Evolution of Color and Pattern in Etheostomatinae (Darter Fishes)

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ABSTRACT

Often directly related to non-random mating, kin recognition, predator avoidance, and other life strategies, diversification of coloration may be an important factor in evolutionary processes like speciation and adaptive radiation. Darters (Etheostomatinae) are a speciose clade of freshwater fishes belonging to family Percidae that show remarkable diversity in color and color pattern, including species with bold and complex patterns. Their high variation in coloration coupled with species richness makes Darters an excellent group for understanding the macroevolutionary and ecological factor driving the evolution of color. Using recent developments in color and pattern analysis such as *recolorize* in R Studio and adjacency analysis, we were able more objectively and accurately quantify darter color patterns than any prior study of this clade. Using multivariate approaches for understanding the variation in complex traits and their relationship to other factors (e.g., habitat) and phylogenetic comparative methods such as and evolutionary model fitting, we were able to quantitatively describe trends in color pattern and color diversification in this clade. Our data set was comprised of 107 species of darters and 352 photos from field sites and online databases. We found that almost 40% of color variation in Etheostomatinae can be described across the first four PC axes, which varied primarily in factors like the presence of conspicuous colors like red and blue, the complexity of color pattern, the presence of melanistic colors (blacks) and the strength of patch boundaries. Modern darter color diversity appears to have originated towards the present, rather than early in the radiation of darters. Color also seems to be evolving rapidly in younger clades such as *Etheostoma* and *Nothonotus* and is much slower in basal groups likes *Ammocrypta, Crystallaria,* and *Percina*. Using canonical correspondence analysis we found trends of brighter, more conspicuous color and pattern were associated with shallow, upland streams and riffles, with more dull colors and patterns associated with lower elevation, soft substrates, large river and pool environments. Maximum likelihood model fitting also showed that small rivers and riffle environments were associated with broadly accelerated evolution in diverse color traits, with fast color evolution in large rivers being restricted to dark-light color characteristics. In addition to niche partitioning among different habitat environments (sand, rock, vegetation), color diversification could be the result of differing photic environments (light spectrum changes, water clarity, etc) and differing female preferences in sexual selection. Our results are consistent with natural selection on darter color in small river and shallow riffle environments, likely driven by changes in predator abundance and/or differences in photic conditions. Enhanced color diversification in some river habitats (streams and riffles) may indirectly contribute to reproductive isolation in darters through reinforcing sexual signaling in secondary contact between lineages. Further expansion of this data increase taxonomic coverage could help to further test for relationships between darter coloration and lineage diversification (speciation and extinction). Our work helps to understand the origins of the varied and brilliant coloration of darters and illustrates how new statistical toolkits can expand our evolutionary understanding of organismal color diversity.

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INTRODUCTION

The Importance of Color to Organismal Biology

Coloration is an essential trait for organismal performance, and different clades across the tree of life have evolved an extraordinary diversity of color and patterns associated with different environments, adaptations and life histories. However, the diversity of coloration is not even across the tree of life, with some clades such as Dendrobatidae (poison dart frogs), showing a dizzying array of varying bright and complex color patterns, while other clades such as Ursidae (bears) have more simple or homogenous color patterns. As one of the main components of visual perception in vertebrates, coloration serves many functions among different clades of animals. Predator avoidance, intraspecific communication, thermoregulation, and non-random mating are just a few of life strategies in which color plays a direct role. However, these different selective factors may select for colors or patterns in opposition, and thus incur trade-offs between these essential life functions.

Cryptic coloration, for example, includes those patterns and colors that provide camouflage against the natural environment, and can be important to both predator avoidance in prey and in concealment in predators during hunting behaviors (Stevens and Merilaita, 2008). While cryptic coloration is often associated with irregular/patchy patterns and dull/pale colors (depending on the environment/ecosystem), crypsis is benefitted by low environmental heterogeneity. In highly variable environments, other strategies for minimizing recognition by prey/predators may include “disruptive” elements like bars/stripes to break up the overall outline of the fish and impair pattern matching by other organisms; this type of coloration is common in many coral reef fish such as the bluefaced angelfish (*Pomacanthus xanthometopon*) (see bottom right of Figure 1 for juvenile coloration). Color may also impact thermal ecology and colors useful for blending into the environment may impose physiological costs (Smith, 2016).

Figure 1. Examples of coloration types in fishes. Cryptic coloration (top left), countershading (top right), conspicuous coloration (bottom left), and disruptive coloration (bottom right).

In contrast with needs of avoidance and concealment, sexual selection on color often favors conspicuous colors that are bright and highly contrast with the surrounding environment are commonly favored by females rather than cryptic coloration (Maan, 2006). Organisms with conspicuous colors such as reds and yellows often synthesize these colors from carotenoids that are stored from the environment; flamingos and yellow finches are just a couple examples of the many species that derive pigmentation from diet (Brush, 1990). Maintaining coloration derived from diet may indicate to females of a species that an individual is competent in sequestering nutrients from the environment, therein possibly driving sexual selection of brightly colored mates in dichromatic species. Previous studies of Bahama mosquito fish and Cardueline finches support this proposition of females preferring brightly colored males to those less chromatic (Badyaev and Ghalambor, 1998, Heinen-Kay et al, 2015). The tradeoffs imposed by these selective factors on organismal coloration may vary with animal behavior, environment and life stage (Endler, 1991, Medina, 2020) – for example crypsis may be more important in juveniles while conspicuous colors are developed in adulthood to signal sex and advertise fitness to mates (Heinen-Kay et al, 2015).

The Functional Biology of Color

Diversity in colors and patterns among animals are made possible by chromatophores, pigment-containing cells that lie within the dermis and epidermis (Obika, 1990). Chromatophores can be further categorized into different cell types dependent on what pigments they contain: melanophores (black/brown), xanthophores (yellow), cyanophores (blue), erythrophores (red), leucophores (white), and iridophores (reflective/iridescent). Melanophores, melanin-containing chromatophores, are the most common pigment cells, while xanthophores and erythrophores are slightly less abundant, but not uncommon (Burton, 2002). Cyanophores, cyanosome-containing chromatophores, however, are among the rarest: only having been observed in a few fish species (Goda, 1995). Most blue coloration in animals has been attributed to well-studied iridophores, chromatophores that lack pigment cells within but instead contain light refracting guanine crystal platelets that result in shades of iridescent whites, blues and purples depending on the angle at which light strikes (Cloney, 1983). Combinations of these cell types within the dermis of animals results in spectacular displays of color and pattern across groups of organisms. Certain groups of organisms (i.e., fish, lizards and some invertebrates) are able distribute said pigments throughout dendritic extensions of the chromatophores via fine muscle movements regulated via neural and/or hormonal cues (Aspengren et al, 2008, Fujii, 1993, Raposo and Marks, 2007).

As previously discussed, the diversity of chromatophores and pigments within them possessed by an organism is influenced by many factors including sexual and natural selection as well as environment. Specificities of life histories among groups drive diversification of photic/visual systems. Genes controlling either the functionality of opsin, the ubiquitous photoreceptor protein in the animal kingdom, or other molecules and receptors further down the visual transduction pathway, may undergo adaptive changes to better suit the photic environment where the organism resides (Hauser and Chang, 2017) As a result, visual systems of groups can vary in terms of number of cones, rods, and density of retinal ganglion cells (Hitchcock, 1986, Sanes and Masland, 2015, Shand, 2000).

Environmental properties may also impact the visualization of these different types of coloration. The effects of light attenuation, loss of intensity of light as it travels away from the source and through a medium, in water are far greater than in air. Spectrums of visible light change rapidly in the water column (Lythgoe, J.N., 1988), especially in turbid waters (i.e., rivers, estuaries, lakes) where suspended particles scatter and absorb light (Peng, 2009). This scatter and absorption of light affects the visual clarity and light penetration at different depths, resulting in adaptive evolution of signal propagation and visual systems of organisms residing at different depths. For example, despite its relatively commonness as a conspicuous color in fishes, red light is absorbed at shallow depths and poorly visualized in deeper waters. A previous study (Morrongiello et al., 2010) stated nuptial coloration in the southern pygmy perch (*Nannoperca australis* Günther) was positively correlated with which wavelengths of light are most present in the environment. As signaling is influenced by light, so are visual systems. Species existing in shallower waters where visual clarity is high, such as darters, may rely more heavily on color perception, therein possess more classes of pigment containing cells or visual cones (photoreceptor cells responsible for color vision), whereas deep sea/benthic species may have higher densities of rods (photoreceptor cells responsible for low-light vision) to allow better vision in low-light environments. (Levine and MacNichol, 1979; Loew and Lythgoe, 1978; Wagner, 1998)

The Evolutionary Importance of Organismal Color

Due to the importance of color in sexual selection in many species, color can play a significant role in the establishment of reproductive isolation. In Guadeloupean anoles (*Anolis marmoratus* ssp.), adaptation to differing light environments seems to be driving the divergence of a population into two subspecies (Crawford, 2023). In cases of sympatric speciation, sexual color characteristics may be the only or one of a limited set of factors preventing mating between diverging populations (Wagner, 2012). Adaptations associated with changing environments or ecological roles may also predispose some clades to diverging along lines of sexually selected color morphs (Maan, 2006). Coloration may also reinforce speciation in allopatry, for example if different habitats or geographic regions vary in predator abundance (Muñoz, 2013) or in cases of sensory drive where sexual signals experience selection for optimizing in different environmental conditions (Jenck, 2020).

Color may also play an important role in the development of biodiversity across broader macroevolutionary processes. Adaptive radiation is the process by which clades radiate explosively into new species as a result of adaptation into available ecological niches (Rundell and Price, 2009). This type of speciation is especially exemplified in isolated populations such as the Malawi African rift lake cichlids and Galapagos finches but may contribute to the origin of diverse clades across the tree of life. The "Stages" model of adaptive radiation proposed by Streelman and Danley (2003) suggests that a transition from natural selection on ecological factors like habitat and dietary niches, to sexual selection on "communication" characteristics like coloration may be a critical process in the diversification of clades undergoing adaptive radiation.

Darter Fishes (Etheostomatinae)

Fishes show a remarkable diversity of color and pattern, with many clades varying substantially in the complexity of patterns, proportions of cryptic and conspicuous colors and degree of sexual dichromatism. Nestled within the Percidae phylogeny, darters (Etheostomatinae) (see Figure 2) are a colorful, diverse lineage of small freshwater fishes native to North America, dating back around 30-38 mya to the early Oligocene (Near et al, 2011). With over 200 described species, darters comprise nearly 20% of North American freshwater fish diversity (Page, 2011). Most of this diversity is concentrated in the southeastern United States, a region renowned for its biological diversity across many flora and fauna, especially in freshwater systems (Collen et al, 2014); however, there are darter species found as far north as Canada and as far south as Mexico.

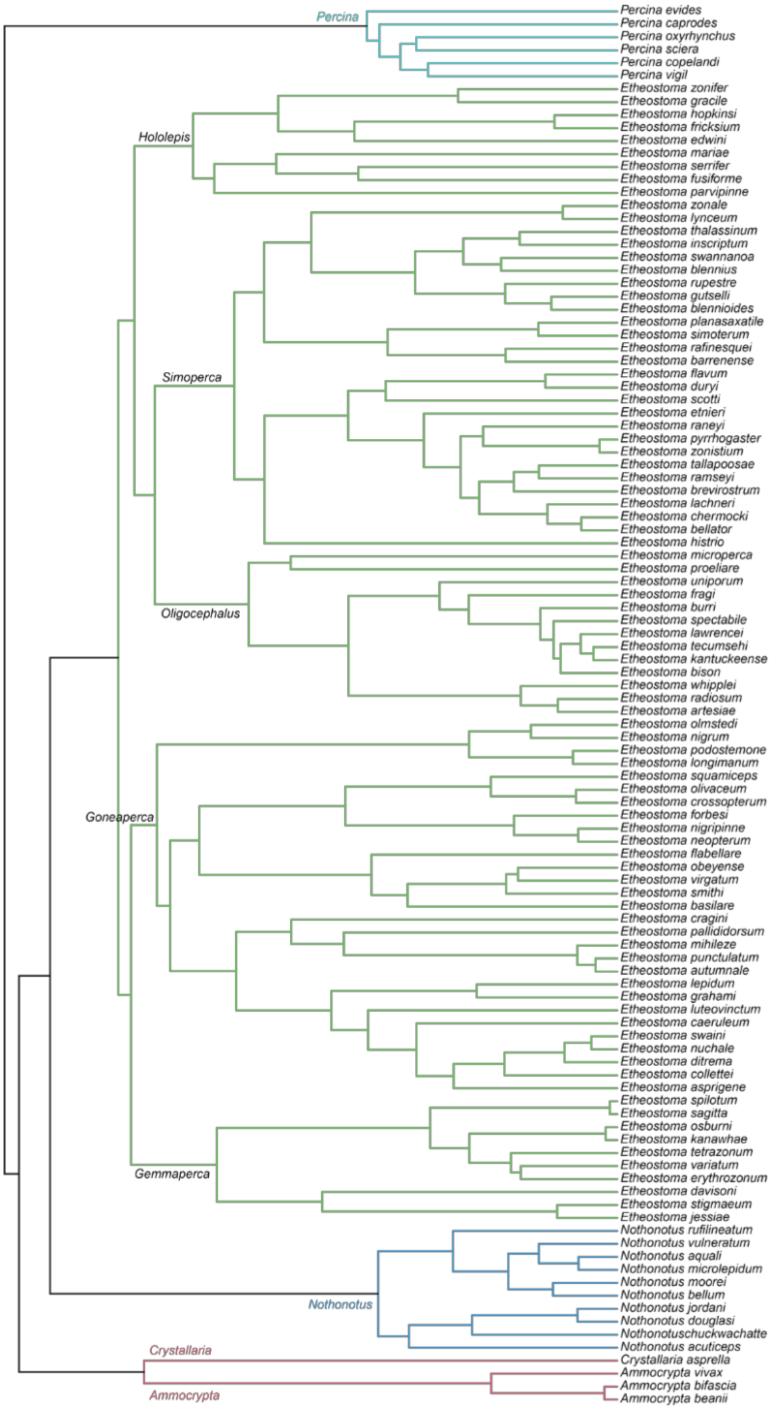


Figure 2. Phylogeny of the darters, *Etheostomatinae* (species highlighted in this study). Branches are colored by genera and clades (e.g., subgenera) highlighted in the text are noted.

Darter species are typically benthic or more rarely hyperbenthic (live on or in close association with the substrate) and are largely lotic fishes, living in environments ranging from small spring-fed streams to large, high velocity rivers, with very few living partially in lentic habitats. Within these flowing water habitats, darters occupy various microhabitats such as sandy pools, rocky riffles, and shallow vegetation-filled runs (Carlson & Wainwright, 2010). Aside from some pool-specialist species, darters are most often found in high current microhabitats along the benthos. Existing at or below the benthic boundary layer (the region of reduced current flow near the substrate) is likely the reason many species in this clade have extremely reduced swim bladders or completely lack the organ (Evans & Page, 2003). Darters feed primarily on non-evasive prey; primarily larvae from aquatic invertebrate groups such as ephemeropterans (mayflies), chironomids (midges), and trichopterans (caddisflies) (Wynes & Wissing, 1982) with some specialist species feeding solely on gastropods (i.e., Snail Darter – *Percina tanasi*) (Starnes, 1977), though less frequently vegetation, detritus and small fish/larval fish may comprise elements of their diet (Turner, 1921). Darters fall prey to larger, piscivorous fish such as smallmouth bass (*Micropterus dolomieu*), largemouth bass (*Micropterus salmoides*) and spotted bass (*Micropterus punctulatus*) (Lachner, 1950). Dwelling in shallower waters and riffles aids darters in predator avoidance, since these larger and deeper-bodied fishes cannot maneuver as easily in shallow, rapid waters.

