

CONVERGENCE IN A MECHANICALLY COMPLEX PHENOTYPE: DETECTING STRUCTURAL ADAPTATIONS FOR CRUSHING IN CICHLID FISH

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Morphological convergence provides strong evidence that evolution is adaptive. However, putatively convergent morphology is often examined in two dimensions with no explicit model of function. In this study, we investigated structural and mechanical similarities of the lower pharyngeal jaw (LPJ) in cichlid fish that have evolved the ability to crush hard-shelled molluscs. Using a novel phylogeny, we demonstrated molluscivory has been gained and/or been lost numerous times in Heroine cichlids. Within this comparative framework, we produced three-dimensional computed tomography (CT) scans for LPJs of both morphotypes in the trophically polymorphic *Herichthys minckleyi* and six evolutionarily independent pairs of closely related species. Like *H. minckleyi*, these species exhibit divergence between a molluscivore and a nonmolluscivore. Using the CT scans, we generated finite element models of papilliform *H. minckleyi* LPJs to determine where stress would concentrate in a jaw not modified to crush molluscs. Then, we examined whether stress in the papilliform LPJ predicted structural modifications in molariform *H. minckleyi* and other molluscivorous species. Despite potential constraints, stresses imposed during prey processing explain 40% of LPJ variation in mollusc crushing species. The structural and mechanical analyses also suggest divergence found in polymorphic species could provide the substrate for trophic differences found in reproductively isolated cichlids.

KEY WORDS: Cuatro Ciénegas, feeding, functional morphology, molluscivory, speciation, trophic polymorphism.

Morphological similarities shared among phylogenetically independent lineages strongly suggest evolution is adaptive. However, phenotypic similarities may exist because of common genetic (McGuigan and Blows 2007), developmental (Hodin 2000), scaling (Nummela and Sanchez-Villagra 2006; Savage et al. 2007),

and functional (Wainwright et al. 2001) constraints and might only rarely arise because of similar adaptations to a shared environmental challenge. For instance, despite the likely adaptive divergence that has occurred between humans and giraffes during the evolution of their necks, these species and virtually all other mammals

have seven cervical vertebrae (Narita and Kuratani 2005). Even the diversification of innovative structures such as the pharyngeal jaw apparatus of cichlid fish may be heavily influenced by non-adaptive constraints. Although the cichlid pharyngeal jaw likely permits the processing of an unparalleled array of prey (Liem 1973), characteristics ranging from the sites of muscle attachment (Stiassny and Jensen 1987; Wainwright 2006) to the expression of genes responsible for the development of these highly modified gill arches (Hulsey et al. 2005b) are likely conserved among all cichlids. Delineating what phenotypic similarities in the cichlid pharyngeal jaw are readily adapted to the mechanical challenges of feeding on a novel prey type would facilitate the identification of what components of the pharyngeal jaw are most easily modified during evolution. Using a novel phylogeny, computed tomography (CT), and finite element modeling, we test whether the mechanical demands of crushing hard-shelled molluscs predict the repeated evolution of structural similarities during cichlid lower pharyngeal jaw (LPJ) diversification.

The enhanced ability to efficiently crush hard-shelled prey may be a key functional innovation of the cichlid pharyngeal jaw (Hulsey 2006). In contrast to many types of ecological specialization such as piscivory (Wainwright et al. 2001; Chakrabarty 2005), the ability to break down durable prey is likely accompanied by structural divergence with highly predictable mechanical consequences. Whereas tensile and shear stress may dominate the forces exerted on many skeletal elements, durophagous organisms must produce and resist exceptional compressive forces during the processing of their heavily defended prey (Wainwright 1987; Grubich 2003). Hard-shelled molluscs likely require the greatest amounts of compressive force to breakdown of any freshwater prey type that cichlids exploit (Vermeij 1987). Therefore, when comparing cichlids that differ in the extent to which they crush molluscs, we would expect that the bony elements of the pharyngeal jaw (Fig. 1), where most prey processing occurs, should exhibit divergent modifications for resisting compressive stress. Furthermore, cichlid fish that have independently diverged in the ability to crush molluscs would be expected to have highly similar jaw modifications in areas in which stress is concentrated when prey are crushed.

Convergence in trophic morphology is thought to be a ubiquitous component of cichlid diversification (Fryer and Iles 1972; Lazzro 1991; Winemiller et al. 1995; Ruber and Adams 2001). Molecular phylogenetic information has been invaluable in assessing whether similarities in cichlid morphology and ecology are similar because of shared phylogenetic history or are independently derived. Cichlids in the tribe Heroini that are endemic to the Neotropics potentially provide an ideal group to examine the repeated evolution of structural modifications associated with durophagy because of the large number of times this trophic specialization has putatively evolved (Hulsey 2006). However, the

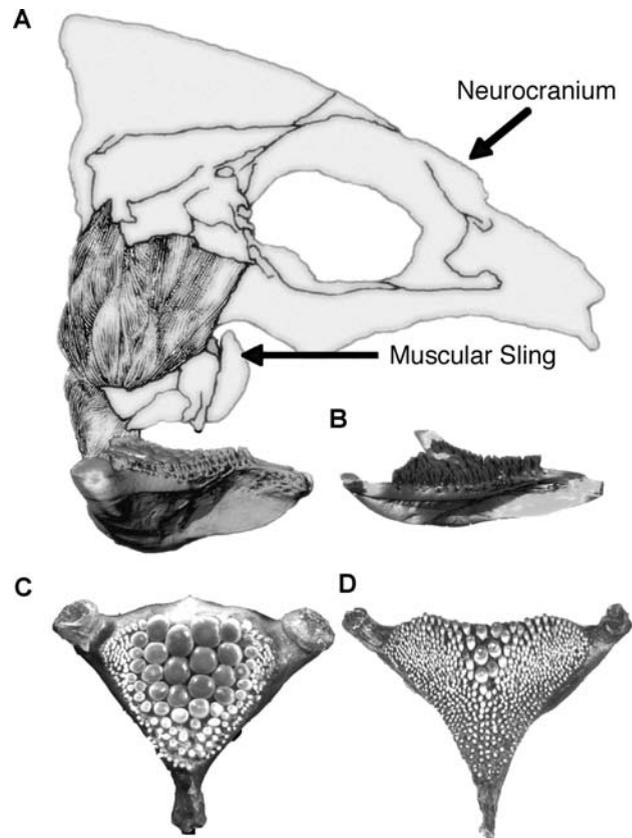


Figure 1. Several orientations of molluscivorous and nonmolluscivorous lower pharyngeal jaws (LPJs) are shown. The LPJ of a molariform *H. minckleyi* (A) is depicted ventrally attached to the neurocranium via the cichlid pharyngeal muscular sling. Contrasting the ventral view of a papilliform (B) with a molariform (A) the difference in the thickness of the jaw (z-axis in text) is apparent. The dorsal, or prey's eye, view of a molariform (C) and papilliform (D) *H. minckleyi* exhibit obvious phenotypic differences. However, the overall shape of the pharyngeal jaws in this orientation (the x and y plane in the text) is highly similar facilitating a robust quantification of phenotypic differences between molluscivores and nonmolluscivores in the z-axis.

