

# Ecomorphological patterns linking morphology and diet across three populations of pumpkinseed sunfish (*Lepomis gibbosus*)

A.E. Berchtold, S.F. Colborne, F.J. Longstaffe, and B.D. Neff

**Abstract:** Relationships between morphological traits and their ecological function frequently result in patterns that are consistently observed within taxa. Across fishes, the field of ecomorphology has identified a number of morphological traits linked to foraging tactic. Here we examined the links between morphology and diet in pumpkinseed sunfish (*Lepomis gibbosus* (L., 1758)) from three temperate freshwater lakes. We focused on morphological variation in pharyngeal jaw and gill raker structures, both involved with the processing of prey after capture, in relation to diet. Using stomach contents and stable isotope analysis, we established mean resource use estimates for both populations and individual fish. Among populations and individuals within lakes, we observed that pharyngeal jaw size and gill raker spacing increased with the consumption of littoral prey (e.g., hard-shelled snails) relative to pelagic prey (e.g., zooplankton), but the morphological changes were greater for the pharyngeal jaws. Overall, the relationships that we observed between morphology and foraging tactic were consistent with patterns observed in pumpkinseed and across other fishes. Individual-level diet variation associated with morphology may result in phenotypic diversity within populations that has multiple ecological and evolutionary implications for these populations.

**Key words:** *Dreissena polymorpha*, ecomorphology, gill rakers, internal morphology, *Lepomis gibbosus*, pharyngeal jaw, phenotypic plasticity, resource polymorphism, stable isotopes.

**Résumé :** Les liens entre les caractères morphologiques et leur fonction écologique se traduisent souvent par des motifs uniformément observés au sein de taxons. En ce qui concerne les poissons, l'écologie a cerné un certain nombre de caractères morphologiques associés aux tactiques d'alimentation. Nous examinons les liens entre la morphologie et le régime alimentaire chez des crapets-soleil (*Lepomis gibbosus* (L., 1758)) de trois lacs d'eau douce tempérés. Nous nous penchons plus particulièrement sur les variations morphologiques en fonction du régime alimentaire des structures de la mâchoire pharyngienne et des branchiospines, qui interviennent toutes deux dans la transformation des proies après leur capture. À la lumière de contenus stomacaux et de l'analyse d'isotopes stables, nous avons établi des estimations de l'utilisation moyenne des ressources tant pour les populations que les poissons individuels. Entre les populations et entre les individus d'un même lac, nous avons observé que plus la consommation de proies littorales (p. ex. escargots à coquille dure) était grande par rapport à celle de proies pélagiques (p. ex. zooplancton), plus la taille des mâchoires pharyngiennes et l'espacement des branchiospines étaient importants, cette variation morphologique étant toutefois plus grande pour les mâchoires pharyngiennes. Globalement, les relations que nous avons observées entre la morphologie et les tactiques d'alimentation concordent avec les motifs déjà observés chez les crapets-soleil et chez d'autres poissons. Les variations du régime alimentaire associées à la morphologie entre individus pourraient se traduire par une diversité phénotypique au sein des populations qui aurait de multiples conséquences en ce qui concerne l'écologie et l'évolution de ces populations. [Traduit par la Rédaction]

**Mots-clés :** *Dreissena polymorpha*, écomorphologie, branchiospines, morphologie interne, *Lepomis gibbosus*, mâchoire pharyngienne, plasticité phénotypique, polymorphisme des ressources, isotopes stables.

## Introduction

The field of ecomorphology identifies relationships between morphological traits and ecological processes, such as resource use (e.g., Motta and Kotrschal 1991; Wainwright 1996). Biologists have identified patterns between morphological features and foraging tactic (e.g., Delariva and Agostinho 2001; Kassam et al. 2003) and related variation in morphology across populations and species to their local ecology, inferring adaptation. These patterns have also been used to predict ecological characteristics of species and individuals based on their morphology (e.g., Griffen and

Mosblack 2011). By utilizing an ecomorphological approach, we can yield a greater understanding of variation within species and of selection processes, such as local adaptation.

In fishes, patterns relating morphology, diet, and habitat use have been well studied (e.g., Wainwright 1996; Amundsen et al. 2004; Hendry et al. 2009). Pharyngeal jaws, for example, vary considerably in size, shape, and dentition across species, producing an array of jaw forms that accommodate feeding actions (i.e., grinding, filtering, or cutting) suitable to particular prey (for a description of such diversity in Centrarchidae and other temperate fishes see Keast 1978a). In particular, pharyngeal jaw size is

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associated with the ability to crush hard prey items, such as the shells of molluscs, with hypertrophied jaws found in populations that consume large quantities of hard-shelled prey types (e.g., Wainwright 1991; Huckins 1997; Osenberg et al. 2004). Across species, patterns have also been found in pharyngeal jaw dentition with shell-crushing fish typically having large molariform teeth, linked to increased grinding surface area, compared with consumers of soft tissues that have finer needle-like teeth linked to piercing and tearing (Keast 1978b; Burress 2015). Furthermore, gill raker length and density have been associated with degree of zooplanktivory because dense gill rakers are thought to improve filtering capacity of smaller food particles, e.g., pelagic zooplankton, which may otherwise escape through the gill if the rakers were shorter or less densely packed (Werner and Hall 1976; Malmquist 1992; Amundsen et al. 2004). In addition to interspecific diversity, variation of these morphological features is also common within fish populations and individual trait differences typically correspond to differences in foraging efficiency (Robinson and Schluter 2000). In some populations, variation of feeding morphology defines distinct groups that display corresponding divergence in habitat and resource use, commonly referred to as resource or trophic polymorphisms (Smith and Skulason 1996; Robinson and Schluter 2000). Consistency in such patterns relating morphological traits to foraging ecology therefore provides a predictive framework from which to study not only individual variation across populations but also variation within populations.