Darter species show an extremely high variability in color composition and pattern, with a diversity comparable to more tropical and marine clades of fishes. More basal clades of darters, like the genera *Percina* (more hyperbenthic species), *Ammocrypta* and *Crystallaria* show cryptic coloration, comprising simple patterns of tans and browns, consistent with the sandy, silty or in some cases rocky environments they inhabit. However, the genera *Etheostoma* (the most species rich genus) and *Nothonotus* are often sexually dichromatic, with bright and bold "nuptial" coloration and pattern developing on males (see Figure 3).

Figure 3. Examples of differing coloration in male darter species. Species show, left (top to bottom): *Crystallaria asprella, Nothonotus rufilineatus, Etheostoma lachneri*, middle: *Etheostoma barrenense, Etheostoma gore, Etheostoma lynceum*, right: *Etheostoma nigripinne*, *Etheostoma derivativum*, *Etheostoma kenicotti*. Note the plain/simple colors and patterns on *Crystallaria asprella* (top left) as compared with the complex patterns and reds of *Etheostoma barrenense* (middle right) and the bright greens of *Etheostoma lynceum* (bottom middle). Other common features include vertical stripes (*E. lynceum*, *E. lachneri*), horizontal bars/blotches (*E. barrenense, E. gore*), saddles (*C. asprella*), rows of small spots/squares (*E. deriviativum, N. rufilineatus*) as well as stripes or banding on the fins.

Indeed, for many darter species, the only reliable distinguishing characters for identification are nuptial colors of males, with females possessing much simpler cryptic patterns and primarily browns, tans and blacks. Nuptial coloration varies greatly with species-specific colors ranging from bright reds and oranges to striking blues and greens, as well as well-defined banding across the bodies and fins, vertical stripes and saddles formation (Bossu and Near, 2015). Nuptial colors also develop and are at their most conspicuous during the spawning season for darters, which begins in early spring and extends into mid-summer, although timing may vary slightly by species and latitude (Hubbs, 1985).

The diversity of species with strong sexual dichromatism suggests that color is important to sexual selection in the majority of darter species. Darters' vision is comprised of a 2-cone system capable of detecting red, blue, green, and small amounts of purple light but not ultraviolet wavelengths (Moran and Fuller, 2018). Previous research has shown that male coloration may be an important to access to mates in species like the orange throat darter, with more colorful males more likely to "win" access to females (Zhou, 2016). Darter species also seem to prefer aspects of coloration from their own species (Williams and Mendelson, 2013). Sexual selection for conspecific coloration characteristics may also facilitate reproductive isolation in some darter species (Williams, 2013). However, the strength of selection for conspicuous and dichromatic colors varies across darter species (Williams and Mendelson, 2013). Additionally, the more cryptic and simple colors of females and the reduction of conspicuous colors during the non-spawning months suggests a distinct fitness trade-off associated with these bright and bold male colors. As a result, selection on darter coloration is likely complex and mediated by multiple ecological and sexual factors.

Darters as a Model System for the Analysis of Color Evolution

Why are darters so unusually diverse in their coloration and exhibit such bright and contrasting colors compared to other North American ichthyofauna? Given their high species richness, variety of coloration (including both chromatic and structural colors), and environmental variability, darters represent an ideal system for studying the evolutionary origins of color and pattern diversity. Most studies of darter coloration have focused on single species, species pairs/complexes, or simply a small fraction of darter species (Gumm and Mendelson, 2011; Zhou, 2014). A species rich and colorfully diverse clade such as darters presents an opportunity to investigate the macroevolutionary development of color diversity on a large scale using comparative approaches. In recent years, however, advances in comparative analyses have opened doors for more comprehensive studies regarding color evolution since this earlier work was published.

Advances in the Quantification of Organismal Color

In previous studies, color scoring methods for broad macroevolutionary studies have been predominantly subjective. Many of these studies bin highly variable color data into a limited number of color groups, like red, blue and black (Cicotto and Mendelson 2016). In addition to limited groupings, the values assigned to these colors in different regions of the specimens were solely binary (i.e., present or absent). As a result of binary scoring of color presence and limited color groupings the proportional representations of color, adjacent color groups, strength of color boundaries, and many valuable color data that lie between peaks of red and blue are lost.

In the past 5-10 years, more advanced methods have been developed to ensure color spectrums are represented properly when conducting evolutionary studies of color and pattern.

These methods include R packages such as “recolorize”, “pavo”, and “colordistance”, as well as statistical approaches such as adjacency analysis (Endler, 2018). The R package “recolorize” (Weller, 2024) allows a more objective approach to categorization of color than in any approaches before. Zone maps, originally described in 1984 (Endler, 1984), result in pixelated images which, with “recolorize”, can be assigned a color class number. The number of color classes in a dataset are first binned using a histogram method and then can be manually reduced to a more meaningful representation of the dataset using the ‘recluster’ function (Weller, 2024).

Pixels of an image with an assigned color class number allow statistical analyses to be ran on otherwise largely subjective data such as color. Adjacency analysis, a function within the aforementioned pavo package, can then be conducted on the dataset to acquire 16 useful color metric summaries such as adjacency, aspect ratio, and proportions of color classes in each image (Endler 2012; Endler 2018; Endler and Mielke, 2005). Multivariate analyses such as principal component analysis (PCA) (Wold, 1987) and phylogenetic comparative methods such as disparity through time analysis (DTT) (Guillerme et al., 2020) and Brownian motion evolutionary models (Slater, 2010) can then be utilized on the data set to describe trends in color evolution.

Objectives

The purpose of this study was to understand the major trends in darter coloration and its evolution. The specific objectives addressed in this study are: 1) Quantify the major patterns of color variation in darters using more quantitative/advanced techniques and tools such as “recolorize” and “adjacency analysis”. 2) Examine the patterns of macroevolution in

Etheostomatinae using phylogentic approaches including disparity through time analysis and rate-model fitting to test selection on specific traits in the clade. Has color evolution evolved steadily through the radiation of darters, or is there evidence of changing rates/selection? 3) Test whether selective factors such as habitat have shaped the diversification of color by comparing evolutionary rate models.

MATERIALS AND METHODS

Specimen and Photograph Collection

Photographs of live darter specimens were obtained from live field photography and through open-source online databases. Thirty-three field sites were visited from November 2021 to May 2024. Sampling occurred across the state of Tennessee in Cumberland, Ohio, Mississippi, and Tennessee river drainages primarily in spring and summer during peak coloration. Fishes were collected using an *LR-24 Smith-Root* electrofishing backpack. Voltage and frequency settings were adjusted at each site to account for differences in water conductivity.

Any individuals not belonging to family Percidae were immediately released upon recovery from the electroshock. Members of Percidae were kept and identified to species level using *Peterson* field guides and other guides curated by Jessica Arbour. Males in nuptial coloration were preferred; however, some females and males not in coloration were also collected, along with rare species or individuals without significant sexual dichromatism. Specimens were euthanized using a clove oil solution and a solution of 10% formalin was painted onto fin bases after pinning in preparation for photographing. Specimen photos from FishBase, Florida Museum Ichthyology Collection, Illinois Natural History Survey, and iNaturalist were used for species not included in the collection permit and/or for species we were unable to locate at field sites. An appendix with a comprehensive list of species collected from the field and from the listed databases is included at the end of this document.

Table 1. List of all species collected from field and databases. Sources include the Arbour Lab, FishBase, iDigBio, Florida Museum of Natural History (FMNH), Illinoi Natural History Survey (INHS), iNaturalist, Native American Fisheries Association (NANFA), and

Species Name	*Number Collected*	*Source(s)*
Ammocrypta beanii	1	INHS
Ammocrypta bifascia	1	FMNH
Ammocrypta vivax	2	INHS
Crystallaria asprella	2	FMNH, INHS
Etheostoma artesiae	5	iNaturalist
Etheostoma asprigene	2	iNaturalist
Etheostoma autumnale	1	NANFA
Etheostoma barrenense	4	Arbour Lab
Etheostoma basilare	1	Arbour Lab
Etheostoma bellator	1	iNaturalist
Etheostoma bellum	1	INHS
Etheostoma bison	3	Arbour Lab
Etheostoma blennioides	9	Arbour Lab
Etheostoma blennius	12	Arbour Lab
Etheostoma brevirostrum	2	SFC
Etheostoma burri	2	iNaturalist
Etheostoma caeruleum	7	Arbour Lab
Etheostoma chermocki	1	iNaturalist
Etheostoma chuckwachatte	1	iNaturalist
Etheostoma cinereum	2	iNaturalist, SFC
Etheostoma collettei	3	iNaturalist
Etheostoma colorosum	4	iNaturalist
Etheostoma coosae	3	iNaturalist, SFC

Etheostoma corona	2	FMNH, iNaturalist
Etheostoma cragini	1	NANFA
Etheostoma crossopterum	7	Arbour Lab
Etheostoma davisoni	2	FishBase, iDigBio
Etheostoma derivativum	3	Arbour Lab
Etheostoma ditrema	1	INHS
Etheostoma duryi	5	Arbour Lab
Etheostoma edwini	3	INHS, iNaturalist
Etheostoma erythrozonum	1	Robert M. Wood
Etheostoma etnieri	6	Arbour Lab
Etheostoma exile	3	INHS
Etheostoma flabellare	8	Arbour Lab
Etheostoma flavum	10	Arbour Lab
Etheostoma forbesi	1	iNaturalist
Etheostoma fragi	2	iNaturalist
Etheostoma fricksium	2	INHS, iNaturalist
Etheostoma fusiforme	1	FMNH
Etheostoma gore	10	Arbour Lab
Etheostoma gracile	2	Arbour Lab
Etheostoma grahami	1	INHS
Etheostoma gutselli	2	iNaturalist
Etheostoma histrio	3	FMNH, iNaturalist
Etheostoma hopkinsi	2	FishBase, iNaturalist
Etheostoma inscriptum	2	iNaturalist
Etheostoma jessiae	1	INHS
Etheostoma jordani	1	FishBase
Etheostoma kanawhae	2	INHS
Etheostoma kantuckeense	1	Arbour Lab
Etheostoma lachneri	1	iNaturalist
Etheostoma lawrencei	1	iNaturalist
Etheostoma lepidum	1	FishBase
Etheostoma longnimanum	2	FishBase, iNaturalist
Etheostoma luteovinctum	4	Arbour Lab
Etheostoma lynceum	9	Arbour Lab
Etheostoma mariae	2	iNaturalist
Etheostoma microperca	3	iNaturalist
Etheostoma mihileze	1	FishBase
Etheostoma moorei	1	INHS
Etheostoma neopterum	1	iNaturalist
Etheostoma nigripinne	5	Arbour Lab

Etheostoma nigrum	3	Arbour Lab
Etheostoma nuchale	1	INHS
Etheostoma obama	1	Arbour Lab
Etheostoma obeyense	5	Arbour Lab
Etheostoma olivaceum	3	Arbour Lab
Etheostoma olmstedi	2	FishBase
Etheostoma orientale	1	iNaturalist
Etheostoma osburni	2	iNaturalist
Etheostoma pallididorsum	2	iNaturalist
Etheostoma parvipinne	3	iNaturalist
Etheostoma planasaxatile	1	iNaturalist
Etheostoma podostemone	2	FishBase, iNaturalist
Etheostoma proliare	2	FMNH, iNaturalist
Etheostoma punctulatum	2	NANFA
Etheostoma pyrrhogaster	1	iNaturalist
Etheostoma radiosum	4	iNaturalist
Etheostoma rafinesquei	1	iNaturalist
Etheostoma ramseyi	1	FishBase
Etheostoma raneyi	1	iNaturalist
Etheostoma rupestre	2	FishBase, iNaturalist
Etheostoma sagitta	3	iNaturalist, INHS
Etheostoma scotti	3	FishBase, iNaturalist
Etheostoma serrifer	2	iNaturalist
Etheostoma simoterum	13	Arbour Lab
Etheostoma smithi	3	FishBase, iNaturalist
Etheostoma spectabile	8	Arbour Lab
Etheostoma spilotum	1	iNaturalist
Etheostoma squamiceps	1	iNaturalist
Etheostoma stigmaeum	4	Arbour Lab
Etheostoma swaini	9	Arbour Lab, iNaturalist
Etheostoma swannanoa	2	FishBase, iNaturalist
Etheostoma tallapoosae	1	FishBase
Etheostoma tecumsehi	1	FishBase
Etheostoma tetrazonum	3	iNaturalist, INHS
Etheostoma thalassinum	2	iNaturalist
Etheostoma uniporum	3	iNaturalist
Etheostoma variatum	2	INHS
Etheostoma virgatum	2	iNaturalist
Etheostoma whipplei	3	iNaturalist
Etheostoma zonale	6	Arbour Lab

Etheostoma zonifer	1	FMNH
Etheostoma zonistium	6	Arbour Lab
Nothonotus acuticeps	1	FishBase
Nothonotus aquali	2	Arbour Lab, iNaturalist
Nothonotus bellum	3	Arbour Lab
Nothonotus camurum	3	Arbour Lab
Nothonotus douglasi	1	iNaturalist
Nothonotus jordani	1	FishBase
Nothonotus microlepidum	4	Arbour Lab
Nothonotus moorei	1	INHS
Nothonotus rufilineatum	10	Arbour Lab
Nothonotus sanguifluum	3	Arbour Lab
Nothonotus vulneratum	1	iNaturalist
Percina caprodes	1	Arbour Lab
Percina copelandi	2	Arbour Lab
Percina evides	2	Arbour Lab
Percina oxyrhychus	4	Arbour Lab
Percina sciera	7	Arbour Lab
Percina vigil	1	Arbour Lab

Individuals were photographed with a Nikkon D7500 DX and using a Nikkon Nikkor AFS-DX 85mm macro photography lens. Photographs were taken in a photo-tank with a black background and *Calibrite ColorChecker* to ensure a standardization across images regardless of outdoor conditions (see Figure 4 below). Field photos were converted into digital negative files

