vol. 204, no. 4 the american naturalist october 2024

Freshwater Habitats Promote Rapid Rates of Phenotypic Evolution in Sculpin Fishes (Perciformes: Cottoidea)

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Submitted July 19, 2023; Accepted May 21, 2024; Electronically published August 19, 2024

Online enhancements: supplemental PDF.

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abstract: Invasions of freshwater habitats by marine fishes provide exceptional cases of habitat-driven biological diversification. Freshwater habitats make up less than 1% of aquatic habitats but contain ~50% of fish species. However, while the dominant group of freshwater fishes (Otophysi) is older than that of most marine fishes (Percomorphaceae), it is less morphologically diverse. Classically, scientists have invoked differences in the tempo and/or mode of evolution to explain such cases of unequal morphological diversification. We tested for evidence of these phenomena in the superfamily Cottoidea (sculpins), which contains substantial radiations of marine and freshwater fishes. We find that the morphology of freshwater sculpins evolves faster but under higher constraint than that of marine sculpins, causing widespread convergence in freshwater sculpins and more morphological disparity in marine sculpins. The endemic freshwater sculpins of Lake Baikal, Siberia, are exceptions that demonstrate elevated novelty akin to that of marine sculpins. Several tantalizing factors may explain these findings, such as differences in habitat stability and/or habitat connectivity between marine and freshwater systems.

Keywords: geometric morphometrics, macroevolution, micro–computed tomography (μCT), niche conservatism.

Introduction

Transitions to novel habitats have catalyzed a highlight reel of biological diversification (Simpson 1953; Schluter 2000; Stroud and Losos 2016). Bellflowers (subfamily Lobelioideae) radiating on the Hawaiian archipelago, cichlids (subfamily Pseudocrenilabrinae) in African rift lakes, and vanga birds (family Vangidae) on Madagascar are classic examples (Yamagishi et al. 2001; Seehausen 2006; Givnish et al. 2009). Freshwater invasions by marine fishes provide extreme examples of such habitat-driven diversification (Carrete Vega and Wiens 2012). Despite containing only 0.01% of the habitable aquatic habitats on Earth, freshwater ecosystems contain roughly the same number of species of fishes as marine systems (Dawson 2012). Most living freshwater fishes belong to a single evolutionary lineage (Otophysi), which evolved from a marine ancestor approximately 224 million years ago (Hughes et al. 2018; Cassemiro et al. 2023). However, many other lineages of fishes have also transitioned from marine to freshwater habitats, followed by varying degrees of species proliferation and morphological diversification (Bloom and Lovejoy 2017; Kolmann et al. 2020; Rabosky 2020; de Brito et al. 2022). On a global scale, a distinctive pattern has emerged between marine and freshwater fishes: despite the roughly equal species richness in each environment, marine taxa as a whole are more morphologically diverse, and many freshwater fishes seemingly converge on a subset of potential morphologies (Friedman et al. 2022). Unequal ages cannot explain this global pattern because Otophysi, the dominant group of freshwater fishes, is substantially older than Percomorphaceae, the dominant group of marine fishes (Carrete Vega and Wiens 2012; Hughes et al. 2018). Overall, freshwater habitats are rich in species but poor in morphological innovation compared with the oceans.

Unequal diversification between two groups may be explained by (1) differences in the rates of morphological evolution (i.e., differences in tempo) and/or (2) differences in the strengths of constraints on morphological evolution, which may reduce the potential for morphological innovation in one group (i.e., differences in mode; see Sidlauskas 2008). Here we evaluate these hypotheses in a diverse clade of fishes (sculpins) that spans both marine and freshwater habitats, and we test for the effect of habitat on the rate (tempo) and mode of morphological evolution. The ~300

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sculpin species in the superfamily Cottoidea primarily inhabit high latitudes across the Northern Hemisphere (Yabe 1985; Knope 2013; Smith and Busby 2014). Although Cottoidea contains five families, three dominate its diversity: Cottidae, Agonidae, and Psychrolutidae. Cottidae is an almost entirely freshwater group of ∼100 species that includes an adaptive radiation within Lake Baikal, Siberia (Goto et al. 2015). *Cottus* is the most speciose and broadly distributed cottid genus (∼70 species, Eurasian and North American distribution) and is infamous for the morphological similarity of its constituent species (Young et al. 2022). Psychrolutidae (∼210 species) is mostly composed of marine species, and Agonidae (∼50 species) is entirely marine (Smith and Busby 2014; Nelson et al. 2016). Most sculpins are small (<20 cm), cryptic, benthic species that feed primarily on benthic invertebrates (Buser et al. 2019). However, each major family contains many exceptional taxa, including piscivores, pelagic and/or semipelagic species, and large-bodied species. Each of these atypical sculpin types has analogs in both freshwater and saltwater. In fact, freshwater and marine sculpins overlap quite substantially in trophic ecology (see Buser et al. 2019, figs. 4 and 5 and the discussion section). Consequently, Cottoidea provides a fascinating group in which to test for unequal diversification between freshwater and saltwater taxa: it contains numerous species in both environments, with equivalent trophic diversity, and anecdotal evidence of high morphological similarity among freshwater species. Do sculpins follow global trends in fish morphology and show higher morphological diversity in the marine versus freshwater portions of the radiation? If so, do unequal rates of evolution or, alternatively, unequal levels of evolutionary constraint best explain the disparity? To answer these questions confidently, we analyzed a structure that contains an abundance of ecologically and evolutionarily informative morphological information: the skull.

The skull is a dynamic and multifunctional structure that has been one of the focal points of vertebrate morphology and evolutionary biology since the founding of the field nearly two centuries ago (Darwin 1859). The teleost fish skull is particularly interesting because of its overwhelming complexity and the number of simultaneous functions that it has evolved to perform, including, but not limited to, feeding, respiration, communication, defense, prey detection, orientation, and even locomotion (Webb 1984; Fish 1987; Westneat 1994; Westneat 2004; Price et al. 2010; Collar et al. 2014; Fine and Parmentier 2015; Konow et al. 2017; Boyle and Herrel 2018; Evans et al. 2019*a*, 2019*c*, 2021, 2023; Hu et al. 2022; Larouche et al. 2022; Knapp et al. 2023). In addition to the vertebrate skull's multiple functions, studies have found strong relationships between the shape of the skull and ecological factors like diet and habitat (Dumont et al. 2016; Foth et al. 2017). Among fishes, habitat exerts strong selective pressure on the skull shapes of clades as disparate as snailfishes (Gerringer et al. 2021), eels (Kaifu et al. 2013), sticklebacks (Garduno-Paz et al. 2010), minnows (Akin and Geheber 2020), cichlids (Gilbert et al. 2020), and sculpins (Kerfoot and Schaefer 2006). The pattern even holds for marine fishes generally (Friedman et al. 2020), with differences in depth, flow regime, and foraging habitat typically assuming greatest importance. Marine and freshwater habitats have also been shown to drive differences in skull shape and foraging ecologies. For example, marine stickleback populations have larger gapes, more protrusible jaws, and faster ram speed and cranial rotation than closely related freshwater populations (Higham et al. 2017).

