	OR, RL, SQ, TR

Note: Differences due to population (Otonabee River, OR; Rice Lake, RL; Ter River, TR; Susqueda Reservoir, SQ) were examined with Tukey's HSD multiple comparison tests, while differences due to habitat type (littoral, pelagic) were identified through separate Student-Neuman-Keuls analyses. Letters earlier in the alphabet represent higher means and identical uppercase letters indicate no significant difference. The expected, optimal adaptive state (i.e. position or size) of each trait in littoral and pelagic environments is listed; populations exhibiting a pattern of morphological change that was in a direction that is consistent with the optimal state, regardless of statistically significant differentiation between treatments, are also provided.

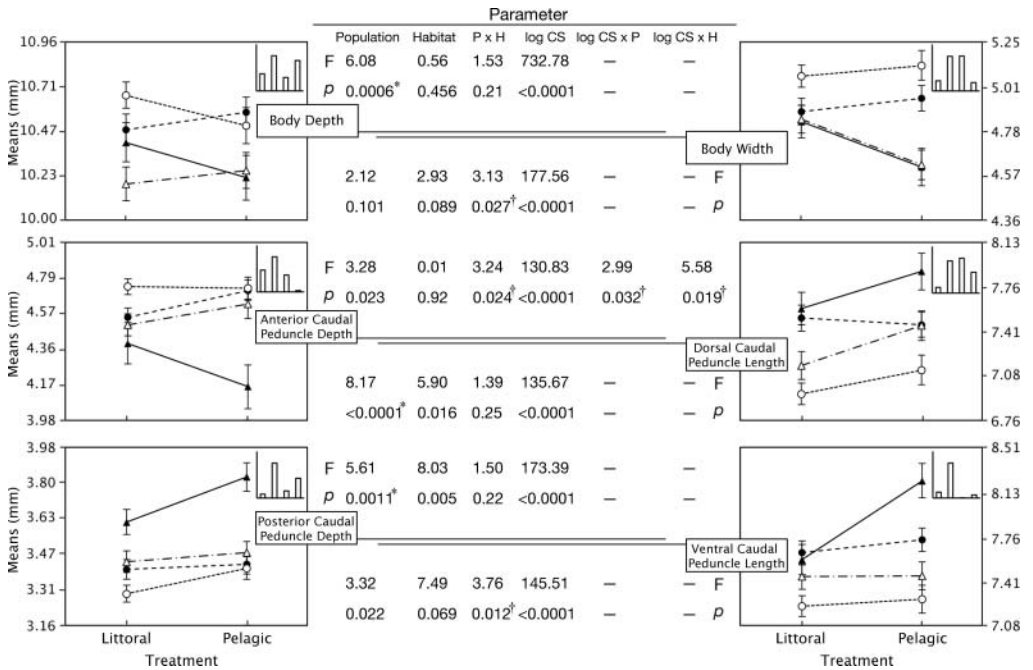

Fig. 3. Reaction norm plots to changes in habitat type for six body and caudal peduncle traits, identified as having functional significance for feeding and foraging. Log_{10}-transformed measurements were regressed against the log_{10}-transformed centroid (CS) in an ANCOVA. Interactions between CS and a main effect (P, population; H, habitat type) that were not significant (i.e. homogeneous slopes) were removed from the analysis, and probabilities were Bonferroni-adjusted. Pumpkinseed populations were Otonabee River (● and - - -), Rice Lake (▲ and ——), Ter River (○ and · · · ·), and Susqueda Reservoir (△ and — -). The y-axis is plotted in log_{10} scale with back-calculated values. Insets provide a graphical representation of the magnitude of the slope (i.e. absolute value of the difference between the pelagic and littoral treatment) for each population (left to right: Otonabee, Rice, Susqueda, Ter).

to Canadian pumpkinseed, the caudal peduncle of non-native juveniles was shorter along the dorsal side and narrower at the posterior. Under pelagic treatments, individuals from Rice and Susqueda, both of which are lacustrine populations, exhibited narrower body depths than the riverine populations (Otonabee and Ter); riverine populations exhibited shorter anal fin base lengths than the lacustrine populations. Juveniles from the Rice, Susqueda, and Ter populations exhibited longer dorsal fin base lengths than fish from the Otonabee population, and while not statistically significant, juveniles from Rice and Susqueda exhibited greater body widths than those from Otonabee and Ter. Juveniles from lacustrine populations reared in pelagic enclosures exhibited narrower bodies and longer dorsal caudal peduncle lengths than individuals from the same populations held in littoral enclosures. Juveniles from Otonabee and Susqueda exhibited greater body depths when held in pelagic enclosures than they did in littoral enclosures, whereas the pattern for this trait was reversed for fish from Rice and Ter. In all populations, juveniles held in pelagic enclosures exhibited a smaller anal fin base, a shorter pre-dorsal and pre-pectoral, and a greater posterior caudal peduncle depth and ventral caudal peduncle length than those held in littoral enclosures. With the exception of fish from Ter, pumpkinseed in littoral