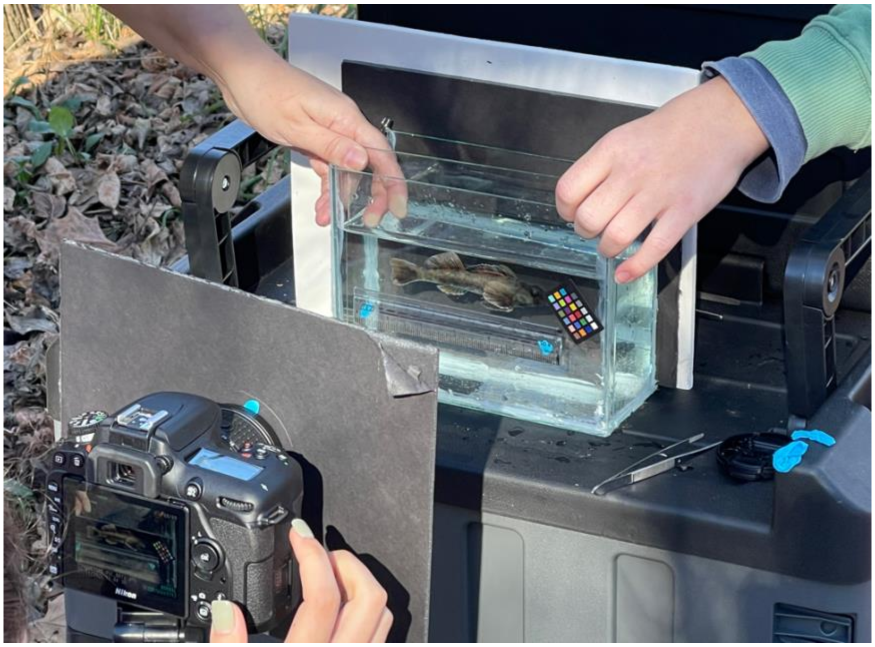
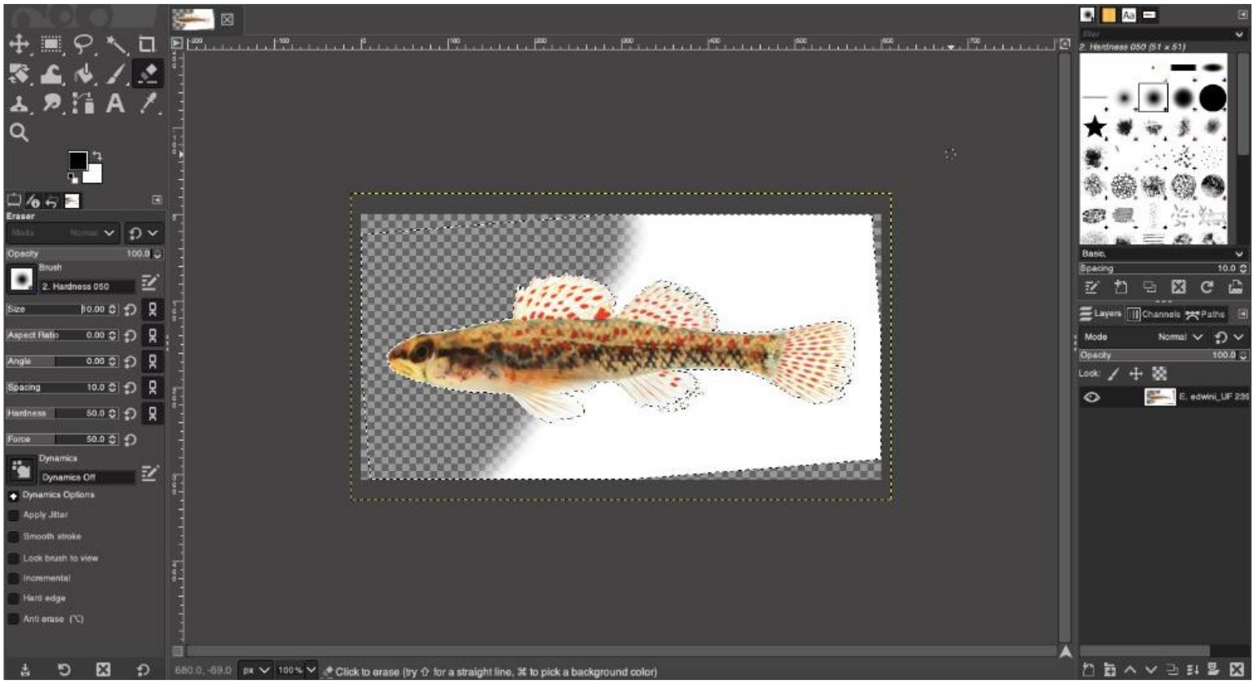


Figure 4. Field photo tank setup (species pictured: *Etheostoma blennioides*)

and individual color profiles were created for each image using *ColorChecker* software. Color profiles were assigned to their respective photos and all images were exported as tagged image files (TIFs). The background was removed from images using the image editing software *GIMP* (see Figure 5). The resulting images of the fishes were exported as portable network graphic files (PNGs) in order to retain as much image data as possible within reasonably sized files suited for



Eraser

Opacity 100.0

Brush 2. Hardness 050

Size 10.00

Aspect Ratio 0.00

Angle 0.00

Spacing 10.0

Hardness 50.0

Force 50.0

Dynamics Dynamics Off

Click to erase (try ⇧ for a straight line, ⌘ to pick a background color)

Figure 5. Image background removal process in GIMP photo editing software (species pictured: *Etheostoma edwini*)

analyses in R and to permit transparent backgrounds. A total of 352 photos were acquired across 107 species from the field and online databases.

Quantification of Darter Color

In order to quantify color variation across darter species, images were processed using several packages in R Studio. Most color quantification analyses require colors to be grouped into a discrete set of major color classes rather than the full spectrum of RGB (or similar values) for each pixel. We used the *R* package *Recolorize* to provide a quantitative approach to the simplification of raw color data into a set of discrete color classes, as it is uses well established clustering approaches and was developed to be resistance to small changes in lighting between photographs (Weller, 2024). Although largely automatic, segmentation of colors using the *recolorize* function requires some preliminary parameters to be manually set. The 'method' argument within the function must be set to either a k-means or histogram clustering method. For the purposes of this project, the histogram method was chosen, as k-means clustering can vary across different runs due to its iterative natures, whereas histogram clustering remains constant across iterations (Weller, 2024). We also found that histogram clustering methods were more effective at detecting conspicuous colors (e.g., red) that make up a smaller portion of the overall "surface" area of the fish but may be important to sexual selection or visibility to predators (e.g., small red spots on some *Nothonotus* species). Using the histogram clustering method, pixels are assigned to one of *n* bins within each axis of the colorspace ("sRGB"), and the average sRGB composition per bin is obtained. To ensure colors across all images were represented during the clustering, a reference image of 40 individuals from both database and field photos containing representative colors was created and used for the initial clustering and classing of colors. Five bins across each axis in an sRGB colorspace (e.g. 5^3) were selected for this data set, resulting in an initial fit of 125 color groupings. These initial color classes were narrowed down to a refined fit of 14 classes that were representative of cryptic (black, brown, grey, tan, white) and conspicuous colors (green, blue, red, orange) throughout the photos. Within *recolorize*, the 'cutoff' argument was set to *48* and the histogram clustering method *ward.D2*, a clustering method that aims to minimize variance within clusters (Ward, 1963), was used to achieve the refined fit. This set of color classes was mapped to all subsequent specimen photographs using the *R* function *impose colors*, ensuring that all specimen color maps would be directly comparable.

Once the images were simplified to include only colors generate by the refined fit in *recolorize* (see Figure 6), adjacency analysis (Endler, 2012) was conducted on the data set to extract color metric data from images by assigning each pixel a numeric value associated with the color groups gathered from *Recolorize*; for this data, pixels were assigned a number 1-14 corresponding to the color mappings carried out above. Adjacency analysis utilizes the frequency of transitions between different color states moving from one pixel to each of its adjacent pixels to quantify different aspects of color pattern and diversity. We focused on the following color data metrics from an adjacency analysis of each mapped specimen photo for this study: overall transition density (m) – which is believed to be higher when patterns are more complex; Simpson

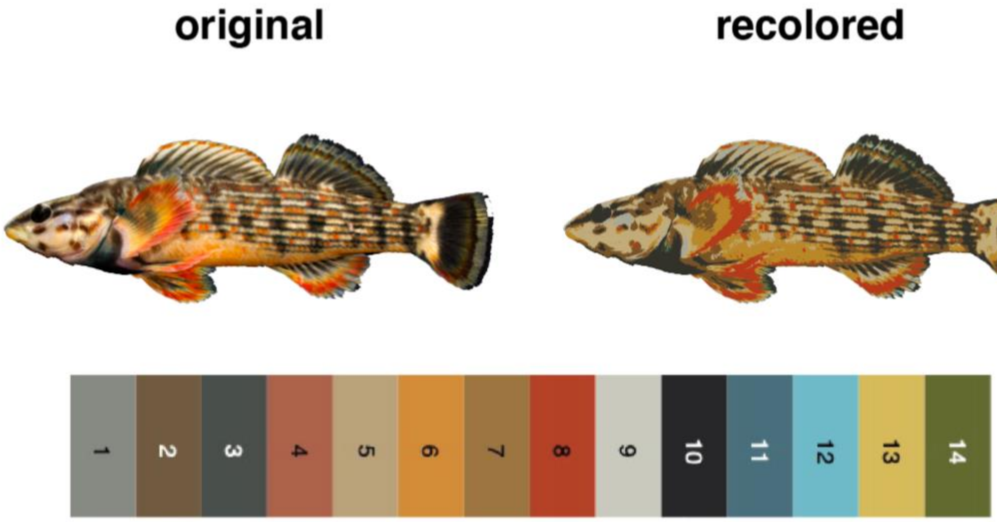


Figure 6. Original image and recolorized image after being processed in R studio. Color simplification maintained the major patterns across the fish, while allowing color classes to be directly compared across darter images.

color class diversity (*Sc*), and Simpson transition diversity (*St*) – these are maximized when the proportion of colors or transitions is equally spread across all possible colors/transitions; Aspect ratio (A) the ratio of horizontal to vertical color class transitions - <1 indicate horizontal banding (more transitions moving vertically) and >1 indicates more vertical banding (more transitions moving horizontally); boundary strengths (are color patches sharply delimited or gradually fading together) for black/white/grey tones (achromatic boundary strength - *m_dL*) vs red/blue/green/other bright colors (chromatic boundary strength - *m_dS*). We also included the proportion of each of the detected color classes in the image (*p_i*).

Evolutionary Trends in Darter Coloration

We used Principal Component Analysis (PCA) to quantify major patterns of color variation in Etheostomatinae. This approach reduces a multivariate data set to a set of axes describing the major relationships between variables, as well as the proportion of overall variation explained by each axis. We determined the number of critical components (those representing non-random variation) using parallel analysis, an approach which contrasts the eigenvalues from the observed data (~ the proportion of variation explain by each axis) to a distribution generated from random, non-correlated data. We investigate overall patterns of color variation across the darter phylogeny using the R function *phylomorphospace* (package “phytools”), which projects the branches of the darter phylogeny onto the PC axis scores (Revell, 2012). A recently updated, time calibrated *Percidae* phylogeny was used for all phylogenetic analyses (Arbour, 2021), here and below.

We used two approaches to investigate overall trends in the evolution of darter coloration. Using the *R* package *Geiger* (Pennell, 2014; Harmon, 2008), disparity through time

(DTT) analyses were ran on the resulting PC scores to test for whether color diversity evolved early, late or steadily over the darter radiation (see Figure 11). DTT analyses calculate the average disparity (trait diversity) of all subclades present at a time in a dated-phylogeny, and this is plotted across the evolutionary history of the clade. Disparity was calculated as the pairwise Euclidean distance across the critical PC axes. This was contrasted with a series of simulated character histories generated under a model of constant rate evolutionary model – Brownian Motion (BM). BM models represent a random walk process governed by a single rate parameter (σ^2), akin to a trait evolving under genetic drift. We produced 1000 BM simulated character sets (Fig. , gray area) and determined the average BM DTT curve (Fig. , dashed line). The pattern of color evolution was quantified using the Morphological Disparity Index, which is the area between the observed DTT curve and the average BM simulated curve. Negative values of MDI are associated with early bursts of trait evolution, while positive values are associated with strong selection towards a global optimum or increasing rates of evolution towards the present (see Figure 7). DTT analysis was carried out using the function *dtt* in the R package *Geiger*.

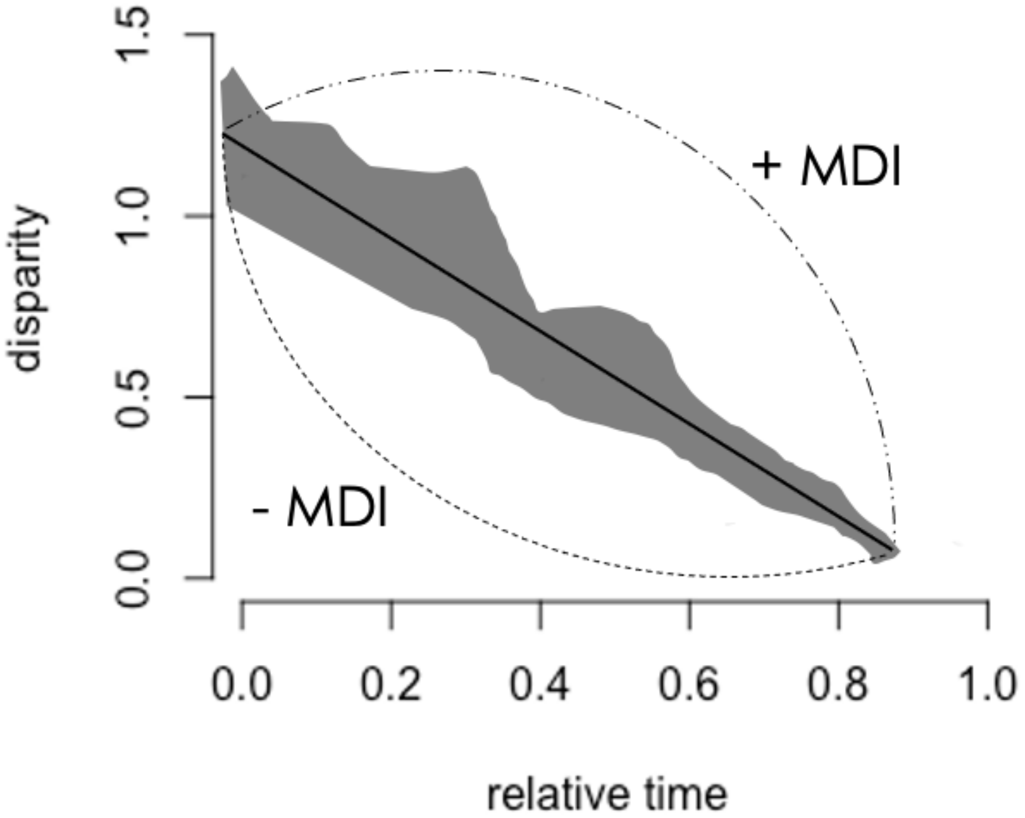


Figure 7. Disparity through time (DTT) analysis sample plot. Dotted-dashed line represents a positive MDI, indicating faster trait evolution towards the present or trait selection occurring within the clade. The dashed line represents a negative MDI, which indicates early diversification of the clade (early burst model).

We used the R function “multirateBM” to visualize changes in the evolutionary rate of each PC axis across the branches of the darter phylogeny. This approach allows the BM evolutionary rate parameter (σ^2) to vary across the branches of a tree according to expectations under a random walk model. We calculated a multirate BM model for each of the four critical PC axes and plotted these rates across the corresponding branches in the darter phylogeny.

Ecological Selection on Darter Coloration

Coloration may serve to hide species from predators or to advertise traits (sex, age, status) to other members of a species. We investigated whether coloration in darters is linked to adaptation to varying habitats. Regional habitat data was acquired from *FishNet2* (all records for each species) using a custom script by Maya Stokes (pers. comm.). Median values for the following habitat metrics were obtained for each species: discharge, drainage size, elevation, slope, velocity and proportional abundance of substrate type (e.g., sand, limestone, shale, silt/detritus) across fishnet2 records. We also obtained a binary character for use of pool vs. riffle in streams by darter species from (Ciccotto and Mendelson, 2016) (missing entries were interpreted from species descriptions and natural history guides), reflecting the use of local, micro-habitat types by darters.

Canonical Correspondence Analysis (CCA) was used to test for an association between color and habitat datasets. CCA is a multivariate data reducing technique similar to PCA, but is used to detect the major relationships between two datasets and is typically used in cases of environmental data (Legendre and Legendre, 2013). We used function 'cca' in the R package *vegan* (Oksanen, 2022) with the habitat dataset as the constraining matrix and used 'anova.cca' to test for a significant relationship between color and habitat. We determined the amount of color variation explained as the proportion of constrained to total inertia (data variability).

We investigated whether habitat has impacted the rate of diversification in darter coloration. We compared both the larger scale, "macro-habitat" data (i.e., river properties above, including drainage size, elevation and substrate) to local habitat use, or "micro-habitat" variable (use of pools vs. riffles within a river system). In order to simplify macro-habitat data for further analyses with color, we first produced an ordination of the environmental data using correspondence analysis, since data was mixed (proportions, heavily skewed environmental variables, differing scales, etc.). We then used a Cluster Analysis (CA) was used to create discrete habitat groups based on the scores of the ordination, using a Euclidean distance matrix. We then applied a hierarchical cluster analysis to the distance matrix using Ward's algorithm to minimize within habitat cluster variance, using the R function *hclust*. We considered the major division between habitat types (large, sandy rivers vs. smaller rocky streams, see results), as well as subtypes within larger vs. smaller river habitats (four total habitat types), in the following analyses.

