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Do constructional constraints influence cyprinid (Cyprinidae: Leuciscinae) craniofacial coevolution?

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Constraints on form may determine how organisms diversify. As a result of competition for the limited space within the body, investment in adjacent structures could represent an evolutionary compromise. For example, evolutionary trade-offs resulting from limited space in the head could have influenced how the sizes of the jaw muscle, as well as the eyes, evolved in North American cyprinid fishes. To test the evolutionary independence of the size of these structures, we measured the mass of the three major adductor mandibulae muscles and determined the eye volume in 36 cyprinid species. Using a novel phylogeny, we tested the hypotheses that the sizes of these four structures were negatively correlated with each other during cyprinid evolution. We found that evolutionary change in the adductor mandibulae muscles was generally positively and/or not correlated, suggesting that competition for space among cyprinid jaw muscles has not influenced their evolution. However, there was a negative relationship between mass of adductor mandibulae 1 and eye volume, indicating that change in these physically adjacent structures is consistent with an evolutionary constructional constraint. *Biological Journal of the Linnean Society*, 2011, **103**, 136–146.

ADDITIONAL KEYWORDS: ecomorphology – freshwater fish – integration – modularity – North America – phylogenetic comparative method.

INTRODUCTION

For many morphological phenotypes, allocation to one structure may compromise investment in another structure. These trade-offs operate at many levels of biological organization and among diverse components of organismal design (Garamszegi, Moller & Erritzoe, 2002; Striedter & Northcutt, 2006). From a developmental perspective, if extensive somatic investment is made in one structure, this could limit soma dedicated to the formation of another structure (Emlen, 2001; Moczek & Nijhout, 2004). It is also possible that constructional trade-offs constrain investment in phenotypes because the structural space in organisms is limiting (Barel, 1983; Herrel, Aerts & De Vree, 2000; Devaere *et al*., 2006). If one structure is enlarged, then it could limit the size of other structures, especially adjacent ones. For example, debate continues regarding the modular versus compensatory evolution of mammalian brains (Barton & Harvey, 2000; de Winter & Oxnard, 2001). Similarly, a recent study of the craniofacial morphology of cichlid fishes found little evidence to bolster the frequently cited idea that craniofacial constructional constraints were evolutionarily important in teleost fish (Hulsey, Mims & Streelman, 2007). However, the absence of constructional constraints in this unique cichlid lineage might not generally characterize diversification in other diverse groups such as cyprinid fishes. To examine a set of long postulated constructional constraints, we quantified morphology, reconstructed a novel phylogeny, and then employed independent contrasts to examine whether there are any negative correlations among adductor mandibulae (AM) muscle masses and/or eye volume in North American cyprinid fishes.

Cyprinid fishes represent one of the most species rich groups of teleosts (Strange & Mayden, 2009). In species-rich lineages, identifying what components of

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the phenotype evolve independently can provide insight into the forces structuring their diversification (Liem, 1973; Albertson *et al*., 2005; Hulsey, 2006; Hulsey, Garcia de Leon & Rodiles-Hernandez, 2006; Diogo, Hinits & Hughes, 2008). The identification of genetic, developmental, functional, and/or constructional constraints in the cyprinid trophic apparatus could identify the aspects of the teleost head that are more evolvable, as well as the elements that generally change in a more integrated manner. For example, the three AM muscles might co-evolve in several ways. Adult AMs might be strongly positively correlated because in cypriniform fishes, such as the genetic model system *Danio rerio*, these muscles differentiate from the same progenitor muscle mass early in ontogeny (Hernandez, Patterson & Devoto, 2005). Similarly, functional studies might lead to the inference that change in one muscle might be positively correlated with change in another to coordinate the forces exerted when the upper and lower jaws are closed during feeding (Takahasi, 1925; Anker, 1978; Wainwright *et al*., 2004). It is also possible that fish AM subdivisions could evolve independently. Although AM1 and AM2 function primarily during feeding, AM3 might largely function during respiration (Osse, 1969), indicating AM3 might be functionally modular from these other adductor muscles.

Alternatively, because the AM muscles are confined to the cheek region just posterior to the jaw, these muscles may compete extensively for space in this constrained region of the head. In tetraodontiform fishes, where there has been extensive duplication of AM muscles, total AM mass does not increase as the number of muscles increases. Therefore, the overall volume available for divergence in the AMs could be constrained (Friel & Wainwright, 1997). However, all three AMs appear to be positively correlated in cichlid fishes, suggesting that the muscles show little evidence of constructional constraints in perciform fishes (Hulsey *et al*., 2007). Regardless, because the mass of a muscle strongly predicts its volume and the mechanical properties of striated muscle can generally be estimated accurately if mass and pinnation angle are known (Calow & Alexander, 1973), muscle mass informs both the spatial and functional properties of the AMs. If constructional competition for space were to influence AM mass and function in cyprinids, we might expect a negative correlation among the masses of the adductor muscles during evolution.