Here, we studied pumpkinseed sunfish (*Lepomis gibbosus* (L., 1758)), a member of the North American Centrarchidae family. Pumpkinseed are typically molluscivores (e.g., Gastropoda) inhabiting littoral zones of freshwater lakes (e.g., Keast 1978a); they possess hypertrophied pharyngeal jaws compared with other sunfishes, which enable them to produce a bite force strong enough to crush hard-shelled prey (Lauder 1983; Wainwright 1996). In most lakes, pumpkinseed are sympatric with bluegill (*Lepomis macrochirus* Rafinesque, 1819), but exhibit resource partitioning as a result of interspecific competition between the two species (Mittelbach 1984). Bluegill typically consume zooplankton (Copepoda and Cladocera) and, consistent with morphological patterns expected for fish, possess pharyngeal jaw structures that are small relative to those of molluscivorous pumpkinseed (Keast 1978b; Collar and Wainwright 2009).

If morphological relationships across species relating jaw and gill raker structures to diet are robust, then they should also be found when there is individual-level diet variation within a species. Indeed, across separate populations of pumpkinseed, jaw size has been correlated to the local density of snails relative to softer benthic invertebrates: lower snail densities corresponded to decreased jaw size (Wainwright et al. 1991). Additionally, based on diet alone, littoral–pelagic resource polymorphisms have been identified in approximately 30 populations of pumpkinseed, typically in the absence of bluegill (Robinson et al. 1993; Gillespie and Fox 2003; Jastrebski and Robinson 2004). Past comparisons between littoral pumpkinseed morphs that forage on benthic invertebrates, e.g., snails, and zooplankton-consuming pelagic morphs showed that (i) gill raker length increased with consumption of zooplankton (three populations examined) and (ii) jaw width increased with littoral foraging (one population examined) (Robinson et al. 1993; Jastrebski and Robinson 2004). These studies provide support for the expected relationships between morphology and diet at the level of individual fish but, with the exception of one study on an invasive pumpkinseed population in Europe (Bhagat et al. 2011), not all of the feeding traits expected to be part of this relationship have been investigated, e.g., total jaw size and associated musculature. Furthermore, past studies correlated morphological traits with diet based on stomach contents alone (e.g., Jastrebski and Robinson 2004). Although stomach contents provide accurate estimates of recent diet, i.e., hours to days, they cannot provide estimates of long-term diet and may mask longer

term resource variation that could have important implications for the relationships of interest.

In this study, we further examined the patterns between resource use and morphological traits in pumpkinseed by examining variation in the pharyngeal jaws (size and related musculature), gill rakers (size and number), and diet among populations from three lakes, including one lake that exhibits a littoral–pelagic resource polymorphism. Here, we inferred resource use by coupling stomach content analysis with longer term diet estimates available through stable isotope analysis. We also examined pumpkinseed from three lakes that, while relatively close to each other, varied in two environmental factors: (1) the presence or absence of zebra mussels (*Dreissena polymorpha* (Pallas, 1771)) and (2) the presence or absence of competing bluegill. Choosing communities that varied in composition provided an opportunity to examine relationships between individual form and diet across a range of environmental conditions. Within lakes, we expected pharyngeal jaw and gill raker morphology to reflect the diets of individual pumpkinseed such that individuals consuming more littoral resources, e.g., snails, would have larger pharyngeal jaws (both jaw size and related musculature) and less densely packed gill rakers relative to consumers of pelagic resources, e.g., zooplankton. Furthermore, we expected that these relationships would be robust among the lakes, reflecting the general ecomorphological relationships of fishes.

## Materials and methods

### Study sites and sample collection

Pumpkinseed were collected from three study lakes (Opinicon, Lower Beverley, and Ashby) over the period of 26 May – 28 June 2011 (for lake coordinates see Table 1). Opinicon and Lower Beverley lakes have both been invaded by zebra mussels and contain bluegill, a close relative of pumpkinseed, but Ashby Lake lacks both bluegill and zebra mussels. Fish were caught either by angling with a small piece of worm (2–3 cm) or dip-netting directly from the water column. Only fish with a total body length of 120–180 mm were kept to ensure that sampled fish were (i) adults past an ontogenetic niche shift that occurs between juvenile and adult life stages in sunfish (Mittelbach 1984) and (ii) of overlapping body sizes across the sampled lakes. Fish were immediately euthanized using clove oil. Stomach contents and livers were extracted and preserved by freezing at –20 °C in separate 1.5 mL microcentrifuge tubes for diet analysis. Whole fish were then preserved at –20 °C for morphological analysis. Ashby Lake pumpkinseed were classified into littoral- and pelagic-caught groups based on their collection habitat (see Jastrebski and Robinson 2004). Pumpkinseed from Opinicon Lake and Lower Beverley Lake contain only a single, littoral resource morph.

### Morphological analysis

We examined pharyngeal jaw morphology by measuring (i) the mass of the pharyngeal jaw bones and (ii) the mass and length of the levator posterior muscle after blind-coding all samples to avoid potential observer bias. The fifth ceratobranchial (lower jaw bone; LJ) and third pharyngobranchial (upper jaw bone; UJ) (Wainwright 1989) were used for measures of the pharyngeal jaw bone size. The right UJ and LJ bones were removed from each fish, patted dry, and weighed twice to the nearest 0.1 mg to provide a mean dry mass of each bone, which was then used for subsequent analyses (e.g., Wainwright et al. 1991). Jaw musculature was examined using the levator posterior muscle (LP), which is the primary muscle that exerts crushing force by moving the UJ and LJ towards each other. The length and mass of the LP provide a proxy for the overall bite force an individual is capable of producing (Wainwright 1989; Galis and Drucker 1996; Osenberg et al. 2004). The length of the right LP muscle was measured by photographing the muscle with an Olympus Stylus Tough-6000 (10 megapixel) digital camera

**Table 1.** Summary information of pumpkinseed sunfish (*Lepomis gibbosus*) sampled from three lakes.

Lake	Collection habitat	Coordinates	n	Total body length (mm)	Wet mass (g)
Opinicon	Littoral	44°34'N, 76°19'W	15	167±14	103±26
Lower Beverley	Littoral	44°60'N, 76°14'W	17	157±19	93±37
Ashby	Littoral	45°05'N, 77°21'W	11	128±7	36±7
	Pelagic		19	148±11	58±13

**Note:** Lake names, collection habitat, number of fish collected (n), total body length (mean ± SD), and wet mass (mean ± SD) are included in the table.

while the muscle was still attached to the fish and then using ImageJ version 1.46 software (National Institutes of Health, Bethesda, Maryland, USA) to measure its length to the nearest 0.001 mm. The LP muscles were then extracted from the fish, dried at 55 °C for 24 h, and weighed to the nearest 0.1 mg.