The very complexity that makes the skull so rich in ecological, functional, and evolutionarily informative characters has restricted its consideration in the world of modern quantitative approaches to comparative anatomy. For example, many morphometric studies rely on one-dimensional or two-dimensional abstractions of shape, but this can limit and/or bias the characterization of the head, which in many fishes is highly three dimensional (3D; see Buser et al. 2018). An avenue to study the head more fully and accurately is provided by 3D geometric morphometrics, but the complex arrangement of mobile elements in the skull of fishes introduces a great deal of potential sources of nontarget variation that must be taken into account. For example, in fishes the upper jaw is generally attached to the neurocranium only by soft tissue and is free to project away from the head and rotate relative to the neurocranium (Hulsey et al. 2005; Westneat 2005; Holzman et al. 2008; Bellwood et al. 2015). Standardizing the posture of the bones of the skull is therefore prerequisite to studying the shape of the head using geometric morphometrics, and this has been a bottleneck to the broad application of this technique in fishes. Consequently, previous studies of evolutionary habitat transitions in fishes have been restricted to aspects of gross morphology, such as body length (de Brito et al. 2022) or linear morphometric abstractions of body shape (Buser et al. 2019; Friedman et al. 2022), leaving a great wealth of potentially informative variation unconsidered. For example, the elements that make up the floor of the mouth (hyoid apparatus) and the "gill plate" (opercular series) of fish heads drive feeding strikes and respiration but are not captured by body shape or other gross aspects of morphology (Farina et al. 2019).

In this study, we compare the rates and patterns of morphological diversification in the freshwater and marine portions of the sculpin radiation. We used 3D geometric morphometrics techniques to capture the shape of the skull, then quantified and compared variation across the superfamily. We accounted for the inherent variation in posture of the elements of the highly mobile teleostean fish skull by imposing a standard position of each bony element prior to shape analysis of the overall skull. We calculated rates of morphological evolution and fit evolutionary models for freshwater and marine sculpins to test whether relatively low rates of morphological evolution (i.e., differences in tempo) or constrained morphological evolution (i.e., differences in mode) better explain any differences in shape variation between these groups. We also compared patterns of diversity through time, lineage density, and evolutionary allometry among and within the three major families that constitute Cottoidea: the predominantly freshwater family Cottidae and the two primarily marine families Agonidae and Psychrolutidae. Combined, the results illuminate whether a detailed, fine-scale history of diversification in a prominent clade of freshwater and marine fishes mirrors suspected macroevolutionary trends at the scale of all fishes.

Methods

Taxon Sampling and Phylogenetic Hypothesis

We selected taxa to represent all major lineages of the superfamily Cottoidea, including the three major constituent families, Agonidae, Cottidae, and Psychrolutidae, and the minor families Jordaniidae and Rhamphocottidae (sensu Smith and Busby 2014). Species were selected to represent as many major lineages within each cottoid family as possible given the constraints imposed by the availability of museum specimens and species represented in available phylogenetic hypotheses (supplementary table 1 in Buser 2024*c*). We included representatives of all major subfamilies in each family and, in the case of the Cottidae, diverse representatives of the Lake Baikal radiation and all major lineages within the genus *Cottus* (see Goto et al. 2015). We gathered ecosystem data for each species from Buser et al. (2019) as well as from Eschmeyer et al. (1983) through the FishBase information system via the R package rFishBase (ver. 4.1.2; Boettiger et al. 2012; Froese and Pauly 2023; see the supplementary R script in Buser 2024*a*). We used a subtree of the phylogenetic hypothesis of fishes published in Rabosky (2020) to represent the phylogenetic relationships of cottoid species. We used the taxonomic framework described in Smith and Busby (2014) because it is the most consistent with the phylogenetic relationships of the cottoids represented in the Rabosky (2020) phylogenetic hypothesis and is the classification scheme used by cottoid taxonomists and systematists (e.g., Buser and Lopez 2015; Orr et al. 2019; Love et al. 2021; Young et al. 2022). We pruned the subtree to include only species from Cottoidea for which adequate museum specimens were available using functions from the R package phytools (ver. 1.2-0; Revell 2012; fig. 1). The resulting taxonomic sample included 105 species of cottoids (approximately one-third of total species in Cottoidea) and the out-group

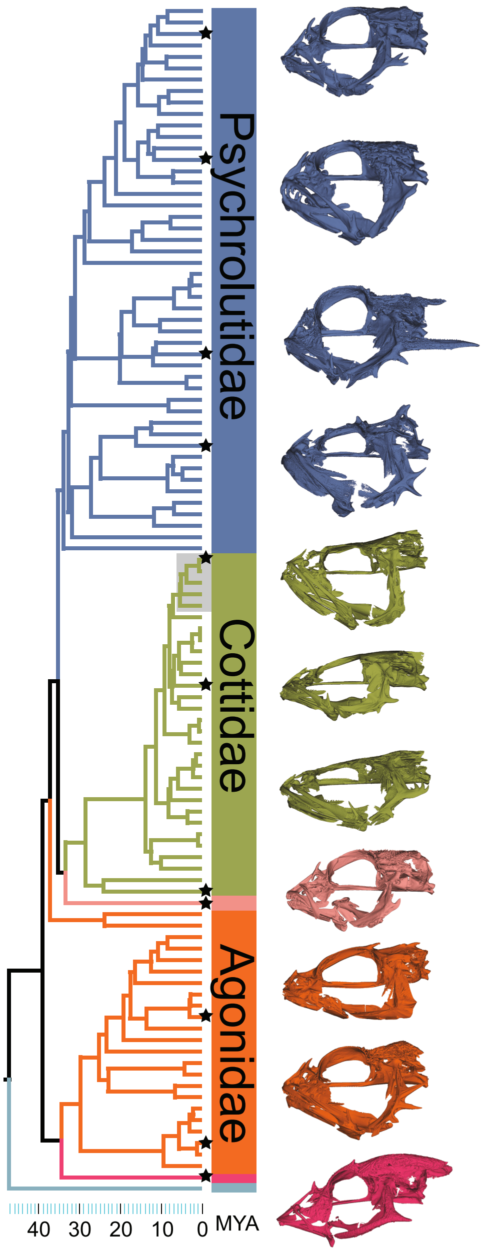

Figure 1: Phylogenetic hypothesis of sculpins with representative skulls color coded by family. The Baikal sculpin clade within the family Cottidae is shaded with a gray background. Time is indicated in millions of years ago (MYA). Each illustrated skull represents a tip in the phylogeny, the latter indicated with a black star. From top to bottom, the illustrated skulls and corresponding starred tips in the phylogeny are as follows: *Oligocottus rubellio* (OS Uncat), *Artedius harringtoni* (OS 4533), *Enophrys bison* (OS 5915), *Dasycottus setiger* (CUMV 97976), *Cottocomephorus grewingkii* (OS 4244), *Cottus cognatus* (CUMV 78131), *Leptocottus armatus* (OS Uncat), *Jordania zonope* (CUMV 97998), *Xeneretmus latifrons* (UW 155979), *Hemilepidotus hemilepidotus* (CUMV 98219), and *Rhamphocottus richardsonii* (UW 16400).

taxon *Trichodon trichodon*. All tree manipulations were performed in the R statistical environment, and an annotated R script that contains all analyses and pertinent data is included in Buser (2024*a*).

Anatomical Data

To quantify patterns of morphological diversity across sculpins, we studied the skull and jaws using 3D geometric morphometrics. For each species in our dataset, we constructed digital models of the bones of the cranium using micro–computed tomographic (μCT) data and represented the morphology of the cranium using 3D coordinate positions of anatomical landmarks (see below).