Vision has been critically important to the diversification of cyprinid fishes (Huber & Rylander, 1992a, b), and several visual abilities may be tied to the size of the cyprinid eye (Otten, 1981; Land, 2000). In fish, eye size could also influence the AM muscle mass through constructional constraints (Barel, 1984; Strauss, 1984; Gosline, 1989) although a recent study of this relationship found little evidence for constructional constraints (Hulsey *et al*., 2007). Notably, developmental studies also suggest that a trade-off exists between ocular and mandibular arch musculature (von Scheven *et al*., 2006). Were eye size to commonly limit AM size during trophic diversification, we would expect change in these muscles and change in eye size to have a negative correlation in cyprinid fishes. If eye size increases and constructional constraints operate, this change should result in relatively smaller jaw closing muscles. Alternatively, if there were a positive or no correlation between eye volume and the masses of the muscles, this would suggest there is little constraint imposed by eye size on AM size in cyprinid fishes.

When assessing the correlated evolution of characters, past examinations of constructional constraints have generally not adequately accounted for the effects of body size in comparisons (Strauss, 1984). However, morphological phenotypes generally change extensively as organisms grow (Schmidt-Nielsen, 1984). Constructional studies have also rarely accounted for the potential influence of shared evolutionary history on the associations among characters. Species are not evolutionarily independent data points and incorporating phylogenetic hypotheses into the analyses of associations among characters provides a means to more robustly determine how characters coevolve (Felsenstein, 1985). To determine the constructional constraints among eye size and the size of the AMs, it is critical to evaluate the influence of both body size and phylogenetic history on the correlations recovered among these structures.

We first documented variation in AM and eye size within a diverse subset of North American cyprinids. Then, we generated hypotheses for the phylogenetic relationships among the species examined using information from the cytochrome *b* (*cytb*) mitochondrial gene combined with the nuclear *enc1* gene. Finally, using independent contrast analyses, we examined the evolutionary correlations among the AMs and eye volume in North American cyprinids. If no correlation or a positive correlation exists among these structures during minnow evolution, this would be consistent with these structures not extensively competing for space during cyprinid craniofacial evolution. Alternatively, if constructional constraints have generally been critical, we would expect a negative correlation among the sizes of these structures such that, if one structure increased in size, this would come at the cost of decreasing the size of another structure during the evolution of these cyprinid craniofacial structures.

MATERIAL AND METHODS

MORPHOLOGY

We utilized cyprinid specimens collected from the wild and also specimens accessioned into the University of Tennessee Etnier Ichthyology Collection. Collection localities are available from the lead author (C.D.H.). All fish used in the morphological analyses were originally preserved in formalin and then transferred to 70% ethanol for long-term storage.

For three specimens of each species, head length (HL) was measured as the length of the head between the posterior edge of the preopercle and the tip of the upper jaw (Fig. 1). Before carrying out eye measurements and muscle dissection, the eye was removed from the orbit. Three measurements were made on the orbit with calipers in order to estimate the volume of the eye (Fig. 1). Both the width and height of the orbit were measured as the orbit can deviate from a perfect circle (Barel, 1984). To measure the depth of the eye, the protruding end of dial calipers was inserted into the eye. The depth of the eye was measured as the point at which further increase in length of the calipers was impeded by the bones separating the two eyes of the fish. When removing the eyes of the minnows, it was clear the eyes were not spherical. The depth of the cylindrically shaped minnow eyes was consistently smaller than the estimated width and height of the external orbit. Therefore, we used the average of eye width and height as a measure of diameter, as well as eye depth, to parameterize the formula for the volume of a cylinder and estimate the volume of the eye.

For all individuals, the mass of each of the three AMs was measured to estimate the volume of each muscle. Because of the conserved structural properties of vertebrate skeletal muscles, mass provides a robust estimate of muscle volume (Calow & Alexander, 1973). The AM1, AM2, and AM3 were dissected whole from the head (Fig. 1) and placed into 70% ethanol. Before measurement, each muscle was removed from its vial, patted twice on paper towel, and immediately weighed. The mass of each AM muscle was thereby determined to the nearest 0.1 mg using a digital balance.

DNA ISOLATION AND SEQUENCING

To generate a phylogeny of the species examined morphologically, cyprinid fin clips were collected from several locations in the southeastern USA (available from lead author, C.D.H.) and stored in 100% ethanol. To provide a phylogenetic hypothesis of the relationships of the species examined, the *cytb* mitochondrial gene and the nuclear gene ectodermal-neural cortex 1 (*enc1*) of 36 cypriniform species (GenBank accession numbers: HQ446741-HQ446799) were sequenced (Table 1). Sequences of *cytb* for several species were obtained from previous studies (Bielawski & Gold, 2001; Mayden, 2002; Blum *et al*., 2008; Schonhuth *et al*., 2008; Strange & Mayden, 2009; Schonhuth & Mayden, 2010). Primers for the new *cytb* sequences (1140 bp) were obtained from (Schmidt & Gold, 1993) and primers for *enc1* (810 bp) were obtained from Li *et al*. (2007). GenBank sequences from *Danio rerio* and novel sequences of the catastomid *Catostomus commersoni* were included as outgroups in the phylogenetic analysis.