To assess morphological variation of gill rakers, the first right branchial gill raker arch was removed and stained with Alizarin Red S (Sigma–Aldrich Canada, Ltd.) in a 75% ethyl alcohol solution for 24 h (Springer and Johnson 2000; Jastrebski and Robinson 2004). Only the first arch was assessed because it can account for up to 60% of the total filtering area in fishes; therefore, it is considered to be the most relevant to overall filtering performance (e.g., Gibson 1988). As with the jaw bones and muscles, all gill arches were blind-coded prior to measuring. The total number of gill rakers was counted using a dissection microscope. Photographs (see camera details above) of each arch were captured and the length of the second raker was measured from the photographs using ImageJ. Also using ImageJ, distances between each of the first four rakers from the apex of each arch were measured to the nearest 0.001 mm; mean distance between the four focal rakers (“mean raker gap”) was then calculated.

Correction for the possible effects of allometric growth on pharyngeal jaw and gill raker measurements was made using ordinary least-squares regression on each morphological variable with body length as the predictor variable. All mass-based morphological variables (LP, UJ, and LJ masses) were log-transformed prior to regression to ensure linearity. Residual values resulting from these regressions were then used for all further analyses (see Schulte-Hostedde et al. 2005).

### Diet analysis

Stomach contents were collected from each fish to provide a detailed summary of foraging tactic in the sampling groups; of the 62 fish collected, 57 (92%) had measurable stomach contents at the time of collection and were included in this analysis. Contents were classified into one of four prey types: (1) snails (Gastropoda), (2) zebra mussels, (3) zooplankton (primarily Copepoda and Cladocera), and (4) other (including other benthic invertebrates, plant material, fish eggs, and unidentifiable stomach contents). The stomach contents were dried at 55 °C for 24 h and weighed to the nearest 0.1 mg. Diet was recorded as the proportion that each prey type contributed to the total dry mass for each individual. All proportions were arcsine square-root transformed to pass the assumptions of normality and equal variances required for statistical analysis.

To provide inferences of longer term resource use, stable isotope analysis data were gathered from other simultaneously occurring studies of sunfish diet that used the same fish analyzed here (S.F. Colborne, B.D. Neff, and F.J. Longstaffe, unpublished data). In brief, the ratio of heavy to light stable isotopes, most commonly carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ), contained in the proteins of an organism predictably reflects isotopic composition of the proteins of its diet (DeNiro and Epstein 1981; Hobson and Clark 1992). In this study, we used the stable isotope measures of liver tissue, providing an estimate of diet over a 1–7 week period prior to tissue collection (Thomas and Cahoon 1993; Buchheister

and Latour 2010). As stable isotope ratios can vary temporally and spatially (e.g., Bearhop et al. 2004), resource baseline values were determined for pelagic (zooplankton) and littoral (snails and mussels) resources in each lake over the same period that fish were being sampled. Littoral benthic invertebrates were collected using D-net sweeps through the vegetation and upper layer of sediment and then hand sorted through a series of nested sieves. Pelagic zooplankton were collected using vertical tows (80  $\mu\text{m}$  mesh size) through the upper 3–5 m of the water column. All samples were then frozen at –20 °C until further processing in the laboratory. Snails and mussels were manually removed from their shells prior to isotopic analysis. The same collection and preparation techniques were used for all of the isotopic measures reported in this study.

Fish liver tissue and reference prey samples were freeze-dried at –50 °C for 24 h and then manually ground using a mortar and pestle. Ratios of  $^{13}\text{C}:^{12}\text{C}$  and  $^{15}\text{N}:^{14}\text{N}$  in each sample were determined using continuous-flow mass spectrometry in the Laboratory for Stable Isotope Science at The University of Western Ontario (London, Ontario, Canada). Stable isotope ratios are expressed as the difference from a standard reference material in parts per thousand (‰):

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

where  $X$  is  $^{13}\text{C}$  or  $^{15}\text{N}$ ,  $R$  is the ratio of  $^{13}\text{C}:^{12}\text{C}$  or  $^{15}\text{N}:^{14}\text{N}$ , and  $\delta$  is the ratio of heavy to light isotope in the sample. The values of both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  used here were gathered from larger data sets that were calibrated to VPDB (Vienna Pee Dee Belemnite) and atmospheric nitrogen (AIR), respectively, for each lake using two-point calibration curves with the international standards USGS-40 ( $\delta^{13}\text{C}$  accepted value –26.39‰;  $\delta^{15}\text{N}$  accepted value –4.52‰) and USGS-41 ( $\delta^{13}\text{C}$  accepted value +37.63‰;  $\delta^{15}\text{N}$  accepted value +47.57‰). Additionally, internal laboratory standards of keratin ( $\delta^{13}\text{C}$  accepted value –24.04‰;  $\delta^{15}\text{N}$  accepted value –6.36‰) and IAEA-CH-6 ( $\delta^{13}\text{C}$  only; accepted value –10.45‰) or IAEA-N2 ( $\delta^{15}\text{N}$  only; accepted value +20.30‰) were used to monitor the precision and accuracy of isotopic measurements. Measured values for keratin ( $\delta^{13}\text{C}$ : –24.1‰ ± 0.1‰;  $\delta^{15}\text{N}$ : +6.34‰ ± 0.2‰), IAEA-CH-6 ( $\delta^{13}\text{C}$ : –10.5‰ ± 0.1‰), and IAEA-N2 ( $\delta^{15}\text{N}$ : +20.4‰ ± 0.2‰) were all within the acceptable ranges for each standard. Across 57 duplicate samples of fish tissue and prey sources, the mean reproducibility was ±0.1‰ for both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , which was within the acceptable ±0.2‰ range for such isotopic analyses.