CT Data. We gathered μCT scan data from museum specimens representing all 105 species of cottoids in our phylogenetic hypothesis ($n = 1$–6 individuals per species; median: 1). We conducted all μCT scanning and reconstruction at the Karl F. Liem Bioimaging Center at Friday Harbor Laboratories (Friday Harbor, WA) using an 1173 Bruker Skyscan μCT system. Scanning parameters ranged from 60 to 75 kV and from 100 to 133 μA, and resolution ranged from 18.1 to 54.7 μm (voxel size). We used a 1-mm aluminum filter on all scans. We reconstructed the raw image stacks using NRecon (Bruker microCT, Kontich, Belgium, 2016). We supplemented this dataset with previously published CT reconstructions of cottoid species (Buser et al. 2018, 2019, 2023; Farina et al. 2019; Blackburn et al. 2024) downloaded from https://www.morphosource.org, for a total sample size of 215 individuals representing 105 species (supplementary table 1 in Buser 2024*c*).

Landmark Scheme. We developed a landmark scheme for capturing the shape of the skull of fishes broadly, as well as that of the highly modified preopercular bone found in sculpins (supplementary figs. 1–11, supplementary table 2 in Buser 2024*c*). The preopercle in sculpins possesses enlarged spines, which are associated with defense, intraspecific competition, and habitat (Buser et al. 2023). We modified previously published landmark schemes for the neurocranium and preopercle (Evans et al. 2019*b*; Buser et al. 2023) and also identified landmarks on other important bones of the skull, including those of the oral jaws and hyoid apparatus (Anderson et al. 2013; Buser et al. 2019). The landmarks thus represent many important elements in the functional morphology of the skull (e.g., oral jaw mechanics), the sensory anatomy (e.g., eye size), and other evolutionarily meaningful aspects of variation. The landmarks used in this study can translate to other percomorph fishes and could serve as a template for broad comparisons of skull shape across ray-finned fishes. Supplementary table 1 in Buser (2024*c*) provides an anatomical description of each landmark, and supplementary figures 1–11 in Buser (2024*c*) indicate where each landmark lies on an example skull. For all bilaterally symmetric structures, we placed landmarks on the left and right sides of each specimen. In total, our landmark scheme included 156 anatomical landmarks as well as 271 semilandmarks distributed across 31 anatomical curves. Semilandmark positions carry no assumption of anatomical homology across specimens, but the curves they define are presumably homologous (Zelditch et al. 2012). We used the program 3D Slicer (ver. 5.6.2) with the SlicerMorph extension (Fedorov et al. 2012; Rolfe et al. 2021) to manipulate the CT data and record the position of each of the anatomical landmarks, following the workflow described in Buser et al. (2020). We included a Slicer file in Buser (2024*b*) that shows all landmarks and semilandmarks used in this study on an example skull. The file includes color-coded partitioning of each pertinent skeletal element in the skull and complements supplementary figures 2–11 in Buser (2024*c*). Each legend for supplementary figures 2–11 describes how to reproduce the views in each figure using the Slicer file.

Analysis of Shape

Local Superimposition. All remaining analyses were performed in the R statistical environment (ver. 4.2.3) using the R Studio interface (ver. 2023.12.1+402), with functions from previous studies and functions from the packages abind (ver. 1.4-5), geiger (ver. 2.0.10), geomorph (ver. 4.0.5), Morpho (ver. 2.11), and motmot (ver. 2.1.3; Harmon et al. 2008; Adams and Otárola-Castillo 2013; Pennell et al. 2014; Plate and Heiberger 2016; Schlager 2017; Buser et al. 2018, 2019; R Studio Team 2018; Puttick et al. 2019; R Core Team 2019). For specific steps and functions used in our analyses, see the supplementary R script in Buser (2024*a*).

Preliminary results showed that the Lake Baikal sculpin lineage, which is nested within the freshwater sculpin family Cottidae, diverged substantially not only from the mean of the dataset but also from the mean shape of other members of its own family. A strong outlier such as this has the potential to impact our results in significant ways, and we therefore created two permutations of the raw coordinate dataset: one with the Baikal taxa and one without the Baikal taxa. We performed all of the following analyses on each dataset and assessed the effects of including the Baikal taxa on our results.

We quantified and analyzed the variation in skull shape using standard 3D geometric morphometrics techniques, including general Procrustes analysis to remove nonshape variation from our dataset and principal component analysis (PCA) to visualize the variance in shape (Zelditch et al. 2012). We used only the symmetric component of shape to represent each of the symmetric structures, which we extracted using the bilat.symmery function in geomorph (Mardia et al. 2000; Klingenberg et al. 2002).

We used the local superimposition approach as described by Rhoda et al. (2021*a*, 2021*b*) as a means to control for some of the variation that may result from postural differences and/or preservation artifacts. In brief, the full landmark configuration is separated into subsets corresponding to mobile elements (i.e., individual articulated cranial bones). Each of these subsets of landmarks is then individually superimposed to its corresponding landmarks in a template specimen, which in this case was the mean landmark configuration following a generalized Procrustes superimposition on the entire dataset (i.e., all specimens and all landmarks). This method has been optimized to handle the articulated complexity of teleost fish skulls (Larouche et al. 2022).

Phylogenetic Comparative Methods. We visualized the major axes of skull shape variation identified by the PCA by first warping a model of the skull of *Artedius harringtoni* (OS 5360) to take on the average shape of all skulls in our dataset (which is equivalent to the shape represented by a PC score of zero on each PC axis) and then warped it from there to show the shape represented by the extremes of each PC axis. To visualize trends in morphological variation, we used the phylomorphospace approach described by Sidlauskas (2008). That method plots each species within a morphospace defined by pairwise combinations of PC axes and overlays the phylogenetic relationships of the taxa, including the inferred morphology of the ancestors. We tested for patterns in the distribution of taxa in the shape space using a phylogenetic multivariate analysis of variance (phyMANOVA) on the shape variables, with environment (i.e., fresh vs. salt; shape ~ environment) or taxonomic family (shape ~ family) as the grouping variable. We had no reason a priori to believe that the average shape of freshwater taxa would differ from that of saltwater taxa, nor that the average shape of each family would differ from one another per se, as our hypothesis framework centers on shape disparity (see below), not average shape. Rather, we used the phyMANOVA to complement our visual assessment of the phylomorphospace.

Tempo and Mode of Shape Evolution

This study asks whether marine and freshwater sculpins differ in the rate (tempo) or model (mode) of shape evolution. To answer that question, we first tested for differences in morphological variation between freshwater and marine sculpins. Then we tested whether the mechanisms we hypothesized could explain such a phenomenon (i.e., differences in tempo or mode).

We compared levels of morphological variation between freshwater and marine sculpins by calculating mean disparities in skull shape. The cottid genus *Cottus* dominates freshwater sculpin diversity (Goto et al. 2015). Based on our earlier anatomical investigations and the well-known morphological similarity of species in the genus *Cottus* (Young et al. 2022), we hypothesized that skulls of the freshwater sculpins would exhibit lower mean disparity than skulls of marine sculpins. We used the morphol.disparity function in the R package geomorph to calculate the mean disparity of each group and test for statistically significant differences. Given the significant result from that test (see below), we also tested whether both major marine clades differed from the freshwater Cottidae in disparity by repeating this analysis with taxonomic family as the grouping variable. We also calculated and visualized how morphological disparity likely varied through time for each of the three major taxonomic families using the dtt function from the package geiger and compared that to a null distribution of 1,000 simulations (see Harmon et al. 2003). We did not construct a separate "marine" diversity-through-time plot because this analysis is designed to take whole clades as input, and freshwater sculpins are nested inside of marine ones. We tested for differences in tempo and mode of evolution as described in the following.