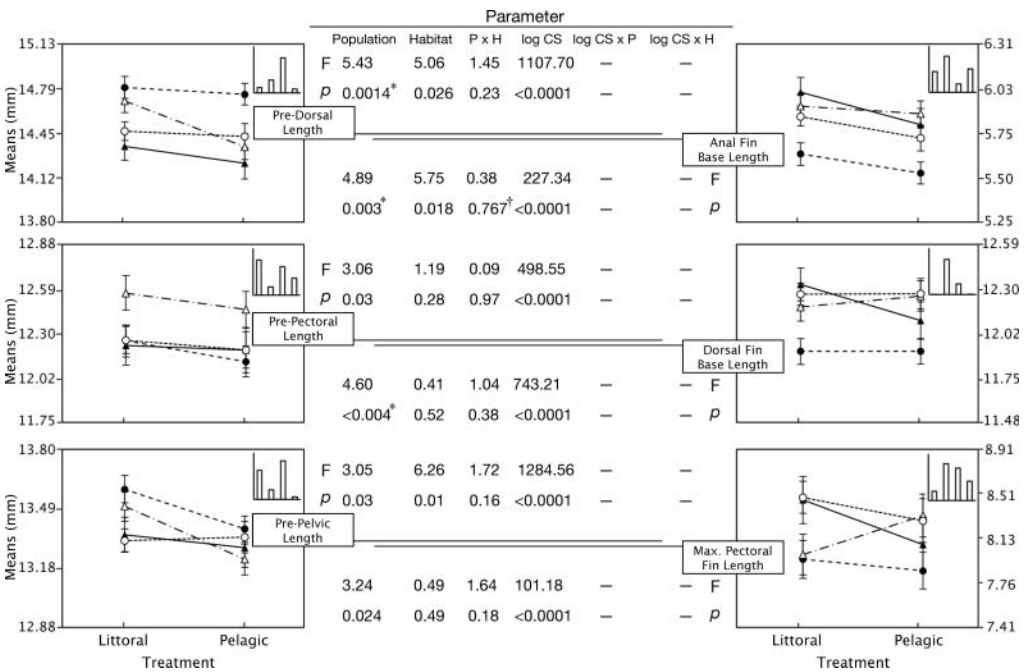

Fig. 4. Reaction norm plots to changes in habitat type for six median and paired fin traits, identified as having functional significance for feeding and foraging. As for Fig. 3, measurements were transformed and probabilities were Bonferroni-adjusted; symbols, line designations, and y-axis scaling also correspond to those in Fig. 3.

enclosures exhibited shorter pre-pelvic lengths than fish held in pelagic enclosures. Relative to the fish held in littoral enclosures, three populations exhibited greater anterior caudal peduncle depths when held in pelagic enclosures (Fig. 3).

Canadian and Iberian populations generally exhibited shorter gill rakers, along with longer and wider jawbones when restricted to littoral conditions (Fig. 5). Riverine populations exhibited shorter lower jaw (ceratobranchial 5) widths and gill raker lengths, although the difference was not statistically significant in the latter (Table 2). For all internal traits, interaction terms between the main effects were not significant; morphological differentiation between littoral and pelagic treatments occurred in the same direction in all of the study populations, regardless of geographic or habitat origin. Under littoral conditions, the lower jaw was longer and wider in all populations. Native populations exhibited a pattern of morphological change that is consistent with functional expectation in five internal traits, while non-native populations exhibited a pattern that is consistent with functional expectations in four internal traits (Table 2).