current evidence for the evolution of molluscivory in Heroines is largely based on mitochondrial DNA (Hulsey et al. 2004). Further phylogenetic analyses of Heroine cichlids that include information from the nuclear genome would greatly improve our confidence that trophic specializations such as molluscivory have evolved convergently.

The importance of feeding specialization to cichlid diversification is vividly demonstrated by trophically polymorphic species such as *Herichthys minckleyi* that exhibit clear divergence in their pharyngeal jaws along a force production axis. In *H. minckleyi*, there are two co-occurring and interbreeding pharyngeal jaw morphotypes (Kornfield and Taylor 1983) that differ in the extent that they exploit snails (Hulsey et al. 2005a,b). Molariform *H. minckleyi* have robust jaws and teeth putatively adapted to crush

molluscs whereas papilliforms, individuals with more gracile pharyngeal jaws, are specialized to masticate less durable prey (Hulsey et al. 2006a,b). Discrete trophic polymorphism as found in the pharyngeal jaws of *H. minckleyi* is rare in extant taxa, but may be an intermediate step in the diversification of many cichlid species (Meyer 1990; Barluenga et al. 2006). Generally, the relationship between intraspecific polymorphism and interspecific divergence remains unclear. However, if trophic polymorphism represents a transient stage through which species diverge, we would expect the structural and mechanical modifications found in *H. minckleyi* to be common among closely related but divergent cichlid species that differ in the extent to which they feed on molluscs.

Studies of convergence often focus on morphologically simple structures that can be characterized by linear external measurements (Winemiller et al. 1995; Ruber et al. 1999; Leal et al. 2002; Stayton 2006). However, organisms are three-dimensional and their phenotypes are often complex and difficult to describe using traditional morphometrics. For instance, skeletal elements such as the LPJ often contain fenestrae or holes, are attached to other structures, and integrated via suturing making robust comparisons among different species difficult. However, with the advent of three-dimensional scanning techniques such as CT x-ray scans (Stock 1999), the three-dimensional morphology of structures can now be ascertained. If homology can be estimated, it should be possible to evaluate convergence in not only the external, but also the internal structure of complex phenotypes such as the cichlid LPJ.

Once the three-dimensional structure of a putatively convergent phenotype has been characterized, it would be ideal to assess if the mechanical consequences of variation in this structure might also be similar. Mechanical models of jaw lever systems such as the lower oral jaw (Wainwright and Richard 1995) and anterior jaw four-bar linkage (Westneat 1995; Hulsey and García de León 2005) often use linear measurements to provide functional insight into how the size and shape of skeletal elements translate into ecological specialization. However, skeletal elements such as the cichlid LPJ likely exhibit mechanically complex properties that are difficult to infer from linear measurements alone (Bayraktar et al. 2004; Richmond et al. 2005). Assessing the links between morphological and mechanical convergence may be especially important given the potential for decoupling in these levels of mechanical design (Alfaro et al. 2005). Furthermore, most skeletal elements are not materially uniform solids that exhibit consistent material properties that can easily be tested empirically. Assessing how a complex and integrated structure such as the cichlid lower pharyngeal jaw (LPJ) enhances mechanical abilities necessitates analytically complex models (Daegling and Hylander 1997). Fortunately, a method developed in engineering known as Finite Element Analysis (FEA) can now be used to examine how stress is

transmitted through structurally complex solids. This method that decomposes three-dimensional objects into a mesh made up of a large number of interconnected finite polygons provides computational approximations of how forces applied to skeletal elements translate into stress. This method has been increasingly used to understand the mechanical properties of bony elements (Korioth and Versluis 1997; Dejak et al. 2003; Rayfield 2004; Richmond et al. 2005; Mchenry et al. 2006; Al-Sukhun et al. 2007), and FEA could provide substantial insight into how forces experienced during snail crushing stress components of the cichlid LPJ. By combining CT scans with FEA, we can examine the structural as well as the mechanical basis of evolutionarily replicated modifications in the cichlid LPJ.

In this study, we provide an integrative examination of putative convergence in the cichlid LPJ. Using phylogenetic information obtained from both the mitochondrial and nuclear genomes, we evaluated whether molluscivory has evolved numerous times in Heroine cichlids. Within this comparative framework, we produced three-dimensional CT scans of the LPJ structures of both morphotypes of the trophically polymorphic *H. minckleyi* and six evolutionarily independent pairs of closely related species. These species pairs, such as *H. minckleyi*, exhibit divergence between a molluscivore and a nonmolluscivore. Using the CT scans as input, we generated finite element models of an ontogenetic series of papilliform *H. minckleyi* to determine where stress should be concentrated in an LPJ not modified to crush molluscs. Then, we examined whether the location of stress in the papilliforms predicted (1) structural modifications to the molariform *H. minckleyi* pharyngeal jaw and (2) morphological differences between closely related molluscivorous and nonmolluscivorous species pairs. Using this framework, we assessed the extent to which structural similarities in the LPJ likely represent adaptations to compressive stresses experienced during prey processing by molluscivorous cichlids.

