

# Ecological clustering within a diverse minnow assemblage according to morphological, dietary and isotopic data

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## SUMMARY

1. The ecological role of an organism is a complex interaction between many factors, such as morphology, behaviour and physiology, which can act as functional constraints upon how an organism engages with the environment.
2. We examined the ecological roles, guild structure and ecological convergence among 15 sympatric minnow species (Cyprinidae) in the New River (North Carolina, U.S.A.). Minnows often dominate fish species diversity and biomass in North American streams.
3. We evaluated the ecological relationships among minnows using popular methods that correspond to different aspects of minnow biology: geometric morphometrics (landmark-based PCA), gut content analysis and stable isotopes (<sup>13</sup>C, <sup>15</sup>N, <sup>2</sup>H). We then tested for clustering (i.e. guilds) and convergence among minnows using these traits.
4. Important shape variation among species was primarily associated with mouth orientation and caudal peduncle morphology, implicating the importance of trophic and activity patterns. Minnows clustered into five guilds based on body shape. Gut contents revealed three discrete guilds: pelagic- and benthic-oriented invertivory and benthic herbivory. Stable isotopes revealed only two discrete guilds: algivory and invertivory; however, all minnows preferentially assimilated invertebrates.
5. Minnows thus exhibit dynamic ecologies based largely on utilisation of the benthic–pelagic resource axis.

*Keywords:* Cyprinidae, geometric morphometrics, gut contents, niche, stable isotope

## Introduction

Minnows often dominate fish species diversity and biomass in North American streams (Boschung & Mayden, 2004). Minnow assemblages are generally polyphyletic such that members of most major clades are often represented in any particular stream. This pattern may be due in part to historically different basin configurations, particularly those of the present day Tennessee and Mobile basins of the southeastern United States. For example, ancient rivers such as White's River and the Appalachian River may have served as the sites of early minnow diversification and assemblage formation during periods of rapidly fluctuating sea levels during the late Pliocene and early Pleistocene (2–3 Mya; Nagle & Simons, 2012). One result of this type of distribution and

evolutionary history is that sympatric minnows often have considerably different ecologies. The benthic–pelagic axis has been particularly important during the proliferation of minnows. For example, clades consist largely of either benthic or pelagic species and Hollingsworth *et al.* (2013) hypothesised that an ancestral benthic to pelagic shift coincided with a burst in minnow diversification.

Most benthic minnows are distributed within a few species-poor clades such as those containing the genera *Semotilus* (4 species), *Campostoma* (6), *Nocomis* (7), *Phenacobius* (5), *Exoglossum* (2) and *Rhinichthys* (9; Hollingsworth *et al.*, 2013; www.fishbase.org). Despite their common association with the benthos, these species exhibit different body shape and diets. *Campostoma* and some *Nocomis* consume mostly filamentous algae

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(Power, Matthews & Stewart, 1985; Power, Stewart & Matthews, 1988; German *et al.*, 2009), whereas the others consume mostly benthic insects (Hambrick, Jenkins & Wilson, 1975; Pappantoniou & Dale, 1982; German *et al.*, 2009). In contrast, the pelagic species are distributed across several more speciose clades such as those containing *Notropis* (90), *Luxilus* (9) and *Cyprinella* (32), among others (Hollingsworth *et al.*, 2013; www.fish-base.org). These species are generally small, tend to aggregate into schools and may feed from the surface, the water column (e.g. drift feeding) or the benthos (Boschung & Mayden, 2004). All minnows are generally elongate, but they may differ in relative body depth, lateral compression, caudal peduncle depth and orientation of the mouth (Menhinick, 1991; Boschung & Mayden, 2004). Despite these generalities, the ecological roles and relationships among many minnows are poorly understood, in part due to their sheer diversity. For example, the Leuciscinae (i.e. minnows) consists of approximately 570 species distributed throughout Eurasia and North America (Imoto *et al.*, 2013). Furthermore, they are frequently overlooked in lieu of more charismatic or economically important species that inhabit the same environments, such as basses and sunfishes (Centrarchidae) and trout (Salmonidae).

Consumer-resource dynamics can be mediated by many factors. The most prominent factor may be functional limitations associated with morphology that can facilitate exploitation of certain resources and preclude exploitation of others. For example, mouth orientation is often associated with how and from where species feed (Gerking, 1994). Long guts facilitate the assimilation of difficult to digest food resources such as those with complex structures or cellulose (Wagner *et al.*, 2009). Specialised jaws often allow exploitation of hard-shelled resources such as molluscs (Mittelbach, 1984; Meyer, 1989) or facilitate assimilation of nutrient-poor resources by rupturing cells during mastication (Xie, 2001). Beyond these functional constraints, what species actually consume is perhaps the most basic metric used to quantify the dietary niche of that organism (Elton, 1927; Hutchinson, 1957). For example, fish consumers may display a proclivity for benthic or surface-oriented foraging, which will mediate the importance of particular prey items associated with different habitats (López-Fernández *et al.*, 2012). Among fish, this often results in disparity in the relative importance of autochthonous and allochthonous resources. Physiological processes that mediate assimilation are also important. For example, teleost fish intestines do not produce enzymes capable of breaking down cell walls (i.e. cellulose), and

therefore to facilitate digestion of these food resources, they rely on microorganisms to produce necessary enzymes, mechanical processing to physically rupture cell walls and/or long digestive tracts to maximise transport time (Sibbing & Nagelkerke, 2001; German *et al.*, 2009; Wagner *et al.*, 2009; Pelster *et al.*, 2015).

We investigated the ecological roles of a diverse assemblage of 15 minnows, and particularly how species partition into guilds based on commonly used metrics: geometric morphometrics, gut contents and stable isotopes. These metrics are functionally related in that they reflect (i) how species are equipped to utilise resources (i.e. morphology), (ii) how species utilise resources (i.e. behaviour) and (iii) how species assimilate resources (i.e. physiology). Second, because most of these species are disparately related, yet co-occur and thus experience similar environmental conditions, we evaluated the degree of ecological convergence.