Multivariate analysis

There was significant morphological differentiation among populations and between habitat types (two-factor MANOVA of size-adjusted measurements: population, Wilk's $\lambda = 0.369$, $F = 4.33$, $P < 0.0001$; habitat type, Wilk's $\lambda = 0.713$, $F = 4.65$, $P < 0.0001$), and

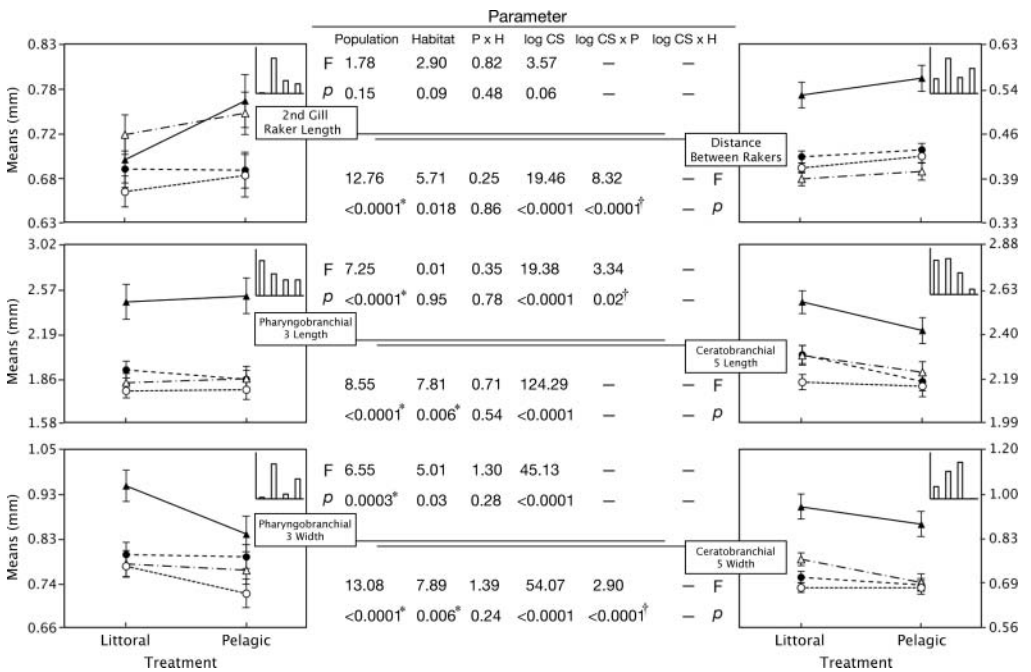

Fig. 5. Reaction norm plots to changes in habitat type for six internal morphological traits identified as having functional significance for feeding and foraging. As for Fig. 3, measurements were transformed and probabilities were Bonferroni-adjusted; symbols, line designations, and y-axis scaling also correspond to those in Fig. 3.

also a significant interaction between population and habitat type (Wilk's $\lambda = 0.641$, $F = 1.86$, $P = 0.002$). Significant morphological differentiation among the four populations and two treatments was then detected in the multivariate DFA (Wilk's $\lambda = 0.20$, $F = 3.10$, $P < 0.0001$). The first canonical axis separated Canadian and Iberian populations, but the second axis did not appear to separate groups by either geographic origin or habitat treatment (Fig. 6). Mahalanobis distances indicated that littoral/pelagic treatments did not produce morphological differentiation in either the Otonabee ($P = 0.65$) or Ter ($P = 0.09$) populations, whereas significant morphological differentiation was detected in the two lacustrine populations ($P < 0.0001$). The first two canonical axes explained 71% of the total variation, and the third explained an additional 13% (total of 84%) (Table 3). The dorsal and ventral lengths of the caudal peduncle were significantly correlated with the first canonical axis, the position of the dorsal and pelvic fins were significantly correlated with the second canonical axis, and body depth, body width, and the posterior caudal peduncle were significantly correlated with the third canonical axis.

For internal traits, there was also significant morphological differentiation among populations and between habitat types (two-factor MANOVA of size-adjusted measurements: population, Wilk's $\lambda = 0.384$, $F = 9.17$, $P < 0.0001$; habitat type, Wilk's $\lambda = 0.890$, $F = 2.96$, $P = 0.009$), but no significant interaction between population and habitat type (Wilk's $\lambda = 0.870$, $F = 1.47$, $P = 0.30$). In the subsequent multivariate DFA (scatterplot not provided; Wilk's $\lambda = 0.30$, $F = 4.74$, $P < 0.0001$), neither the first nor second canonical axis appeared to separate groups by population origin or habitat treatment. Mahalanobis