The  $\delta^{13}\text{C}$  values of tissues can be negatively biased due to higher lipid content compared with pure protein samples; thus,  $\delta^{13}\text{C}$  measures of fish liver tissues were lipid-corrected using a mathematical correction model refined for aquatic organisms (Kiljunen et al. 2006):

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

and

**Table 2.** Morphological analysis of pumpkinseed sunfish (*Lepomis gibbosus*).

Lake	Collection habitat	Pharyngeal jaw				Gill raker		
		LP mass (g)	LP length (mm)	UJ mass (g)	LJ mass (g)	Total no. of rakers	Mean raker gap (mm)	Raker length (mm)
Opinicon	Littoral	0.011±0.004a	10±1a	0.08±0.03a	0.15±0.05a	12±1ab	0.9±0.1b	1.3±0.2a
Lower Beverley	Littoral	0.010±0.005a	9±1a	0.07±0.03a	0.14±0.06a	11±1b	1.0±0.2a	1.2±0.3a
Ashby	Littoral	0.003±0.002a	8±1a	0.03±0.01a	0.04±0.01a	10±1b	0.7±0.1b	1.0±0.2a
	Pelagic	0.003±0.002b	8±1a	0.03±0.01b	0.05±0.02b	12±1a	0.7±0.1b	1.2±0.3a

**Note:** All analyses were performed on morphological measures corrected for allometry using ordinary least-squares regression (see Materials and methods), but values presented here represent raw data. Lake name, collection habitat, levator posterior muscle (LP) mass and length, upper (UJ) and lower (LJ) pharyngeal jaw bone mass, total number of gill rakers, mean gap between gill rakers, and gill raker length are included in the table. All values are means ± 1 SD. Letters indicate significant differences among lakes and collection habitats ( $P < 0.05$ ) according to Tukey's post hoc comparison test.

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

where  $L$  is the estimated lipid content of each sample based on the atomic ratio of carbon and nitrogen (C:N),  $\delta^{13}C'$  is the lipid-corrected value of a sample,  $\delta^{13}C$  is the measured value of each sample,  $D$  is a constant (7.02‰) representing the mean isotopic difference between lipids and pure protein, and  $I$  is a constant (0.05) (Kiljunen et al. 2006).

### Statistical analysis

To assess variation of morphology across lakes (and habitats for Ashby Lake), multivariate analysis of variance (MANOVA) was used (response variable: LP dry mass, LP length, UJ mass, LJ mass, total number of rakers, mean raker gap, and raker length; fixed factor: lake and habitat). Those traits that were found to differ between lakes and habitats were further examined using univariate analyses and Tukey's post hoc comparisons. Based on the variables that were found to differ significantly in the MANOVA (see above), a discriminant function analysis (DFA) was performed to determine which suites of traits characterized the morphological variation. Subsequent univariate analyses were then performed to assess how sampling groups differed based on the composite morphological variables, e.g., DFA 1, provided by the DFA. Only DFA axes that explained at least 10% of the total variation were considered significant and used for further analyses.

Diet was assessed using both stomach content and stable isotope data. First, the stomach content proportions were used in a two-factor ANOVA to determine variation in diet among pumpkinseed from each lake (response variable: stomach content proportions; fixed factors: prey type, lake, and habitat). Second, stable isotope compositions were used to estimate the contribution of different potential prey types to pumpkinseed diet using two-source stable isotope analysis in R (SIAR) mixing models (R version 3.0.1; Parnell et al. 2010; R Development Core Team 2012). The liver tissue  $\delta^{13}C$  and  $\delta^{15}N$  values (mean ± 1 SD) of each fish were used in separate models for each lake. Based on the prior examination of stomach contents, the SIAR models for Opinicon and Lower Beverley lakes did not include zooplankton as a variable due to their absence from the stomach contents (see Results). Also, the SIAR models for Ashby Lake (littoral- and pelagic-caught fish) did not include zebra mussels as a potential prey group because these mussels are absent from that lake. Consequently, all SIAR models were two-source mixing models comparing either (i) littoral benthic prey and zebra mussels (Opinicon and Lower Beverley lakes) or (ii) littoral benthic prey and pelagic zooplankton (Ashby Lake). The isotopic composition of the prey samples collected at the same time as the fish were used as the "source" estimates for each model. Snails were used as a proxy for littoral benthic invertebrates in general because they are relatively long-lived integrators of littoral resources and are considered accurate proxies for the littoral community of temperate freshwater lakes (e.g., Post 2002;

**Table 3.** Discriminant function analysis (DFA) coefficients for pumpkinseed sunfish (*Lepomis gibbosus*).

Variable	DFA 1	DFA 2
Levator posterior muscle mass	1.2	1.4
Upper pharyngeal jaw mass	3.5	-3.9
Lower pharyngeal jaw mass	2.5	6.7
Number of gill rakers	-0.3	0.2
Gill raker gap	3.2	-9.9

**Note:** Pharyngeal jaw (size and musculature) and gill raker morphological traits are included in the table. DFA 1 and DFA 2 explained 91% and 8% of the total between-class variance, respectively.