We reconstructed the evolutionary history of the discrete habitat classes across the darter phylogeny using stochastic character mapping, aka SIMMAP (*R* function 'make.simmap'), which creates an evolutionary history for a discrete character based on the states at the tips and an estimate transition rate matrix (Huelsenbeck et al. 2003; Bollback 2006). We accounted for uncertainty in the estimation of ancestral characters by running all analyses on 100 separate SIMMAP reconstructions. We considered both the major split between large and small rivers, the subtypes within larger and smaller rivers (four habitat groups, see results) and we also reconstructed the evolutionary history of pool vs. riffle use 100 times using SIMMAP. We also reconstructed the history of a combined term, incorporating river size and riffle/pool use to incorporate habitat selection at the macro- and micro- scale. We only created a combined term for the two-group large vs. small rivers, and not the four habitat subtypes as the resulting models were highly parameterized (8 total groups produced >50 parameters in the resulting model) and some grouping possessed very few taxa, which can bias model fitting approaches. However, we found that the results of the four habitat classes and the model incorporating habitat type and local habitat use were highly complementary (see results)

After habitat classes were created and mapped onto the evolutionary history of the darter radiation, the *mvMorph* package (Clavel et al. 2015) in *R* was used to test whether evolutionary rates across PCs varied across habitat classes. 'mvMORPH' allows for multivariate BM models of continuous trait evolution, which include both an evolutionary rate per trait, but also evolutionary covariances between traits, to be fit to a multivariate dataset using a maximum likelihood approach. We fit several models of evolution onto the 4 PC axes (and see results), including 1) a multivariate BM model with a single rate for each PC axis (e.g., the null model, in which habitat does not impact color diversification, 2) a BM model with varying rates for the 2 major discrete habitat classes across all PC axes, 3) a BM model varying rates across four habitat types, 4) a BM model with varying evolutionary rates associated with pool and riffle use, and lastly 5) a model incorporating both river size (large vs. small) and local habitat use (pool vs. riffle). We compared the resulting maximum likelihood fits across each SIMMAP tree using Akaike Information Criterion (AIC). We calculated ΔAIC as the AIC of each of the three models (per SIMMAP tree) minus the lowest observed AIC value for that reconstruction, such that the best fit model per tree has a ΔAIC. Following Burnham and Anderson (2002) we considered models with ΔAIC > 2 to be unlikely to be the best fit model. We determined ΔAIC for each model across all 100 SIMMAP reconstructions and determined the percentage of reconstructions in which the three models were determined to be the best fit.

RESULTS

Color Variation Across Darter Fishes

Phylogenetically corrected PCA performed on species' wide averages of color metrics obtained via adjacency analysis on photos revealed four PC axes represent 62.8% of variation within the data (see Table 2) Across PC1 (23.6%) higher loadings were associated with achromatic colors (grays and blacks) and higher achromatic boundary strength (*m_dL*) while lower loadings associated with chromatic boundary strength (*m_dS*) and transition diversity (*St*). Overall PC1 represented a gradient between fish with complex patterns with clearly defined boundaries of color patches vs species with simple patterns and cryptic colors. In Figure 9, the example species for low PC1 scores shows bright reds and blues, as well as well-defined bands of colors in the fins. Across PC2 (15.5%), species with positive scores tend to possess pale coloration (tans and yellows) and species with negative scores show dark, melanistic coloration (browns, blacks).

Species with positive PC3 (13.6%) were found to have either more vertical striping and complex patterns and transitions or blue coloration, while negative PC3 scores comprised species with more cryptic colors, less complex patterns and more horizontal stripes and greater definition of black/brown patches. PC4 (10.1%) represents a gradient where higher loadings tend to contain conspicuous colors such as red, blue, and light blue while lower loadings represent species that may have more complex patterns and/or brown and green present.

Table 2. Summary of color metric loadings as associated with each PC axis. Important loadings for each axis are bolded (see Figure 8 below for color associations with values *p_i*).

Color Metric	***PC Axis Loadings***			
	PC1	**PC2**	**PC3**	**PC4**
p_1 (gray)	0.242	0.206	0.060	0.104
p_2 (brown)	-0.111	**-0.344**	-0.147	**-0.310**
p_3 (dark gray)	**0.268**	-0.094	**0.361**	-0.188
p_4 (dull red)	-0.246	**-0.328**	-0.037	0.230
p_5 (beige)	-0.129	**0.382**	-0.243	-0.049
p_6 (gold)	**-0.321**	0.022	0.064	0.019
p_7 (tan)	**-0.345**	0.065	-0.091	-0.265
p_8 (bright red)	**-0.293**	-0.201	0.116	0.221
p_9 (white)	0.104	**0.253**	-0.232	**0.278**
p_10 (black)	0.079	**-0.441**	0.151	-0.073
p_11 (blue)	0.105	0.156	**0.395**	**0.335**
p_12 (bright blue)	-0.062	0.037	**0.315**	**0.438**
p_13 (yellow)	**-0.254**	**0.334**	0.048	-0.059
p_14 (green)	-0.167	0.138	0.239	**-0.273**
m (transition density)	-0.008	0.100	**0.343**	**-0.278**
A (aspect ratio)	0.058	0.110	**0.383**	-0.217

Sc (color diversity)	-0.173	-0.173	0.190	0.012
St (transition diversity)	**-0.327**	0.009	0.210	0.211
m_dS (chromatic boundary strength)	**-0.416**	0.015	-0.019	0.071
m_dL (achromatic boundary strength)	0.186	**-0.253**	-0.149	0.212

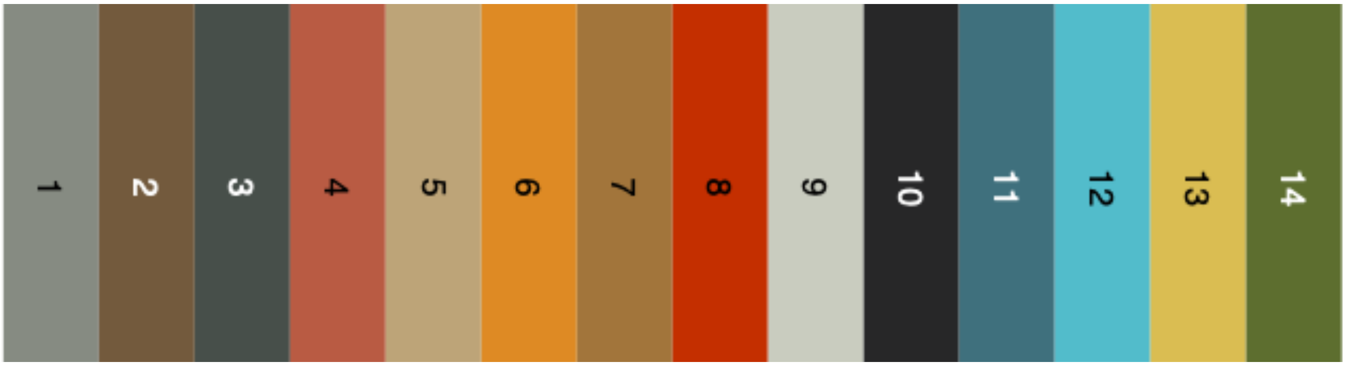


Figure 8. Colors as associated with color metric values *p_i* in Table 2 above.

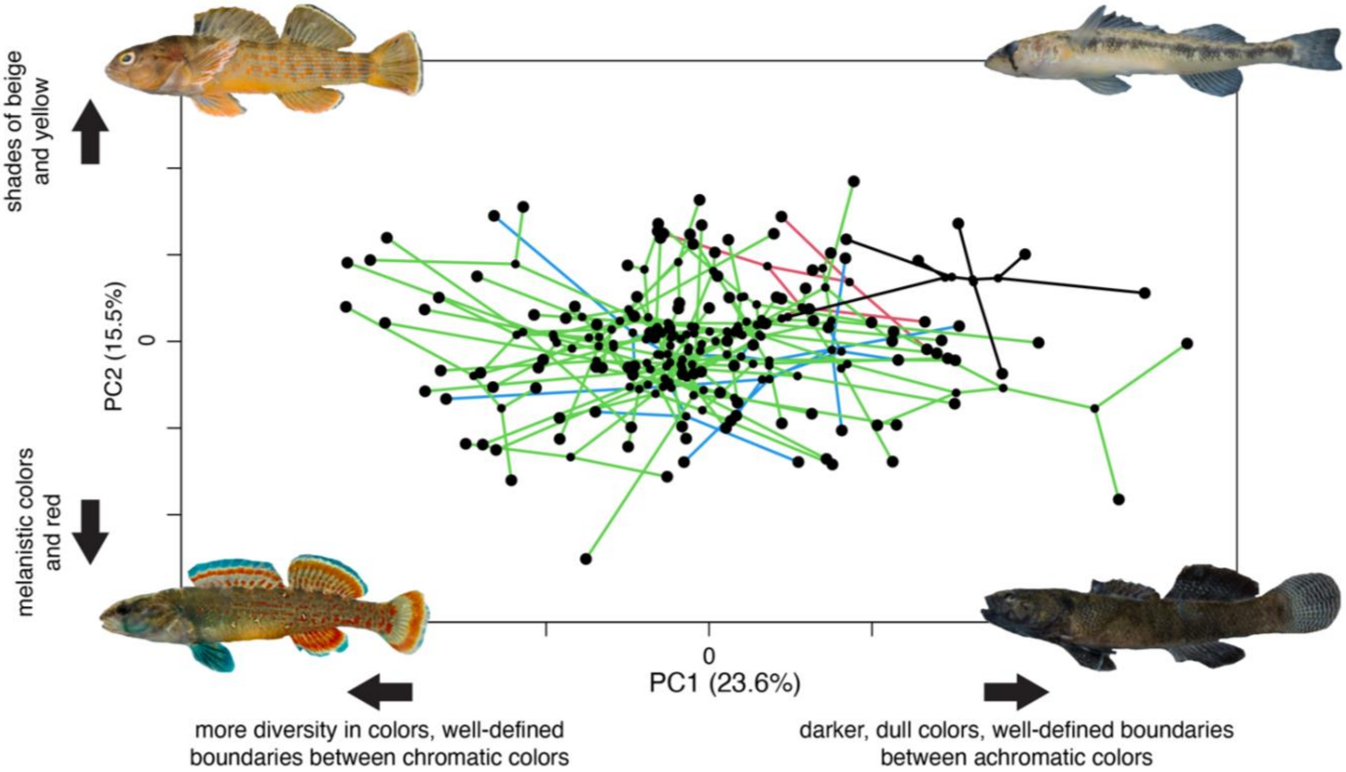


Figure 9. Phylomorphospace plot displaying PCA results along axes 1 and 2 results. Species included for reference photos: *N. camurum* (top left), *P. sciera* (top right), *E. whipplei* (bottom left), and *E. nigripinne* (bottom right). Lines show branches of the evolutionary tree connecting the species, with ancestral values at nodes estimated. Lines are colored by genera: Green = *Etheostoma*, Blue = *Nothonotus*, Black = *Percina*, Red = *Ammocrypta* and *Crystallaria*.

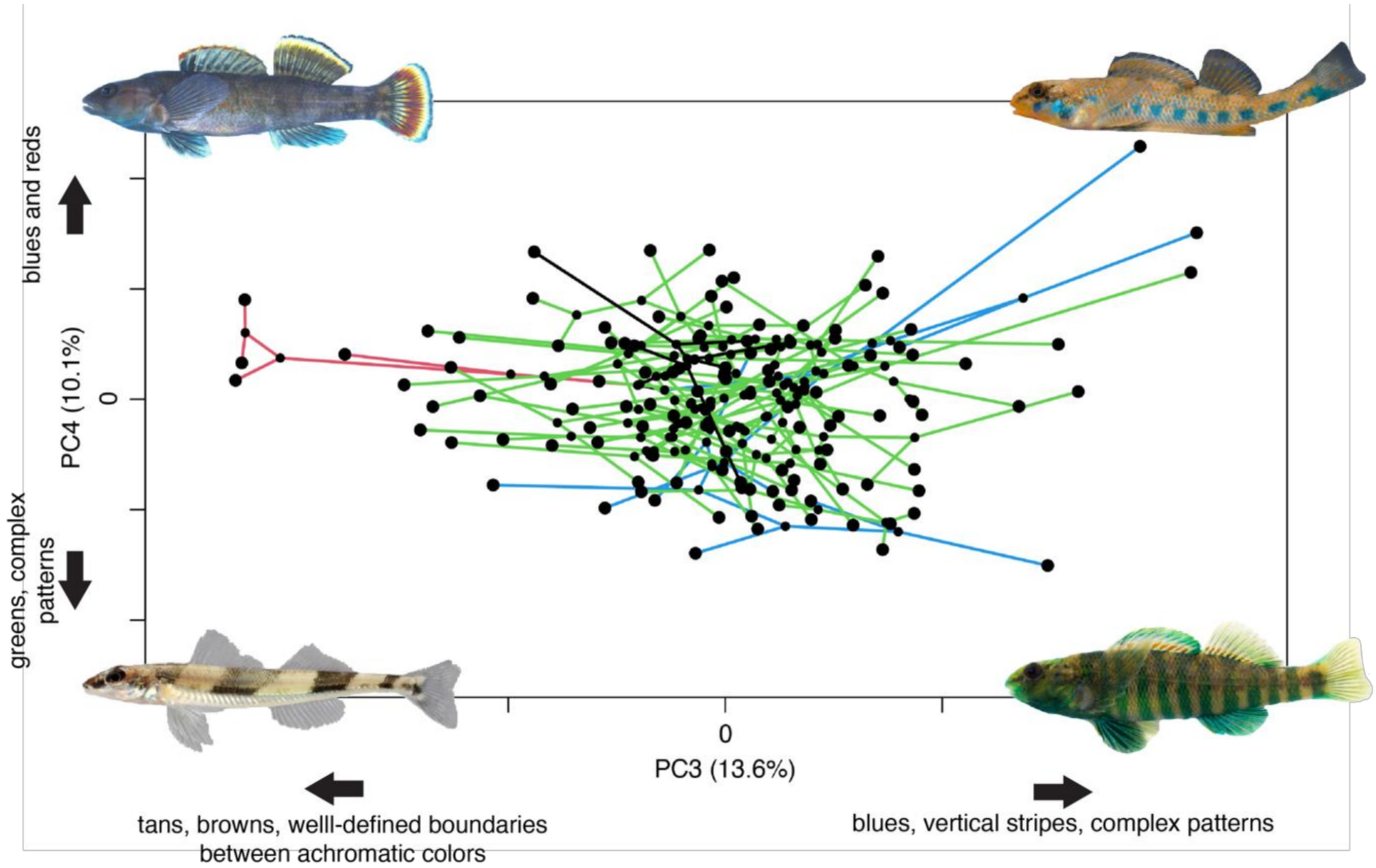


Figure 10. Phylomorphospace plot displaying PCA results along axes 3 and 4. Species included for reference photos: *E. jordani* (top left), *E. gore* (top right), *C. asprella* (bottom left), and *E. zonale* (bottom right) See Figure X for line color legend.