For sequencing, total genomic DNA was isolated from fin clips. An aliquot of 1 μL from this 50 ng μL^{-1} solution was used to provide a DNA template for the polymerase chain reaction (PCR). Amplifications were carried out in an Eppendorf EP Gradient thermocycler and conditions consisted of an initial denaturation step of 94 °C (2.0 min) followed by 30 cycles alternating between 54 °C (1.0 min) and 72 °C (1.5 min). A final incubation of 72 °C for 4 min was added to ensure complete extension of products. Subsequently, the PCR products were separated from unincorporated primers and dNTPs using electrophoresis in Tris-acetate buffered agarose gels with

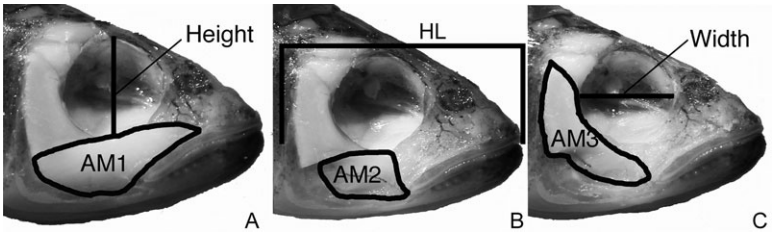

Figure 1. Craniofacial measurements. The three adductor mandibulae, AM1 (A), AM2 (B), and AM3 (C), were dissected from the head of the cyprinid specimens. The outline of the muscles is shown to highlight their position in the cyprinid head. Then, the height and width of the orbit were measured. In cyprinids, the interior components of the two eyes meet in the centre of the head. The third dimension of the cylindrically shaped eye was measured as the width of the head at the center of the eye socket. This third measurement was then combined with the height and width to estimate the eye volume. All of theses measurements were transformed and adjusted for size using head length (HL).

Table 1. GenBank sequences for cytochrome *b* (*cytb*) and ectodermal-neural cortex 1 (*enc1*) for 36 cyprinids species and two outgroups

Species	*Cytb*	*Enc1*
Campostoma anomalum	DQ486799	HQ446786
Campostoma oligolepis	HQ446741	HQ446787
Clinostomus funduloides	HQ446742	HQ446796
Cyprinella callistia	HQ446743	HQ446768
Cyprinella chloristia	HQ446744	HQ446793
Cyprinella galactura	GQ275225	HQ446765
Cyprinella spiloptera	GQ275223	HQ446774
Cyprinella trichoristia	HQ446745	HQ446766
Erimystax dissimilis	HQ446746	HQ446798
Hemitremia flammea	AY281054	HQ446788
Hybopsis amblops	HQ446747	HQ446784
Luxilus chrysocephalus	GQ275161	HQ446778
Luxilus coccogenis	HQ446748	HQ446772
Lythrurus fasciolaris	HQ446749	HQ446791
Lythrurus lirus	U17273	HQ446790
Nocomis effusus	HQ446750	HQ446782
Nocomis leptocephalus	EU082468	HQ446770
Nocomis micropogon	GQ275148	HQ446771
Notropis serralatus	HQ446755	HQ446785
Notropis asperifrons	HQ446751	HQ446773
Notropis boops	AF352261	HQ446775
Notropis buccatus	GQ275154	HQ446763
Notropis hudsonius	HQ446752	HQ446795
Notropis leuciodus	HQ446753	HQ446783
Notropis rupestris	HQ446754	HQ446789
Notropis scepticus	AF352283	HQ446794
Notropis spectrunculus	HQ446756	HQ446764
Notropis telescopus	AF352290	HQ446769
Notropis volucellus	AF352268	HQ446792
Notropis xaenocephalus	HQ446757	HQ446767
Phenacobius catostomus	HQ446758	HQ446799
Chrosomus erythrogaster	EU755055	HQ446780
Pimephales notatus	HQ446759	HQ446776
Rhinichthys cataractae	HQ446760	HQ446779
Rhinichthys obtusus	DQ990250	HQ446777
Semotilus atromaculatus	HQ446761	HQ446781
Danio rerio	AC024175	EF032975
Catostomus commersoni	HQ446762	HQ446797

ethidium bromide (1.5 mg μL^{-1}) added for visualization. Positively-amplified DNA was then purified using an enzymatic combination of 1 μL of exonuclease I (10.0 U μL^{-1}) and 1 μL^{-1} shrimp alkaline phosphatase (2.0 U μL^{-1}) per 10 μL of PCR product. Treated PCR products were sequenced by the High Throughput DNA Sequencing Facility at the University of Washington using the same primers utilized for amplification. Complete gene sequences were assembled from individual reactions using SEQUENCHER, version 4.6 (Gene Codes). Sequences were aligned using Clustal X (Thompson, Plewniak & Poch, 1999) and codon positions were defined using MACCLADE, version 4.0 (Maddison & Maddison, 2000).