## Methods

### Study species

We used an assemblage of 15 sympatric minnows in the New River (Watauga County, NC, U.S.A; 36.21°N, 81.65°W), which encompasses several major lineages and variable ecologies. This assemblage includes widely distributed species that occur throughout much of North America (e.g. *R. cataractae* and *Semotilus atromaculatus*), throughout Southeastern or Eastern North America (e.g. *Nocomis leptocephalus* and *R. atratulus*), as well as several species that are endemic to the New River drainage (e.g. *Phenacobius teretulus*, *Nocomis platyrhynchus* and *Exoglossum laurae*; Table 1). We recognise that *N. rubellus* from the New River basin is currently considered an undescribed species (*N. sp. cf. rubellus*, Kanawha Rosyface Shiner; Berendzen *et al.*, 2008), but for simplicity, will refer to it as *N. rubellus*.

### Sampling

We sampled fish in December (2011), March, July and December (2012) from the New River using a LR-24 Electro-fisher (Smith-Root, Inc.). All fish were identified according to Menhinick (1991). Specimens were temporarily stored on ice in the field and later stored in a -80°C freezer in the laboratory. Additionally, we manually sampled invertebrates that represent potential prey items, including (i) benthic taxa (*Ceratopsyche*, *Maccaffertium*, *Isonychia*, *Elimia*, *Chironomus*) and (ii) terrestrial taxa (adult *Ceratopsyche* and *Chironomus*). We also

**Table 1** New River minnow assemblage metrics, their status in the New River and the proportion (%) of the assemblage that each species represents. Species codes correspond to the abbreviations used in Figs 3 and 5

Taxa	Common Name	Code	Status	Proportion
<i>Camptostoma anomalum</i>	Central Stoneroller	Cam_ano	Native	0.36
<i>Clinostomus funduloides</i>	Rosyside Dace	Cli_fun	Native	0.05
<i>Cyprinella spiloptera</i>	Spotfin Shiner	Cyp_spl	Native	0.01
<i>Exoglossum laurae</i>	Tongue-Tied Minnow	Exo_lau	Endemic	0.01
<i>Luxilus coccogenis</i>	Warpaint Shiner	Lux_coc	Introduced	0.04
<i>Nocomis leptocephalus</i>	Bluehead Chub	Noc_lep	Native	0.18
<i>Nocomis platyrhynchus</i>	Bigmouth Chub	Noc_pla	Endemic	0.02
<i>Notropis photogenis</i>	Silver Shiner	Not_pho	Native	0.003
<i>Notropis rubellus</i>	Rosyface Shiner	Not_rub	Native	0.02
<i>Notropis scabriceps</i>	New River Shiner	Not_sca	Native	0.14
<i>Phenacobius teretulus</i>	Kanawha Minnow	Phe_ter	Endemic	0.01
<i>Pimephales notatus</i>	Blunthead Minnow	Pim_not	Native	0.10
<i>Rhinichthys atratulus</i>	Blacknose Dace	Rhi_atr	Native	0.05
<i>Rhinichthys cataractae</i>	Longnose Dace	Rhi_cat	Native	0.01
<i>Semotilus atromaculatus</i>	Creek Chub	Sem_atr	Native	0.003

sampled autochthonous (e.g. algae) and allochthonous (terrestrial leaves) production sources. Voucher specimens of all fish were deposited in the Auburn University Museum of Natural History (Auburn, Alabama, U.S.A.). All individuals used for analyses were representative of adult size classes. Fish from all sampling periods were used for morphometric and gut content analyses. Only specimens from March and July sampling periods were utilised for isotopic analyses to reduce temporal variation.

#### Geometric morphometric analysis

The left sides of preserved fish were photographed in lateral aspect using a mounted Nikon D5100 digital camera. We used 18 biologically homologous landmarks (Fig. S1) that describe the lateral body shape of cypriniform fish (Armbruster, 2012). Photographs were digitised and landmarked using tpsUTL and tpsDIG2 (Rohlf, 2006), respectively. Landmarks were superimposed and aligned to correct for size, rotation and translation using MorphoJ (Klingenberg, 2011). Principal components were then used to describe major axes of shape variation among species.

#### Gut content analysis

Fish were thawed, and the entire GI tract was dissected and preserved in 10% formalin. Contents from the oesophagus to the posterior end of the anterior bulb (i.e. the foregut) were removed (Sternler & George, 2000), examined under a microscope and identified. Prey items were grouped into ten functional categories: aquatic or terrestrial insects, molluscs (snails), crustaceans, algae (including diatoms), amorphous detritus, vegetation,

wood, seeds and sediment (i.e. sand and silt). Aquatic and terrestrial insects were discriminated with the aid of morphological keys and illustrations (Merritt, Cummins & Berg, 2008). Volumetric proportions of each category were estimated as described by Winemiller (1990). Briefly, the contents were spread on slides and their area compared to that of material of known volume. We then performed principal component analysis to describe major variation among minnow diets using the princomp command in R (R Core Development Team, 2011).

#### Isotopic analyses

Caudal muscle samples were dissected in the laboratory. Muscle samples were lyophilised until dry (i.e. asymptotic mass), ground into a homogenous powder and weighed into tin (CN) or silver (H) capsules. Invertebrates and basal resources were lyophilised whole and homogenised. Samples were analysed for CN at the University of Georgia stable isotope laboratory (Athens, Georgia, U.S.A.) and for H at the Colorado Plateau Stable Isotope Laboratory (Flagstaff, Arizona, U.S.A.). Delta notation represents the deviation of stable isotope ratios ( $^{13}\text{C}:^{12}\text{C}$ ,  $^{15}\text{N}:^{14}\text{N}$  and  $^2\text{H}:^1\text{H}$ ) from universal standards: PDB limestone (C), atmospheric nitrogen (N) and standard mean ocean water (H). Lipid content can bias  $^{13}\text{C}$  analysis (Post *et al.*, 2007) because lipids are  $^{13}\text{C}$ -depleted relative to proteins and carbohydrates. However, minnow C:N ratios were less than 3.5 indicating that lipid content was sufficiently low to prevent bias (Post *et al.*, 2007), so we did not correct for lipid content. Furthermore, lipid content estimated from C:N was similar among minnow species suggesting uniform effects (if any) from lipids, and thus limiting potential bias of our comparative analyses.