The diversity of color differed across the genera of darters (as represented by area occupied in Figure 9), with *Etheostoma* being the most diverse. Overall, *Etheostoma* is distributed widely across PC1 (complex, conspicuous colors and well-defined patches) and PC2 axes (pale-dark), with a wide range of species coloration. *Etheostoma* species range from species with bright, complex banding (e.g., *E. caeruleum*), to species in *Goneaperca* like *E. crossopterum* and *E. nigripinne*, with largely dark, simple patterns (with the exception of tail banding). *Nothonotus'* range is similar to that of *Etheostoma*, with a slightly smaller and more moderate distribution across PC1 (no extreme positive or negative PC scores) and is slightly more associated with negative PC2 loadings that represent dark coloration in the data. *Percina* is situated along the positive values of PC 1 and 2 axes, representing pale and less complex, more poorly defined patterns. *Ammocrypta* and *Crystallaria* are similar to *Percina* in their position in the phylomorphospace but are associated slightly more with positive PC2 scores than any of the other genera. Basal genera like *Percina* and *Ammocrypta* show low overlap with "derived" genera like *Etheostoma* and *Nothonotus* in PC1 and PC2 space.

On PC axes 3 and 4, *Etheostoma* is once again widely distributed, aside from trend in occupying slightly negative value space along PC4 (more greens and complex patterns, such as in *E. zonale* and *E. lynceum*) (see Figure 10). *Nothonotus* has a spread-out distribution across both PC axes including two species, *N. rufilineatum* and *N. douglasi* which occupy areas with extreme positive values on both axes. *Percina* is largely situated around the midpoint of both axes, save *Percina copelandi* which occupies a space with negative PC3 values and slightly positive PC4 values. *Ammocrypta* and *Crysallaria* are situated at negative values along PC3 and occupy space along PC4 near zero. *Percina* shows greater overlap with *Etheostoma* on PC3 and PC4, while *Ammocrypta/Crystallaria* remains well separated from *Etheostoma* and *Nothonotus* on PC3.

Macroevolutionary Trends in Darter Color

Disparity through time analyses conducted across all PC axes revealed positive MDI values (Figure 11). This may indicate either increasing rates of evolution towards the present or strong selection towards a single optimum value. PCs 1 and 3 subclade disparities dropped below the DTT curve of BM simulated character histories for a brief time before maintaining higher subclade disparity values than that of BM evolution towards the present. PCs 2 and 4 subclade disparity was found be higher than subclade disparity under BM evolution from the beginning of relative time for the clade. The calculated MDI value across all PCs is 0.216 (per axis values: PC1-MDI = 0.148, PC2-MDI = 0.380, PC3-MDI = 0.230, and PC4-MDI = 0.230) (see Figure X.). These overwhelmingly positive MDI values suggests that subclade disparity (i.e., trait evolution) has increased towards the present. P-values associated with each PC axis were all equal to 1 (based on a lower tail test). Overall, modern color diversity in darters likely originated recently, rather than in early divergence events between the different genera.

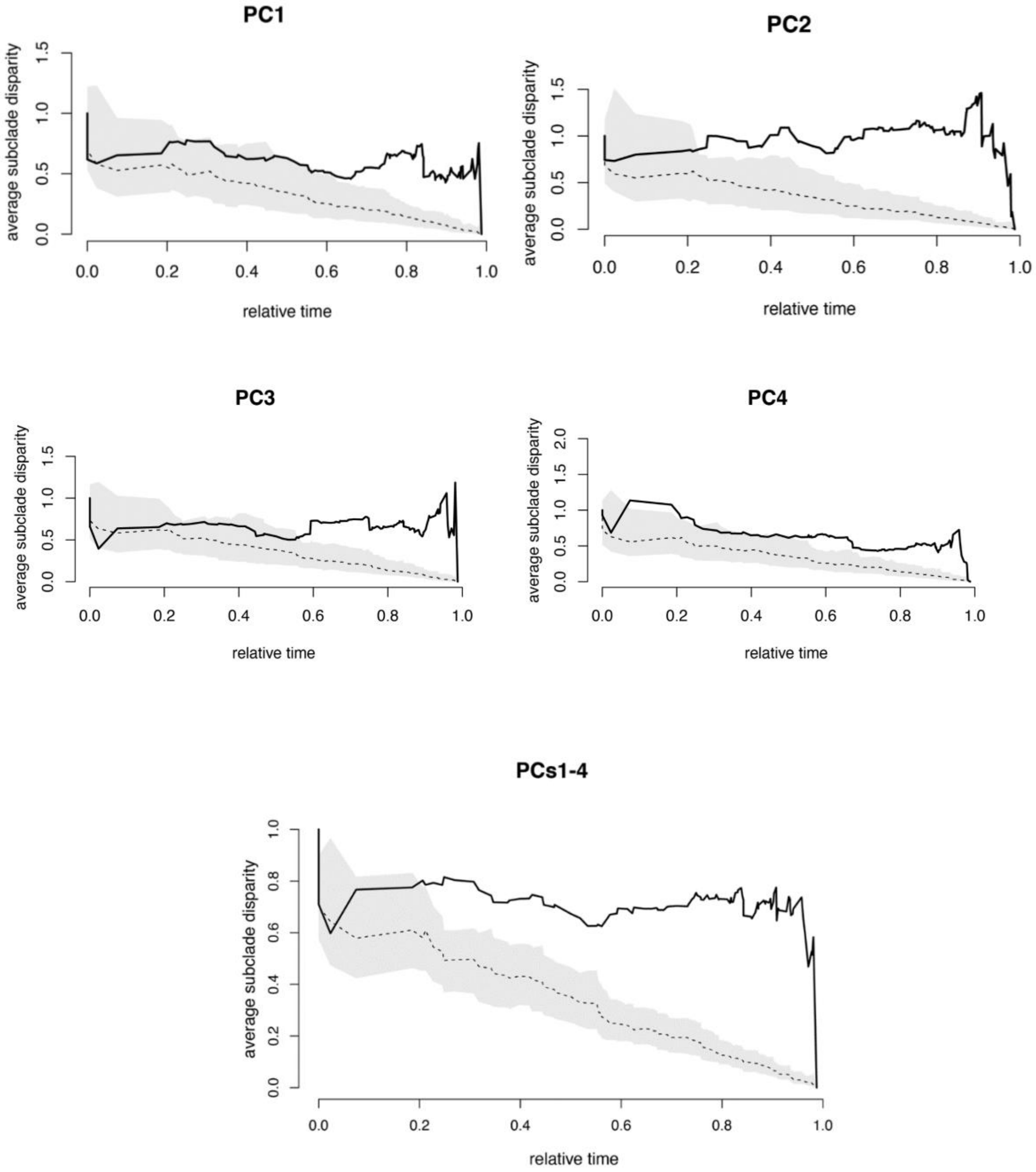


Figure 11. Disparity through time analysis plots across all four PC axes. Positive MDI values across all PC axes indicate subclade disparity has increased towards the present.

Multirate BM models allowing a variable evolutionary rate parameter across the darter phylogeny reveal variable rates of color evolution across the various clades of darters. Overall, across all four axes, rates of evolution were highest among recent clades, rather than among basal branches. This is consistent with our prior DTT results indicating greater diversification towards the present. The highest rates of PC1 trait evolution are seen in subclade Gemmaperca and other fast-water species of *Etheostoma* such as the firebelly and bandfin darters (*E. pyrroghaster* and *E. zonistium*) and genus *Nothonotus* (see Figure 12). PC2 rates are more variable across the phylogeny than seen in PC1 (see Figure 13). PC2 evolutionary rates were highest in *Ammocrypta* species and dispersed seemingly randomly throughout *Etheostoma*. Lowest rates are observed in genus *Crystallaria* as well as three *Etheostoma* species (*E. mariae, E. serrifer,* and *E. fusiforme)*. PC3 rates are substantially slower across the phylogeny than in PCs 1 and 2, with fast rates present in only a handful of *Etheostoma* and *Nothonotus* species (see Figure 14). Rates are lowest in the basal genera of *Ammocrypta* and all but two species in *Percina*. Trait evolution across PC4 shows high rates returning to genus *Nothonotus* as seen in PC1 (see Figure 15). Lowest rates were low in *Crystallaria* and all species of *Percina* but two, and randomly dispersed randomly throughout *Etheostoma*.

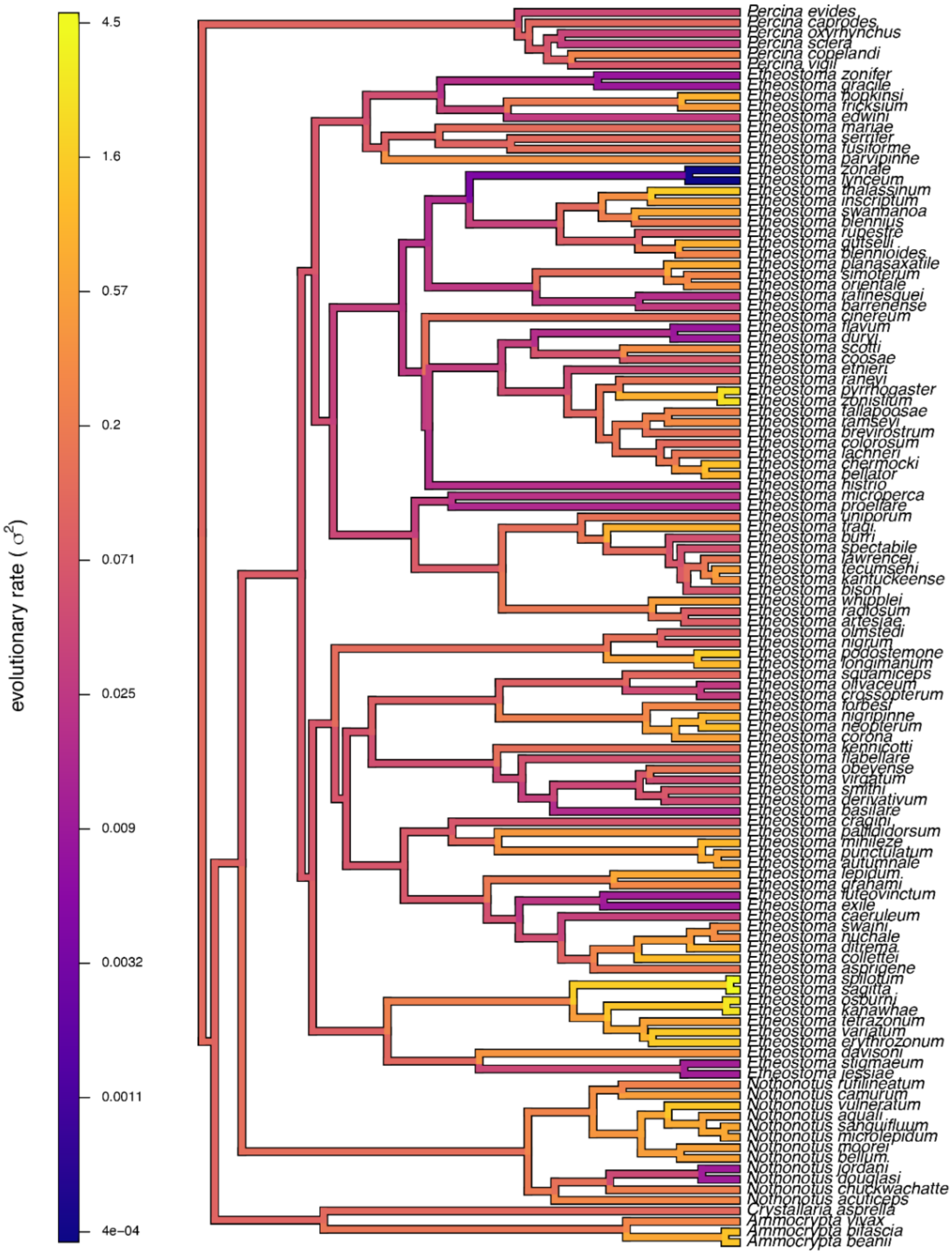


Figure 12. Evolutionary rates across PC1 demonstrate trends of fast rates largely across Gemmaperca and other fast-water species in genus *Nothonotus*.

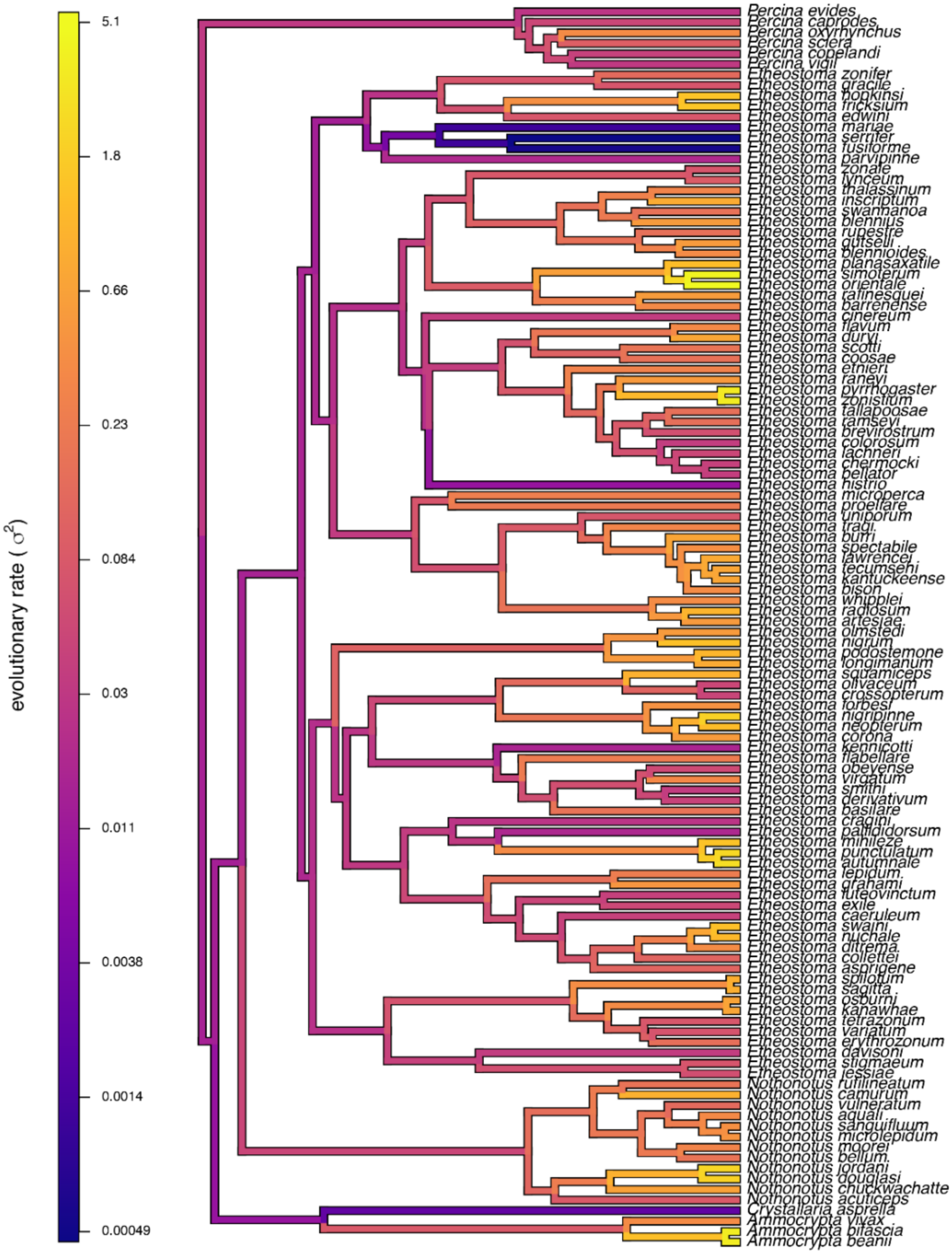


Figure 13. Evolutionary rates across PC2 demonstrate trends of fast rates more randomly dispersed throughout the phylogeny, with particularly high rates in *Ammocrypta* and in some *Etheostoma* species that are habitat specialists.

