ENVIRONMENT AND PHYLOGENY INFLUENCE SCALE SHAPE VARIATION IN ETHEOSTOMATINAE DARTERS

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I wish to dedicate this thesis as a humble gift to all those whose love, generosity, passion, patience and support was a catalyst of my ardor for the wild things of this world, and helped me to achieve everything that I have against all odds. Those who should be specially named are my grandparents (Fred R. I & Winifred A. Rodkey and Mildred & Virgil R. Crisman), my parents (Fred R. II & Karyn S. Rodkey), my husband (Nicholas Grady), Patrick Zollner, Courtney Mycroft, Amy & Larry Peters, and Rebecca E. Blanton Johansen.

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ABSTRACT

Variation in scale number and type has been well studied in Teleost fishes however, variation in scale shape has received less attention. Given the well-documented role of environment and phylogeny on multiple aspects of phenotype, we evaluated the impact of both on scale shape variation in darters (Percidae: Etheostomatinae). We predicted that darters with close phylogenetic relationships and/or shared ecologies would have more similar scale shapes, but this relationship would be mediated by their use of the boundary layer. We used geometric morphometrics and seven homologous scale landmarks from 30 individuals each for 92 species of darters representing all genera and terminal clades. Variables describing habitat, spawning mode, maximum body size (Size), and phylogeny were summarized from the literature. We used ordinations to examine scale shape variation among phylogenetic and ecological groups. To test for relationships between scale shape and ecological characteristics we conducted Partial Least Squares and Phylogenetic Generalized Least Squares analyses. Scale shape variation occurred within and among darter clades, and was significantly related to phylogeny, suggesting some variation is evolutionarily constrained. However, after accounting for phylogenetic signal, Size and water column position (WCP) were related to scale shape such that extra-large, midwater species had longer, narrower scales that may decrease laminar drag, and sub-benthic darters had scales that were narrower at the anterior insertion, had longer scale bodies and longer, wider ctenial margins that may facilitate burying. Among benthic darters, Size was significantly related to scale shape and may indicate that boundary layer use reduces selective pressures of drag. Consistency between our results and others from the literature provide support for environmental influences on scale shape in Teleost fishes.

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CHAPTER I: INTRODUCTION

Phylogeny imposes constraints on morphology (Guill et al. 2003a; Rodríguez-González et al. 2017; Ospina-Garcés et al. 2018), but numerous studies have also demonstrated the importance of ecological drivers of morphological diversity in both terrestrial and aquatic species (Losos et al. 1998; Zelditch et al. 2017; Watanabe et al. 2019). Fishes are the most speciose vertebrate group and correspondingly possess a diverse array of phenotypes (Moyle and Cech Jr. 2004). Variation in body size, body shape, and feeding structures are often best explained by phylogeny (Knouft and Page 2003; Ciccotto and Mendelson 2015; Geheber and Frenette 2016), although the physical properties of water, such as viscosity and incompressibility, also have a strong influence on species traits (Imre et al. 2002; Moyle and Cech Jr. 2004; Bower and Piller 2015).

Environmental influences on fish morphology often leverage predictable modifications to fins or body shape to maximize unsteady-state (highly maneuverable) swimming in systems with low or unpredictable flow (ponds, lakes, oxbows, or pools of streams/tidal zones or shallow riffles of streams) or to maximize steady-state (highly streamlined) swimming in rapid and/or unidirectional flow environments (Brinsmead and Fox 2002; Langerhans 2008; Foster et al. 2015; Wainwright 2019). However, the degree to which flow influences morphological traits can be constrained by evolutionary history with outcomes that vary among species, including those within the same aquatic systems (Krabbenhoft et al. 2009; Foster et al. 2015). Behavior may also influence the interactions between environment and morphology, as behavioral plasticity of fishes in low versus high-flow environments have been known to reverse the link between

steady/unsteady-state morphologies and local flow regimes (Krabbenhoft et al. 2009; Meyers and Belk 2014; Bower and Piller 2015).