Hypothesis 1: Differing Rates (Tempo) of Morphological Evolution. Testing for differing rates of morphological evolution was a multistep process. First, we found the best-supported model of trait evolution for our skull shape dataset. Next, we tested for differences in the rate parameter of this model in freshwater versus marine sculpins. Finally, we estimated branch-specific rates of evolution across the sculpin phylogeny.

We determined the best-fitting model of morphological evolution by fitting four different models to the first 27 PC axes of our trait data, which were the maximum number of trait variables that were feasible to analyze given finite computational resources and together represent ~92% of the total variance of the dataset. The first model was a single-rate Brownian motion model that assumes a single global rate of trait evolution across the entire tree. The second model that was evaluated was a variable-rate Brownian motion model that allows rates of trait evolution to vary between freshwater and marine sculpins. The third model that was evaluated was a single-rate, single-peak Ornstein-Uhlenbeck process that models stabilizing selection around an adaptive optimum. This process has been shown to be particularly relevant when studying functional traits like the skull and jaws (Larouche et al. 2022). Previous studies have shown that freshwater fishes typically evolve along more morphologically constrained evolutionary trajectories (Friedman et al. 2022). It is therefore possible that the freshwater sculpins are evolving under a different morphological optimum than are the marine species. We evaluated this hypothesis with our fourth model: a two-peak Ornstein-Uhlenbeck model where freshwater and marine sculpins were each assigned their own trait optimum. All model fitting analyses were performed in BayesTraits (ver. 4; Pagel et al. 2004; Venditti et al. 2011), and model fit was assessed by comparing Bayes factors calculated from log-transformed marginal likelihoods.

Preliminary results showed that a variable-rate Brownian motion model of evolution was the best fit to our data, so we compared the best-supported rates of morphological evolution for freshwater versus marine sculpins using the compare.evol.rates function from geomorph and assessed significance by specifying the simulation method within that function, which then compares our empirical results with a null distribution obtained by simulating the landmark data under a Brownian motion model with a single evolutionary rate across the phylogeny (Adams and Collyer 2018). We further specified 1,000 iterations of this procedure to estimate significance.

To determine which lineages were driving the differences in rates among groups, we estimated branch-specific rates of skull shape evolution using a multirate Brownian motion model (variable-rate model) implemented in BayesTraits (Venditti et al. 2011). This program implements a reversible-jump Markov chain Monte Carlo approach to estimate branch-specific rate shift probabilities across a phylogeny. To reduce computational time, we limited the dimensionality of our data and used only the first 27 PC axes, as in our model-fitting analysis above. Although the PC axes are mathematically independent, the traits they represent may be evolutionarily correlated (see Revell and Collar 2009; Adams and Collyer 2018). To account for potential evolutionary correlations among our trait axes, we used the TestCorrel function, which constrains the correlation between separate trait axes to zero. We ran our BayesTraits analysis using uniform, noninformative priors and ran two independent chains each for 200,000,000 generations, discarding the first 60,000,000 as burn-in. Each chain was sampled every 1,400,000 generations after burn-in using a stepping-stone sampling method. Model convergence was evaluated by confirming that both chains reached stationarity at the same marginal likelihoods using Tracer (ver. 1.7.2; Rambaut et al. 2018). Such a variable-rate analysis outputs a set of phylogenies wherein each branch has been scaled by its Brownian motion rate of evolution.

Hypothesis 2: Differing Levels of Constraint (Mode) of Morphological Innovation. We tested whether the freshwater Cottidae experienced more morphological constraint than did the major marine sculpin families using the lineage density analysis described in Sidlauskas (2008). "Lineage density" is a representation of the efficiency with which a clade expanded throughout a region of morphospace. It is calculated by comparing the total magnitude of morphological change within a clade to its diversity of morphologies. Clades with low lineage density diversify with minimal convergence and tend toward morphological innovation. Clades with high lineage density frequently retrace evolutionary pathways and tend toward morphological conservatism. Such groups may have high per-lineage rates of evolutionary change but tend to oscillate around a morphological optimum or within a restricted region of morphospace. Importantly, lineage density is typically conceived as a property of an entire clade or subclade and is not clearly defined for a paraphyletic group. Given that freshwater sculpins nest within the radiation of marine ones, we calculated separate estimates of lineage density for Psychrolutidae and Agonidae and did not estimate lineage density for marine sculpins as a whole.

We calculated the lineage density of each family by measuring the morphological branch length (i.e., the sum of the Euclidean distance between each pair of nodes in the clade across all pertinent axes of the morphospace) and that clade's occupied ellipsoid volume of morphospace following Sidlauskas (2008). Because volumes can easily collapse to zero when subclades differ minimally on any morphospace axes, we also used the alternative formulation of lineage density, which treats volume as the sum (rather than the product) of ranges (Sidlauskas 2008). We tested for statistical differences in our lineage density results by comparing the observed results to results simulated from a null model. Under the null model, we simulated the morphological evolution of the entire superfamily under a single Brownian motion model of evolution using the methods described in Sidlauskas (2008). We parameterized the Brownian motion model with our observed data using functions from the package geiger. We repeated this process 1,000 times and compared the results with those obtained from the observed data. We checked for the presence of morphological outliers, which may heavily influence volumetric measures, using the plot.outliers function from geomorph. See the supplementary R script in Buser (2024*a*) for further details on this analysis.

Evolutionary Allometry

While the body size of sculpins is generally small, freshwater sculpins as a whole are less variable in size than their marine counterparts (de Brito et al. 2022). Body size often covaries with shape on both intraspecific (i.e., static allometry) and interspecific (i.e., evolutionary allometry) levels (Gould 1966; Klingenberg and Zimmermann 1992; Lande 1979). If freshwater and marine sculpins differ in how shape varies with size across species (i.e., interspecific allometric trajectories), it could help to explain any differences in shape evolution between the two groups (for a review, see Sherratt et al. 2022). We therefore also considered the potential effects of size variation on morphological evolution in cottoids by testing for differences in interspecific allometric trajectories among the major cottoid families. To derive coefficients for evolutionary allometry and assess tribe-specific evolutionary allometries, we employed phylogenetic analysis of covariance, applying two models. The first model encompassed two principal effects: the factor "family" and the covariate "size," measured as the natural logarithm–transformed maximum body sizes of the species available in FishBase (Eschmeyer et al. 1983; Froese and Pauly 2023). Similarly, the second model has the factor "family," but the covariate "size" was measured as the natural logarithm-transformed centroid size. For both models, we expect interaction between factors. Considering the possible confounding nature of the phylogeny, we employed type II sums of squares for statistical tests of model terms (Adams and Collyer 2018). We applied the methodological approach proposed by Zelditch and Swiderski (2023): given the species nonindependence, we reduced the ordinary least squares residuals through PCA up to the first 12 PCs. Early attempts revealed that 12 PC axes was the maximum number of variables that could be considered by this method, given finite computational resources. Subsequently, we estimated Pagel's λ for both multivariate datasets, using the transformPhylo.ML function in motmot. Finally, we transformed the phylogeny by the λ value using the rescale function in geiger. Tests of statistical significance were conducted through permutations of the residuals from the reduced model. The statistical significance of the angle was determined by comparing it to the distribution of random values. The phylogenetic ANCOVA was fitted using the procD.pgls function in geomorph, and pairwise comparisons between evolutionary allometries were conducted using the pairwise function in RRPP. Beyond assessing the null hypothesis that the angles differ no more than expected by chance, we also examined the hypothesis that they are no more similar than anticipated by chance. Allometric axes were then plotted on the first PC, along with scores relative to the size axis, using the plotAllometry function in geomorph with the method set to Predline.