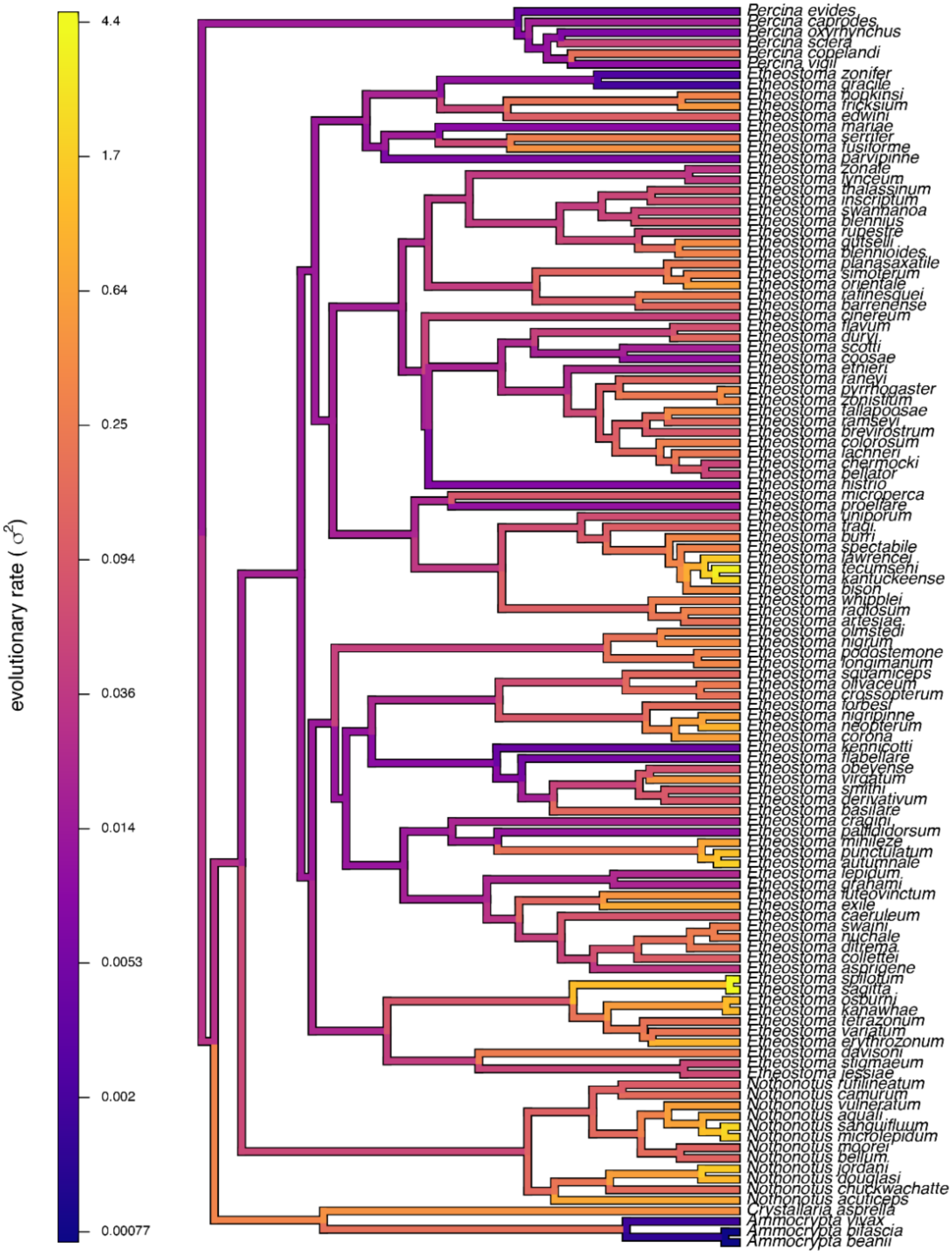


Figure 14. Evolutionary rates across PC3 are less variable than across PC1 and PC2 axes. Rates in basal groups such as *Ammocrypta* and some *Percina* species are lowest.

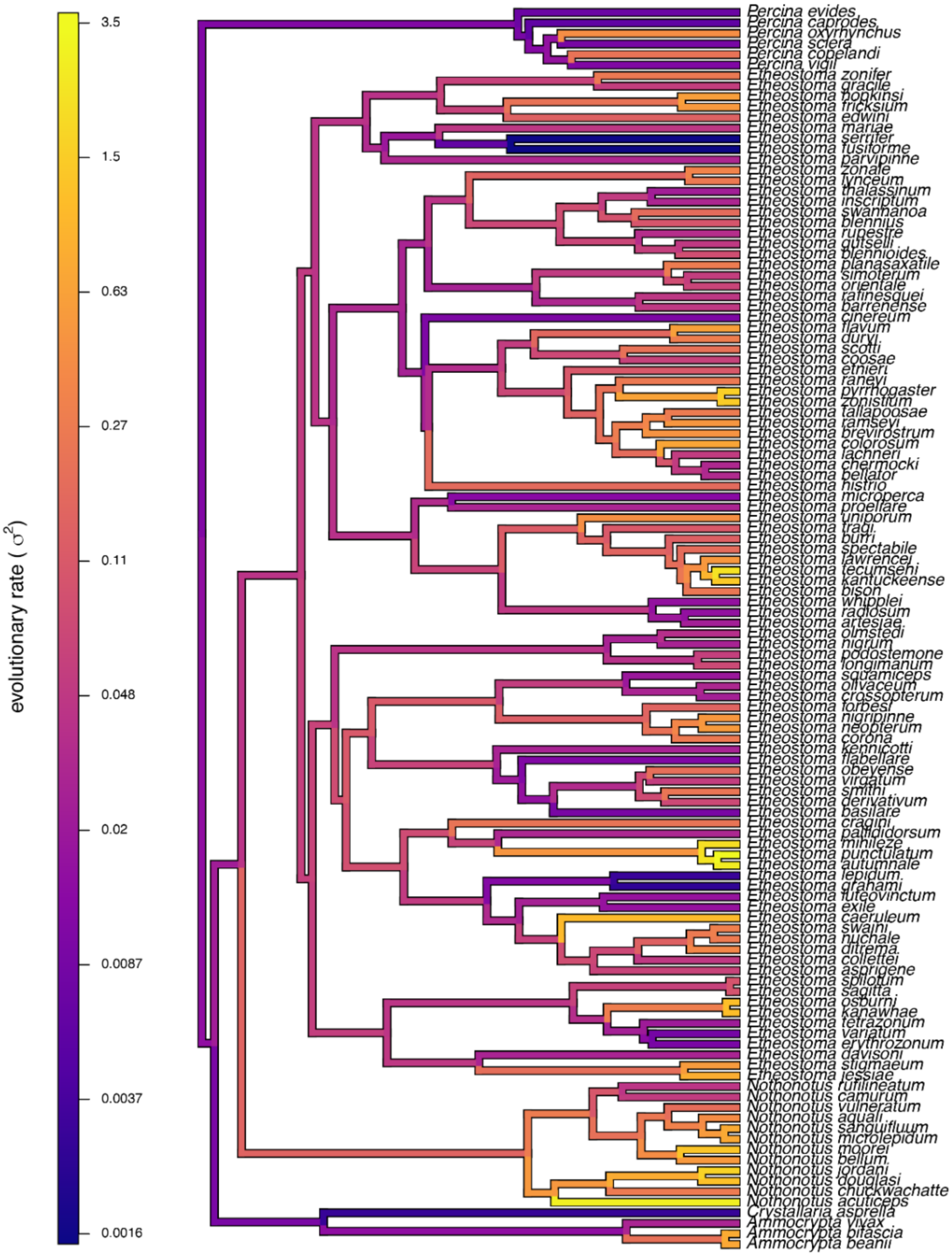


Figure 15. Evolutionary rates across PC4 demonstrate high rates in *Nothonotus* and low rates in *Crystallaria*, some *Etheostoma* species, and two *Percina* species.

The Impact of Habitat on Darter Color Diversification

We found that habitat was a significant predictor of darter color (p = 0.03). The canonical correspondence analysis conducted on the habitat and color data suggests that habitat explained ~33.6% of color variation across darter species. Figure 16 illustrates the various habitat variable relationships with color metric data from adjacency analysis. Habitats with high discharge, velocity, and drainage values (i.e., large rivers) were associated with achromatic color metrics (grey/white/black colors and patterns comprising dark and light contrasts), blues and to a lesser extent more vertical banding. Exemplars of such habitat-color relationships would include the vertically striped, pale/tan logperch (*Percina caprodes*), as well as the pale, saddled (vertical bands) Crystal Darter (*Crystallaria asprella*). Habitats with characteristics such as high slope, high elevation, and rockier bottoms (small, upland, rocky streams) were associated with more bright colors (i.e., green, red, gold, etc.), horizontal banding and higher transition diversity (*St*) representing varying and complex patterns. Example species from these habitats include the splendid darter (*E. barrenense*) with bright red and a horizontal mid-lateral band, as well as rainbow darters with red, blue, orange and grey colors in alternating patches with varying orientations on the body and fins.

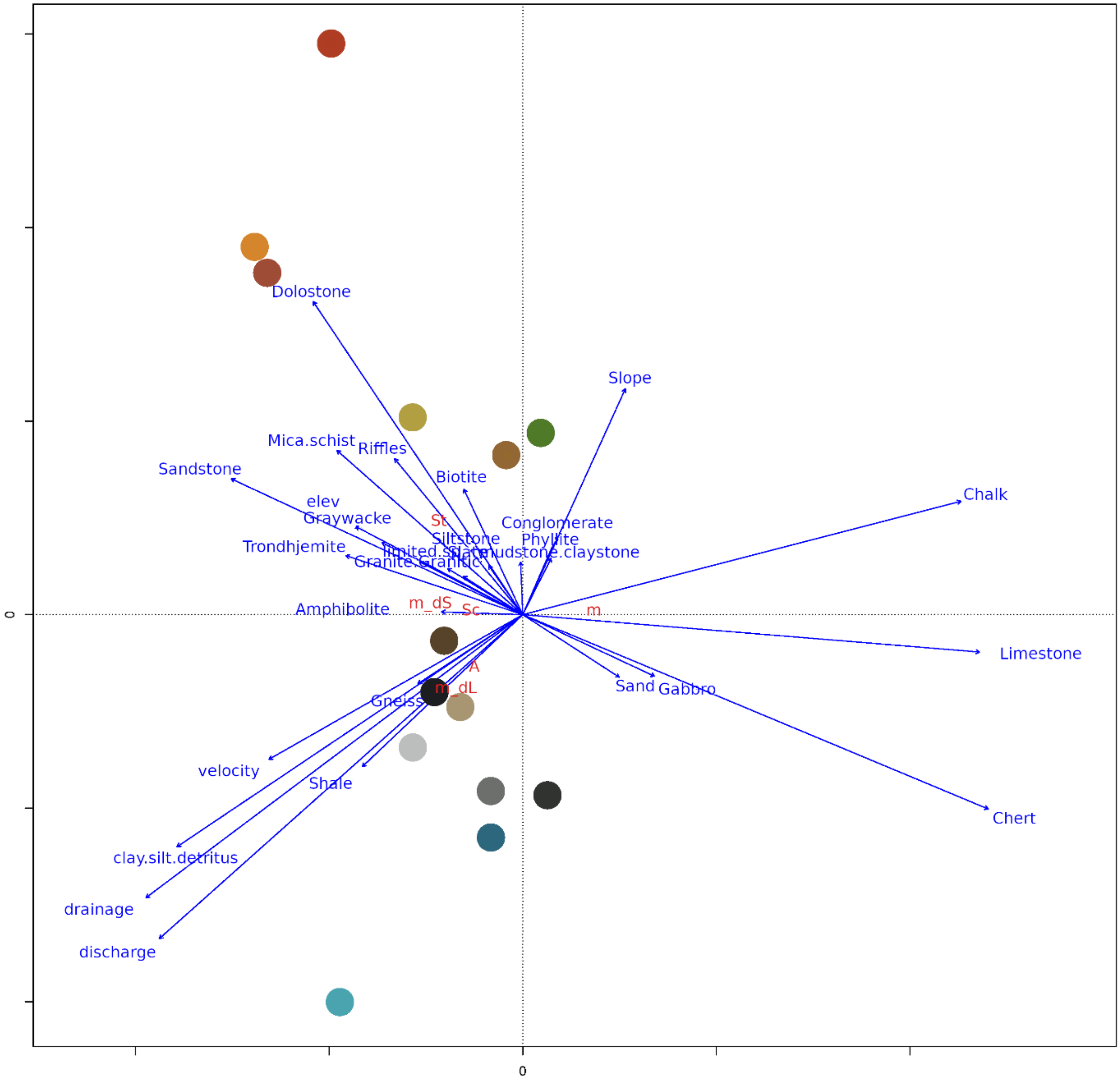


Figure 16. Canonical correspondence analysis biplot representing correlations between habitat (blue arrows) and color pattern metrics (points and red letters).

The cluster analysis initially separated larger rivers with softer sediments (sand, silt, detritus) from small rivers with rocky substrates (limestone, shale, dolostone, etc.). Within these two groups, rivers were further divided into: large, low elevation rivers with soft sediment bottoms, medium rivers with shale, small rivers at high elevation, and extremely small rivers with limestone bottoms. Using the *phytools* package, a SIMMAP ancestral state reconstruction was created of the four habitat groups onto the Percidae phylogeny (see Figure 17). We found mixed support for a given ancestral habitat in the darter clade, although our sampling across the different clades is incomplete.; throughout radiation of the Percidae phylogeny, we see large and medium sized rivers become common among the basal lineages of *Percina, Ammocrypta,* (large rivers) and a few *Nothonotus* species (medium sized rivers). *Etheostoma* species are largely associated with the small high elevation streams and tiny limestone streams, with a small number of reversals to larger river systems (e.g., among some lineages of *Hololepis*, the ‘swamp’ or ‘backwater’ darters).

Our ancestral reconstruction of large vs. small rivers (referred here as rivers vs. streams) incorporating riffle and pool use showed a similar pattern to the four-habitat-classes results above. *Percina/Ammocrypta* and *Crystallaria* were more likely to be found in (large) rivers, while *Etheostoma* was typically found in (small) streams, although some species in *Etheostoma* were also associated with rivers (see Figure 18). However, pool and riffle species were more phylogenetically clustered within large and small river habitats. For example, stream-pool species were common among the Oopareia clade in *Etheostoma* (the “barcheek” darters, *E. basilare* and *E. smithi*), while all *Nothonotus* were consistently reconstructed as river-riffle species.