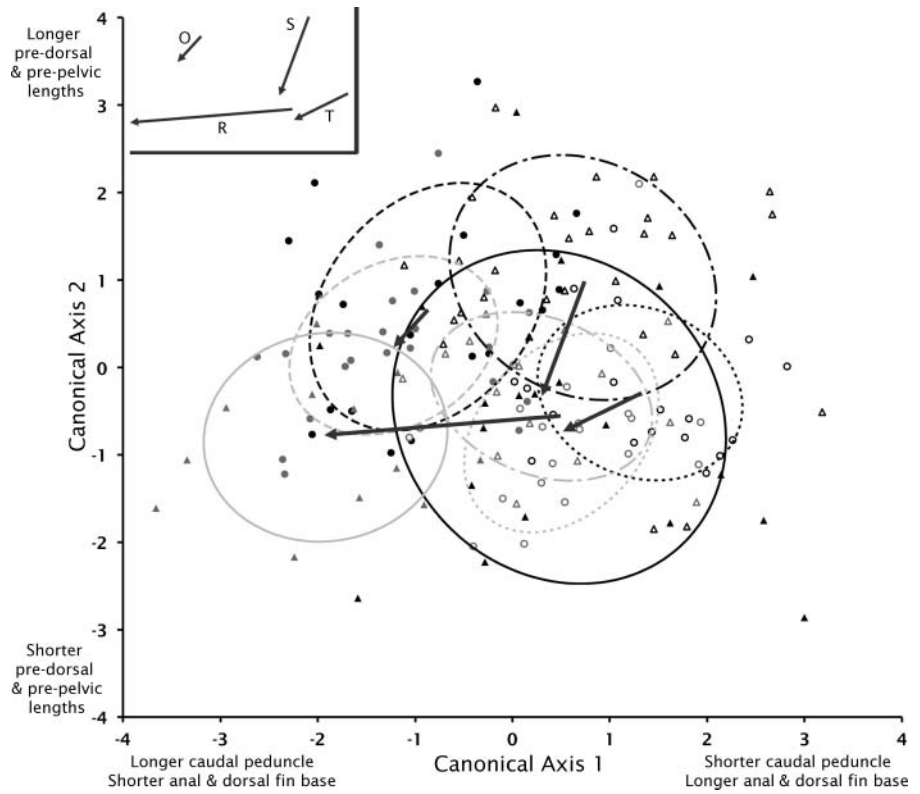

Fig. 6. Scatterplot of pumpkinseed (*Lepomis gibbosus*) canonical scores from the multivariate DFA, performed on twelve size-adjusted external morphological traits, with 50% ellipsoids around the centroid of each group for littoral (black) and pelagic (grey) trophic treatments. Arrows denote the shift of the centroid between littoral and pelagic treatments for each population, and are recreated in the inset for clarity. Symbol and line designations correspond to those in Fig. 3.

distances indicated that littoral/pelagic treatments for Otonabee ($P = 0.35$), Ter ($P = 0.97$), and Susqueda ($P = 0.07$) did not produce significant morphological differentiation, whereas the differentiation was significant in Rice ($P < 0.01$). The first three canonical axes explained 97% of the total variation (Table 3). The width of the upper jaw, distance between gill rakers, and gill raker length were significantly correlated with axes 1, 2, and 3, respectively.

Phenotypic plasticity

Analyses of variance of the canonical scores, separated by geographic origin, revealed that both population factors and habitat type influenced the first axis for non-native populations, whereas only habitat type influenced the axis for native pumpkinseed (Table 4). Habitat type continued to influence both axes 2 and 3 for non-native fish, while only population influenced axis 2 for native populations. Based on the variance components of the DFA, the percent variation of morphology explained by population-based genetic factors and phenotypic plasticity was 26% and 18% for Canadian populations, respectively.

Table 3. Canonical loadings (i.e. correlations) between the 18 morphological traits and each canonical axis in the overall multivariate discriminant function analysis (DFA)

	External traits				Internal traits		
Morphological trait	Axis 1	Axis 2	Axis 3	Morphological trait	Axis 1	Axis 2	Axis 3
Body depth	−0.002	−0.099	**0.578**	Second gill raker length	−0.073	−0.128	**0.814**
Body width	0.175	0.023	**0.672**	Distance between rakers	−0.083	**0.794**	0.129
Anterior caudal peduncle depth	0.118	−0.204	0.290	Ceratobranchial 5 width	−0.262	−0.210	−0.356
Posterior caudal peduncle depth	−0.283	−0.305	**−0.424**	Ceratobranchial 5 length	−0.204	−0.046	−0.584
Dorsal caudal peduncle length	**−0.435**	−0.130	−0.171	Pharyngobranchial 3 width	**0.610**	−0.168	−0.334
Ventral caudal peduncle length	**−0.512**	0.018	−0.207	Pharyngobranchial 3 length	−0.086	0.259	0.170
Anal fin base length	0.363	−0.027	−0.350				
Dorsal fin base length	0.357	−0.172	−0.274				
Maximum pectoral length	0.210	−0.283	0.013				
Pre-dorsal length	−0.091	**0.770**	0.188				
Pre-pectoral length	0.159	0.392	−0.456				
Pre-pelvic length	−0.034	**0.568**	0.108				
Cumulative % of total variance explained	*50.3%*	*71.3%*	*84.3%*		*74.2%*	*89.8%*	*96.8%*

Note: Traits with correlations/loadings that are greater than or equal to an absolute value of 0.4 are shown in **bold** font, and are considered to have biologically significant relationships (McGarigal *et al.*, 2000).