For benthic fishes the effects of flow on morphology may be less predictable than for fishes living in the water column (Langerhans 2008). Benthic fishes like South American riverine catfishes that occupy high-flow environments have unique morphological adaptations for station-holding such as suckers, enlarged pectoral fins, and pectoral spines (Casatti and Castro 2006; Leal et al. 2011; Pagotto et al. 2011). Wainwright (2019) found evidence of rougher scale surfaces in benthic damselfish compared to those living in the water column, and his results suggest that divergent flow types in each water column position – laminar vs. turbulent – may impact scale traits in these fishes. However, many benthic riverine fishes seek shelter in the substrate boundary layer, where they experience reduced flow across the body, and likely a reduction in the expected selective pressures of flow on morphological traits (Page and Swofford 1984; Langerhans 2008; Carlson and Lauder 2011).

In the 1800s Agassiz defined scales types (placoid, ganoid, ctenoid, and cycloid) that distinguish some of the major taxonomic groups of fishes, (Helfman et al. 2009) and modern systematists commonly use variation in scale counts, breeding scale color, and scale arrangement as diagnostic traits of systematic relationships, species diversification, and species identification (Raney and Suttkus 1964; Page 1981; Blanton and Jenkins 2008; Layman and Mayden 2012). Taxonomic and phylogenetic utility of scales suggests some degree of phylogenetic constrain on scale morphology. However, variation in other aspects of scale morphology, such as shape and meristic features, can be species-specific (Lippitsch 1990; Coburn and Gaglione 1992; Masood et al. 2015; Ibáñez 2015), which may imply that factors other than phylogeny are impacting fish

scales. One possibility is that scales may function to reduce drag in Teleost fishes, similar to placoid scales in sharks (Oeffner and Lauder 2012). Recent studies in Teleosts quantify scale topography and scale variation along the body (Wainwright and Lauder 2016; Wainwright et al. 2017), and hypothesize that the ctenii may reduce turbulent flow along the body as the fish moves (Fletcher et al. 2014; Lauder et al. 2016; Wainwright and Lauder 2018). Differential levels of scale rugosity between species occupying laminar (in pelagic damselfishes) versus turbulent (in benthic damselfishes) flow regimes suggests that scale topography plays a role in drag reduction (Wainwright 2019). Muthuramalingam et al. (2019) note reduced measures of skin friction drag due to the overlapping arrangement of scales during steady swimming in European Sea Bass replicas.

With over 250 species described, Darters (Percidae: Etheostomatinae) are an ideal clade of fishes in which to examine relationships between phylogeny, a benthic environment, and scale shape (Near et al. 2011). These typically benthic fishes are known for their flashy breeding colors and darting movements on the bottom of streams. They have a diversity of habitat requirements and morphologies (Kuehne and Barbour 1983; Page 1983; Carlson and Wainwright 2010; Geheber and Frenette 2016). In general, body morphology and ecology have been described as conserved within clades of darters (Guill et al. 2003a). However, in some cases, sister species are highly divergent in both (Geheber and Frenette 2016). Variation in some aspects of darter morphology have been related to factors other than phylogeny including breeding behaviors, habitat, and prey acquisition strategies (Paine et al. 1982; Page and Swofford 1984; Guill et al. 2003a; Carlson and Wainwright 2010; Martin and Page 2015). Additionally, convergence in morphology relating to ecological factors has been noted (Page and Swofford

1984; Guill et al. 2003a; Carlson and Wainwright 2010; Geheber and Frenette 2016). For

example, Page and Swofford (1984) described six major darter ecomorphs: "Gravel-run" darters

(the large, midwater species of *Percina*); "Riffle" darters (benthic residents with stocky bodies

and vibrant sexually dimorphic breeding coloration); "Sand-run" darters (including *Ammocrypta*,