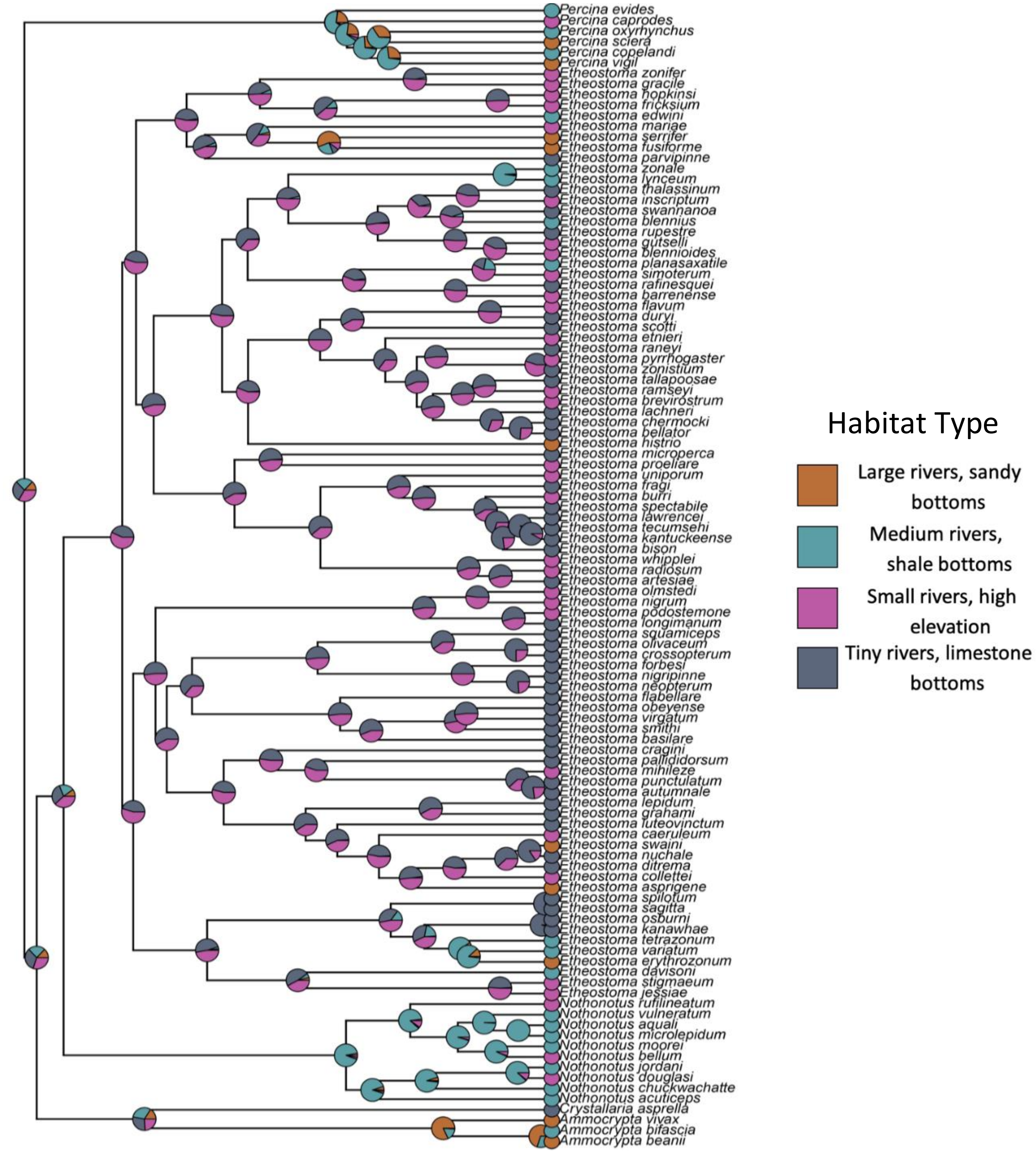


Figure 17. Phylogenetic tree produced by “make.simmap” in the R package “phytools” demonstrates shifts in habitat trends across the *Etheostomatinae* phylogeny using four major habitats classifications selected using cluster analysis.

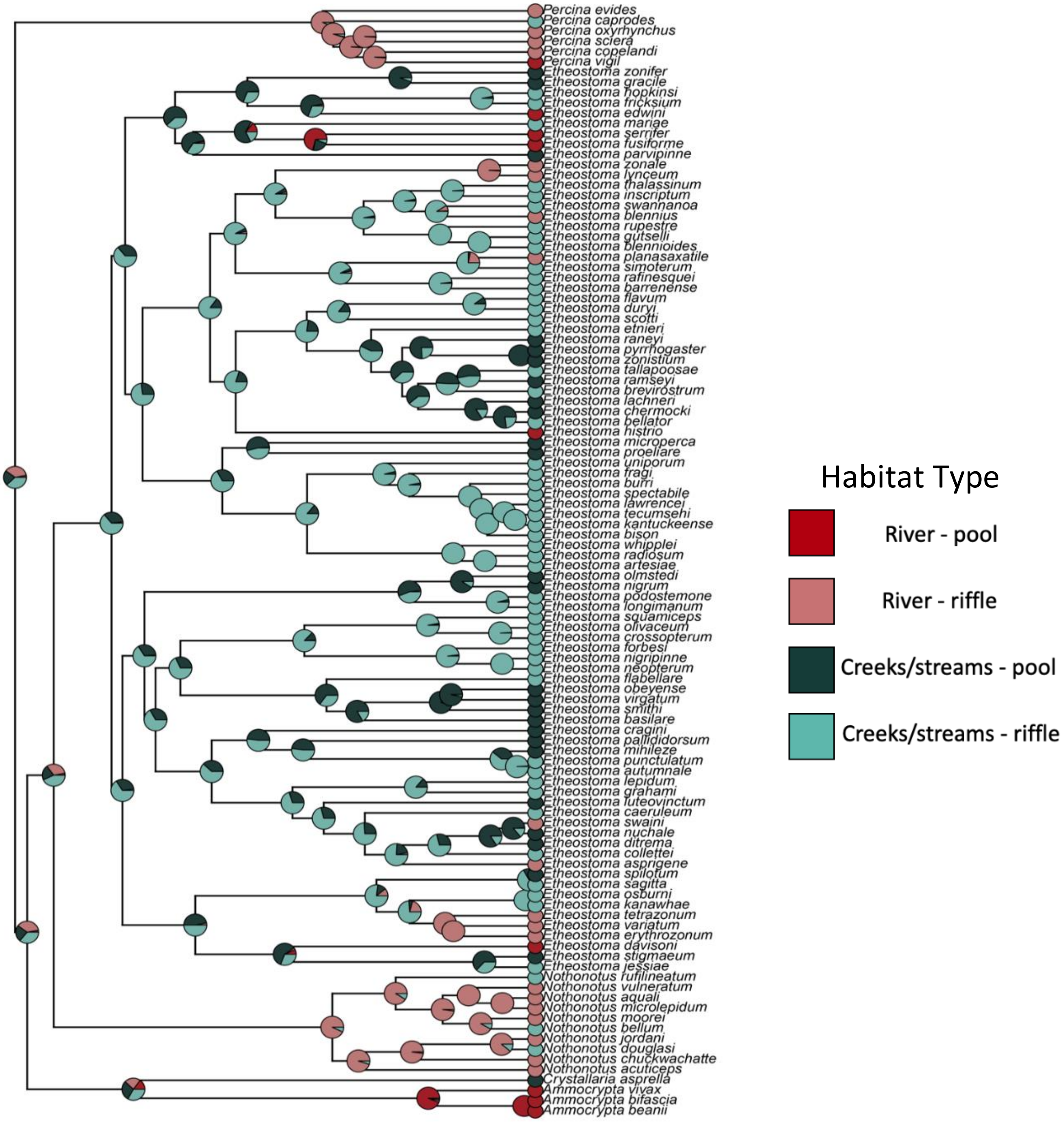


Figure 18. Phylogenetic tree produced by “make.simmap” in the R package “phytools” demonstrates shifts in habitat trends across the *Etheostomatinae* phylogeny using river size (large vs. small) combined with pool and riffle habitat as characters.

We found strong evidence that habitat impacts the rate of diversification of color pattern in darters. A single rate multivariate BM model was never supported over the habitat-dependent diversification models tested (Table 3). We found similar support for the four-habitat-classes model (large, medium, small and tiny) as well as the model incorporating river size and riffle/pool use (e.g., large river riffle, large river pool, etc.). In the model incorporating four habitats (large rivers to tiny limestone streams), diversification rates were elevated in the most extreme habitats. Rates were elevated across all four PC axes in tiny, limestone streams, and along PC1 and 2 in large habitats. Rates were similar to each other in medium, shale-bottomed rivers and small, high elevation streams (Figure 19). This observation was surprising, given the prevalence of relatively plain, pale species in large rivers in *Percina* and *Ammocrypta* (Figures 17 and 18) and the limited PC area occupation of these clades. Indeed, in comparing the complexity of color (*Sc*), the complexity of pattern (*m*) and the proportion of conspicuous colors like blue, red and green, all three shows low values in large rivers (albeit with substantial variability in color diversity). However, some large river species in riffles show more complex patterns and colors (e.g., *Etheostoma erythrozonum* and *Etheostoma swaini*).

Table 3. ΔAIC values from trait evolution model fitting using mvMORPH. Bold values indicate those showing strong support (ΔAIC across a large portion of habitat reconstructions).

Model	**Median ΔAIC (min, max)**	**Percent Best Supported Model (ΔAIC = 0)**
BM1 – Brownian Motion	.92 (16.37, 74.56)	0%
BMV2– Riffle/Pool	37.79 (7.52, 73.60)	0%
BMV2 – 2 Habitats: Rivers & Creeks/Streams	39.69 (11.81, 70.39)	0%
BMV4 – 4 Habitats (large medium, small and tiny rivers)	2.59 (0, 43.71)	**47%**
BMV4 – 2 Habitats: Rivers & Creeks/ Streams + riffle & pool in each	0 (0, 48.31)	**53%**

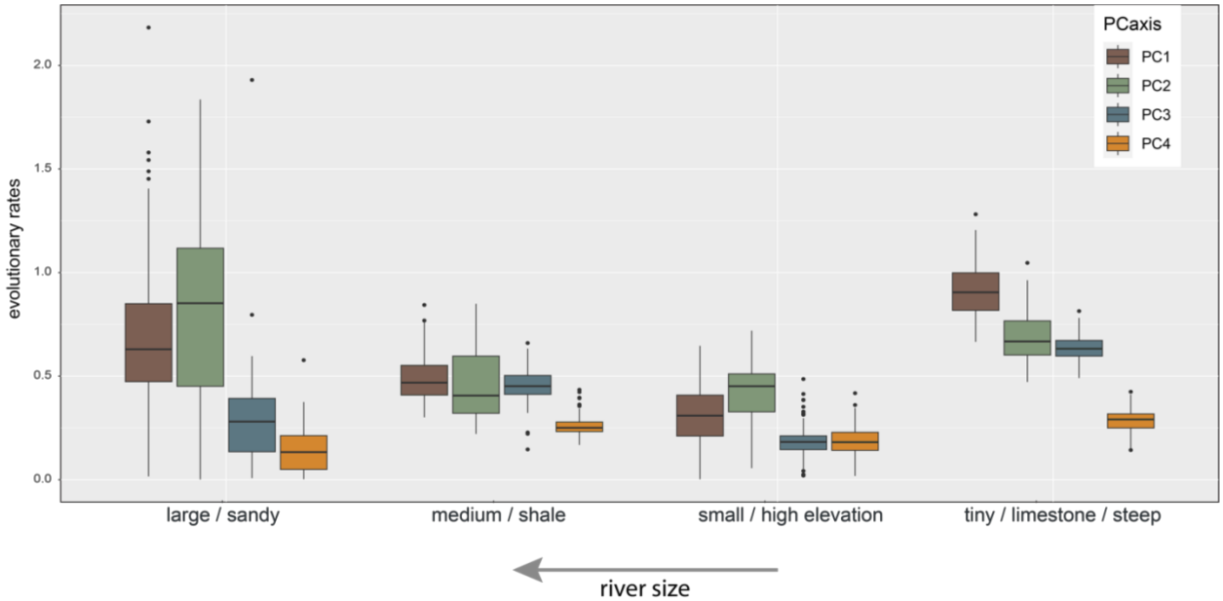


Figure 19. Evolutionary rates across the four-habitat-classes model (BMV). Rates across PC axes 1, 3, and 4 are highest in tiny streams. PC2 rates are highest in large rivers with sandy bottoms.

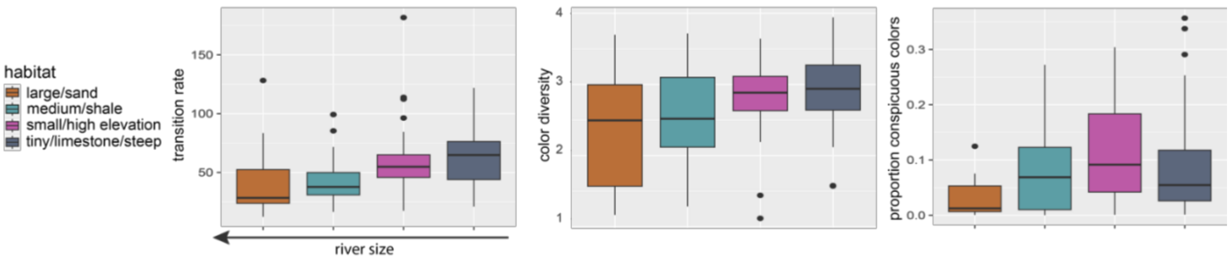


Figure 20. Habitat and color metric interactions. Color diversity tends to be associated with small high elevation rivers and tiny rivers with limestone bottoms. Large rivers with sandy bottoms tend to have small proportions of conspicuous colors and fewer transitions between colors than other habitats.

Riffle and pool habitats showed differing rates of color diversification, but the patterns diverge between riffle and pool habitats in each case. In small creeks and streams, riffle habitats showed consistently high rates of color evolution compared to pool habitats (Figure 21). Also riffle habitats in large and small streams showed overlap in rates of evolution, in particular across the two most important axes (PC1 – diverse colors, complex and distinct patterns, and PC2 – pale vs. dark coloration), though PC3 (blues, complex patterns, vertical stripes) was consistently elevated in small streams compared to large rivers.

However, diversification rates varied greatly in large river pool habitats by comparisons (Figure 21). Evolutionary rates in river pools were low compared both to large river riffles and small stream habitats, with the sole exception of PC2 (pale vs. dark coloration). On PC2 large river-pools showed highly elevated rates of evolution compared to all other habitats. Therefore, large river-pool species diversify primarily on an axis of color darkness, in contrast with all other darter habitats, which diversify more broadly on axes incorporating pattern complexity, color diversity and bright colors (Figure 22). Interestingly, while we find pattern complexity, color diversity, and conspicuous colors were both slightly elevated in smalls streams compared to large river habitats. However, riffle species showed markedly higher proportions of conspicuous colors (red, blue, green) compared with pool species, regardless of river size. Very few large river-pool species showed conspicuous colors.

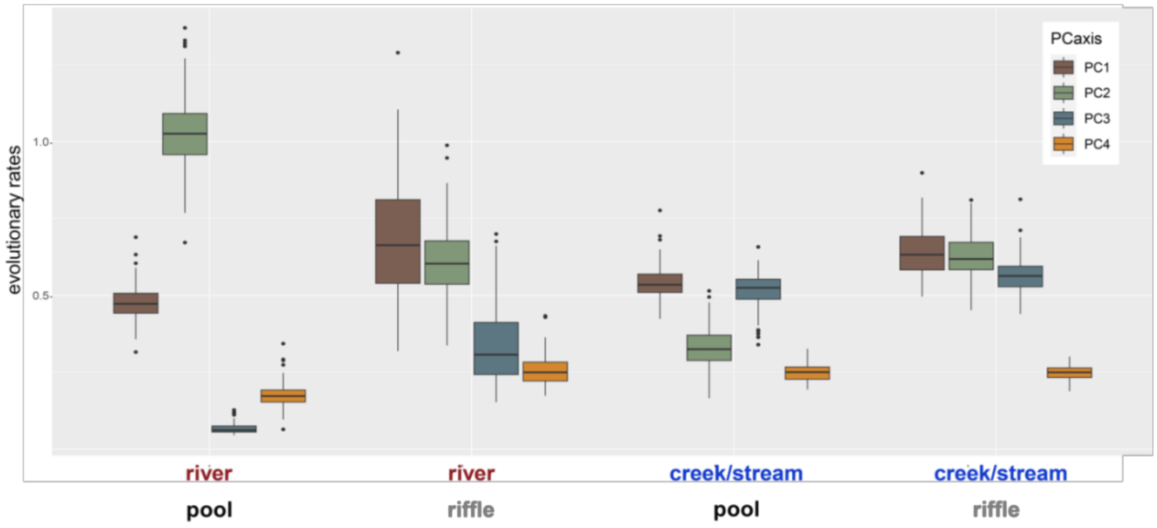


Figure 21. Evolutionary rates across the river size and riffle/pool use model. Rates are highest overall in riffle habitats of both sizes except for PC2 rates in river-pools.