Results

Skull Shape Variation

The primary axis (PC1) of skull variation in Cottoidea primarily corresponds to elongation, ranging from relatively short (anteroposteriorly contracted) skulls to relatively long (anteroposteriorly elongated), narrow (laterally compressed) skulls. The length of the uppermost preopercular spine is associated with the elongation of the skull, such that an elongate skull associates with an elongate spine and short skulls associate with short spines. The secondary axis (PC2) of skull variation pertains to the width of the skull and the orientation of the orbits, such that wide (laterally expanded) skulls have dorsally oriented orbits while narrow (laterally compressed) skulls have laterally oriented orbits. The curvature of the uppermost preopercular spine covaries, such that laterally expanded skulls tend to have highly curved spines while laterally compressed skulls usually have straight spines. The shape variation captured by these and all remaining PC axes can be visualized using the supplementary R script in Buser (2024*a*) and associated data.

Freshwater sculpins occupy a narrow subset of the morphospace occupied by marine sculpins, and the mean shapes of the two groups are not statistically significantly different (see fig. 2; supplementary fig. 12 in Buser 2024*c*). Freshwater sculpins mostly have wide, dorsoventrally compressed skulls with short preopercular spines, while marine sculpins exhibit this morphology along with many others. The distributions of the three major families overlap in the morphospace of the first two PC axes at the centroid of overall distribution (fig. 2; supplementary fig. 12 in Buser 2024*c*). This overlap is substantial enough that there is no statistically significant difference in the average shapes of the taxonomic families, akin to the trend observed in freshwater versus marine sculpins as a whole. However, each family occupies distinct areas of morphospace through one or more lineages. The *Enophrys* lineage within the marine family Psychrolutidae is the sole occupant of an area of morphospace characterized by long, laterally compressed skulls with highly elongate, straight uppermost preopercular spines. The *Hemitripterus* lineage within the marine family Agonidae is the only occupant of a region of morphospace characterized by short, laterally compressed skulls, with short, straight uppermost preopercular spines. The *Comephorus* lineage within the freshwater family Cottidae is alone in an area of morphospace characterized by short, laterally expanded skulls with short, curved uppermost preopercular spines. *Comephorus* is a component of the Lake Baikal radiation within Cottidae, and outside of the Lake Baikal sculpins the remainder of Cottidae shows a restricted distribution in morphospace. The pattern of their distribution shows substantial crossing of evolutionary trajectories and overlap of the subclades within Cottidae (supplementary fig. 13 in Buser 2024*c*). This pattern contrasts with the other families, whose constituents are spread evenly in morphospace. Together, these results support our hypotheses that marine lineages would explore novel areas of morphospace and that the freshwater lineage would be more restricted. However, the Baikal sculpins explored morphologies unique among sculpins and substantially expanded the morphospace occupied by Cottidae. Indeed, *Comephorus* was the only taxon flagged in our outlier analysis (see the supplementary R script in Buser 2024*a*).

The results of the morphological disparity analysis reinforce the pattern of reduced morphological variation in the non-Baikal cottids relative to the primarily marine families. Freshwater sculpins as a whole have lower mean disparity than marine sculpins, and the difference is statistically significant ($P < .001$). Cottidae shows the lowest mean disparity of the three major families, but only when the Baikal taxa are removed does their difference from the mean disparity of Psychrolutidae and Agonidae become statistically significant (see supplementary table 4 in Buser 2024*c*). This shows that the freshwater taxa do indeed exhibit reduced morphological variation relative to the marine taxa but that the diversity found within one freshwater subclade (i.e., the Baikal sculpins) is substantial enough to complicate what would otherwise be a simple story. Likewise, the morphological disparity of the marine families Agonidae and Psycholutidae is higher than that predicted by a null model throughout their history, while that of the freshwater Cottidae are consistent with the null model until only recently, and this pattern is found in both the Baikal-inclusive and the Baikal-exclusive datasets (supplementary fig. 14 in Buser 2024*c*).

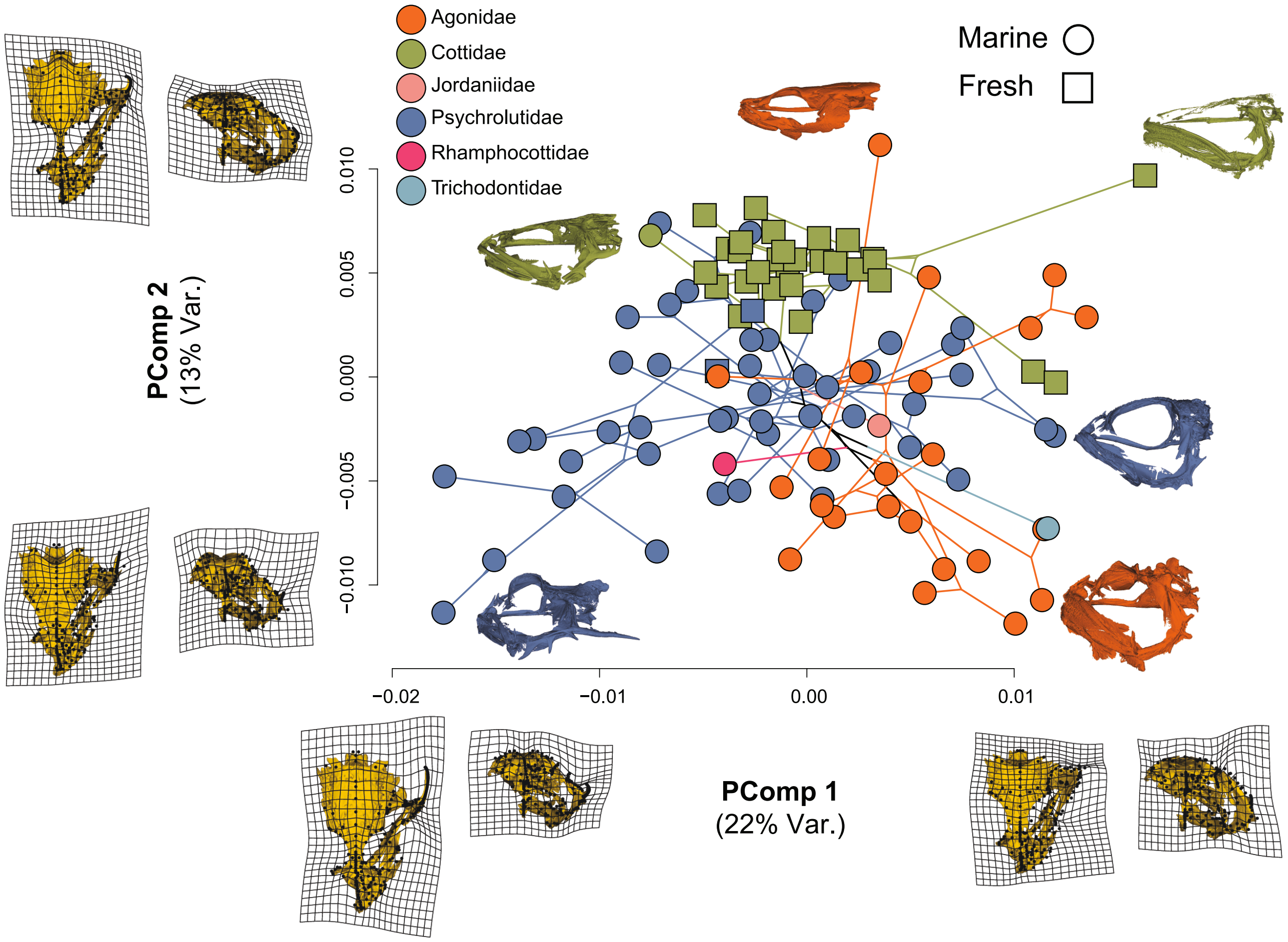

Figure 2: Phylomorphospace of PC1 and PC2 of the skull of Cottoidea. The proportion of total variation captured by each principal component axis is indicated in parentheses. Filled circles and squares represent the average shape of a species, and lines represent the phylogenetic relationships of taxa. Circles indicate species that live predominantly in the marine environment, and squares indicate species that live predominantly in the freshwater environment. Each circle, square, and line are colored according to the taxonomic family as in figure 1. The extremes of the morphospace are represented with specimens from each of the three major families, color coded according to family (clockwise, from top): *Anoplagonus inermis* (UW Uncat), *Comephorus baikalensis* (USNM 362046), *Triglops nybelini* (UW 40354), *Hemitripterus bolini* (UW 42659), *Enophrys diceraus* (OS 113), and *Leptocottus armatus* (OS Uncat).