Table 4. Results of analyses of variance (ANOVA) on the canonical scores for DFA axes, after controlling for population origin (i.e. performing separate DFAs on native and non-native populations, and analysing those canonical scores independently)

	Axis 1			Axis 2			Axis 3		
Model parameter	*F*	*P*	Components of variance	*F*	*P*	Components of variance	*F*	*P*	Components of variance
Native populations									
Population	0.63	0.47	0	21.04	0.01	0.792 (75.4)	0.00	0.99	0
Habitat type	25.85	0.007	0.583 (30.1)	2.87	0.17	0.108 (10.3)	0.63	0.47	0
Population × habitat type	13.43	0.02	1.168 (60.2)	0.00	0.98	0	1.23	0.33	0.033 (10.5)
Error			0.188 (9.7)			0.151 (14.3)			0.280 (89.5)
Total			**1.939 (100)**			**1.051 (100)**			**0.313 (100)**
Non-native populations									
Population	34.88	0.004	1.155 (77.8)	3.40	0.14	0	3.34	0.14	0
Habitat type	6.18	0.06	0.195 (13.1)	4.87	0.09	0	16.72	0.01	0.228 (30.3)
Population × habitat type	0.36	0.58	0	11.91	0.03	1.367 (84.5)	8.47	0.04	0.413 (54.9)
Error			0.134 (9.1)			0.250 (15.5)			0.111 (14.8)
Total			**1.484 (100)**			**1.617 (100)**			**0.752 (100)**

Note: The contribution towards morphological differentiation in native and non-native pumpkinseed is based on the discriminatory power (i.e. the percentage of total variance explained) of the DFA, following an adjustment to reflect the variance components on each axis.

The analysis for Iberian pumpkinseed revealed that those populations exhibited 27% less plasticity (13% explained by habitat type) and 46% more apparent genetic differentiation (38% explained by population-based factors).

DISCUSSION

While the results of the present study provide support for our hypothesis that native and non-native pumpkinseed would exhibit contrasting levels of phenotypic plasticity in response to habitat type, there was limited evidence that non-native fish exhibit greater plasticity than conspecifics from the native range. For instance, Iberian populations that were held under pelagic conditions exhibited a smaller degree of lengthening in their caudal peduncles than native fish; in multivariate space, morphological separation between littoral and pelagic treatments was not detected for the internal traits of either Iberian population. Since its initial introduction into Europe, the pumpkinseed has become established in numerous lakes and rivers, and the species continues to undergo range expansion through rivers and artificial reservoirs in Spain and Portugal (Clavero *et al.*, 2013). These systems are unlike the Canadian water bodies that native pumpkinseed inhabit: the Iberian Peninsula experiences strong annual droughts, lowered water levels, and, consequently, reduced availability of littoral habitats. The successful spread of pumpkinseed through Iberia may be linked to their ability to transition to an alternate habitat, which would necessitate exhibiting strong levels of phenotypic plasticity in response to habitat type. While this study confirmed that Iberian pumpkinseed are nevertheless capable of exhibiting morphological plasticity to littoral and pelagic conditions, they display substantially less plasticity than fish of native Canadian origin.