Materials and Methods

SPECIMENS

The LPJ of both morphotypes of *H. minckleyi* and 12 other species of Heroine cichlids were dissected from the buccal cavity of individuals caught from the wild. All fish were preserved in formalin and transferred to 70% ethanol for long-term storage prior to dissection. Once dissected, the LPJs were cleaned of all muscle and the jaws were allowed to dry. Species examined were chosen because they likely represent independent evolutionary origins of closely related molluscivorous and nonmolluscivorous species in the tribe Heroini (Hulsey et al. 2004; Winemiller et al. 1995; Hulsey 2006). The LPJs examined ranged in width from 7.1 mm to 17.6 mm and were obtained from adult individuals ranging from 65 mm to 146 mm Standard Length (SL). The LPJs of approximately three individuals per species (Table 1) were examined

Table 1. The contrasting *H. minckleyi* morphotypes and molluscivorous species followed by its nonmolluscivorous closely related morphotype/species are listed.

Taxon	Diet	<i>n</i>	Average cell volume (mm ³)	Average cell contrast (mm ³)	<i>t</i> -test <i>P</i> -value
<i>Herichthys minckleyi</i> molariform	M	10	27.9	9.2 ± 0.5	<0.001
vs. <i>Herichthys minckleyi</i> papilliform	P	9	18.7		
<i>Herichthys labridens</i>	M	5	25.1	11.5 ± 1.2	<0.001
vs. <i>Herichthys bartoni</i>	D	6	13.6		
<i>Veija maculicauda</i>	M	7	16.1	8.4 ± 0.8	<0.001
vs. <i>Paraneetroplus bulleri</i>	I	1	7.7		
<i>Thorichthys ellioti</i>	M	1	17.2	10.2 ± 3.0	<0.001
vs. <i>Thorichthys helleri</i>	I	3	7.0		
<i>Astatheros macracanthus</i>	M	3	18.7	6.2 ± 0.7	<0.001
vs. " <i>Cichlasoma</i> " <i>octofasciatum</i>	P	4	12.4		
<i>Nandopsis urophthalmus</i>	M	5	14.6	5.9 ± 1.1	<0.001
vs. <i>Petenia splendida</i>	PV	2	8.7		
<i>Nandopsis trimaculatum</i>	M	5	13.9	0.5 ± 0.5	0.01
vs. <i>Archocentrus centrarchus</i>	A	7	13.4		

The prey type that contributes most to the diet of the taxa are taken from Hulsey (2006) or references therein: M, molluscivore; P, plant feeder; D, detritus feeder; A, arthropods; and PV, piscivore. The number of LPJs per species examined (*n*) and average cell volume for the LPJ of a 77-mm individual is given (mean). The average difference in the contrast between the molluscivorous and nonmolluscivorous relatives (mean ± SE) is also depicted.

along with an ontogenetic series of pharyngeal jaws obtained for molariform (10 individuals) and papilliform (nine individuals) *H. minckleyi*.

DNA ISOLATION AND SEQUENCING

To provide an improved phylogenetic hypothesis of the species examined, sequences (GenBank accession numbers: EU620420-EU620426) of 504 base pairs of the S7 Ribosomal intron one (Chow and Hazama 1998) were combined with 1137 base pairs of the mitochondrial cytochrome *b* gene and sequences of the S7 intron that were previously generated. Sequences from the South American cichlids *Mesonauta festivus* and *Geophagus steindachneri* were included as outgroups. All individuals sequenced for this study were collected in the wild from locations reported in Hulsey et al. (2004). Conditions for the polymerase chain reaction (PCR) and sequencing were similar to those used in previous studies (Hulsey et al. 2004; Higham et al. 2007). Gene sequences were assembled from individual reactions using the program Sequencher version 4.6 (Gene Codes, Ann Arbor, MI). Sequences were aligned using Clustal X (Thompson et al. 1999), trimmed to ensure complete sequences for all individuals, and codon positions in cytochrome *b* were defined using MacClade 4.0 (Maddison and Maddison 2000).

PHYLOGENETIC ANALYSIS

For the phylogenetic analysis, relationships among the 15 species of Neotropical cichlids were examined in a Bayesian framework. ModelTest 3.06 (Posada and Crandall 1998) was used to identify the best model of molecular evolution for the S7 intron as

a single partition and for each codon site in the cytochrome *b* gene. Two separate Bayesian analyses were executed to find approximations of the maximum-likelihood tree using MrBayes 3.0 (Ronquist and Huelsenbeck 2003) with the S7 intron alone and the cytochrome *b* gene and S7 intron combined. The analyses treated the transition-transversion matrices, number of invariant sites, and gamma-shape parameters as unlinked or independent for the intron and each codon site of cytochrome *b*. Flat prior probability distributions for all parameters were assumed before analysis. We ran 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 (MCMC) 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, the equilibrium was reached at approximately 50,000 generations, and sample points prior to 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 (Fig. 2).

3-D STRUCTURE ACQUISITION

CT scans of LPJs were obtained using a Scanco Viva/Micro-CT x-ray scanner (Scanco Medical, Southeastern, PA) and workstation at sampling intervals ranging from 0.016 mm to 0.060 mm

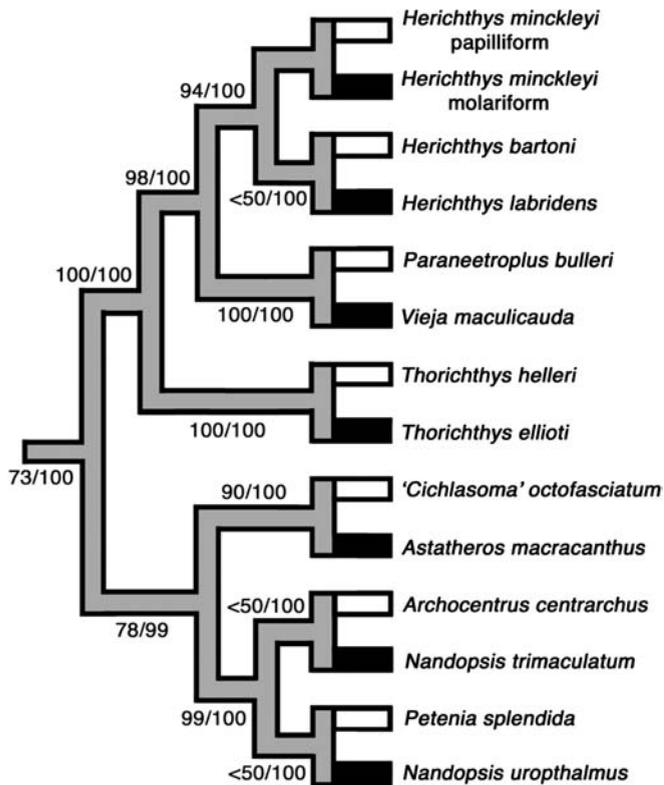


Figure 2. The phylogenetic topology of the best Bayesian tree recovered from the combined analysis of the cytochrome *b* gene and *S7* ribosomal intron fragment is depicted. Bayesian posterior probabilities of the nuclear intron followed by the posterior values of both genes analyzed simultaneously are depicted behind the respective node they support. The topologies did not differ greatly although *H. bartoni* showed closer affinities to *H. minckleyi* and there was a lack of resolution among *Nandopsis trimaculatum*, *Archocentrus centrarchus*, *Nandopsis urophthalmus*, and *Petenia splendida* when the *S7* intron was analyzed alone. Species that feed extensively on molluscs (black) and species that do not exploit molluscs (white) are depicted on the topology, as is the presumed ambiguity (gray) of the ancestral condition among Heroine cichlids.