Phylogenetic analysis

Before the phylogenetic analyses, MODELTEST, version 3.06 (Posada & Crandall, 1998) was used to identify the best model of molecular evolution for each codon site in *cytb* and *enc1*. With each gene partitioned into its codon sites, five independent Bayesian analyses were executed to determine approximations of the maximum likelihood phylogeny for the two concatenated genes using MrBayes, version 3.0 (Ronquist & Huelsenbeck, 2003). The analyses treated the transition–transversion matrices, number of invariant sites, and gamma shape parameters as unlinked or independent for each codon site. Flat prior probability distributions for all parameters were assumed before analysis. We ran the five separate Bayesian analyses for 1 000 000 generations with four Markov chains in each run. We sampled trees from the Markov chain Monte Carlo search algorithm every 100 generations. After each analysis, the log-likelihood scores were plotted against generation to identify the point at which likelihood values reached equilibrium. In all five analyses, the equilibrium was reached at approximately 50 000 generations, and sample points before generation 100 000 in each run were discarded as 'burn-in' samples. The remaining samples from all runs combined were used to produce a majority rule consensus tree in PAUP* 4.0b10 (Swofford, 2002). The percentage of all trees that recovered a particular clade (the clade's posterior probability) was depicted on the best likelihood topology found during the Bayesian analyses.

Comparative analysis

Because species are not evolutionarily independent (Felsenstein, 1985), we also performed an independent contrast analysis using our single best phylogenetic topology. For each analysis of contrasts, PDAP software (Midford, Garland & Maddison, 2008), as implemented in MESQUITE (Maddison & Maddison, 2005), was used to generate contrasts.

To estimate the relationships among the muscle masses and eye volume, the measurements were first transformed and then adjusted by head length in a phylogenetic context. Because mass generally scales with the third power of length, the cube root of the masses of the muscle and eye sizes were first determined. Then, the mass of AM1, AM2, AM3, their combined mass, and the volume of the eye were $\log_{10}$-transformed to account for an observed increase

in variance in our morphological measurements that would be expected when body size increases (Schmidt-Nielsen, 1984). Subsequently, measurements were size corrected using HL *sensu* Blomberg, Garland & Ives (2003). In brief, we determined the independent contrast correlation for each variable and $\log_{10}$ HL and estimated the slope between these contrasts. To generate the HL-corrected residuals for each variable, we utilized reduced major axis regression to fit the intercept and then the contrast-derived slope was forced onto the species correlation between each variable and HL. The independent contrast correlations among the three HL-corrected AM mass residuals, total AM mass residuals, and eye size residuals were then examined.

RESULTS

The mass of AM2 was consistently the smallest of the three AMs in all the cyprinids examined (Table 2). Both the AM1 and AM3 were sometimes found to be the largest muscle among the species examined. The

Table 2. Morphometrics of adductor mandibulae (AM) mass and eye size for the North American cyprinid fish examined