Correa et al. 2012; Jones et al. 2013). Mean trophic enrichment factors (TEFs) were based on multiple freshwater fish species from northern temperate lakes ( $\delta^{13}C$ :  $0.47\% \pm 1.23\%$  and  $\delta^{15}N$ :  $3.23\% \pm 0.41\%$ ; Vander Zanden and Rasmussen 2001) because species-specific TEFs were unavailable for pumpkinseed.

The resource use of individual fish was then estimated for each lake using the SIARsolo command and the same model variables as presented above for overall lake estimates. The individual estimates of littoral contribution to diet were then used in an analysis of covariance (ANCOVA) to examine the relationship between diet and morphology among the three sample lakes (response variable: proportion of littoral resources in diet; fixed factor: lake; covariate: DFA score).

All statistical analyses were performed using R versions 2.14.2 or 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria) or JMP version 10.0.0 (SAS Institute Inc., Cary, North Carolina, USA) with  $\alpha = 0.05$ . All means are presented with ±1 SD.

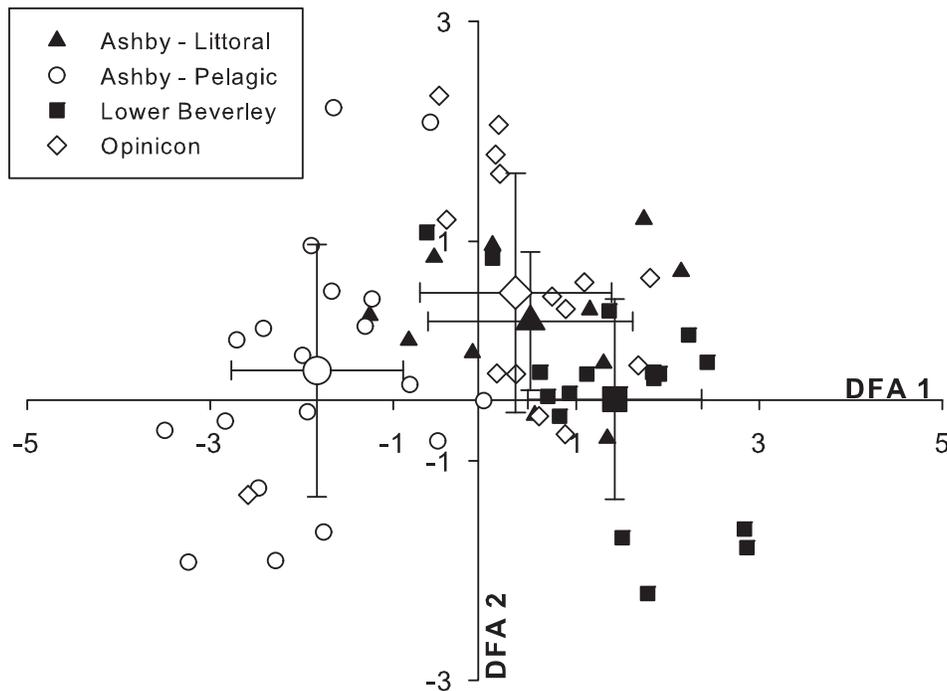
## Results

### Morphological analysis

Significant differences were observed among pumpkinseed groups in the morphological traits measured (MANOVA,  $\lambda = 0.9$ ,  $F_{[21,162]} = 3.1$ ,  $P < 0.001$ ). Univariate analysis of each morphological variable revealed differences among sampling groups in all pharyngeal jaw and gill raker traits, except LP and gill raker lengths (Table 2). Subsequent Tukey's post hoc comparisons demonstrated that all pharyngeal jaw measures, except LP length, distinguished littoral and pelagic foraging fish (Table 2).

Based on the results of the MANOVA, DFA was performed on all morphological traits except LP and gill raker lengths. The first and second discriminant functions (DFA 1 and DFA 2) explained 91% and 8% of the between-class variance, respectively. DFA 1 loaded positively for LP, UJ, and LJ masses and mean raker gap; in contrast, the number of rakers loaded negatively on the same axis. DFA 2 did not meet the 10% threshold, so it was not considered for further analysis of the morphological variables (Table 3). Examination of DFA 1 scores found that there were overall morphological

**Fig. 1.** Discriminant function analysis (DFA) results for pumpkinseed sunfish (*Lepomis gibbosus*) with pharyngeal jaw and gill raker morphological characteristics as predictors for sampling location. Centroids are represented by the larger symbols for each lake and habitat and indicate mean ( $\pm 1$  SD) DFA values for each group; also shown are individual DFA scores for each fish.



differences among pumpkinseed collected from different lakes and habitats (ANOVA,  $F_{[3,58]} = 34.2$ ,  $P < 0.001$ ). The Ashby Lake pelagic-caught fish had significantly lower scores than all other groups (Tukey's post hoc comparison test, all  $P < 0.001$ ; Fig. 1).

### Diet analysis

Stomach content analysis revealed significant differences in resource groups both within and among lakes. Across the three lakes, there were significant differences in the stomach content proportions among the prey-type categories (ANOVA,  $F_{[3,185]} = 40.3$ ,  $P < 0.001$ ), but no evidence of a lake and habitat effect ( $F_{[3,185]} = 1.2$ ,  $P = 0.3$ ). There was a significant interaction between prey type and lake and habitat group ( $F_{[7,185]} = 6.5$ ,  $P < 0.001$ ). The greatest concentrations of zooplankton were found in the stomach contents of Ashby Lake pelagic-caught fish (38%) compared with the stomachs of Opinicon Lake and Lower Beverley Lake pumpkinseed, which had no zooplankton but contained more zebra mussels (59% and 90%, respectively) than any other prey type (for details see Table 4).

SIAR models of diet based on liver tissue isotopic composition indicated that in Opinicon and Lower Beverley lakes, more than 60% of the diet was composed of zebra mussels compared with other benthic invertebrates. In comparison, estimates of Ashby Lake pumpkinseed indicated that diet was dependent on the collection habitat type; littoral-caught pumpkinseed consumed over 80% benthic invertebrates, but there was a nearly equal split between resource types for pelagic-caught fish (Table 5, Fig. 2).