(Fig. 3). The resulting image layers were then stacked into a single file and adjusted by their sampling intervals to standardize the LPJ volumes. The compiled images were then rotated on a coordinate system. The stacked images were adjusted so that the two horns of the LPJ (Fig. 4A), where the primary pharyngeal crushing muscles attach, were positioned parallel to the x-axis and the central keel of the LPJ was bisected by the y-axis. Once rotated, three homologous points were identified on each LPJ: (1) center of the right horn, (2) center of the left horn, and (3) the tip of the keel. We used these landmarks to extrapolate homologous sampling regions for all pharyngeal jaws examined. These sample regions were located at the centers of the cells of a grid that was overlaid parallel to the tooth surface of each LPJ. The x-axis of

the grid was overlaid on the LPJs in the region connecting the centers of the two horns. Twenty-one grid lines, evenly spaced, were vertically placed along this axis, between the two horn centers. The y-axis, orthogonal to the first, was placed between the posterior-most point along the midline of the LPJ and the most anterior point on the keel. Twenty-one grid lines, evenly spaced along this y-axis, spanned from the posterior midline to the center of the keel. The individual sample regions, located at the center of the cells where the gridlines overlapped, were represented by squares with sides of 0.4-mm length. The volume ventral to these square grid cells, in the z-plane, was estimated for each LPJ.

To obtain comparative data for the approximately 100 sample regions on each LPJ, correlations were generated between the volume measurements taken from each sample region and SL of individuals within a species. Because the volume of the LPJ and other morphological components of the jaw scale strongly with size, it was necessary to standardize the sampled volume measurements to a single SL for intermorph and interspecific comparisons. Linear measurements such as SL should scale with the square root of area and the cube root of volume. Therefore, because we kept the square sample regions the same area among different sized individuals, we were able to compare the sampled volumes in each region using linear correlations. Once a correlation was generated for each sample region in a given species, the volume for a 77-mm SL individual was estimated. We chose to standardize values to those of an individual of 77-mm SL because our specimens generally spanned this size, it was the approximate SL for the two species that had low samples sizes, and individuals have likely undergone any ontogenetic shift to utilizing hard-shelled prey at this SL (Hulsey et al. 2006a). Because of our size correction, the particular size of individuals examined in comparisons also should not influence the generality of the morphological or mechanical conclusions drawn. All volumetric data acquisition was performed using code written in Matlab.

For the analysis of replicated morphological evolution, only the sample regions that contained some portion of the LPJ between species were examined. The average volume estimated at 77-mm SL for all sampling regions containing structure, was compared between molluscivorous and nonmolluscivorous morphotypes and species to determine if molluscivores generally had more robust LPJ structure. For our comparative analysis, we only used differences between the species pairs because of the difficulty in reconstructing ancestral states of the phenotypes examined. Despite a likely lack of spatial and mechanical independence, sample regions were treated as independent units of replication to test for morphological similarities. To determine if molluscivorous versus nonmolluscivorous morphotypes and species pairs differed in the volume of their LPJs, *t*-tests on estimated volumes at all putatively homologous sampling regions were performed for closely related trophic pairs.

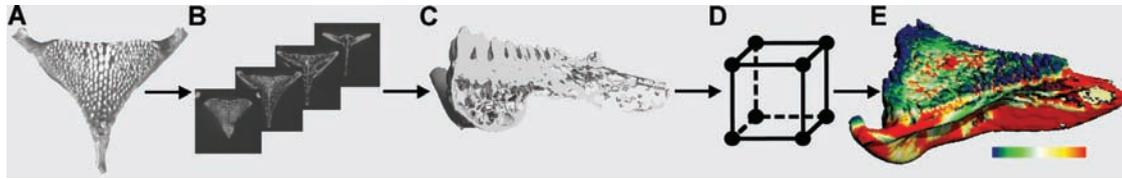


Figure 3. Cichlid LPJs were used to generate 3D computed tomography (CT) scans and finite element analyses (FEA). The LPJ (A) of 15 species of cichlids were dissected from the buccal apparatus, cleaned of attached muscles, and allowed to dry. These LPJs were then scanned (B) and the stacks shown here as a series of dorsal to ventral slices were assembled into 3D models (C) such as the one here shown from a lateral perspective. These models were then rendered into several hundred thousand tetrahedral finite elements (D) forming a 3D mesh. The elements in this mesh were given mechanical properties that are thought to generally characterize vertebrate teeth and bone. The models were constrained structurally at three points (1) left pharyngeal musculature attachment (2) right pharyngeal musculature attachment (3) tip of the keel where it attaches long the midline of the buccal apparatus. Subsequently, a computationally generated “snail” was used to simulate the forces applied to the toothed surface of the three-dimensional model of the LPJs of papilliform *H. minckleyi*. The stresses with warmer colors representing high stress were then recorded in the finite element model (E).

FINITE ELEMENT ANALYSIS

To evaluate which components of the pharyngeal jaw likely experience the most stress during compressive prey processing, we performed an FEA on the ontogenetic series of papilliform *H. minckleyi* CT scans. Scans containing the x-ray attenuation of every solid voxel in the jaw that was embedded within empty space were parameterized for the FEA. Attenuation is directly related to structural density (Stock 1999), and because of a clear bimodality in the x-ray attenuation of the LPJ structure, voxels were assigned to one of two groups. The first group consisted of voxels with attenuation values below 4.56 cm^{-1} and the second was comprised of structures whose attenuation values were greater than this value. The high-attenuation structures were confined to the high-density tooth caps that likely contain sequestered metals (Suga et al. 1992) resulting in higher density material analogous to enamel in mammalian teeth. Lower-density structures comprised the rest of the teeth and the jaw. All segmentation and model manipulation was performed using the Scanco IPL software.