Crystallaria, and *Etheostoma vitreum* that all frequently bury themselves in sandy runs, have

slender, pale-colored bodies); "Quiet-pool" darters (benthic residents, but in constrast to "Riffle"

darters, have smaller body sizes, are only somewhat stocky in body shape, and maintain cryptic

coloration Page and Swofford 1984); and "Midwater flowing-pool" or "Benthic flowing-pool"

darters that straddle descriptions of "Gravel-run" or "Riffle" darters, depending on their position

in the water column (Page and Swofford 1984). Considering evidence for both phylogenetic and

environmental influences on darter morphological phenoytpes, we evaluate the relative influence

of these forces on darter scale shape variation. We hypothesized that scale shape variation is

influenced jointly by phylogeny and ecological factors (Guill et al. 2003a). We predict that

exposure to different flow regimes, body size, and spawning behavior influence scale shape

among darters (Page and Swofford 1984; Carlson and Wainwright 2010; Bossu and Near 2015;

Geheber and Frenette 2016). We predicted that darters with close phylogenetic relationships

and/or similar ecologies would have more similar scale shapes than more distantly related and/or

ecologically dissimilar species, and the relationship between scale shape and ecology should be

stronger among darters which do not utilize the boundary layer of streams. Our results will

provide new insights into phenotypic diversity in this speciose group of fishes and the complex

nature of the relationship between form, evolutionary history, and environment in benthic fishes.

CHAPTER II: MATERIALS AND METHODS

Species and ecological variable selection

We first evaluated the presence of scale shape variation within and among genera and terminal clades of darters. To capture the potential range of scale variation, we selected at least one species from each genus and terminal clade within Etheostomatinae (92 total), following the phylogeny of Near et al. (2011). However, we labelled the previously unresolved *E. maydeni* as *Allohistium maydeni*, following the recommendation of MacGuigan and Near (2018).

We assessed whether variation was explained by ecological characteristics of our selected species including water column position (WCP), environment type, microhabitat, spawning mode, substrate size, and maximum body size (Size) of darters (classifications in Appendix 1). WCP influences the level of exposure to predators and flow, potentially necessitating modification to scale shape for drag reduction. We classified species based on Bossu and Near (2015) as benthic (those primarily living on the substrate) or midwater (living primarily above the substrate), but added the category of sub-benthic for those that bury frequently in the substrate outside the spawning season (Kuehne and Barbour 1983; Page 1983; Page and Burr 2011a).

Considering variation in flow type and level experienced by fishes in different environments, more specialized scale shapes may be beneficial within lotic environments (flowing systems, probably requiring more steady-state swimming) by reducing increased drag experienced compared to fishes living in lentic environments (slower or non-flowing systems, probably requiring more unsteady-state swimming). We classified darters as either lotic specialists or generalists in environment type (Appendix 1). We sampled only three lentic

specialists (*E. fusiforme*, *E. proeliare*, *E. nuchale*), which we grouped with the generalists, since they likely experience flows more similar to generalists than to lotic specialists.

Microhabitat, a metric that captures both flow and depth, may also influence the relative amount of viscous drag a darter experiences, and has been linked with variation in other morphological traits in darters (Kuehne and Barbour 1983; Page and Swofford 1984). We generalized microhabitat categories into "pool" (slower or non-flowing, deeper waters), "run" (moderate flow and depth), "riffle" (faster flowing, shallow waters) (Page and Burr 2011b), or "generalists" (species which frequent multiple microhabitat types; Appendix 1).

Spawning mode and associated behaviors may contribute to scale shape modifications to reduce drag, maintain body contact during mating, affect the successful display of seasonal breeding colors and/or skin modifications, or to facilitate egg burying. We included four spawning modes recognized by Page (1983): egg "attachers", "buriers", "clusterers", and "clumpers" (Appendix 1). We were only able to sample one species from the clumping category (*Nothonotus microlepidus*), which we placed into the clusterer group for analyses. The clumping behavior may be more similar to that of clusterers than buriers as far as the fish body's interaction with flow and substrate are concerned (see Page and Swofford 1984), even though it has been hypothesized that clumping behaviors are derived from burying eggs (Page and Swofford 1984).