To estimate the relative assimilation of prey items, we employed a three-isotope Bayesian mixing model (Hopkins & Ferguson, 2012). Potential prey items were partitioned into four functional groups; however, to account for all items individually, they were coded as sub-sources in the mixing model (Table S1). We incorporated error associated with (i) fractionation estimates, (ii) variation in consumer and source isotope ratios, (iii) study-wide error among standards, (iv) source concentration (of C and N) and (v) digestibility of sources (see below for details). We corrected fish isotopic signatures using generic fractionation rates supported by meta-analyses (Post, 2002): 3.5‰ and 0.5‰ for  $^{15}\text{N}$  and  $^{13}\text{C}$ , respectively. Sterner and George (2000) demonstrated that these rates are appropriate for cyprinid fish by analysing the isotopic discrepancy between consumer muscle tissues and foregut contents. We incorporated error (e.g. SD) associated with these rates as they were reported in Post (2002): 0.98‰ ( $^{13}\text{N}$ ) and 1.3‰ ( $^{13}\text{C}$ ). Fractionation of  $^2\text{H}$  was considered to be 0.0‰ because previous estimates have not been different from zero (Jardine, Kidd & Cunjak, 2009), particularly when lipid content is low. We also incorporated the error among standards:  $\pm 0.10\%$  ( $^{15}\text{N}$ ),  $\pm 0.09\%$  ( $^{13}\text{C}$ ) and  $\pm 2.5\%$  ( $^2\text{H}$ ). Source digestibility was incorporated using prior literature (Whitledge & Rabeni, 1997; Roth, Hein & Vander Zanden, 2006): plants (41%), detritus (14%), algae (39%) and invertebrates (92%). Finally, we used uninformative priors (i) because IsotopeR is robust to uninformative priors (Hopkins & Ferguson, 2012) and (ii) to provide estimates that are independent of the gut content analysis. The model was run using three MCMC chains and 10 000 MCMC runs with 10% burnin.

#### Trait correlation

We tested for correlations among the three trait matrices: (i)  $^{15}\text{N}$  and  $^{13}\text{C}$ , (ii) morphometric PC1 and PC2 and (iii) dietary PC1 and PC2 using Mantel tests with 10 000 permutations using the mantel function in the 'vegan' R package (Legendre & Legendre, 2012). Note that  $^2\text{H}$  is not included so that the three matrices have the same dimensionality. As we were interested in each pairwise comparison, we used multiple Mantel tests rather than a partial Mantel test, which can be difficult to interpret (Legendre & Legendre, 2012).

#### Cluster analysis

We tested for the presence of guilds defined by each of the aforementioned traits using Ward's minimum

variance clustering. Clusters were evaluated using confidence intervals derived from 10 000 multiscale bootstrap resampling. Cluster analysis was performed and *P*-values were calculated using the R package pvclust (Suzuki & Shimodaira, 2011). Approximately unbiased *P*-values are between 0 and 1 and reflect how strongly the cluster is supported by the data (Suzuki & Shimodaira, 2011). We tested for clusters using geometric morphometrics (PCs), gut contents (PCs) and stable isotope ratios.

#### Phylogenetic analyses

We inferred the evolutionary relationships among minnows in the New River using published sequence data: Cytochrome b (cytb), Cytochrome C oxidase subunit 1 (CO1) and nuclear Recombination-Activating Gene 1 (RAG1; See Table S2 for accession numbers). Sequences were available for all species except RAG1 for *Cyprinella spiloptera* and *Notropis rubellus*, for which RAG1 sequences from the closely related *C. lutrensis* and *N. micropteryx* were substituted, respectively (Hollingsworth *et al.*, 2013). *Chrosomus erythrogaster* was included as an outgroup. Sequences were visually aligned and trimmed to make each gene the same length (cyb: 1137 bp; CO1: 652 bp and RAG1: 1490 bp), and the three genes were concatenated in Mesquite ver. 2.75 (Maddison & Maddison, 2011). We used maximum likelihood analysis in RAxML ver. 7.2.8 (Stamatakis, 2006) using the GTR +  $\Gamma$  model. To test for convergence, we mapped the phylogeny onto the morphometric (PC1 and PC2), dietary (PC1 and PC2) and isotopic ( $^{13}\text{C}$  and  $^{15}\text{N}$ ) variables using Mesquite.