FEA makes estimation of stress accumulation in complex three-dimensional structures possible by decomposing an object

into a mesh of finite components. The Scanco Finite Element (FE) solver was used to create tetrahedral meshes from the segmented volumes of the LPJs. The meshes were composed of a large number of tetrahedral elements connected together at their vertices. The resulting collection of contiguous tetrahedral elements and their vertices were used to determine the stress in response to loading. The Scanco 3D viewer software was used to measure distances on the scans and determine site coordinates. To keep computation time tractable, LPJ scans were scaled to sizes between 750,000 voxels and 4,000,000 voxels, resulting in a resolution of 0.042 mm/voxel. Scans were iteratively scaled to this range to reduce distortion of small structural elements and keep resolutions the same across models.

Once the mesh was created, we assigned material properties to the elements in each of the two attenuation groups. The exact mechanical attributes of the two structures in cichlid pharyngeal jaws are currently unknown, and therefore, the two groups were assigned mechanical properties documented for similar types of skeletal structures in other vertebrates. The Young’s modulus, which is the inverse ratio of how much a material deforms to how

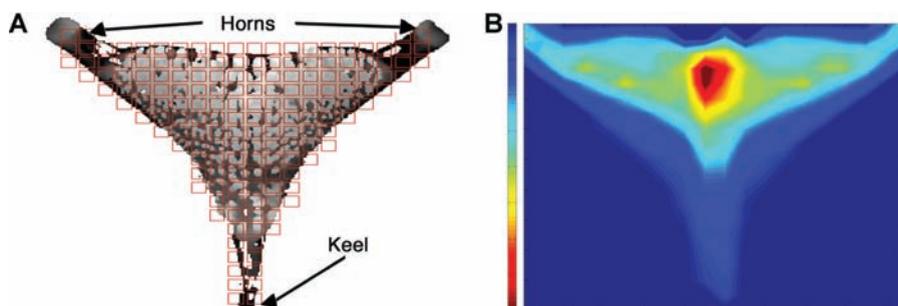


Figure 4. Both the amount of structure and stress in different areas of the jaw were sampled using a grid (A) of similar sized squares that were overlaid on the dorsal surface of the LPJ. The combined stress concentrations (B) in different regions of the LPJ inferred from the FEAs of an ontogenetic series of papilliform *H. minckleyi* are depicted. High stress (red) is centered along the posterior midline of the LPJ and grades into medium levels of stress (green) along the arms of the jaw connecting the “horns,” sites of muscles attachment. Generally the upper parts of the horns and the keel do not appear to experience substantial amounts of stress (blue).

much mechanical stress is applied to it, was given a different value for the two sets of structures. A Young's modulus of 17 gigaPascals (GPa), an average of several measurements for that of cortical bone (Ashman et al. 1984) was assigned to the lower-density portions of the jaw. A modulus of 80 GPa, that of the enamel of a human molar (Mahoney et al. 2000), was assigned to the higher-density tooth cap portions. The Poisson's ratio, which is the ratio of how much a material expands orthogonally to the direction in which a force is applied, for both components of the LPJ was kept at 0.3, a value commonly used for vertebrate jaws and teeth (Erickson et al. 2002; Dumont et al. 2005).

In all FEA models, boundary constraints must be imposed on the structure to ensure the model does not "move" and to ensure a unique solution can be obtained. A boundary condition file was created for each model, describing three anchoring sites for each LPJ. The LPJs were anchored at the lateral extremes of both the left and right horn and at the end of the keel. These sites were chosen because the horns of the jaw are the sites where the primary pharyngeal force producing muscles attach, and the tip of the keel is ligamentously attached to the midline of the pharyngeal apparatus. These points are the primary anchoring points of the jaw during prey processing and allowed the remainder of the jaw to be included in the FEA analysis. One voxel at each site was used to anchor the jaws during the FEA. Using a minimal number of voxels at the anchoring sites was necessary so that the sites would act only as pivoting supports, and not computationally resist torques from the bending of the jaw. Anchoring of these very small regions caused the voxels near the anchoring sites to experience extremely high and likely unrealistic amounts of stress. However, because these effects were limited to such small areas and the stress metric described below is not significantly affected by overestimation of stresses in a small number of voxels, the contribution of the unrealistically stressed voxels at the three anchoring sites likely had minimal influence on the results.

To solve the FEA models, the force applied must also be specified. The forces applied to the papilliform FEA models was 1600 Newtons, approximately three times the maximum force large molariform *H. minckleyi* resist before their jaws split (Hulsey 2006). Because we were interested in relative stress throughout the LPJ, the application of this extensive force was necessary to allow us to evaluate not only the one or two weakest regions of the jaw but stress accumulation throughout the jaw. If lower stresses had been applied, we would have had reduced power to detect mechanical weakness and the potential relationship between stress concentrations and remodeling among evolutionarily independent lineages of cichlids (see below). If much greater stresses had been applied, extreme stresses would have been concentrated in all regions of the jaw making the FEA virtually uninformative. For consistency, the force used was applied to each papilliform LPJ on the same area of the tooth bed. Forces were not applied directly

to tooth tips to avoid unrealistically high stress concentrations in single tooth. Instead, a cylindrical "snail shell" was modeled and oriented perpendicular to the tooth bed. The surface of the shell in contact with the tooth bed was made to take the exact contours of the surface. The shell was given a Young's modulus of 1.0 GPa, much lower than that of the tooth surface, to eliminate sharp edge effects at the circumference of the cylinder. Eliminating these edge effects in the teeth allowed us to study the stress deeper in the jaw, unaffected by irregular stress concentrations propagating down the tooth bed that appeared to be difficult to model accurately. The shell was positioned on the centerline of each papilliform jaw and contacted the four largest teeth on each jaw, the area that is likely the most common location for prey processing.