Testing for Differences in Tempo and Mode of Shape Evolution

Hypothesis 1: Differing Rates of Morphological Evolution. A variable rate Brownian motion model best explains the morphological evolution of skull shapes in Cottoidea (supplementary table 3 in Buser 2024*c*). Results are similar for both the Baikal-inclusive and the Baikal-exclusive permutations of the dataset, indicating that the Baikal taxa neither exert an outsized influence on the results nor deviate substantially from the typical evolutionary rates of Cottidae. While freshwater and marine sculpins differ significantly in their rate of morphological evolution ($P = .001$), the results differ from the prediction of slower morphological evolution in the freshwater taxa. Rather, the skulls of freshwater sculpins evolve faster (supplementary table 4 in Buser 2024*c*). Within the dominant group of freshwater sculpins (i.e., Cottidae), the highest rates of skull shape evolution appear throughout the Baikal clade and, outside that, near the tips of the phylogeny (fig. 3). This pattern indicates accelerated morphological diversification in the Pleistocene or late Pliocene for the non-Baikal cottids and implies an initial period of relative evolutionary stasis followed by a recent accelerated morphological diversification in Cottidae outside of Baikal. Conversely, the primarily marine Agonidae and Psychrolutidae show consistent moderate and slow rates, respectively, of morphological evolution through time. This combination of relatively low morphological diversity but high rates of morphological evolution in non-Baikal freshwater sculpins contextualizes the overlap of evolutionary trajectories seen in the morphospace and further supports the idea that most freshwater sculpins are constrained to vary within a restricted area of morphospace.

Hypothesis 2: Differing Levels of Constraint of Morphological Innovation

When the Lake Baikal cottoids are included in the analysis, the three major sculpin families do not differ significantly in lineage density ($P > .17$). However, when the Baikal cottoids are excluded, the freshwater sculpin family Cottidae shows the highest lineage density of any of the families, and this finding is statistically significant for both measures of lineage density ($P < .01$). This result indicates that the non-Baikal freshwater sculpins are indeed primarily retreading the same area of morphospace, while the marine families (and Baikal) are under no such constraint (supplementary table 4 in Buser 2024*c*). The Lake Baikal cottids evolved in a pattern more similar to that observed in marine lineages rather than the other freshwater lineages, but, as noted above, the Baikal sculpins are generally evolving at a faster rate than are the marine groups.

Evolutionary Allometry

There were no significant effects of size (maximum body size or centroid size) or differences among families under evolutionary allometry trajectories, indicating that the distinct patterns of morphological disparity between freshwater and marine sculpins noted above are not due to differences in allometric trajectories in the constituent taxa (supplementary tables 5 and 6, supplementary fig. 15 in Buser 2024*c*).

Discussion

Tempo and Mode of Evolution in Cottoidea

The morphological evolution of sculpins played out very differently in freshwater and marine environments. Marine sculpins diversified their morphologies slowly and steadily, while freshwater sculpins evolved under a faster rate but did not innovate morphologically outside of Lake Baikal. Rather, constraints appear to have caused most freshwater sculpins to continuously retread the same areas of morphospace. Those constraints have led to a remarkable and unexpected inverse relationship between evolutionary rate and disparity in the morphological diversity of these fishes. While we have identified the mechanisms that underlie this pattern in sculpins, the question remains: what drives the differences in tempo and mode of evolution between these freshwater and marine radiations? We propose that ecological opportunity and habitat connectivity have tuned the tempo and mode of morphological evolution in sculpins and present these factors as a model with which to explore the evolution of freshwater versus marine taxa broadly.

Evolution in Freshwater: Niche Conservatism and (Limited) Ecological Opportunity

Overall, there appears to be fewer realized skull shapes for freshwater sculpins. Freshwater sculpin lineages appear tethered to a limited area of morphospace and rarely escape the apparent pull of this region, despite high rates of morphological evolution. This conservatism mirrors patterns observed in diet and functional feeding morphology in cottoids, which likewise show high levels of constraint (Buser et al. 2019).

There is, of course, an exception to this generality, namely, the radiation of freshwater sculpins in Lake Baikal. The Baikal cottoids have been described as an adaptive radiation by many researchers (Sideleva 2003; Goto et al. 2015; St. John et al. 2022), and our results support this conclusion. Rather than oscillating within a restricted area of morphospace, the Baikal cottoids evolved diverse morphologies, including the truly bizarre morphology of the Baikal oilfishes (genus *Comephorus*), which is unique not only