Aquatic ecosystems possess distinct areas that differ in structural complexity and foraging resources. Within these environments, fishes have a well-documented tendency to develop phenotypic characteristics that facilitate the acquisition of food, and help to reduce energy expended for manoeuvring and foraging. For example, pelagic habitats are large, open-water environments with few aquatic macrophytes and limited benthic or hard-bodied invertebrates. Bluegill, European whitefish (*Coregonus lavaretus*), and threespine stickleback (*Gasterosteus aculeatus*) from pelagic zones develop fusiform bodies to reduce drag for open-water locomotion (Ehlinger and Wilson, 1988; Cresko and Baker, 1996; Kahilainen *et al.*, 2011). When restricted to a pelagic habitat, all four pumpkinseed populations developed morphological changes that enhance sustained locomotion and foraging in an open-water environment. Relative to pumpkinseed held under littoral conditions, pelagic fish exhibited dorsal and pelvic fins that were more anteriorly positioned and caudal peduncles that were longer (Table 2). In natural systems, pumpkinseed caught from pelagic areas also exhibit longer caudal peduncles, and more anteriorly located median fins than fish caught from littoral habitats (Gillespie and Fox, 2003; Bhagat *et al.*, 2006). The lengthening of the caudal peduncle is comparable to the morphological form exhibited by pelagic taxa such as Scombridae; long caudal peduncles increase the undulation amplitude of their caudal fin to maximize swimming performance during long periods of sustained locomotion (Fisher and Hogan, 2007). In pelagic environments, median fins that are positioned away from the caudal peduncle are analogous to a decrease in body depth in that region, reducing drag forces where propulsive forces are generated through a decreased body silhouette (Webb, 1984). Our results suggest that, as with their native counterparts, non-native pumpkinseed are capable of exhibiting phenotypic plasticity and developing external morphological traits that are beneficial

during periods of increased locomotion, which likely helps to preserve energy while moving through open water in search of prey.

Both Canadian and Iberian populations exhibited longer anal fin base lengths in littoral treatments, and shorter anal fin base lengths in pelagic treatments. Habitat complexity is governed by physical attributes within the environment, and littoral environments are discernibly more complex than the open water. Fishes that occupy littoral areas must negotiate around rocks, protruding structures, and aquatic macrophytes, which can be facilitated by possessing morphological characteristics that are optimal for manoeuvrability (e.g. Garduño-Paz *et al.*, 2010). In gibbose species, the anal fin is essential for orientating the body within the water column. Standen and Lauder (2005) conducted a detailed study of the role of median fins in the three-dimensional kinematics of a centrarchid. The authors found that as individuals increase their swimming speed, they begin to depress their median fin to temporarily reduce its surface area. As water velocity increases, a smaller fin can produce the same amount of lateral force necessary to generate roll and yaw manoeuvres as a large fin in stagnant water (Drucker and Lauder, 2001). In our study, aquatic macrophytes grew through the littoral enclosures, and so pumpkinseed within these treatments would need to manoeuvre through the vegetation in search of prey. Moreover, as the water velocity within these enclosures was negligible, fish with larger anal fins would be capable of generating stronger side forces for navigating around the complex vegetation, without experiencing an increasing drag force that typically accompanies morphological structures with higher surface areas (*sensu* Sagnes *et al.*, 2000).

Contrary to functional expectations and empirical studies of other fishes, body depth increased in progeny from Susqueda Reservoir and the Otonabee River when these fish were held under pelagic conditions. Pelagic enclosures also induced body width decreases in both lacustrine populations, while riverine fish developed wider bodies. Fishes that occupy pelagic zones typically develop narrower, more streamlined body forms, which are considered to be more adaptive for cruising through open water (Webb, 1984). While it is difficult to ascertain why some of the populations exhibited morphological responses that are not consistent with predicted functional expectations, dissimilar morphological change between littoral and pelagic pumpkinseed is not uncommon. Januszkiewicz and Robinson (2007) exposed pumpkinseed to predator odours and observed that pelagic fish exhibited more pronounced morphological change than fish caught from littoral areas. Given that the effects of both deeper and narrower body forms on fitness are unknown, the disparate responses that we presently observe could still have a favourable effect on fitness during later stages of life. Further study is needed to determine how changes to the external morphology of pumpkinseed translate into changes in evolutionary fitness.

While many external morphological traits in fish are functionally significant for detecting, pursuing, and capturing prey (Higham, 2007), internal traits are used for feeding, and they can significantly influence the ability of fish to handle, manipulate, and consume prey. In our study, littoral treatments induced pumpkinseed populations to develop upper and lower jaw structures that were larger (length and width) than those of pumpkinseed from pelagic enclosures. Empirical work on numerous species, including several centrarchids, has demonstrated that the pharyngeal jaw apparatus is used to crush prey, and that a smaller apparatus is a morphological constraint for consuming gastropods and larger invertebrates (Wainwright, 1988; Mittelbach *et al.*, 1999).

Littoral habitats can support a greater diversity of taxa than pelagic environments, including a higher proportion of macroinvertebrates (e.g. Havens *et al.*, 1996); instead of

consuming small zooplankton, fish that inhabit near-shore areas can maximize their energy intake by consuming larger invertebrates. Werner and Hall (1974) found that bluegill, which are morphologically similar to pumpkinseed, adhere to optimal foraging theory and select larger sized prey when available. In a littoral environment, pumpkinseed would benefit from possessing larger jaw structures, as this would facilitate the manipulation of larger, more energy-rich prey items such as amphipods or ephemeropterans.