Species	SL (mm)	HL (mm)	AM1 (mg)	AM2 (mg)	AM3 (mg)	ED (mm)	EW (mm)	EH (mm)	EV (mm^3)
Notropis scepticus	51.0	9.8	1.22	0.67	2.07	2.9	5.2	5.2	61.9
Notropis hudsonius	69.0	12.3	6.47	2.46	4.10	3.5	5.9	5.6	92.2
Clinostomus funduloides	70.4	14.7	7.92	2.81	7.42	4.1	6.3	5.9	120.6
Luxilus chrysocephalus	70.9	14.2	5.59	3.11	6.84	3.4	6.4	6.4	111.2
Notropis telescopus	57.9	10.5	1.57	0.46	1.96	2.8	5.3	5.4	61.4
Luxilus coccogenis	67.4	12.2	3.28	1.28	4.27	3.2	5.8	5.7	82.1
Notropis leuciodus	51.7	8.7	1.44	0.48	1.85	2.7	4.3	4.4	41.0
Notropis xaenocephalus	56.9	10.4	2.35	0.74	3.54	2.6	5.5	5.1	56.9
Notropis volucellus	53.7	10.0	2.18	0.67	1.89	2.7	5.0	4.9	52.0
Cyprinella callistia	74.5	12.8	10.19	4.59	7.94	3.4	5.7	5.4	82.3
Hybopsis amblops	60.9	12.0	4.61	1.68	1.62	3.2	5.6	5.0	69.9
Chrosomus erythrogaster	60.3	10.5	2.57	0.88	3.03	2.8	4.9	4.8	51.5
Erimystax dissimilis	75.8	13.6	11.80	5.61	5.87	3.9	5.6	4.9	84.0
Notropis asperifrons	46.4	8.7	1.12	0.25	1.74	2.7	4.2	3.9	34.3
Semotilus atromaculatus	99.3	18.8	35.51	20.80	29.65	5.6	5.9	5.9	153.9
Notropis spectrunculus	48.1	9.4	2.97	0.80	2.69	2.5	4.7	4.3	39.1
Notropis boops	48.3	8.7	1.01	0.28	1.60	2.4	4.2	4.1	32.3
Cyprinella trichoristia	55.2	10.2	2.25	0.58	4.17	2.9	4.4	4.4	44.3
Nocomis micropogon	86.6	16.4	23.95	8.88	11.59	4.4	5.5	5.7	110.4
Pimephales notatus	72.1	12.1	11.39	3.38	8.44	3.7	4.7	4.4	60.5
Nocomis leptocephalus	90.6	16.7	23.80	9.31	13.50	4.9	5.4	5.5	112.2
Campostoma oligolepis	91.3	15.2	20.21	4.02	4.70	4.2	5.3	5.2	90.6
Campostoma anomalum	91.3	15.1	22.51	6.06	3.83	4.4	5.2	4.9	88.3
Nocomis effusus	81.6	15.7	19.96	11.43	14.39	3.6	5.8	5.7	94.9
Notropis rupestris	48.9	9.2	1.61	0.28	1.33	2.3	4.3	4.2	33.3
Lythrurus fasciolaris	57.4	10.1	1.51	0.38	2.09	2.7	4.2	4.2	38.5
Notropis serralatus	44.8	8.2	1.04	0.28	1.28	1.9	4.1	3.9	24.3
Cyprinella spiloptera	69.6	12.3	4.54	1.85	6.69	3.4	4.4	4.5	53.4
Lythrurus lirus	48.0	8.0	0.63	0.14	1.00	2.1	3.8	3.8	23.0
Cyprinella chloristia	54.5	9.6	1.86	0.48	1.40	2.4	4.1	4.1	32.4
Cyprinella galactura	73.7	13.2	5.50	2.46	6.48	3.5	4.6	4.6	58.6
Notropis buccatus	51.4	11.6	3.11	1.15	1.61	2.8	4.6	4.3	43.5
Hemitremia flammea	44.5	7.8	1.18	0.51	1.45	1.8	3.7	3.6	18.6
Phenacobius catostomus	61.2	10.2	11.06	4.88	2.62	2.5	3.9	3.9	29.8
Rhinichthys obtusus	66.3	13.6	10.15	5.24	6.32	3.2	4.2	4.1	43.8
Rhinichthys cataractae	72.0	13.5	13.23	6.71	4.78	3.0	3.6	3.7	30.7

The standard length (SL) and head length (HL) of fish are given. The mass of the AM1, AM2, and AM3 are given. The eye depth (ED), eye width (EW), and eye height (EH), as well as the calculated eye volume (EV), are also given. The species are placed in order of decreasing HL adjusted eye volumes.

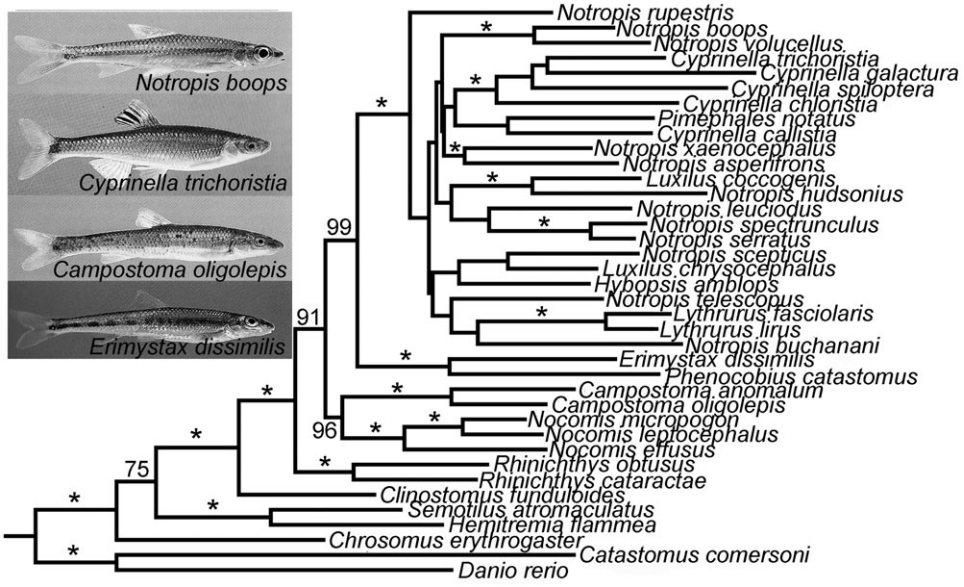

Figure 2. The phylogeny of the cyprinid species examined. The phylogeny was constructed from concatenated sequences of the mitochondrial *cytb* and nuclear *enc1* genes. The single best topology from five Bayesian runs is depicted. Posterior probabilities represent the frequency a node was recovered from all five post burn-in Bayesian runs. Nodes marked with an asterisk (*) are supported with 100% posterior support and all other node support values over 50% are depicted behind the supported node.