## Results

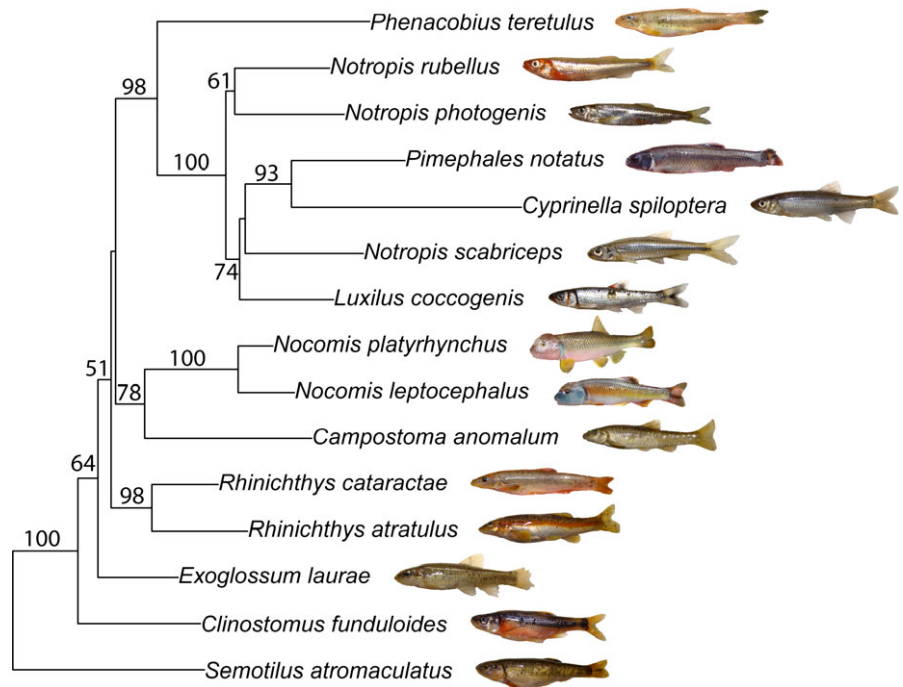
### Phylogeny

The evolutionary relationships inferred from the maximum likelihood tree (Fig. 1) were consistent with previous literature (Hollingsworth *et al.*, 2013). The genus *Notropis* was recovered as non-monophyletic and forms a clade with *Pimephales*, *Luxilus* and *Cyprinella* (Fig. 1). *Campostoma* and *Nocomis* form a clade and *Semotilus* is sister to all other species (Fig. 1).

### Geometric morphometrics

Principal component 1 explained 29.9% of the variation in shape among individuals (Fig. 2a). Negative PC1 values were associated with individuals that had

**Fig. 1** Maximum likelihood phylogeny used to test for convergence in morphology, diet and stable isotope ratios. Bootstrap support is labelled at each node (>50%). The phylogeny is pruned to remove the outgroup taxa. Photos by E.D.B.



inferior-oriented mouths and long caudal peduncle (e.g. *P. teretulus*), whereas positive PC1 values were associated with individuals that had superior-oriented mouths and short caudal peduncles (e.g. *C. funduloides*). PC2 explained 18.0% of the variation in shape among individuals (Fig. 2a). Negative values of PC2 were associated with individuals that had a shallow, short head and slender caudal peduncle (e.g. *P. teretulus*), whereas positive PC2 values were associated with individuals that had a deep, long head and robust caudal peduncle (e.g. *R. cataractae*).

#### Gut contents

Minnows varied primarily in their relative consumption of benthic and terrestrial invertebrates and algae (Table S3). Our PCA analysis resulted in two PCs that described 98.1% of the variation in minnow gut contents (Table S4; Fig. 2c). Most species separated into three discrete clusters by PC1 (76.3%) and PC2 (21.8%): those that consumed primarily terrestrial insects (*N. photogenis*, *N. rubellus*, *N. scabriceps* and *C. funduloides*), aquatic insects (*C. spiloptera*, *Luxilus coccogenis*, *S. atromaculatus* and *E. laurae*) and algae (e.g. *N. leptocephalus*, *N. platyrhynchus* and *C. anomalum*). *Rhinichthys atratulus* exhibited a generalist diet such that they consumed relatively equal fractions of aquatic and terrestrial insects (Table 2; Fig. 2c). *Phenacobius teretulus* and *P. notatus* exhibited omnivorous diets such that they consumed both aquatic insects and algae (Table 2; Fig. 2c). The

aquatic insect category consisted of primarily immature Diptera (Chironomidae and Simuliidae) and smaller fractions of Trichoptera, Ephemeroptera and Plecoptera. The terrestrial insect category consisted primarily of chironomids, trichoptera and ants. The mollusc category consisted entirely of snails and the crustacean category consisted of fragments of juvenile crayfishes.

#### Stable isotopes

Study-wide error (among standards) was  $\pm 0.10\text{‰}$  ( $^{15}\text{N}$ ),  $\pm 0.1\text{‰}$  ( $^{13}\text{C}$ ) and  $\pm 2.5\text{‰}$  ( $^2\text{H}$ ). Production sources displayed discrete  $^{13}\text{C}$  signatures: terrestrial leaves ( $-28.4 \pm 1.4\text{‰}$ ) and algae ( $-16.3 \pm 1.4\text{‰}$ ) and  $^2\text{H}$  signatures: terrestrial leaves ( $-100 \pm 7.9$ ) and algae ( $-218 \pm 26.1$ ). Minnows varied in both their  $^{13}\text{C}$  and  $^{15}\text{N}$  isotope ratios (Fig. 2e). *Campostoma anomalum* and *P. teretulus* were  $^{13}\text{C}$ -enriched relative to all other species (Fig. 2e). Additionally, minnow  $^2\text{H}$  ratios were also widely distributed between those associated with autochthonous (e.g. algae) and allochthonous (e.g. terrestrial leaves) production sources (Fig. 3). Estimates using a three-isotope Bayesian mixing model indicate that all minnows preferentially assimilate nutrients from aquatic invertebrates (Table 2).

#### Trait correlation

Morphometric (PC1  $\times$  PC2) and dietary (PC1  $\times$  PC2) matrices were not correlated ( $r = 0.139$ ;  $P = 0.109$ ).