In a pelagic enclosure, pumpkinseed would not have access to benthic invertebrates. To forage more effectively, individuals would need to develop morphological structures to acquire prey items that occupy the water column. *Coregonus* spp. are widely used for understanding the effects of trophic divergence on morphology, and in numerous independent systems, fish that feed on zooplankton consistently exhibit significant morphological divergence in their pharyngeal gill apparatus (Amundsen *et al.*, 2008; Kahilainen *et al.*, 2011). In our study, pumpkinseed in open-water enclosures tended to develop longer gill rakers than those in littoral enclosures, which would provide individuals with a greater surface area to catch prey items, and increase foraging efficiency (Ruzzante *et al.*, 1998). However, the difference between treatments was not statistically significant. In previous studies, pumpkinseed caught from pelagic habitats have exhibited a significantly smaller distance between individual gill rakers than fish caught from littoral habitats (e.g. Gillespie and Fox, 2003), which can help to filter even smaller prey items (Kahilainen *et al.*, 2011). The results of our study, however, were inconsistent with this pattern, as distance between gill rakers was not significantly different between treatments for any population, and even appeared to increase for pumpkinseed from Rice Lake when those juveniles were held under pelagic conditions.

While the temporal scale of this study may have constrained our ability to detect differences in internal phenotypic divergence between Iberian and Canadian pumpkinseed, this is highly unlikely. In a previous experimental comparison, Hegrenes (2001) reared orangespotted sunfish (*Lepomis humilis*) on different diets for 240 days (three times longer than the present study). While he observed a significant level of morphological change in external traits, there was little divergence in gill rakers or pharyngeal jaw apparatus among treatments. Robinson and Wilson (1996) reared pumpkinseed under differing habitat types for 75 treatment days (five days less than the present study), and observed significant morphological differentiation in both gill raker and jaw morphology. In the present study, significant main effects (before Bonferroni correction) were detected in four of the six traits tested, suggesting that our experimental time frame provides a good representation of the morphological divergence exhibited by pumpkinseed, regardless of their geographic origin.

In two previous comparisons, Yavno and Fox (2013) and Yavno *et al.* (2014) examined differences in phenotypic plasticity between native and non-native pumpkinseed using variable water velocities and competitive interactions, respectively. Those studies revealed that pumpkinseed progeny from the Iberian Peninsula exhibited some morphological changes in response to environmental perturbation that differed in direction from Canadian fish, and that Iberian fish possessed phenotypes that were less 'plastic' [by 32% in Yavno and Fox (2013); by 28% in Yavno *et al.* (2014)] than those of Canadian pumpkinseed. The present study is consistent with those results, as juveniles from Susqueda and Ter exhibited less plasticity than pumpkinseed from Rice or Otonabee (by 27%). However, we were unable to identify any geographic difference in morphological change in response to habitat type: for traits with significant interactive effects, there were no consistent patterns to indicate that non-native pumpkinseed exhibited morphological changes in a direction that was different from that of Canadian fish.

The univariate analysis revealed that Iberian pumpkinseed possess several morphological traits that are less plastic in response to habitat type than those of pumpkinseed from the two Canadian populations. For instance, all populations exhibited an increase in ventral caudal peduncle length when reared under pelagic conditions, but the magnitude of change was smaller in both Iberian populations (Fig. 3); the same pattern is evident for the lengths of both the upper and lower jaw structures (Fig. 5). Yavno and Fox (2013) suggested that the external morphological traits of Iberian pumpkinseed were more canalized, and potentially reflected a pattern of genetic assimilation. In response to environmental perturbations, phenotypic plasticity can produce phenotypic changes in a direction that has a beneficial fitness consequence (Ghalambor *et al.*, 2007), and the genotype then absorbs the state so that the original environmental perturbation is no longer required to induce the phenotypic divergence (Crispo, 2007). In our study, this pattern of genetic assimilation is not supported, as most of the phenotypic changes observed in Canadian pumpkinseed were not towards a state already exhibited by Iberian fish. Instead, the reduction in plasticity might be reflective of differences in environmental stochasticity between the individual water bodies.