Substrate size may determine the type of flow (laminar versus turbulent) in an area of a stream and the relative amount of shelter from flow available therein for darters, thus influencing the amount of consistent flow experienced. We used two substrate type classifications based on Page and Burr (2011) including "Fine" and "Coarse" categories. These serve mainly as a proxy

for the relative size of the boundary layer that is potentially available to a darter and likely the type of flows they will encounter outside of this shelter. Fine substrates (bedrock, sand, and silt) are presumed to have very little boundary layer and more laminar flows, and coarse substrates (rock, gravel, or cobble) are presumed to have at least some amount of accessible boundary layer and more turbulent flows (Carlson and Lauder 2011).

Finally, as size increases so does body surface area, and correspondingly the amount of hydrodynamic drag experienced (Webb 1988). Scale modifications may arise to compensate for increased drag, particularly in large darters that also have a reduced ability to shelter in the boundary layer of a stream. We considered a species "extra large" if maximum body size was between 96-200 mm total length (TL), "large" if maximum body size fell at or between 79 and 95 mm TL, "medium" from 70 and 78 mm TL, and "small" from 45 and 69 mm TL (Appendix 1). These size bins include equal cumulative percentages of species from a frequency distribution of maximum body sizes recorded in literature. In rare instances, classification information for each ecological category was not readily available for a species (due to the recent rise in the descriptions of darter species complexes), and in those cases we estimated values based on data published for its sister taxon.

Specimen and scale selection

Using museum specimens (Appendix 1 and 2), we removed one scale from the right side of each individual, directly above the anal fin origin, one row below the lateral line (Figure 1). This was the only area for all darter species with scales present near a fin origin, eliminating effects of scale location on analysis of shape (Wainwright and Lauder 2016). For most of the 92 species, we extracted scales from 30 adult individuals (Appendix 1 and 2) to minimize potential

effects of ontogenetic growth on scale shape data. We ensured the use of adult specimens by

selecting individuals that were equal to or greater than the average adult standard length for each

species based on published size data, or selected sexually mature individuals (i.e. gravid females

or males with breeding colors or characteristics like tubercles on fins or body, thickened skin on

fins, head, or body, soft knobs on fins, or enlarged genital papilla Page 1983). For each species

we attempted to examine only individuals of a single species from the same location, river

system, or drainage to avoid confounding factors related to intraspecific variation and possible

occurrence of cryptic species (Hopper et al. 2015).

Shape data

After extracting each scale, we removed all soft tissue to enhance consistent placement of

landmarks. The cleaned scale was wet mounted on a slide and imaged with a Nikon confocal

microscope. Scales were centered on the microscope under 10x (for the largest scales, often

species of *Percina*) or 20x magnification with transmitted light. We auto-scaled the images using

NIS Elements b925 (version 4.13.04) before scanning. If scales were not in sharp focus within

NIS Elements, we manually focused them before the image was captured. From these photos we

generated two datasets, one with all species included (92 total) and one including only benthic

species in WCP (i.e., excluding those categorized as midwater and sub-benthic; 72 total). Using

similar methods to Ibáñez (2015), we placed seven geometric morphometric landmarks on each

image using tpsDig2 version 2.26 (Rohlf 2015; Figure 1). We used a Procrustes superimposition

to remove the effects of scale, size, and rotation (R version 3.6.2 package *geomorph* version

3.2.1; Adams et al. 2017, R Core Team 2019). This method aligns the landmarks of all

individuals, creating new Procrustes coordinates for each (Zelditch et al. 2012a). We could not

test for effects of sex on scale shape due to missing sex data or unequal numbers of males and females available. Thus, we averaged Procrustes coordinates within each species to conduct downstream analyses to minimize the potential effect of sex-related variation.

Ordinations and comparative analyses

To examine overall scale shape variation, and to initially guage patterns in scale shape related to phylogeny, we generated a covariance matrix from the averaged Procrustes coordinate shape data for all species to run a Principle Components Analysis (PCA) with default settings in the R package *vegan* version 2.5-6 (Oksanen et al. 2019). Component scores for each species were plotted in PCA morphospace to visualize variation in scale shape among and within genera and terminal clades of darters (Near et al. 2011). Using Mesquite version 3.51 (Maddison and Maddison 2018) we pruned one phylogeny to the full 92 species selected in our study and one which included only those from our selections which were benthic in WCP (72 species total), and tested for a phylogenetic signal in phylomorphospace using the K-statistic "Kmult" (R package *geomorph*; Adams et al. 2017).