species *Luxilus chrysocephalus* and *Notropis telescopus* had the smallest AM1 when adjusted for head length. When adjusted for head size the two species with the two smallest total AM volumes were *Notropis buccata* and *N. telescopus*. *Phenacobius catostomus* exhibited the largest total AM mass and it was followed by *Pimephales notatus*.

The width and height of the eye in all species were very similar, suggesting that the external orbit of the cyprinids examined was generally circular (Table 2). However, the orbit depth was often approximately half the size of the diameter of the external orbit. In *Notropis serralatus*, the eye depth was 46% of the eye width. This suggests that the eye shape of this fish resembles a compact cylinder (i.e. similar to a car tyre). Conversely, *Semotilus atromaculatus* and *Nocomis leptocephalus* exhibited eyes that were almost as deep as they were wide. *Rhinichthys cataractae* and *Rhinichthys obtusus* had the smallest eye volume adjusted for HL of all of the minnows examined. *Notropis scepticus*, *Notropis hudsonius*, and *Clinostomus funduloides* were recovered as having the largest eye size given their HL.

The results from our phylogenetic analysis of the concatenated *cytb* and *enc1* genes (Fig. 2) are largely concordant with previous hypotheses of the relationships of North American cyprinids. *Chrosomus erythrogaster* was recovered as the most basal taxa of the North American minnows. A well-supported clade composed of *S. atromaculatus* and *Hemitremia flammea* were recovered as the next most basal split among the species analyzed. However, the node representing the relationship of this clade to the rest of the cyprinid minnows examined here was not well supported. The next three divergence events recovered in this phylogenetic hypothesis involved the lineages *C. funduloides*, *Rhinichthys* spp., and a clade composed of *Campostoma* spp. + *Nocomis* spp., respectively (Fig. 2). *Erimystax dissimilis* and *Phenacobious catastomus* were supported as forming a clade and this clade was found to be the sister group to the remaining cyprinids analyzed.

Species in the genera *Cyprinella*, *Hybopsis*, *Luxilus*, *Lythrurus*, and *Notropis* were all recovered together in a well-supported clade, However, the relationships within this 'shiner' clade were poorly supported and ambiguous (Fig. 2). In the present analysis, the only currently recognized shiner genus recovered as monophyletic was *Lythrurus*, although we only included two of eleven *Lythrurus* species in this analysis. *Pimephales notatus* was recovered as sister to *Cyprinella callistia*, although this relationship was not supported with greater than 50% posteriors, suggesting that there is little support for this relationship. The remaining *Cyprinella* species were recovered as monophyletic and strongly supported. *Notropis* and *Luxilus* were paraphyletic, with little support for relationships within and