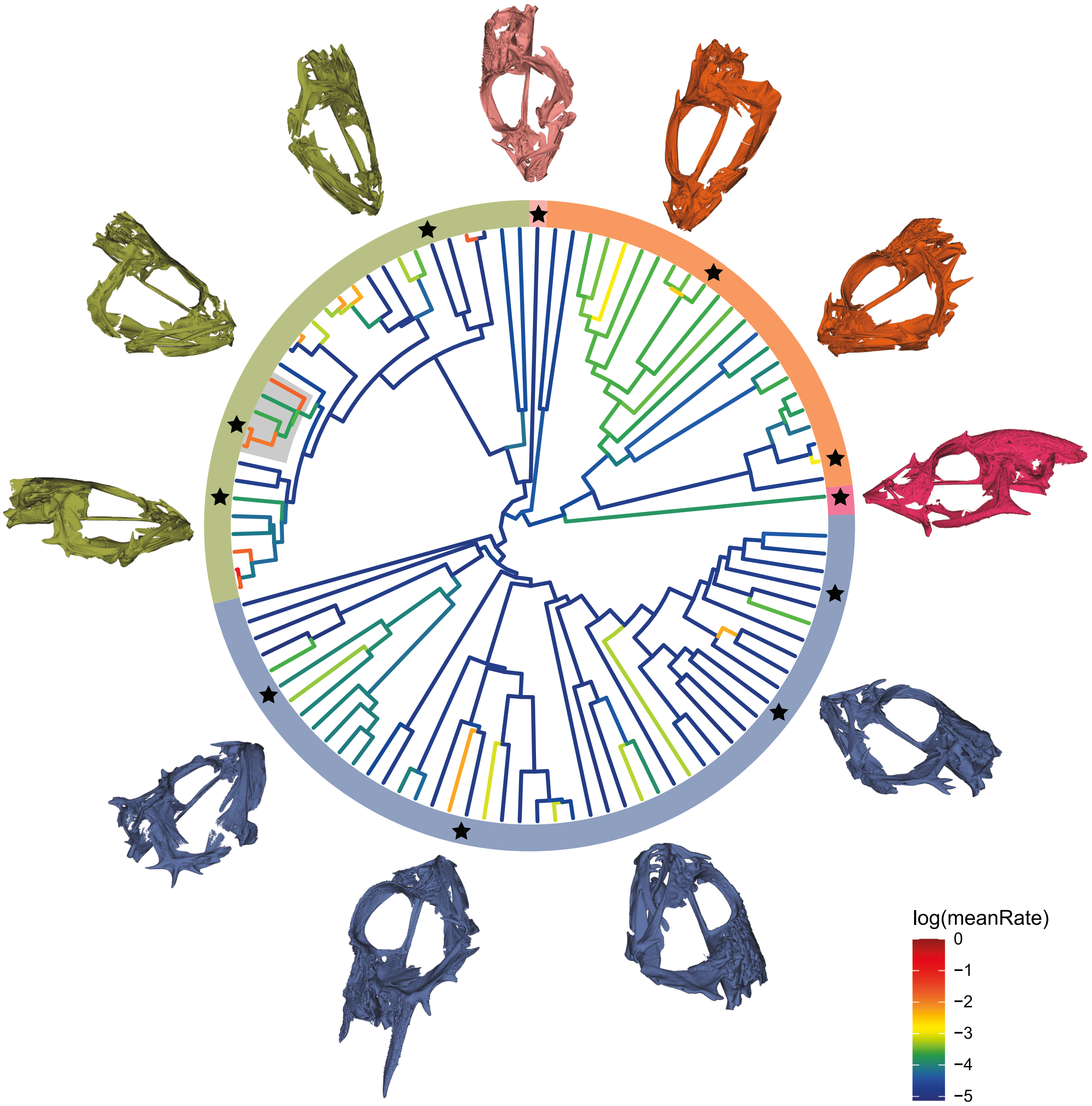

Figure 3: Rates of morphological evolution in the superfamily Cottoidea. Branch lengths are colored by the log of mean rate of morphological evolution. Taxonomic family is color coded around the circumference of the phylogeny, using the same color scheme as in figures 1 and 2. The Baikal sculpin clade within the family Cottidae is shaded with a gray background, as in figure 1. Representative taxa from each family are illustrated and indicated with a black star, as in figure 1. Clockwise from top, the illustrated skulls are as follows: *Jordania zonope* (CUMV 97998), *Xeneretmus latifrons* (UW 155979), *Hemilepidotus hemilepidotus* (CUMV 98219), *Rhamphocottus richardsonii* (UW 16400), *Oligocottus rubellio* (OS Uncat), *Artedius harringtoni* (OS 4533), *Enophrys bison* (OS 5915), *Dasycottus setiger* (CUMV 97976), *Cottus cognatus* (CUMV 78131), *Cottocomephorus grewingkii* (OS 4244), and *Leptocottus armatus* (OS Uncat).

among cottids but also among the entire superfamily Cottoidea (fig. 3). The Baikal sculpins have evolved in a manner more consistent with the marine families, but faster. Lake Baikal is the world's oldest and deepest freshwater lake and hosts truly outlandish habitats to which sculpins have adapted, including hydrothermal vents, cold seeps, and bathypelagic depths (Sideleva 2003). The ecological opportunity presented by the unoccupied niches of Lake Baikal appears to have effectively removed the morphological constraints imposed by the preferred cottid niche.

We conclude that freshwater sculpins tend to invade new freshwater habitats along a line of least evolutionary resistance and are capable of tremendous morphological diversification when given the opportunity. In this interpretation, the dearth of morphological diversity in the non-Baikal cottids simply represents a lack of ecological opportunity. This hypothesis could also help to explain the pattern of body shape evolution of fishes globally, wherein freshwater fishes tend to converge on a subset of the morphologies observed in marine fishes (Friedman et al. 2022).

Despite the morphological conservatism of many freshwater fish lineages, freshwater pufferfishes (Santini et al. 2013), needlefishes (Kolmann et al. 2020), grunters (Davis et al. 2012), and a handful of others have actually out-diversified their closest marine relatives (Bloom and Lovejoy 2017). Here, as we have observed in sculpins, exceptional freshwater diversification hinges on experiencing more ecological opportunity than do co-occurring groups of freshwater taxa that show the more typical pattern of relatively low morphological diversity compared with their marine relatives, such as herrings (Bloom and Lovejoy 2017). It is an open question as to why freshwater habitats present fewer apparent opportunities than does the marine environment. Plausible explanations include more entrenched competition (Betancur-R 2010; Bloom and Lovejoy 2012) and greater homogeneity of the freshwater habitats themselves (see below). Answering this question may be key to identifying the drivers of global patterns of morphological diversification in freshwater versus marine fishes and other instances of disparate morphological evolution.

Evolution in Marine Systems: Habitat Stability and Connectivity

In contrast to the freshwater taxa, marine sculpins spread broadly in morphospace and show stable rates of morphological evolution. This suggests a kind of tortoise-and-hare dynamic, where freshwater taxa accumulate morphological diversity through sudden bursts of diversification while marine lineages accumulate diversity slowly and steadily (supplementary fig. 14 in Buser 2024*c*), akin to the sprinting of the hare compared with the slower but more consistent pace of the tortoise in the analogy. Several compelling scenarios could account for these differences in tempo and mode. First, the pattern may stem from differences in dynamism between freshwater and marine habitats. High-latitude freshwater habitats vary more on short-term (e.g., seasonal) and long-term (e.g., geologic) timescales than do marine ones. For example, the periods of rapid global climate change during the Pleistocene caused the advance and retreat of glaciers, which radically altered the habitable landscape for high-latitude freshwater fishes through both direct (e.g., scouring) and indirect (e.g., continental desertification) means (Miller 1965; Near et al. 2003). Climate fluctuations during the late Pliocene and early Pleistocene likely split some of the major phylogenetic branches of Cottidae (Dalganov and Saveliev 2022) and perhaps spurred the contemporaneous acceleration of morphological evolution in cottids outside of Lake Baikal (figs. 1, 3). Factors such as these may have been more consequential to the speciation/extinction rate of freshwater fishes than to that of marine species, and the disjunct patterns of morphological diversification that we observed may reflect such differences in habitat stability.

Another possibility is that the different connectivity of freshwater versus marine habitats promotes slow and consistent morphological diversification in the marine environment and hampers morphological diversification in freshwater. The combination of high population connectivity and habitat connectivity in the marine realm produces an environment in which populations are less likely to become physically isolated but have greater access to new environments, where they can adapt and morphologically diverge from their relatives. For example, deep-water habitats promote morphological diversification (Martinez et al. 2021) and have been successfully invaded ~300 times by marine fish lineages (Miller et al. 2022) but fewer than 10 times by freshwater fishes (Lowe-McConnell 1987; Page and Burr 1991; Sideleva 2003; Meyer et al. 2015; Stiassny and Alter 2021). There is little reason to believe that freshwater fishes are intrinsically less able to invade and adapt to deep-water habitats. A far more justifiable explanation is that there are only a handful of freshwater habitats that qualify as deep water (usually defined as >200 m deep), and only fish lineages that happen to occupy adjacent habitats could possibly invade them. This physical isolation effectively filters the taxa that have opportunities for habitat transition in a way that the marine environment does not.