### Morphology and diet

We observed a positive relationship between diet (proportion of littoral resources in diet) and morphology (DFA 1 scores; ANCOVA,  $F_{[1,56]} = 5.09$ ,  $P = 0.03$ ), but no evidence of overall variation in diet estimates among the lakes ( $F_{[2,56]} = 1.85$ ,  $P = 0.17$ ; Fig. 3). There was evidence of an interaction effect, indicating that the relationship between diet and morphology varied among the lakes ( $F_{[2,56]} = 3.57$ ,  $P = 0.03$ ).

### Discussion

Based on the patterns relating morphology and resource use across many fish species (e.g., Wainwright 1996; Amundsen et al. 2004; Hendry et al. 2009), we examined three populations of pumpkinseed that were expected to show similar morphological traits depending on the diets of each individual. We found evidence across our three lakes that the size of levator posterior muscles, pharyngeal jaw bones, and gill raker gaps increased as individuals consumed greater quantities of littoral invertebrates (e.g., snails) in their diets. Our stable isotope results provided longer term estimates of resource use and demonstrated similar relationships among diet, pharyngeal jaws, and gill rakers as previous studies that used stomach contents in pumpkinseed (e.g., Wainwright et al. 1991; Robinson et al. 1993; Gillespie and Fox 2003) and other fishes (e.g., Wainwright 1991; Huckins 1997).

Patterns relating morphological traits to diet have been attributed to performance-related consequences of the structures, with these structures then driving character displacement among species. Pumpkinseed with larger pharyngeal jaw bones and levator posterior muscles, for example, demonstrate increased efficiency when crushing hard-shelled molluscs (e.g., Lauder 1983; Osenberg et al. 2004). Consistent with previous pumpkinseed research based on shorter term estimates of diet, our results linked decreased pharyngeal jaw and associated musculature sizes to increased gill raker density, traits associated with feeding on zooplankton (Werner and Hall 1976). Additionally, across all pumpkinseed that we sampled, larger pharyngeal jaw size and smaller gill raker density were correlated with increased long-term littoral resource (e.g., snail) consumption. The tendency for larger jawed pumpkinseed to specialize on hard-shelled prey is understood to reduce interspecific competition (e.g., bluegill; Mittelbach 1984) and be a key contributor to character displacement in the genus. However, the variation in these traits that we detected also within the Ashby Lake pumpkinseed, where bluegill are absent, may specifically reduce intraspecific competition (e.g., Robinson et al. 1996), particularly

**Table 4.** Stomach content analysis of pumpkinseed sunfish (*Lepomis gibbosus*).

Lake	Collection habitat	n	Stomach content			
			Snails	Zebra mussels	Zooplankton	Other
Opinicon	Littoral	12 (out of 15)	0.28±0.35ab	0.59±0.45a	—	0.13±0.20b
Lower Beverley	Littoral	16 (out of 17)	0.06±0.09b	0.90±0.09a	—	0.03±0.06b
Ashby	Littoral	11 (out of 11)	0.14±0.31b	—	0.09±0.30b	0.76±0.40a
	Pelagic	18 (out of 19)	0.16±0.37a	—	0.38±0.41a	0.45±0.43a

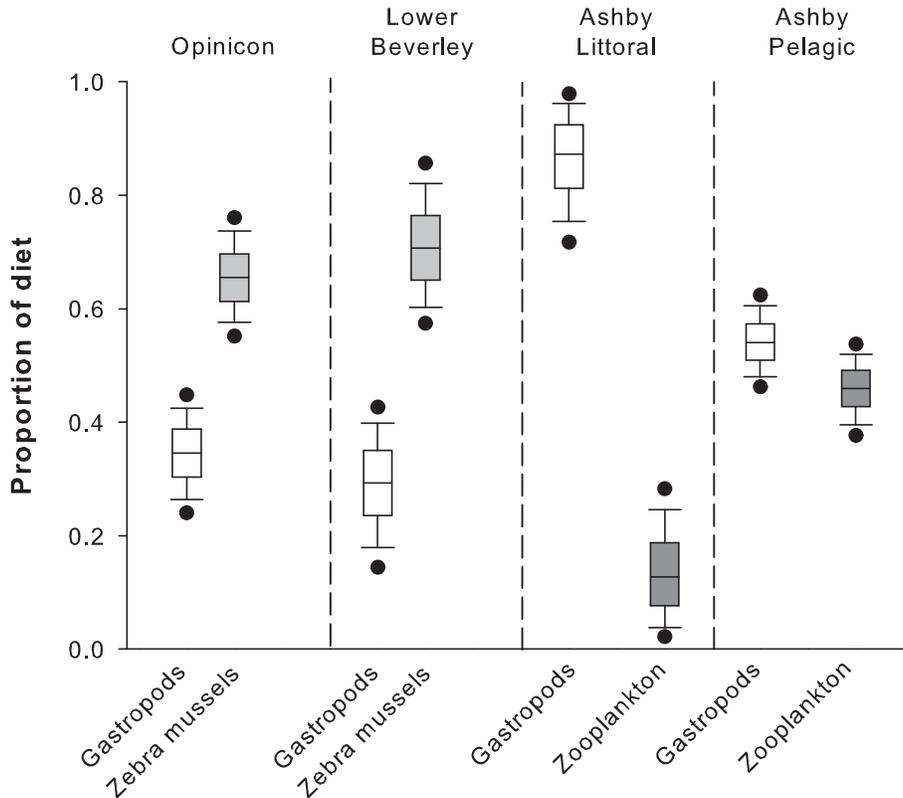
**Note:** Zebra mussels (*Dreissena polymorpha*) are not present in Ashby Lake. Zooplankton are absent from the analysis of Opinicon Lake and Lower Beverley Lake because they were not found in the stomach contents of any fish collected. Some analyses were completed using arcsine square-root transformed data (see Materials and methods), but all proportions presented here represent untransformed data. Contents were identified and sorted into one of four categories: snails (Gastropoda); zebra mussels; zooplankton (Copepoda and Cladocera); or other (including all other benthic invertebrates, plant matter, and unidentifiable contents). Sample size represents the number of fish with stomach contents at the time of collection and the total sample size (in parentheses) for each lake and habitat. All values are means ± 1 SD. Different letters indicate significant differences ( $P < 0.05$ ) among prey types within each lake and habitat group based on Tukey's post hoc comparison test.