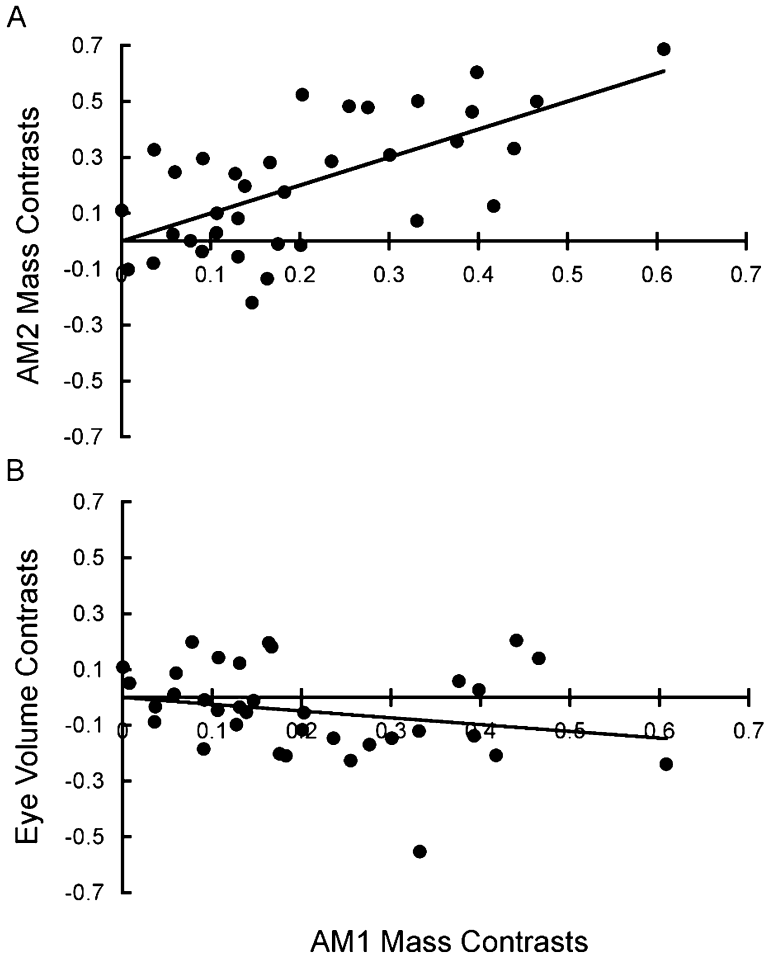

Figure 3. The independent contrasts between masses of the adductor mandibulae, AM1 and AM2 (A), as well as AM1 mass and eye volume (B). There is no support for the AM muscles exhibiting constructional constraints but the negative contrast correlation between AM1 and eye size are consistent with these constraints operating in North American cyprinids.

between these two groups and the remaining shiner species.

Because the number of phylogenetic contrasts in a strictly bifurcating tree is one less than the number of terminal taxa examined (Felsenstein, 1985), all phylogenetically-independent contrast (PIC) correlations in the present study were examined using 35 contrasts. The PIC correlation between the mass of AM1 and AM2 (Fig. 3A) residuals was especially strong and positive ($P < 0.0001$; $r = 0.83$). However, the relationships between AM1 and AM3 ($P = 0.21$; $r = 0.21$) and AM2 and AM3 ($P = 0.32$; $r = 0.06$) were not significant, although they were in a positive direction. When the relationship between the total AM residuals and residuals of eye volume were examined, there was no significant relationship ($P = 0.09$; $r = -0.29$). However, the relationship between AM1, the muscle physically closest to the eye, and eye size (Fig. 3B) was significant and negative ($P = 0.02$; $r = -0.38$)

DISCUSSION

The quantification in the present study of both the mass of the AMs and eye volume provides insights into the biology of some minnow species that might not be apparent without a comparative framework. Additionally, we recovered evidence consistent with both modularity and constructional constraints among phenotypes in the cyprinid traits examined. The positive correlation among the AM1 and AM2 muscles is especially strong, which might have been expected given their shared function during feeding (Osse, 1969) and their common developmental origin (Hatta *et al*., 1990; Hernandez *et al*., 2005). However, AM3 did not appear to be correlated with the other

two AMs examined. Because none of the three AMs showed a positive correlation, cyprinid AMs do not likely compete for space in the head of these fishes. However, AM1 and eye volume did show a negative correlation that is consistent with a constructional constraint. Comparisons between the present study of cyprinids and a recent study of Malawi cichlids (Hulsey *et al*., 2007) indicates that, although there are some interesting exceptions, teleosts could commonly share several patterns of evolution among these crainiofacial structures.

A greater appreciation of the function of the AMs during feeding (Gosline, 1986; Turingan & Wainwright, 1993; Korff & Wainwright, 2004) and how they develop during ontogeny (Hernandez *et al*., 2005) could provide additional insight into the causes of the correlated change among these muscles. Parrotfish species that bite and excavate coral tend to have larger muscles than parrotfish that scrape algae and dead coral (Bellwood & Choat, 1990). However, similar to that found previously in Lake Malawi (Hulsey *et al*., 2007), one of the most herbivorous species examined in the present study, *Campostoma anomalum*, did not have exceptionally large AMs. This could indicate that herbivory and benthic feeding in general is substantially different in freshwater groups such as minnows and cichlids compared to marine groups such as parrotfish. However, many of the minnow species examined in the present study that did have the largest AMs were benthic-feeding fishes such as *Phenacobius catastomus*.

The minnow species at the edges of the phenotypic distributions offer some additional insights into North American cyprinid biology. For example, *Rhinichthys* have exceptionally small eyes and this is likely related to the fact that, unlike most of the cyprinids examined, these species primarily forage at night (Culp, 1989). The species *Notropis buccata* had the lowest total AM mass and this species exhibits unique pearl organs, or extensively developed infraorbital canals, that overlie the AMs (Reno, 1971). It would be interesting to test whether the pearl organs that directly overly the AMs and could require a lot of space in this craniofacial region influence AM size, perhaps through developmental trade-offs. Both *Notropis scepticus* and *Notropis hudsonius* were found to have relatively large eyes and feed in the water column similar to many of the other *Notropis*. This type of feeding behaviour could favour large eyes and also not demand large AM muscles to capture prey (Wainwright *et al*., 2004; Hulsey *et al*., 2007).