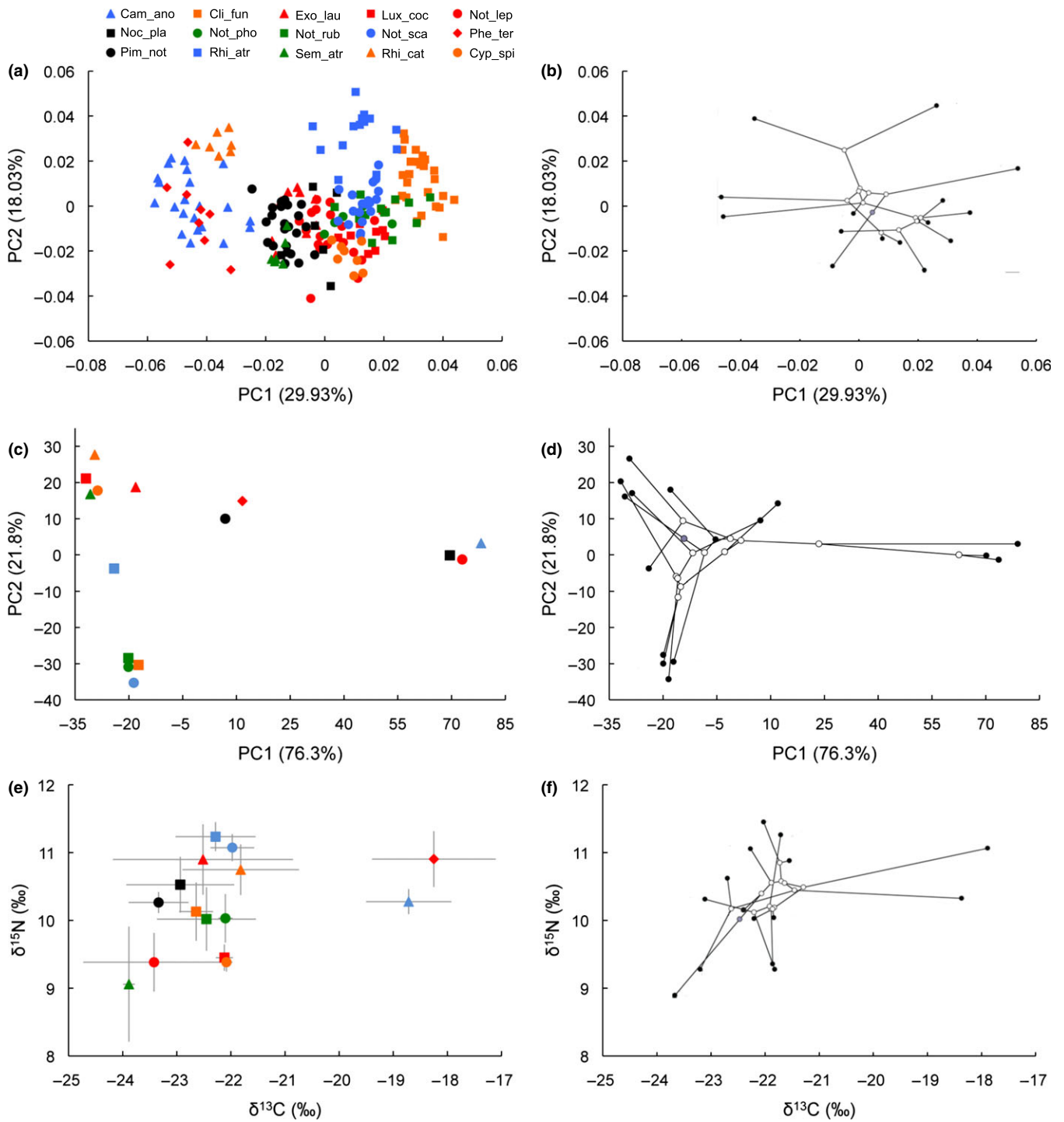


Fig. 2 Shape principal component (PC) analysis (a) showing biologically important body shape differences among minnows with mapped phylogeny (b), principal component analysis showing major variation in minnow diets (c) with mapped phylogeny (d), and mean ( $\pm$ SD) stable isotope ratios (e) with mapped phylogeny (f). Abbreviations follow Table 1.

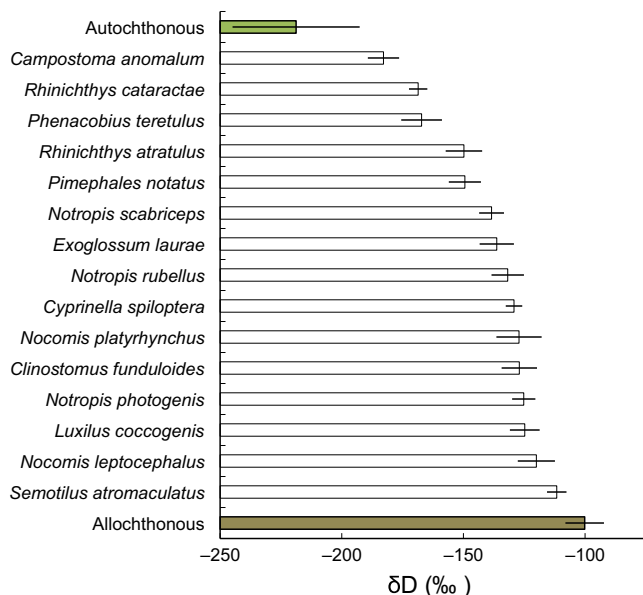
Likewise, dietary and isotopic ( $^{15}\text{N} \times ^{13}\text{C}$ ) matrices were also not correlated ( $r = 0.134$ ;  $P = 0.169$ ). However, morphometric and isotopic matrices were significantly correlated ( $r = 0.344$ ;  $P = 0.0155$ ).

#### Guild structure

Cluster analysis of morphometric data revealed five well-supported groupings of species with similar body

**Table 2** Mean (95% CI) % importance of food resources based on a three-isotope ( $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^2\text{H}$ ) Bayesian mixing model. Values are rounded to the nearest integer