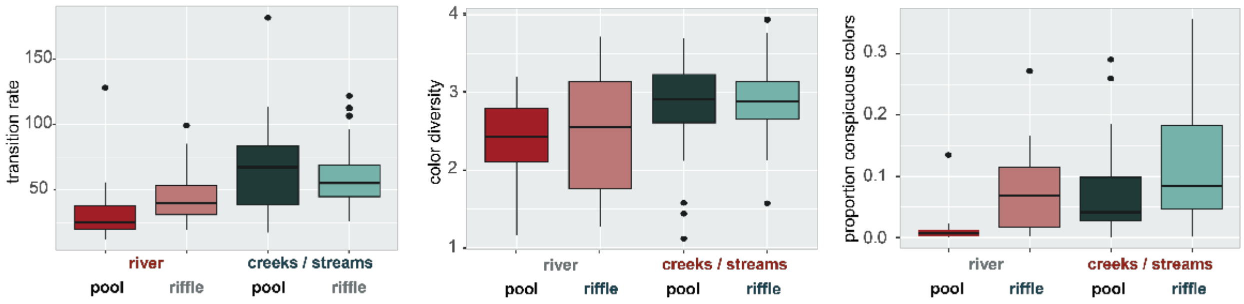


Figure 22. Pool and riffle habitats in both river and creeks/streams and their interactions with color metrics. Proportions of conspicuous color are highest in riffles in both systems. Color diversity and transition rates are higher in creek/stream systems.

DISCUSSSION

Coloration serves as a pillar of an organism's life strategy via communication, whether intraspecific or interspecific, predator avoidance, thermal regulation, sexual selection, and many other facets of existing and succeeding in the natural world. Within fishes, diverse colours and patterns are common across tropical clades in coral reef communities, and some freshwater habitats. Among temperate freshwater radiations, few clades offer the number of species and diversity in color and pattern than in our study group, *Etheostomatinae*. This study's objective was to quantify darter coloration and understand what factors may have contributed to the diverse colors and patterns observed in this remarkable radiation of fishes.

Color Evolution in Darters is Not Neutral

We found that color pattern and diversity was non-randomly distributed across darters. While more "basal" clades like *Percina* and *Ammocrypta* showed simple, pale colors, more "derived" groups like *Etheostoma* and Nothonotus show considerable variation in colors, transitions between colors, conspicuity, pattern complexity and structure and boundary strengths. For example, across PCs 1 and 2, we see *Etheostoma* widely represented. *Etheostoma* is also the most diverse genus within *Etheostomatinae*, whose coloration varies from species with achromatic colors with limited patterns (i.e. *E. crossopterum*) to species with brilliant bright colors and diversity in pattern (i.e. *E. caeruleum*, *E. osburni*). Like *Etheostoma*, *Nothonotus* is broadly distributed across PC1 but slightly more associated with loadings representing dark/bold coloration on PC2. Across PCs 3 and 4, we see *Etheostoma*, again, widely distributed across PC4 aside from some species associated with more greens (i.e., *E. zonale, E. lynceum*) situated at slightly more negative values. *Nothonotus* begins to take on a more spread-out distribution across axes 3 and 4, with some outlier species, *N. rufilineatum* and *N. douglasi* occupying areas of largely positive values across both axes.

Trait diversity may be generated "neutrally" through random walk processes like genetic drift (Wainwright, 2007); under such processes older clades may show greater trait diversity than younger clades. However, our results indicate that diversification has not been even across the evolutionary radiation of the darters. DTT and evolutionary rate model fitting suggest that color and pattern diversity in darters has largely originated recently, potentially through fast evolution in some recent clades. While positive MDI values can be generated under both accelerating rates or strong selection towards a central optimum (Slater et al, 2012), we see evidence of higher rates in both 1) more recently occupied habitats and 2) younger clades, and the strong divergence between the color traits in *Percina/Ammocrypta/Crystallaria* and *Etheostoma/Nothonotus* suggests that selection for colors has varied across the darter radiation. Interestingly, a similar analysis of color pattern in marine butterflyfishes also observed a broad trend of increasing evolutionary rates towards the present (through similar analyses to those observed here) (Alfaro, 2019).

Habitat Structures Color Diversity in Darters

Habitat was associated not only specific coloration attributes (colors, complexity, diversity, etc.) but also with the rate of diversification of color across darters. We found brightly and complexly colored darters (especially with reds and oranges) to be associated with small rocky rivers and riffles, and more achromatic, whereas darters with simple, achromatic colors to be associated with large rivers with softer substrates (sand, silt) or with pools. *Etheostoma* species are often found in small or tiny rivers and streams but can also exist in medium to larger sized rivers. In the subgenus *Hololepsis (Etheostoma)*, the influence of habitat on coloration is apparent; although a closely related group of species, coloration varies greatly. Species dwelling in pool habitats across all river sizes (e.g., *E. zonifer*, *E. parvipinne*) tend to have less conspicuous coloration as compared to riffle specialists within this same subclade (e.g., *E. fricksium* and *E. hopkinsi*). Our results are consistent with previous studies that used qualitatively coded characteristics of darter coloration. Ciccotto and Mendelson (2016) for example, also found red (as a discretely coded character) to be associated with small river size. Similarly, Bossu and Near (2015) found that colorful sexual dichromatism was selected for among the benthic darter clades in *Etheostoma* and *Nothonotu*s, compared to in the more hyperbenthic *Percina* living frequently in larger rivers.

The relationship between darter color and habitat conditions may be mediated by either abiotic (light environment) or biotic (presence of predators) factors. Although brighter coloration is preferred by females in many species (Williams and Mendelson, 2013), and the presence of conspicuous colors like red, blue and green are favored by females of some darter species (Ciccotto and Mendelson, 2016; Williams and Mendelson, 2011), there are also ecological tradeoffs such as the presence of predatory fishes that likely influence the evolution of chromatic colors. Ciccotto and Mendelson (2016) suggested that the relationship between specific darter colors (e.g., red) and river size/substrate was likely driven by a correlation with predator abundance. Our results are consistent with these prior observations; where predators exist in higher concentrations (e.g., pool habitats in rivers and streams), the risk of possessing/evolving bright colors easily seen by the predatory fish may outweigh the development of bright nuptial coloration solely to attract females (as seen in *E. zonifer* and *E.*

parvipinne). Environments where predators are fewer (e.g., shallow riffles), we begin to see more conspicuous colors, in particular red and orange, present across species (e.g., *E. fricksium* and *E. hopkinsi*). Ciccotto and Mendelson (2016) found that largemouth bass (Micropterus salmoides), a major predator of darters, were more likely to attack red targets over black or blue. The small streams and riffles occupied by some species would be difficult for these bass, especially large adults, to navigate and hunt in. Interestingly, the only conspicuous color associated with large rivers and pools in our results was blue, a color which was shown in feeding trials to not be preferred by the largemouth bass (Ciccotto and Mendelson, 2016).

In addition to threat of predation, light penetration in the water and other properties of the environmental media (e.g., clarity/visibility) could also play a part in what colors darters evolve. Light attenuates rapidly in water and colors lost to this attenuation and absorption are reds and oranges/yellows. Blues, violets, and greens can penetrate further into the water column. Many of the riffle specialist species observed (e.g., *N. camurum*, N. *rufilineatum* and *E. simoterum*) possess reds and oranges and in their nuptial coloration. In habitats like riffles and even shallow pools, reds and oranges are common colors, likely because they can be spotted easily by potential mates and/or kin. Colors that penetrate further into the water column (e.g., blues, violets and greens), are less common not only in our study group, but across fish clades in general. Blues, purples, and some greens are structural colors; perceived colors that are a product of light reflection/refraction off guanine crystals within chromatophores. (Bagnara, 2007).

Conspicuous colors and complex patterns were associated with small, upland streams, which would have lower silt loads and often greater visibility than larger downstream rivers.

Improved visibility may also contribute to the rapid evolution of bright colors in small river habitats. In *Pundamilia*, a genus of African lake cichlids, female selection on coloration tends to be influenced by the photic conditions in which they inhabit (Maan, 2010). This concept is impactful to the previous idea that females in species that are sexually dichromatic solely prefer males with brighter coloration (Bossu and Near, 2015). In this study and another performed by Candolin (2016), females from more turbid environments have more relaxed mate selection when encountering bright males. This weakening of sexual selection on male signals could explain hybridization and speciation among species occurring in the same river system.

Among the subgenus *Catonotus* (*Etheostoma*) numerous species tend to be found living in crevices among rocks, such as the fantail darter (*E. flabellare*), fringed darter (*E. crossopterum*) and blackfin darter (*E. nigripinne*). These species do not exhibit conspicuous colors, despite being found in small rivers, but vary predominantly among tan/grey/black colors (especially low on PC2) and appear to evolve more rapidly on PC axis associated with lightness/darkness (see Figure 9) compared to other *Etheostoma* clades. Their more protected location from predators thus does not appear to have facilitated more conspicuous colors, supporting the role for light conditions and visibility in darter color diversification in these habitats as well.

Complex patterns may also improve crypsis against rocky/complex habitats in small, upland streams. Hulse et al (2020) utilized image analyses to compare the Fourier slopes of images containing male darters in nuptial coloration and images of stream background. The Fourier slopes of the images were significantly correlated, indicating that the stream background and the patterns of males in the study were similar in aspect of luminance and other photic properties. Because females of these darter species have often shown selection on males with more complex coloration, it can be speculated that sensory drive, the idea that visual signals are shaped by the receiver's sensory system and the habitat in which they exist, is partially responsible for complex nuptial coloration in many darter species. It is not fully understood as to why females would prefer males with patterns akin to the spatial statistics of their environment, however, Winkielman et al (2003) suggests that efficiently curated pattern (i.e., one similar to natural environments) is most attractive to conspecifics.

The Role of Benthic Lifestyles in Darter Color Evolution

Some macroevolutionary models of trait evolution, like the "radiation in stages model" propose that traits evolve hierarchical, often first along lines of habitat, then diet and lastly traits associated with sexual selection (Streelman and Danley, 2003). Our results are suggestive that the recent diversification of color in the darter clade may have been facilitated by adaptation to prior niches such as habitat. Unlike their darter counterparts, some of the most well-known Percids are large-bodied, pelagic game fishes (Perch, Walleye, Sauger). The comparatively small size of darters is likely an adaptation to benthic living (Carlson and Lauder, 2011) and appears to be under strong evolutionary selection in the darter clade. Further adaptation associated with feeding morphology, including the evolution of more ventral mouths (Clark and Arbour, *in prep*) and large cranial muscles (Arbour et al., *In Review*) facilitated diversification in to small, rocky headwater streams, as are commonly occupied among colorful *Etheostoma* and *Nothonotus* species. Therefore, the prior selection for small body size may have driven the colonization of habitats that facilitated the evolution of diverse and bright coloration among darters.

Additionally, while hyperbenthic species are rare across the darter radiation in general, several (e.g., logperch) are found among the genus *Percina*. These species may possess a rudimentary swim bladder and are able to swim above the bottom, which may improve evasiveness against predators. These species are largely plainly colored, with only the tangerine (*Percina aurantiaca*) and gilt (*Percina evides*) darters, showing substantial conspicuous coloration (yellows and oranges, but not red, blue or green) on the body, with most other species at best possessing a small band of yellow or orange on the concealable dorsal and anal fins. These less colorful darters were often found in large rivers, even though hyperbenthic species may be more evasive and less susceptible to predators (which could reduce selection against conspicuity). This is consistent with our suggestion of an association between benthic-living and colonization of small-stream habitats, which subsequently facilitated color evolution.

While not nearly as diverse as darters, a few non-Etheostomatinae percids are also rheophiles inhabiting fast water environments, such as the Zingel (*Zingel zingel*). While smaller than pelagic predatory percids like walleye, these species are still larger than most darter species and typically found in larger river systems (e.g., Danube) - habitats similar to the larger and simply colored darters in *Percina*. Unlike the colorful darters of *Etheostoma* and *Nothonotus*, these are plainly colored fishes (tans/yellows/beiges) with simple cryptic brown saddle markings. These non-darter rheophiles support our suggestion that benthic-living alone did not drive color evolution in darters, but may have promoted the colonization of small river habitats that enhanced rates of color diversification and selection for bright colors.

The Macroevolutionary Importance of Color Diversification in Darters

Darters have likely speciated largely through allopatry (Harrington and Near, 2012; Hollingsworth and Near, 2009; Kim, 2023; Near and Benard. 2004), as most sister species pairs occur in separate drainages, with evidence in only rare cases for sympatric splits. As such, coloration is unlikely to play a role in the initial establishment of darter species. However, the relationship between the rate of darter color evolution and habitat suggests that color evolution may play a reinforcing role in reproductive isolation in darters.

Secondary contact between related lineages after speciation in allopatry is common in darters, for example in the rainbow darter (*E. caeruluem*) and orangethroat (*E. spectabile*) darter species complexes (Bossu and Near, 2013; Moran et al, 2019) which regularly produce hybrids (and are extremely similar in color pattern). Patterns of genetic introgression have also been repeatedly observed in molecular studies across the subfamily (Near, 2011). Some previous research has indicated that isolation through sexual signaling may be one of the earliest factors to evolve between closely related darter species pairs (Mendelson, 2007). “Results of this study indicate that **sexual isolation** between allopatric populations of *Etheostoma* will tend to evolve to completion faster than **hybrid inviability**” (Mendelson, 2003).

The speed of color evolution is enhanced in small stream and riffle habitats according to our results, and these habitats were particularly associated with the propensity towards bright, contrasting and complicated patterns which would enhance features for conspecific recognition.

As such, small river habitats may help to produce divergent color patterns that aid in sexual isolation if and when lineages (initially speciating in allopatry) come into secondary contact. Indeed, since small, fragmented, upland stream habitats may also be associated with higher rates of allopatry (Cerezer et al, 2023), there may be a synergistic relationship between these factors. Overall, the enhanced rate of color evolution in some habitats may help not only to explain the uneven distribution of color diversity in darters, but also the greater species diversity among some lineages like *Etheostoma*, which are predominantly found in small river habitats compared to their counterparts. However, comparative methods used to test for relationships between rates of speciation/extinction (so called -SSE models like quaSSE) and quantitative traits are strongly impacted by incomplete taxonomic sampling. As such, future work to fully sample color across nearly all darter species would be fruitful for investigating the enhancing impact of color diversification on darter speciation, and its relationship to selective factors like habitat.

Conclusions and Future Directions

This project is the first to quantitatively document the variation and evolution of color in the darter radiation. Trade-offs between bright and bold colors, diverse patterns, distinct boundaries on color patches were important to the diversification of major darter clades. Color across the genera within this clade is non-random; with bright colors more common in younger genera such as *Etheostoma* and *Nothonotus* while more basal genera like *Ammocrypta*, *Crystallaria*, and *Percina* possess more dull coloration. More conspicuous colors, complex colors and fasters trait evolution were associated more with smaller, higher elevation streams which possess shallow water columns, low turbidity and complex rocky substrates. These macroevolutionary patterns may be driven by environmental or biological variables such as photic environment, substrate type, threat of predation, and/or sexual selection by females. However, further studies will be necessary to investigating each of these potential variables in a macroevolutionary context. More complete taxonomic sampling in particular, would be useful in linking darter color characteristics to possible trends in darter speciation rates and species diversity. Understanding how the evolution of coloration interacts with other phenotypic attributes, such as body shape, may also contribute to our understanding of the relationship between habitat use and darter color diversification (Arbour and McQueen, *in prep*). Overall, this work has identified important trends in darter color evolution and provided evidence for selective factors contributing to these patterns and laid useful methodological groundwork for many future projects regarding coloration not only in this clade but clades across all flora and fauna.

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