Once the boundary conditions were set and the forces were applied, the completed model was solved to obtain finite element strains and the resulting stresses. The solution for the FE model is determined by the connections among individual elements via their shared vertices. The edges of the elements function like springs that exhibit strain and result in displacement at the vertices. The equations for every single element are simultaneously solved using algebraic equations that relate all vertex displacements to applied forces via the system stiffness matrix according to the equation

$$\{F\} = [K]\{D\}$$

In this equation, $\{F\}$ is the vector of forces along the vertices, $[K]$ is the system stiffness matrix determined by the mechanical properties and geometry of the LPJ, and $\{D\}$ is the vector of vertex displacements. This computationally intensive step, once solved, provides the means to estimate stress concentrated along individual edges.

To determine the stress in different regions of the jaw, edges were assigned as being either high or low stress according to a threshold value. If examined across the entire LJP, the distribution of stress in individual edges tended to have a large tail with some individual areas exhibiting extremely large stresses as is commonly found in FEA (Dumont et al. 2005). Instead of calculating the total additive stress in regions, we used a cutoff threshold to minimize the influence of these extremely large stress outliers. The computationally estimated amount that finite element edges in the LPJ exceeded 250.0 MPa Von Mises stress was used as the threshold for the concentration of stress in a sample region. A Von Mises stress of 250.0 MPa corresponds to a safety factor of 2.3 above the yield stress of trabecular bone (Bayraktar et al. 2004).

The computationally estimated volume of bone above the stress threshold was determined in each region sampled previously for bone volume in the papilliform *H. minckleyi*. Based on our empirical results and use of a threshold value for assigning stress concentration, we reasoned that the estimated volume above

threshold stress, Y , should decrease exponentially as SL increased and go to zero as SL approaches infinity. This assumption should hold because as a structure gets infinitely large any stress applied should be dissipated throughout the structure and not become concentrated in particular areas. The estimated bone volume above the threshold stress for the ontogenetic series of papilliforms was therefore fit to the equation

$$Y = a * e^{b*SL}$$

A value b determined how steeply Y descended as SL increased in a region, and a estimated a scaling factor specific to each region. Volume above threshold for papilliform *H. minckleyi* was then calculated using equations specific to each sample region for an individual of 77-mm SL to obtain single values of stress at each sample region within the papilliform *H. minckleyi* LPJ.

MECHANICS OF STRUCTURAL CONVERGENCE

We determined how much of the structural differences between molariform and papilliform *H. minckleyi* LPJs could be attributed to mechanical demands for reinforcing the jaw in response to compressive stress. Using the results from the FEA and CT scans, we examined whether LPJ sample region volume increased most substantially between molariforms and papilliforms in those regions in which stress was computationally determined to be highest during simulated snail crushing. Each sample region ($n = 143$) standardized for an individual of 77-mm SL and shared between the molariform and papilliform LPJs was used as a single data-point in a correlation between difference in volume and volume above the stress threshold estimated from the papilliform FEA. This correlation does not take into account the spatial connectedness of the points examined, but the FEA by definition integrates the spatial connectedness of the LPJ morphology when analyzing stress concentrations.

We next tested if stress estimated from simulated mollusc crushing was related to structural changes among molluscivorous cichlid LPJs. To do this, it was determined if FEA-estimated stress in papilliform *H. minckleyi* LPJs explained structural enhancement of the LPJ among independently evolved molluscivorous cichlids. First, we averaged the volumetric divergence recovered between species pairs at 99 sampling sites that all 6 molluscivorous versus nonmolluscivorous species pairs shared in common. We then examined the correlation between volume above threshold stress estimated in the papilliform FEA and the average differences in volume between species pairs at 77-mm SL. A positive and significant correlation between these two variables would indicate that the regions in which stress is concentrated in the nonmolluscivorous papilliform *H. minckleyi* LPJ are those regions that on average exhibited the most structural divergence between closely related molluscivorous and nonmolluscivorous cichlids.

Results

PHYLOGENETIC ANALYSIS

The phylogenetic topology recovered with the S7 intron was largely congruent with previous analyses and with the combined S7 intron plus cytochrome *b* topology recovered here (Fig. 2). For the S7 intron, the maximum sequence divergence within the in-group was 5.7%. One difference between the two topologies was the closer affinity of *Herichthys bartoni* to *H. minckleyi* than to *H. labridens* in the S7 topology. The only other major difference between the two analyses was the lack of resolution for relationships involving *Petenia splendida*, *Nandopsis urophthalmus*, *N. trimaculatum*, and *Archocentrus centrarchus*. Most other relationships showed strong support (> 95% posterior probabilities) and all other sister group relationships used as evolutionarily independent contrasts were supported with greater than 95% posterior probability support in both analyses. The analysis of the S7 intron alone does not reject the contrasts of nonmolluscivorous and molluscivorous species and the combined analysis strongly supports the monophyly of the six species pairs.

MOLLUSCIVORE STRUCTURAL DIVERGENCE

The *H. minckleyi* molariforms exhibited much more extensive structure in almost all components of their LPJ as compared to the LPJ of the papilliforms (Table 1). Likewise, molluscivorous species on average exhibited much greater structural modifications in the LPJ than their nonmolluscivorous close relatives. The contrast between the molluscivorous *H. labridens* and nonmolluscivorous *H. bartoni* showed the greatest structural contrasts with an estimated average difference in volume per cell of 11.5 ± 1.2 SE mm³ for 77-mm SL individuals. The contrast of the molluscivorous *N. trimaculatum* and nonmolluscivorous *A. centrarchus* showed the least amount of structural divergence estimated at an average difference of 0.5 ± 0.5 SE mm³ in volume per cell for 77-mm SL individuals. In general, structural reinforcement in the contrasted species pairs was most pronounced along the midline of the jaw and from this midline to the horns on the jaw where the muscles attach on the LPJ.

FINITE ELEMENT ANALYSIS

For the cells examined for volume above yield stress in papilliform *H. minckleyi*, the average estimated value for sampling regions in a 77-mm individual was found to be 0.13 mm³. Based on the average volume of a cell in the papilliform morphotype this is equivalent to 0.7% of the entire papilliform LPJ being above the threshold yield stress. In general, stress was concentrated both in the region spanning the horns along the midline of the jaw and near the suturing of the two halves of the pharyngeal jaw (Fig. 2B).