Species	Algae	Plant	Detritus	Invertebrate
<i>Rhinichthys cataractae</i>	4 (0–66)	2 (0–33)	0 (0–2)	93 (25–100)
<i>Luxilus coccogenis</i>	4 (0–47)	2 (0–36)	0 (0–5)	93 (41–100)
<i>Semotilus atromaculatus</i>	3 (0–32)	3 (0–40)	0 (0–3)	94 (38–100)
<i>Notropis photogenis</i>	4 (0–56)	3 (0–39)	0 (0–2)	93 (17–100)
<i>Clinostomus funduloides</i>	3 (0–31)	3 (0–35)	0 (0–1)	91 (35–100)
<i>Nocomis leptoccephalus</i>	2 (0–15)	3 (0–47)	1 (0–4)	94 (53–100)
<i>Nocomis platyrhynchus</i>	4 (0–54)	3 (0–47)	1 (0–6)	93 (26–100)
<i>Notropis rubellus</i>	4 (0–35)	3 (0–35)	0 (0–3)	93 (42–100)
<i>Phenacobius teretulus</i>	6 (0–61)	3 (0–40)	0 (0–2)	91 (28–100)
<i>Pimephales notatus</i>	3 (0–29)	3 (0–43)	0 (0–5)	94 (42–100)
<i>Notropis scabriceps</i>	4 (0–50)	4 (0–56)	0 (0–3)	92 (28–100)
<i>Exoglossum laurae</i>	4 (0–49)	3 (0–35)	0 (0–2)	93 (25–100)
<i>Campostoma anomalum</i>	5 (0–59)	3 (0–37)	0 (0–2)	92 (28–100)
<i>Rhinichthys atratulus</i>	3 (0–38)	3 (0–40)	0 (0–1)	94 (36–100)
<i>Cyprinella spiloptera</i>	4 (0–51)	3 (0–39)	0 (0–2)	93 (28–100)



**Fig. 3** Hydrogen isotope ratios (mean  $\pm$  SD) of minnows relative to those of autochthonous (e.g. algae) and allochthonous (e.g. leaf-based detritus) production sources.

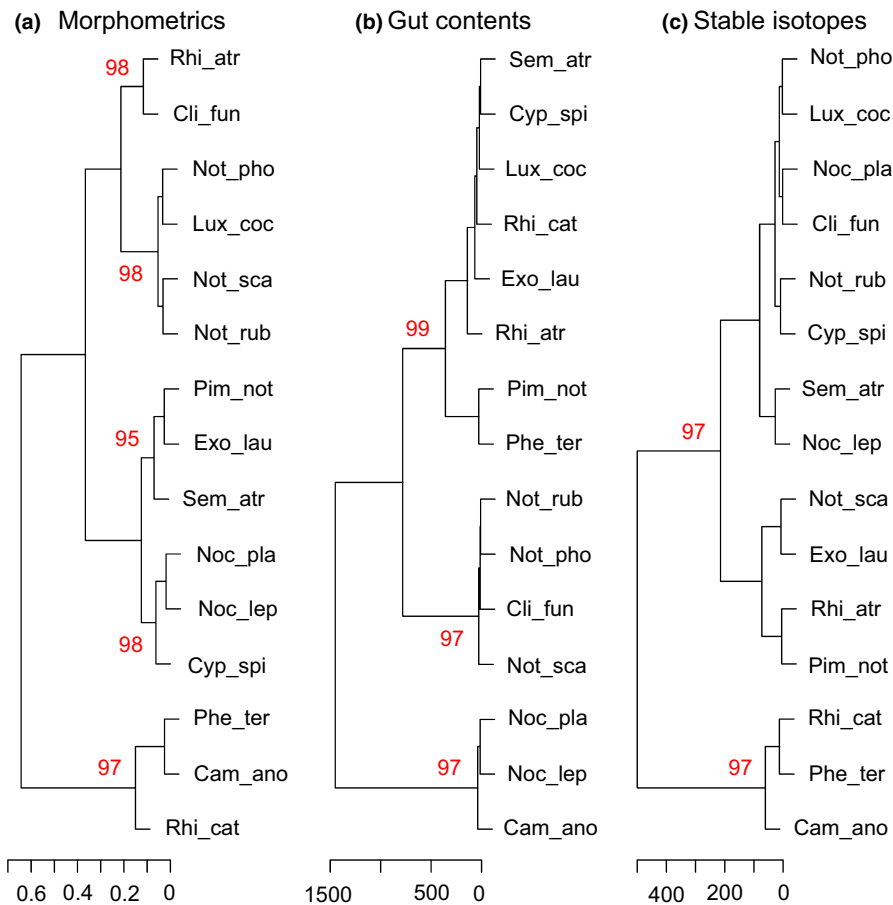
shape (Fig. 4a). The groupings consisted of (i) *Campostoma anomalum*, *R. cataractae* and *P. teretulus* ( $P = 0.97$ ) characterised by inferior mouths, pointed snouts and long caudal peduncle, (ii) *N. platyrhynchus*, *N. leptoccephalus* and *C. spiloptera* ( $P = 0.98$ ) characterised by subterminal mouths and relatively long snouts, (iii) *P. notatus*, *E. laurae* and *S. atromaculatus* ( $P = 0.95$ ) characterised by subterminal mouths and relatively blunt

snouts, (iv) *N. photogenis*, *N. rubellus*, *N. scabriceps* and *L. coccogenis* ( $P = 0.98$ ) characterised by superior mouths and slender caudal peduncle and (v) *R. atratulus* and *C. funduloides* ( $P = 0.98$ ; Fig. 4a) characterised by a robust caudal peduncle. Dietary data revealed three well-supported groupings with similar diets (Fig. 4b). Algivorous species (e.g. *N. leptoccephalus*, *N. platyrhynchus* and *C. anomalum*) were clustered together ( $P = 0.97$ ) as well as species that consumed large fractions of terrestrial insects (e.g. *N. photogenis*, *N. rubellus*, *N. scabriceps* and *C. funduloides*;  $P = 0.97$ ). All other species comprised the third grouping associated with consumption of benthic invertebrates ( $P = 0.99$ ; Fig. 4b). Isotopic data revealed two well-supported groupings with similar isotopic composition (Fig. 4c): *Campostoma anomalum*, *R. cataractae* and *P. teretulus* were grouped together ( $P = 0.97$ ), whereas all other species comprised the second grouping ( $P = 0.97$ ; Fig. 4c). Hereafter, all reference to guilds pertains only to those with at least 95% bootstrap support.