Reed *et al.* (2010) recently modelled the effects of phenotypic plasticity on populations that experience environmental variability. They determined that under highly perturbed conditions, plasticity might lead to the development of traits that surpass the optimum (i.e. maladaptations), which, in turn, decrease fitness and lead to extinction. Under highly variable conditions, the population is more likely to persist if individuals exhibit reduced plasticity, or even canalization. Hallson and Björklund (2012) observed this phenomenon in an experimental comparison using the seed beetle (*Callosobruchus maculatus*); after as little as 18 generations, breeding lines that were subjected to fluctuating temperature gradients exhibited decreased levels of phenotypic plasticity. In our study, pumpkinseed from riverine populations could be exhibiting external locomotory traits that are more canalized as a result of the high degree of perturbation in these systems.

The Otonabee River is part of the Trent-Severn Waterway, which, since the early nineteenth century, has undergone anthropogenic modifications that include the construction of dams to generate electricity and regulate water levels. In the Iberian Peninsula, the Ter River was once a continuous system that originated in the eastern Pyrenees and flowed into the Mediterranean Sea. Over the past century, the Ter River has become segregated by the construction of numerous reservoirs, designed to compensate for the highly variable hydrologic cycle in the region (Puig *et al.*, 1987). The Otonabee River experiences a two-fold increase in water discharge in a single season, whereas discharge in the Ter fluctuates by a factor of 24 (Yavno and Fox, 2013). Pumpkinseed from Ter never exhibited the largest magnitude of phenotypic divergence (i.e. the most plasticity), while those from Otonabee did so for only two morphological traits (one of the twelve external traits and one of the six internal traits; see insets in Figs. 3, 4, and 5). In multivariate space, Mahalanobis distances provide further support for a decrease in plasticity, as separation was not significant between littoral/pelagic treatment pairs for riverine populations in either the external or internal traits. Moreover, given that the mean age of maturity of female pumpkinseed that were sampled from Spain and Canada was 2 and 4 years, respectively (Fox, 1994; Fox *et al.*, 2007), and that the genetic basis for plasticity is subject to selection in some higher-order taxa (e.g. Nussey *et al.*, 2005), pumpkinseed from these riverine populations may have undergone directional selection towards more canalized phenotypes for 50 generations or more.

Susqueda Reservoir also undergoes environmental perturbation. The water body has steep slopes that create a profuse pelagic zone; in a single season, water levels can fluctuate

at least 15 metres, which limits the availability of long-term littoral habitat (Vila-Gispert *et al.*, 2007). Rice Lake, on the other hand, has experienced reduced hydrological perturbation as a result of anthropogenic modifications: the system was prone to large water level changes, but it has stabilized over the past century due to the construction of a downstream dam (Yu and McAndrews, 1994). In the present study, pumpkinseed from Susqueda were less plastic than progeny from Rice. The latter, which were the largest fish used in our study, exhibited the largest phenotypic divergence of any population for seven external and four internal traits, while the former did so for only four external and one internal trait. Again, Mahalanobis distances indicate that littoral/pelagic treatments for Susqueda did not show differentiation for internal traits (but did for external traits), while Rice exhibited strong morphological differentiation between treatment pairs for both external and internal traits. This further supports the notion that the observed reduction in plasticity in Iberian populations could be associated with the levels of environmental perturbation in each aquatic system.

Rivers and reservoirs in Spain and Portugal are unstable aquatic systems that experience a substantial level of biotic and abiotic perturbations (Gasith and Resh, 1999; Santos *et al.*, 2011). In their native range, pumpkinseed are capable of exhibiting strong levels of phenotypic plasticity to both biotic and abiotic factors (Robinson and Wilson, 1996), and some researchers have hypothesized that the spread of some non-indigenous species is due to their ability to exhibit stronger phenotypic plasticity than native counterparts (e.g. Davidson *et al.*, 2011). While pumpkinseed exhibit trophic polymorphism in numerous native and non-native water bodies (Gillespie and Fox, 2003; Vila-Gispert *et al.*, 2007; Bhagat *et al.*, 2011), we were unable to support the prediction that Iberian pumpkinseed would exhibit stronger plasticity in response to habitat-specific factors than native populations. Nevertheless, the reduced levels of plasticity in non-native populations are clearly sufficient to facilitate establishment and survival in rivers and reservoirs in the Iberian Peninsula. In the present study, Iberian pumpkinseed exhibited morphological changes in the same direction as their native counterparts, and towards morphological states that are considered adaptive for navigating through environments with low structural complexity. We suggest that future comparisons incorporate direct measures of heritability and fitness, allowing for a stronger assessment of how morphological changes might affect the invasion success of pumpkinseed in non-native areas.

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