**Table 5.** Summary of pumpkinseed sunfish (*Lepomis gibbosus*) diet analysis using stable isotopes.

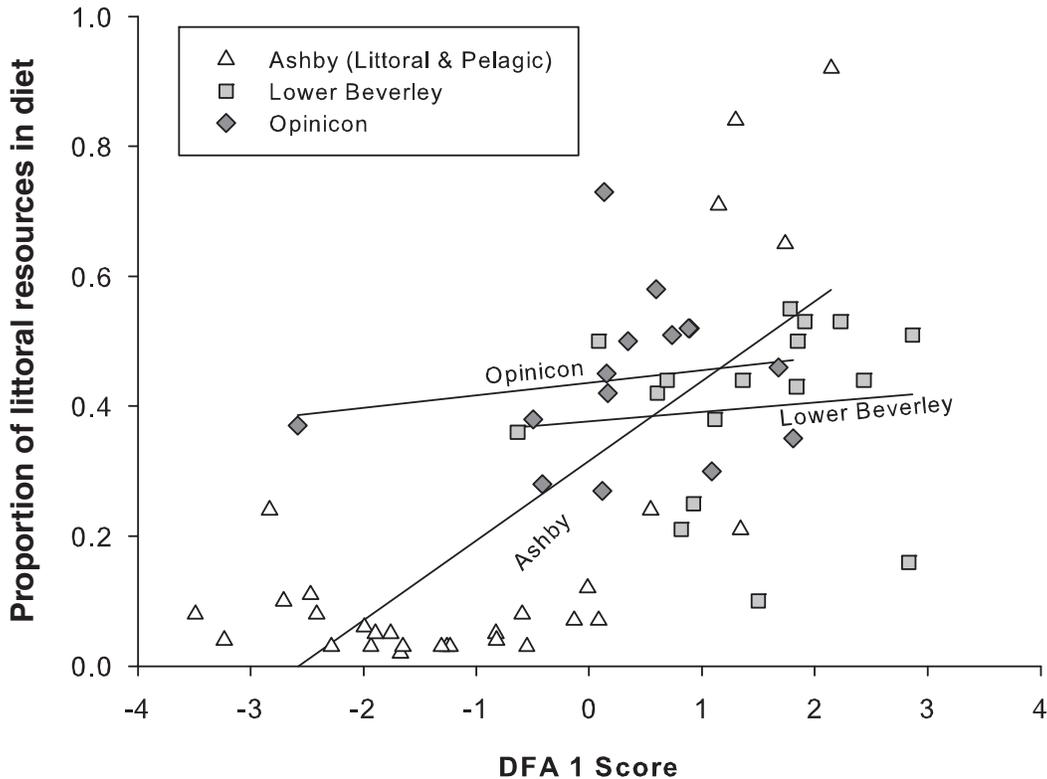
Lake	Collection habitat	n	$\delta^{13}\text{C}$ (‰); VPDB	$\delta^{15}\text{N}$ (‰); AIR	Proportion of diet		
					Snails	Zooplankton	Zebra mussels
Opinicon	Littoral	15	-24.7±2.0	+7.8±0.7	0.35 (0.22–0.47)	—	0.65 (0.53–0.78)
Lower Beverley	Littoral	17	-27.0±1.0	+10.3±1.1	0.29 (0.12–0.46)	—	0.71 (0.54–0.88)
Ashby	Littoral	11	-25.1±2.0	+6.9±1.0	0.86 (0.72–1.0)	0.14 (0.00–0.28)	—
	Pelagic	19	-26.5±1.3	+7.5±0.7	0.54 (0.45–0.64)	0.46 (0.36–0.55)	—

**Note:** Ashby Lake does not contain zebra mussels (*Dreissena polymorpha*). Zooplankton was excluded from the mixing models for Opinicon Lake and Lower Beverley Lake based on the absence of zooplankton in the stomach contents of pumpkinseed sunfish from these lakes (see above).  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values (calibrated against Vienna Pee Dee Belemnite (VPDB) and atmospheric nitrogen (AIR), respectively) for each lake and collection habitat and the results of two-source stable isotope analysis in R (SIAR) mixing models are included in the table. Stable isotope compositions are presented as means ± 1 SD and mixing model estimates are presented as means and 95% Bayesian credibility intervals (in parentheses).

**Fig. 2.** Estimates of pumpkinseed sunfish (*Lepomis gibbosus*) diet using two-source stable isotope analysis in R (SIAR) mixing models. Estimates are based on the isotopic composition of liver tissue collection for fish classified into four groups based on the lake and habitat of collection (Opinicon, Lower Beverley, Ashby Littoral, and Ashby Pelagic). Boxes represent the inner 50% of observations, the line inside a box represents the overall mean value, the whiskers correspond to the 90th and 10th percentiles, and the dots are the 95th and 5th percentiles.



**Fig. 3.** Covariation of pharyngeal jaw and gill raker morphology, indicated by DFA 1 scores, with littoral foraging tactic of four groups of pumpkinseed sunfish (*Lepomis gibbosus*). Symbols are labelled according to the lake of collection with Ashby Lake littoral- and pelagic-caught fish pooled for this analysis (see the Materials and methods). Regression lines of best fit are shown for each lake.



if ontogenetic niche shifts to an adult diet are related to conspecific population density and prey availability during development (Ehlinger and Wilson 1988). Thus, both pharyngeal jaw size and gill raker density appear to be related to individual foraging tactic within pumpkinseed and this pattern may reduce the intensity of not only interspecific competition but also intraspecific competition.