## Discussion

### Ecology of the minnow assemblage

All minnows were generally elongate and many overlapped in morphospace. Most variation was associated with habitat and trophic specialisations, namely caudal peduncle morphology and orientation of the mouth (Gerking, 1994). Deeper, more robust caudal peduncles are associated with burst swimming, usually in structurally complex, lentic habitats, whereas slender caudal peduncles are associated with steady swimming in high-flow conditions (Langerhans & Reznick, 2010). Indeed, minnows with the most robust caudal peduncles (i.e. positive PC1 and PC2 values) were *C. funduloides* and *R. atratulus*, which frequented low-flow areas among littoral vegetation. In contrast, *P. teretulus*, which are riffle specialists (Boschung & Mayden, 2004), had the most slender caudal peduncle (i.e. negative PC1 and PC2 values). The mouth orientation of minnows is also predicted by habitat and trophic characteristics. Species that possess strongly superior mouths (e.g. the three *Notropis* spp and *C. funduloides*) consumed large fractions of terrestrial insects suggesting that they feed from the surface (e.g. Grossman, Moyle & Whitaker, 1982). These species also occasionally consumed seeds, presumably via drift. Several species with weakly superior mouths (e.g. *L. coccogenis* and *C. spiloptera*) largely consumed benthic organisms. Rather than feeding directly from the benthos, these species probably feed via drift (Grossman



**Fig. 4** Cluster analysis of the New River minnow assemblage based on (a) geometric morphometrics (PC scores), (b) gut content analyses (PC scores) and (c) stable isotope ratios ( $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^2\text{H}$ ). Red numbers denote the probability values for clusters with >95% support after 10 000 bootstraps. Scales depict the relative depth of the nodes for the associated plot. Abbreviations follow Table 1.

*et al.*, 1982). Alternatively, species with subterminal or inferior mouths (e.g. *Nocomis* and *Rhinichthys*, among others) exploited aquatic insect larvae and/or algae. The occurrence of fractions of vegetative detritus and sediment in many of these species suggest that they fed in part directly from the benthos.

Most minnow  $^{13}\text{C}$  ratios were associated with those of invertebrates indicating that these are important food sources (Post, 2002). Additionally, minnow  $^{15}\text{N}$  ratios varied by approximately 2.5‰ among species. This range represents a large fraction of differences often used to discriminate trophic levels (2.5–3.4‰; Post, 2002). This variation may reflect the degree to which the consumer engages in omnivory (Post & Takimoto, 2007), differences in the relative assimilation of nutrients (Hopkins & Ferguson, 2012), or differences in the protein content of food resources (Kelly & Martinez del Rio, 2010) all of which can influence isotopic routing. Additionally, isotopic enrichment often occurs during metamorphosis from larval (i.e. aquatic) to adult (i.e. terrestrial) insects (Doi *et al.*, 2007; Tibbetts, Wheelless & Martinez del Rio, 2008). Therefore, variation in the relative importance of aquatic and terrestrial insects may also explain some  $^{15}\text{N}$

variation in minnows. Invertivorous minnows with the lowest apparent trophic positions (TP) possess subterminal mouths, perhaps largely restricting them to benthic foraging and subsequent ingestion of algae and detritus often associated with benthic habitats (i.e. factors that may reduce their apparent TP). *Notropis scabriceps* exhibited the highest apparent TP of all minnows, probably because they exploited primarily surface-derived terrestrial prey (Chippis, Perry & Perry, 1994) that tends to be  $^{15}\text{N}$ -enriched (Doi *et al.*, 2007; Tibbetts *et al.*, 2008) and did not ingest any  $^{15}\text{N}$ -poor materials such as vegetation or detritus. Conspicuous, dorsally positioned eyes that are unique to this species may reflect their surface-oriented foraging (Menhinick, 1991).

Despite consuming different proportions of terrestrial and aquatic insects, algae and sediment, all minnows preferentially assimilated invertebrates. Many minnows probably lack the physiology to assimilate nutrient-poor resources such as algae (German *et al.*, 2009). Mixing models assume that all isotopes are assimilated equally (Hopkins & Ferguson, 2012), which is unlikely to be true for *C. anomalum* and *P. teretulus*. These two species had  $^{13}\text{C}$  signatures similar to those of algae and significantly



different from all other minnows. This suggests that these two species assimilate C from algae, which are C-rich, yet assimilate most of their N and H from invertebrates (i.e. N- and H-rich items). The isotopic signatures of all other species are more congruent with the mixing model results such that all their isotope ratios were similar to those of invertebrates. For example, we expected *N. leptocephalus* to be algivorous (Power *et al.*, 1985, 1988; German *et al.*, 2009), and *N. leptocephalus* in the New River did indeed largely consume green algae; however, their  $^{15}\text{N}$ ,  $^{13}\text{C}$  and  $^2\text{H}$  signatures were more similar to those of invertebrates than algae. Thus, both *Nocomis* species, despite consuming large fractions, probably do not assimilate nutrients from algae. This contrast between *C. anomalum* and *N. leptocephalus* is potentially associated with their foraging mode. *Campostoma anomalum* graze algae (Power *et al.*, 1985) and *Nocomis* presumably engulf it (German *et al.*, 2009). The two genera differ in pharyngeal tooth shape: *Campostoma* possesses flattened teeth and *Nocomis* possesses sharper, curved teeth (German *et al.*, 2009). Pharyngeal teeth are important because the mastication process can make difficult to digest food more digestible (Xie, 2001). Indeed, Power *et al.* (1988) reported that algal cells were ruptured in the guts of *C. anomalum*, which may explain their ability to assimilate algae-derived nutrients. *Phenacobius teretulus* also consumed a significant fraction of algae and their  $^{13}\text{C}$  signatures suggest that they too assimilate some algal-derived nutrients, which contrasts with a previous report that they were selectively invertivorous (Hambrick *et al.*, 1975).