MECHANICS OF STRUCTURAL CONVERGENCE

The molariform *H. minckleyi* had much greater increases in structure as compared to papilliforms (Fig. 5) in the regions in which there were larger amounts of stress concentrated in the jaw ($n = 143$ sampling regions, $r = 0.54$, $P < 0.001$). The molluscivorous Heroine cichlid species also had greater increases in structure as compared to closely related nonmolluscivores in the regions in which there were larger amounts of stress concentrated in the jaw

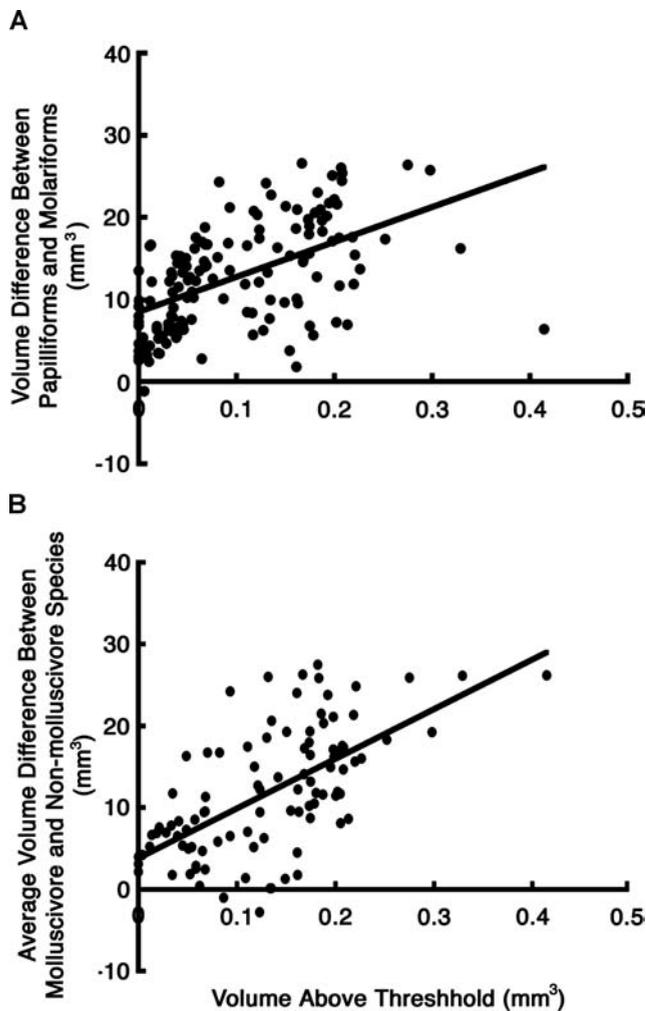


Figure 5. The volume above threshold stress for all of the grid cells containing structure in both molariform and papilliform *H. minckleyi* was determined. For each of the grid cells, the relationship (A) between the amount of stress and the difference in the amount of structure found between molariform and papilliform *H. minckleyi* was then found. Then, the difference in the amount of structure found between the six contrasts of closely related molluscivorous and nonmolluscivorous species was found. The average of the difference for each grid cell for these six contrasts was then taken. This relationship (B) between this average difference and volume above yield stress in papilliform *H. minckleyi* was found for all of the putatively shared and homologous grid cells shared among these species.

of papilliform *H. minckleyi* ($n = 99$ sampling regions, $r = 0.63$, $P < 0.001$).

Discussion

The phylogenetic analysis presented provides further evidence that divergence between a molluscivorous and nonmolluscivorous feeding habit has occurred up to six times independently in the Heroine cichlids of Central America. Although a previous analysis of cytochrome *b* and the *S7* intron demonstrated considerable conflict among these genetic partitions (Higham et al. 2007), our topological results of both the *S7* gene alone and *S7* combined with cytochrome *b* were largely consistent. There were two cases of topological incongruence between the two analyses. When the *S7* intron was analyzed alone, there was a lack of resolution among four species and a slightly different relationship recovered for *H. bartoni* when compared to our combined analysis. However, the numerous transitions between molluscivorous and nonmolluscivorous trophic specialization recovered clearly indicate that Heroine cichlids provide an excellent comparative system for looking at phenotypes associated with the evolution of molluscivory.

Regardless of prey exploited by the close relative, the molluscivorous species in the cichlid species pairs generally exhibited more extensive pharyngeal jaw structure. This supports the notion that hard-shelled molluscs represent some of the most durable prey in freshwater systems and that the LPJs of these fish exhibit enhanced structure because of their trophic specialization. This type of phenotypic similarity among phylogenetically independent lineages that share a feeding habit is likely a common component of cichlid diversity (Fryer and Iles 1972; Winemiller et al. 1995). In the past, much of this phylogenetically independent similarity has been quantified using simple, linear morphometrics that examine structures in which homology is straightforward to assess. However, most organismal structure is three-dimensional and assessment of homology is difficult. Subjecting complexly integrated structures to comparative analysis likely requires novel methods to assess similarity because of the difficulty of establishing homology in a composite structure such as the cichlid LPJ. The method used here that consists of anchoring comparisons to sites with clear homology such as the center of the horns or the tip of the LPJ keel and then interpolating numerous putatively homologous sites could facilitate extensive comparative analyses in structurally complex phenotypes. We have likely only “scratched the surface” in documenting the prevalence of convergence and parallelism in diverse groups such as cichlid fish.

There was only one species contrast of the LPJ we examined that did not differ significantly between a molluscivorous and nonmolluscivorous species. The contrast of the molluscivorous *N. trimaculatum* and nonmolluscivorous *A. centrarchus* LPJs was in the direction predicted for other species pairs, but was not

greatly different in structure. For all of the comparisons, it should probably be kept in mind that these species may not be sister species and this may have reduced our power to detect differences in all cases. Also, one possible explanation for the relative lack of difference is that the snapshot of diet information documenting *N. trimaculatum* as a molluscivore may not be representative of the diet in this species generally. Without extensive sampling of the pharyngeal phenotype of *N. trimaculatum*, it is also not possible to rule out that, like several species of Heroine cichlids (Meyer 1990), it is polymorphic in its pharyngeal jaws and our specimens consisted only of less-robust, nonmolluscivorous individuals. A third possibility is that *N. trimaculatum* feeds extensively on molluscs, but does not crush them. Many cichlids have the ability to suck snails and other molluscs out of their shells, and therefore may be able to exploit this prey type without having to use their pharyngeal jaws to crush hard-shelled prey (Fryer and Iles 1972). This type of many-to-one mapping (Wainwright et al. 2005) of phenotypes to ecology wherein more than one phenotype allows different organisms to solve the same functional problem may be ubiquitous in highly diverse groups such as cichlid fish (Hulsey and Wainwright 2002; Alfaro et al. 2005).