While the greater habitat connectivity of the marine environment creates a continuous opportunity for habitat transitions and subsequent adaptation, it also increases population connectivity and gene flow (Slatkin 1987; DeWoody and Avise 2000; Templeton et al. 2001; Nosil et al. 2019). Higher connectivity could explain why marine taxa evolve slowly but innovate frequently: marine populations have greater access to novel habitats but also experience enough gene flow to effectively slow the rate of evolution. Freshwater habitats are fractal (rivers) or bounded (lakes) and promote much less connectivity among populations. Thus, freshwater populations are more likely to become isolated, but when they do they are often in a similar habitat to that of their ancestors and so do not experience natural pressure to evolve novel morphologies. Rather, they experience pressure to maintain the morphology of their ancestors. So while freshwater fishes are clearly capable of rapid morphological diversification, the opportunities to do so are relatively limited, with chance involved in which lineages effectively stumble upon an ecological opportunity. The high rates of endemism in the Amazon River, the African Rift Lakes, Lake Baikal, the Congo River, and other hyperdiverse freshwater fish assemblages lend support to this idea.

These kinds of chance events that lead to exceptional diversification may also help to explain why some freshwater lineages originating from marine ancestors speciate markedly while other such lineages do not. In pufferfishes, drums, and stingrays, a single clade dominates the freshwater contingent (Bloom and Lovejoy 2017; de Brito et al. 2022). Sculpins also fit this pattern in that most freshwater species belong to family Cottidae (Goto et al. 2015). Why should it be that cottids, the majority of which are doppelgängers of one another, are so speciose, while other sculpin lineages that have transitioned to freshwater, such as the psychrolutid *Myoxocephalus thompsonii* (a freshwater species with no freshwater congeners), are evolutionary singletons? In this study, we have demonstrated that broad-scale patterns of marine versus freshwater fish morphological disparity hold constant at a much smaller phylogenetic scale and in the fine-scale morphology of the skull (not only in more generalized aspects of body shape). Future studies could use the landmark scheme, geometric morphometrics techniques, and hypothesis framework that we describe herein to investigate additional groups of fishes spanning the marine-freshwater divide and look for patterns to help explain why some freshwater invaders blossom into speciose clades (even if the constituents are morphologically indistinguishable) while others never do. Is there some trait that distinguishes the prolific freshwater speciators from their more modest relatives? Or did they simply have a more favorable roll of the proverbial dice?

Conclusion

We conclude that freshwater and marine sculpins differ markedly in the tempo and mode of morphological evolution. Freshwater sculpins evolve faster but appear constrained to a common morphology, while marine sculpins evolve slowly but with little apparent restriction to their morphological diversification. We attribute the higher levels of morphological constraint present in freshwater taxa to niche conservatism and a greater scarcity of ecological opportunity in the freshwater realm. Several tantalizing explanations for these findings, such as differences in habitat stability and/or habitat connectivity between marine and freshwater systems, warrant further consideration and study.

Acknowledgments

J. Andrés López, Benjamin Frable, H. J. Walker, Katherine Maslenikov, Luke Tornabene, and Theodore Pietsch provided access to museum specimens. Dustin Campbell, Russell Campbell, Jennifer Luparell, Michael Hofmann, and Christopher Trail isolated computed tomography data. National Science Foundation (NSF) grant DEB-1745267 to B.L.S. and NSF DEB-2237278 to K.M.E. supported the development of the manuscript.

Statement of Authorship

T.J.B. and K.M.E. conceptualized the project; T.J.B., K.M.E., O.L., M.P.N., B.L.S., and A.P.S. developed the methods and experimental design; T.J.B., M.W.S., and A.A. collected data; T.J.B., K.M.E., O.L., and M.P.N. analyzed the data; K.M.E., M.W.S., A.A., B.L.S., and A.P.S. provided resources; T.J.B., K.M.E., and O.L. wrote the original draft; and all authors reviewed and edited the manuscript.

Data and Code Availability

All supplementary materials are hosted on the Harvard Dataverse repository. The supplementary R script is hosted at https://doi.org/10.7910/DVN/Y0ITLP (Buser 2024*a*). The supplementary Slicer scene is hosted at https://doi.org/10.7910/DVN/RESKOT (Buser 2024*b*). The supplementary tables and figures are hosted at https://doi.org/10.7910/DVN/K5CWI7 (Buser 2024*c*).

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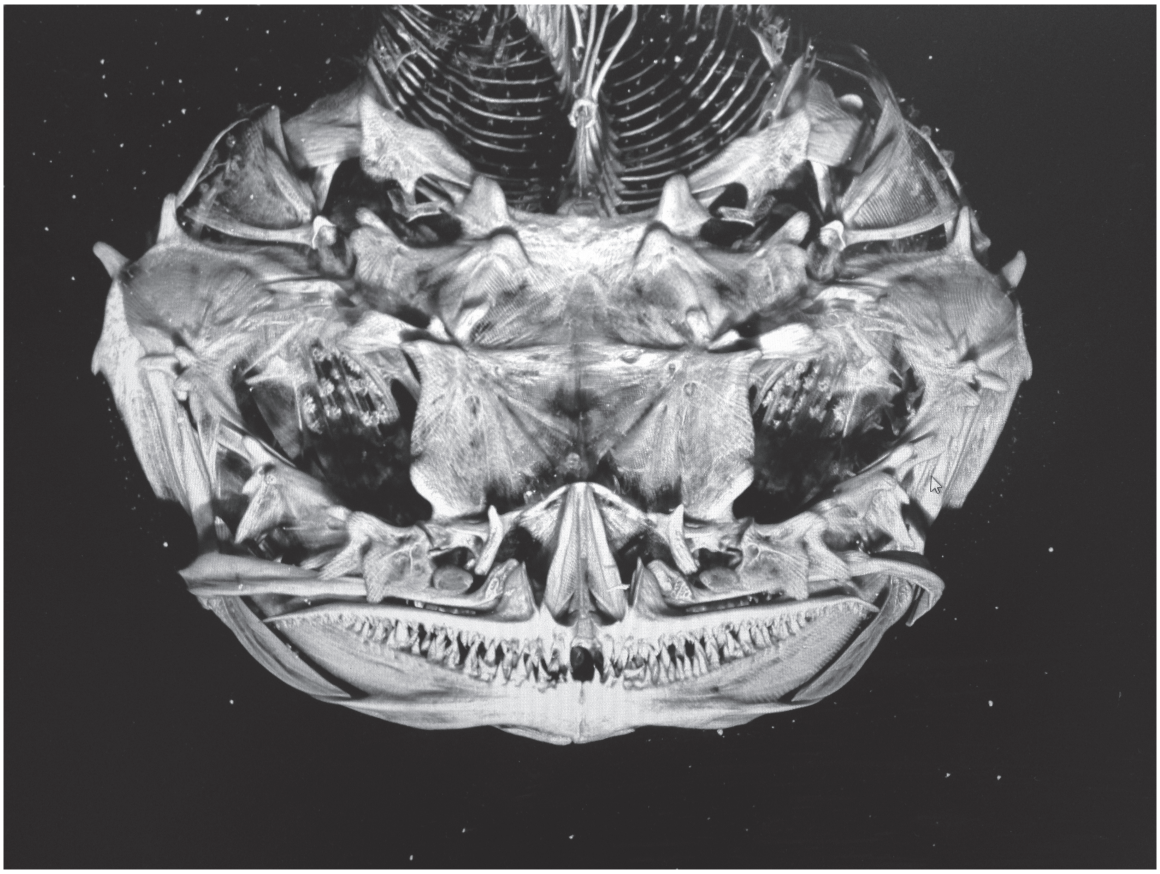

The toothy grin of the bigmouth sculpin (*Hemitripterus bolini*), as seen through a computed tomography reconstruction of its skull. Imaged and photographed by T. Buser.