Minnows are known to have diversified along the benthic to pelagic axis (Hollingsworth *et al.*, 2013). Our morphological and dietary data support a distribution of the New River minnow assemblage along that continuum. For example, robust and slender caudal peduncle morphologies are associated with complex and open habitats, respectively (Langerhans & Reznick, 2010), which also corresponds to benthic and pelagic conditions, respectively. Second, the variable mouth angles and relative exploitation of benthic and terrestrial food items exhibited by this minnow assemblage indicate that the species are distributed along the benthic to pelagic axis. Therefore, in addition to a broad evolutionary importance during the diversification of minnows in streams throughout eastern North America, the benthic to pelagic axis may have implications at assemblage and ecosystem scales as well. For example, the benthic to pelagic axis may be important for resource partitioning, and the ability of individuals or species to move along the axis may be important during seasonal fluctuations in resource availability.

### Convergence of functional traits

There are several examples of convergence in morphology, behaviour and physiology within this minnow community. Despite being distantly related, *C. anomalum* and *P. teretulus* have converged upon similar morphologies, characterised by small, inferior mouths. These species also have similar  $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^2\text{H}$  isotope signatures, which is most likely due to assimilation of algae. In contrast, the distantly related *E. laurae* and *P. notatus* have converged on similar morphology, characterised by relatively blunt heads, yet have different gut contents and isotope ratios. *Phenacobius teretulus* and *P. notatus* have drastically different morphologies and isotope signatures, yet they have converged upon similar diets containing larger fractions of detritus and algae. This probably reflects a shared foraging mode such that they inadvertently ingest, but do not assimilate, detritus while foraging for benthic invertebrates.

*Notropis photogenis*, *N. rubellus*, *N. scabriceps* and *C. funduloides* have evolved independently to exploit terrestrial resources. Terrestrial resources may be consumed directly from the surface or from the water column via drift (Grossman *et al.*, 1982). Alternatively, several species have benthic-oriented diets despite some being evolutionarily divergent. For example, *C. spiloptera*, *L. coccogenis* and *S. atromaculatus* largely consume benthic invertebrates. The likely ancestral condition for this minnow clade was benthic-oriented invertivory (Hollingsworth *et al.*, 2013); therefore, the dietary similarities of these species may not be due to convergence, but merely retention of ancestral behaviour. Many of our distantly related species have similar isotopic compositions, such as *L. coccogenis* and *C. spiloptera*, *C. funduloides* and *N. rubellus*, *S. atromaculatus* and *N. leptocephalus*, and *N. scabriceps*, *R. cataractae* and *R. atratulus*. This is likely to be due to each species having a significant preferential assimilation of invertebrates, which may also reflect ancestral benthic carnivory (German *et al.*, 2009; Hollingsworth *et al.*, 2013). Indeed, the estimated ancestral node values for both diet and isotopic signature were associated with benthic invertivory. Thus, the isotopic similarity among these species probably reflects retention of ancestral physiological traits rather than convergence.

### Guild structure of the minnow assemblage

Our morphological, dietary and isotopic data delineated different numbers of guilds. These guilds also had different compositions. First, two morphometric variables

(PC1 and PC2) that described almost 50% of the variation in minnow body shape partitioned species into five guilds. These guilds probably depict a combination of habitat and dietary preferences considering that the shape variation described by the PCs was associated with mouth orientation and caudal peduncle morphology (Gerking, 1994; Langerhans & Reznick, 2010). In contrast, two dietary variables (PC1 and PC2), which explained approximately 95% of the variation in minnow diets, partitioned species into three guilds corresponding to benthic herbivores, benthic carnivores and pelagic carnivores. Stable C, N and H isotopes partitioned minnows into two guilds. The guild including *C. anomalum*, *R. cataractae* and *P. teretulus* exhibited less negative  $^{13}\text{C}$ , more negative  $^2\text{H}$  and relatively high  $^{15}\text{N}$  signatures. This combination, particularly of  $^{13}\text{C}$  and  $^2\text{H}$ , probably represents an extreme reliance on autochthonous production (e.g. algae). These three species exhibit an affinity for shallow, rocky riffles (Hambrick *et al.*, 1975; Mullen & Burton, 1995), and thus they may be more likely to consume, either directly (e.g. *C. anomalum*) or indirectly (e.g. *R. cataractae* and *P. teretulus*), algae-derived nutrients because light penetration and thereby periphyton growth are concentrated in this habitat (Power *et al.*, 1985, 1988).

Morphology, gut contents and stable isotopes exhibited decreasing discriminatory power, such that they partitioned five, three and two guilds, respectively. In combination, these traits appear to partition three well-supported guilds. The first guild includes largely herbivorous species (i.e. *C. anomalum*, *N. leptocephalus*, and *N. platyrhynchus*). The remaining carnivorous minnows can be further partitioning into benthic and pelagic species. Interestingly, a guild containing *C. anomalum*, *P. teretulus*, and *R. cataractae* was well-supported using morphology and stable isotopes; however, these species exhibit large discrepancies in their gut contents. Furthermore, congeners were generally united within clusters. Thus, perhaps much of the ecological variation exhibited by minnows occurs among genera.

Minnows are a speciose group that are ubiquitous in North American streams (Hollingsworth *et al.*, 2013) and form several discrete guilds such as (i) pelagic carnivores, (ii) benthic carnivores and (iii) benthic herbivores. The contrasting ecologies exhibited by minnows, coupled with their diversity and ubiquity, suggest that minnows will play complex and important roles in the ecosystems they inhabit. For example, the widespread preferential assimilation of invertebrate-derived molecules suggests that minnows may play significant roles in nutrient recycling, and this can be a fruitful area for

future research, particularly regarding their conservation in the face of widespread habitat fragmentation and loss throughout the ecosystems they inhabit (Helms *et al.*, 2011).

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Location of homologous landmarks used for geometric morphometrics.

**Table S1.** Elemental composition (mean  $\pm$  SD) and functional categorisation of potential prey items included in the Bayesian mixing model.

**Table S2.** Genbank accession numbers for gene sequences used to infer minnow phylogenetic relationships.

**Table S3.** Volumetric proportions (%) of generalised prey items in the foregut of syntopic minnows from the New River.

**Table S4.** Factor loadings for the first four principal components describing variation in minnow gut contents.

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