Yet, the structural modifications found in the LPJ of the trophically polymorphic fish *H. minckleyi* do appear to characterize adaptation in numerous other species of cichlid fish. The type of trophic polymorphism found in *H. minckleyi* may often represent an initial stage in organismal diversification (Smith and Skúlason 1996; Proulx and Phillips 2006), and trophic polymorphism may be a transient stage in the divergence of species (Meyer 1990; Smith and Skúlason 1996). Although it is often assumed that the adaptations found in trophically polymorphic species are present in other species within a clade, this assumption is rarely tested. This generality is critical however to the notion that polymorphic species may commonly represent the initial stages of diversification in reproductively isolated species (Barluenga et al. 2006). A recent review of sympatric speciation (Bolnick and Fitzpatrick 2007) suggests that moving beyond traditional geographic analyses of distributional patterns to the evaluation of the underlying assumptions necessary for adaptive speciation might provide increased insight into the feasibility of processes such as sympatric speciation. The pharyngeal jaw of cichlids has long been implicated in the diversification of this incredibly species-rich and morphologically diverse group of fish (Fryer and Iles 1972; Liem 1973). The apparent repeated evolution of structural and mechanical adaptations for molluscivory in clearly divergent cichlid species lends support to the idea that polymorphic differences that initially arise in the trophic apparatus may play a critical role in further cichlid diversification.

One caveat of our study is that we do not know if the morphological differences examined have a substantial genetic basis. Bone, including the pharyngeal jaw of fish, is often thought to re-

model in response to stresses like the loading experienced during mollusc crushing (Woo et al. 1981; Wainwright et al. 1991). It is also known that molariform *H. minckleyi* raised in the laboratory exhibit less robust jaw structure (Trapani 2004). Because we examined wild-caught individuals, the extent that the enhanced bone volumes found in molluscivores are due to genetic differences versus responses to compressive forces experienced during crushing prey is unclear. Future studies that experimentally raise molluscivorous cichlids on softer diets and determine how much of the change in bone volume and mechanics is due to genetic as opposed to plastic responses would help resolve this issue. Yet, if the extensive similarities in molluscivorous cichlids documented here were due primarily to plastic responses, this would suggest an interesting mechanism for the rampant convergence found in cichlids. In fact, the LPJ may be an ideal component of cichlid trophic morphology to test the long-held notion that plasticity has been critical to the evolutionary success of cichlids (Liem and Osse 1975; Meyer 1987).

However, even in cases of putative convergence where lineages have diverged phenotypically for extended periods and subsequently acquired strikingly similar morphology, it is difficult to demonstrate that these adaptations are completely independently derived (Rui 2005). In the case of the LPJ divergence examined here, our incomplete sampling of the over 100 cichlid species in the Heroini and our generally sparse knowledge of their feeding habits complicates the ability to differentiate repeated losses versus recurring gains of trophic habits such as molluscivory (Hulsey 2006). Polarizing the evolution of these rapid losses and gains is also complex because reconstructing ancestral states in adaptively diverging lineages is generally difficult. Also, most extant clades have likely experienced a natural loss of evolutionary information through extinction. Even in cases of putative convergence where lineages have diverged phenotypically for extended periods and subsequently acquired strikingly similar morphologies, totally novel mechanisms may not be responsible for structural resemblances. Parallelism, when phenotypic similarity arises through the same developmental genetic mechanisms, may underlie the shared origin of many traits (Rui 2005). Even characters as phylogenetically disparate as limb loss in snakes and cetaceans may share some of the same genetic underpinnings (Thewissen et al. 2006). Nevertheless, the first step in differentiating whether there are similar mechanistic bases to the origins of traits is establishing phylogenetically independent contrasts of putatively convergent phenotypes. Once we can appreciate the phylogeny and dimensions of structural similarity, adaptations in cichlid fish may offer an ideal system to differentiate whether convergence or parallelism is more commonly responsible for the evolution of similar structures.

Regardless of the genetic basis, the regions experiencing the most stress in papilliforms were the regions in molariform

H. minckleyi that exhibited the most structural enhancement. These morphotypes have clearly diverged along a force production/resistance axis (Hulsey et al. 2005a). When examining convergence in durophagous organisms, one might predict to find some structural modifications in regions in which stresses tend to become concentrated. But, our comparative analysis suggests that an inordinate amount of the structural modifications to the pharyngeal jaw (~40%) found when comparing molluscivores to closely related nonmolluscivores across the Heroine phylogeny can be attributed to mechanical adaptation. These similarities are likely the result of the continuous exertion of compressive forces that are the primary types of forces the pharyngeal jaw should experience during crushing hard-shelled prey (Wainwright 1987).

Interestingly, functional and historical constraints may dictate much of the convergence we have uncovered. All cichlids are characterized by the insertion of the primary pharyngeal crushing muscles on the horns of the LPJ. Ancestrally, these muscles were inserted on the upper pharyngeal jaws (Stiassny and Jensen 1987). The concentration of stress between the horns and the midline of the jaw may reflect a byproduct of the relatively novel insertion of these muscles in this area. Another area in which stress was predicted to be concentrated based on the FEA is along the midline of the jaw. Along this midline, the ancestral gill arches that form the jaw meet and are sutured together (Stiassny and Jensen 1987). The extent of suturing along the midline has been reported previously as critical in adapting the jaws of cichlid fish to the resistance of compressive forces (Hulsey 2006). The papilliform FEA confirms that structural modifications along the sutured LPJ midline in numerous lineages is key to adapting the cichlid pharyngeal jaw to loads experienced during processing durable prey.

Examining structural divergence in a complex phenotype such as the cichlid LPJ within the framework of a mechanical model provided us with the ability to extensively evaluate the cause of repeated evolutionary similarity in this key structure. The coupling of this novel morphological analysis of LPJ structure with the mechanical analysis from the FEA highlights the power of using an explicit mechanical model of three-dimensional structure to understand how morphological divergence translates into the ability to exploit novel prey. As phylogenetic studies continue to delineate how often structural and ecological similarities have arisen independently, studies of adaptation will be greatly facilitated by an increase in the use of mechanically explicit models to test the advantages of three-dimensional structural novelty.

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