Similar to several previous phylogenetic studies, benthic groups such as *Rhinichthys*, *Campostoma*, *Nocomis*, *Erimystax*, and *Phenacobius* were supported as basal to the remaining cyprinids analyzed (Simons & Mayden, 1999; Simons, Berendzen & Mayden, 2003). Species in the genera *Cyprinella*, *Hybopsis*, *Luxilus*, *Lythrurus*, and *Notropis* were all recovered together in a well-supported clade. However, the relationships within this shiner clade were ambiguous (Fig. 2). Support for alternative relationships between these four shiner lineages and the rest of the cyprinids have varied in previous studies (Simons & Mayden, 1999; Simons *et al*., 2003; Bufalino & Mayden, 2010; Schonhuth & Mayden, 2010). *Pimephales notatus* was recovered as sister to *C. callistia* with little support and the remaining *Cyprinella* species were recovered as monophyletic and strongly supported.

The lack of resolution among *Notropis*, *Lythurus*, *Luxilus*, *Cyprinella*, and *Hypobsis* suggests that this clade may have diversified exceedingly rapidly. One potential explanation for this burst of diversification could be the change from the largely benthic lifestyle exhibited in relatively basal clades such as *Rhinichthys*, *Nocomis*, *Campostoma*, *Erimystax*, and *Phenacobious*. Most of the species in the genera *Notropis*, *Luxilus*, *Lythrurus*, and *Cyprinella* feed primarily in the water column. However, it appears that there could have been reversals to a benthic lifestyle even within this shiner clade in groups such as *Hybopsis*. Because of the repeated contrasts between benthic and limnetic feeding species, a further resolved phylogeny of these species might provide an excellent clade to compare morphological feeding adaptations, such as size of the AMs, in species inhabiting these two distinct habitat types. It is also possible that the lack of resolution in this section of the phylogeny is a result of vagaries in the markers that were used to reconstruct relationships in the present study. More mitochondrial sequence data, as well as other nuclear genes with a relatively rapid rate of evolution, would be ideal for resolving these short and generally poorly supported branches in the cyprinid phylogeny. Regardless, resolving relationships within the shiner clade will not only be integral to our understanding of the phylogenetic history of North American minnows, but also to the evolution of this group along a benthic/limnetic habitat continuum. Diversification between benthic and pelagic lifestyles is a ubiquitous component of fish diversification (Robinson & Wilson, 1994).

There are several patterns of craniofacial divergence recovered in the cyprinids examined in the present study and the Lake Malawi cichlids that have recently been examined for the same phenotypes (Hulsey *et al*., 2007) that are notably different. In cichlids, the AM3 is, as a rule, a much smaller muscle than either AM1 or AM2. However, in cyprinids AM3 is large and, in many species examined in the present study, it was the largest muscle of the three AMs. The AM2 was consistently recovered as the smallest

muscle of the three examined here in cyprinids. Additionally, there is no clear correlation between AM3 and either of the other two AMs in cyprinids, although this muscle did show a positive correlation to the other AMs in cichlids. Because the AM1 could function in mouth opening in cyprinids (Sibbing, Osse & Terlouw, 1986) with AM3 functioning extensively in closing the mouth, it is plausible that AM1 might be functionally, as well as evolutionarily, decoupled from AM3. Importantly, because no negative correlation exists between any of the AM muscles in the jaws of either cichlids (Hulsey *et al*., 2007) or the cyprinids examined in the present study, it is likely that these muscles rarely impose constructional constraints on one another during teleost diversification.

The volume of the eye and masses of the AMs cannot become infinitely large, and therefore constructional constraints should operate at some level. Many examinations of eye size have focused on the external size of the eye and made the assumption that the eye is round as it is in humans (Barel, 1984; Strauss, 1984). The depth that the eye protrudes into the cranium is a morphological attribute that has not received much attention, although the results obtained in the present study suggest the eye of the cyprinids examined is not spherical (Table 2). Using our estimates of eye volume, we found some evidence that eye size and AM1 mass do trade-off evolutionarily in cyprinids. Big eyes were associated with relatively small AM1 muscles in our analysis. Interestingly, this trade-off that is consistent with spatial competition between the eye and AM1 was not recovered in Lake Malawi cichlids (Hulsey *et al*., 2007). Similarly, the negative relationship between eye volume and muscle mass observed in the present study should be examined in more fish groups to evaluate whether the cyprinid result is the general rule or whether Lake Malawi cichlids reflect the more common teleost condition.

Identifying what promotes and places limits on phenotypic evolution in radiations such as the cyprinid fishes of North America remains fundamental to an understanding of the rates and pathways underlying how species-rich fish groups have diversified. Constructional trade-offs could be a critical constraint in many groups such as cyprinid fishes. Ultimately, to understand the causes of correlated and independent change among the AMs and other components of fish craniofacial diversity, investigations should continue to examine the integrated evolution of functional, developmental, and genetic components of cyprinid form.

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