Despite the known roles of several morphological traits in foraging efficiency, there may be certain traits that have greater impact than others on performance. Our analyses indicated weaker differentiation of gill raker traits across pumpkinseed foraging tactics relative to what was expected based on studies of other fishes (e.g., Malmquist 1992; Schluter 1995). Other studies of pumpkinseed have also found gill raker morphology to differ subtly or not at all with diet (Robinson et al. 1993; Vila-Gispert et al. 2007; Bhagat et al. 2011), some suggesting that gill raker character release is constrained in this species. Another plausible explanation, however, is that the larger spacing between gill rakers typically associated with littoral foraging on benthic invertebrates (e.g., Werner and Hall 1976) is stronger in groups that consume invertebrates found in the sediment (e.g., amphipods) rather than molluscs, which are typically exposed on rocks and vegetation. Additionally, Watzin et al. (2008) observed that pumpkinseed expel some mollusc shell fragments through their mouth rather than their gill opercula. We also observed crushed shell fragments in the stomach contents and intestines of the pumpkinseed sampled for this study, indicating that shell fragments are also ingested with the softer body tissues after being crushed rather than passing through the gills. Thus, there may be a weaker trade-off between gill raker density and mollusc consumption in pumpkinseed than in other fish species. As such, the selection pressures on gill raker spacing may be lower relative to those affecting the jaw structures that are a primary determinant of shell-crushing ability. Based on our results, we speculate that fil-

tering performance associated with gill raker density is under weaker selection than the pharyngeal jaw structures related to crushing performance.

The ability to accurately infer the relationships between morphological traits and diet may be impacted by the invertebrate communities of each lake and their different foraging tactics. Both Opinicon Lake and Lower Beverley Lake have been invaded by zebra mussels, but Ashby Lake has not. Zebra mussels, which were found in the stomach contents of pumpkinseed from Opinicon and Lower Beverley lakes, reside in the littoral habitat but have isotopic compositions similar to that of pelagic zooplankton (see Appendix Table A1). As a result, for these two lakes, a decrease in  $\delta^{13}\text{C}$  values does not preclude a hard-shelled prey item (zebra mussels) being part of their diet. Indeed, zooplankton were not found in the stomach contents of pumpkinseed from Opinicon Lake or Lower Beverley Lake despite the abundant amounts found in the fish collected from Ashby Lake. Consequently, the associations between morphological traits and isotope-based analysis of diet may be most accurately inferred based on the Ashby Lake fish alone because the resources available vary between soft-bodied pelagic zooplankton and littoral benthic invertebrates.

Resource polymorphisms within a single population resulting from intraspecific competition present the potential for population divergence and sympatric speciation, given strong isolation between resource morphs (Hendry et al. 2009). The divergence seen in pharyngeal jaw and gill raker morphology between foraging groups in Ashby Lake could be phenotypic evidence of disruptive selection that is required to drive sympatric speciation. Because pumpkinseed show strong site fidelity (McCairns and Fox 2004), they are likely to mate in the same lake zone in which they forage. Thus, diet divergence indicates the potential for assortative mating between pumpkinseed resource morphs. However, the strength of selective processes acting on foraging morphology will depend on the heritability of those traits. Previous work has

demonstrated phenotypic plasticity to be the dominant mechanism of pharyngeal jaw differentiation in pumpkinseed (Robinson and Wilson 1996; Mittelbach et al. 1999). Of interest, Parsons and Robinson (2007) demonstrated that divergent foraging morphology in Ashby Lake pumpkinseed was inducible by diet but that such adaptive plasticity might later respond to selection. Thus, while the evolutionary implications of trophic dimorphism may seem to be minimal when phenotypic plasticity occurs, if there is genetic assimilation of these initially plastic differences, then it is possible for evolutionary processes to result in further divergence based on foraging patterns.

In summary, we found evidence across multiple populations and at the individual level within lakes that pumpkinseed show variation in morphological features and resource use that are consistent with the expected ecomorphological relationships across fishes. Although we found evidence that both jaw size and gill raker gaps differed with diet, it appears that in pumpkinseed there may be stronger selection related to jaw morphological traits compared with gill rakers. Overall, the robust relationships between morphology and diet at the individual level within a population suggest that these relationships can be used to further examine ecological and evolutionary processes that may be occurring both within and among populations.

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## Appendix A

**Table A1.** Stable isotope compositions of potential invertebrate prey resource groups from each lake sampled.

Lake	Gastropods			Zooplankton			Zebra mussels			Other littoral		
	$\delta^{13}\text{C}$ (‰); VPDB	$\delta^{15}\text{N}$ (‰); AIR	<i>n</i>									
Opinicon	-20.8±2.9	+3.9±0.5	5	-27.8±1.9	+4.7±0.4	5	-27.4±0.7	+4.7±0.4	5	-21.7±2.5	+3.6±0.9	9
Lower Beverley	-24.7±2.5	+6.0±0.4	4	-28.6±1.1	+7.2±0.6	5	-28.5±2.1	+7.2±0.9	5	-24.7±0.9	+5.9±0.4	9
Ashby	-24.2±1.7	+1.6±0.1	3	-30.4±0.3	+2.0±0.3	4	—	—	—	—	—	—

**Note:** Zebra mussels (*Dreissena polymorpha*) are not present in Ashby Lake. Prey values for “other littoral” invertebrates are not reported for Ashby Lake because insufficient numbers were found in D-net sweeps. All invertebrates were sampled in June 2011, during the same sampling period in which pumpkinseed sunfish (*Lepomis gibbosus*) were collected for the stable isotope analysis of liver tissue.