We used PCA to investigate whether scale shape varied among ecological groups as well. In this case, we coded species scores in morphospace plots by each variable type. Two-block, partial least squares (PLS) analysis (with default settings in program R package *geomorph*) allowed us to identify whether any specific combination of ecological variable types were associated with a particular scale shape (Rohlf and Corti 2000; Zelditch et al. 2012b). To determine whether scale shape covarys with ecological variables after accounting for shared ancestry we conducted a phylogenetic generalized least squares (PGLS; with default settings in

program R package *geomorph*) (Zelditch et al. 2012b, 2017). We carried out all analyses on the

full 92 darter species dataset and the reduced set of the 72 benthic darter species dataset.

CHAPTER III: RESULTS

Scale shape variation and phylogenetic signal

We generated scale shape data from 92 species that represent all six darter genera, all 28

terminal clades *sensu* Near et al. (2011), and all categories (ranging from 2-4) of each ecological

variable examined. The first two axes of the PCA examining scale shape variation among all

species selected for this study explained 82.4% of the variation (Figure 1). Scale shape change

along these axes is summarized by transformation grids, where the anterio-posterior length of the

scale increases and the dorso-ventral width decreases (scales become longer and thinner; Figure

1) along PC1. Scale body length increased (see Figure 1 for scale parts), the anterior scale

insertion narrowed, and the posterior ctenial margin was shorter and wider along PC2 (Figure 1).

Considerable variation was observed in shape both within and among darter genera (Figure 1).

Several taxa had particularly distinct scale shapes, including *Ammocrypta beani* (A01) and *A.*

pellucida (A02), having scales with longer, wider ctenial margins, and very narrow anterior scale

insertions compared to other species. Interestingly, close relatives of these two species (*A. vivax*

(A03), *Crystallaria asprella* (C04)) and one less closely-related but burying species (*E. vitreum*

(E59)), had scale shapes more similar to those found in the genera *Etheostoma, Allohistium*,

Nothonotus, and *Percina* (Figure 1). In the main cluster of species, there was considerable

variation primarily along PC1. Species such as *N. tippecanoe* (N69) and *E. vitreum* (E59) and *E.*

microperca (E38) and *E. proeliare* (E46), had distinctive square-shaped scales recovered close to

the origin of both PC axes (Figure 1). Alternatively, *A. vivax* (A03), *E. sagitta* (E50), and all

members of clade *Richiella* (E13, E31, E51) scored more positively along PC2 than most

species. Several species of genus *Percina* and one species of *Etheostoma* (*E. parvipinne* (E43)) had distinct long, thin scales that scored positively along PC1 (Figure 1).

Our phyloPCA also documented examples of close relatives with similar scale shapes (including the terminal clade *Pagella* from genus *Percina;* terminal clades *Ceasia*, *Oopareia*, *Richiella* and *Microperca* from genus *Etheostoma*; Figure 1). There was also a strong phylogenetic influence on scale shape variation across all darters ($K = 0.6801$, $P = 0.001$) and across benthic-only darters ($K = 0.671$, $P = 0.001$). However, the K-values from the K-mult test ($K<1$) indicate that phylogenetic signal is less than expected under a Brownian motion model of evolution.

Alternatively, several sister species pairs such as *P. palmaris* (P83) and *P. lenticula* (P77), *P. shumardi* (P88) and *P. vigil* (P92), *E. blennius* (E11) and *E. swannannoa* (E56), *N. microlepidus* (N67) and *N. tippecanoe* (N69) had divergent scale shapes (Figure 1). In many cases these sister species were also divergent in one or more ecological variables. Additionally, some distant relatives including species of clades *Adonia* and *Stigmacerca*; species of *Nothonotus* and *Neoetheostoma*; *Crystallaria asprella* (C04) and *E. swannannoa* (E56); *N. tippecanoe* (N69) and *E. vitreum* (E59); *E. baileyi* (E07), *E. fusiforme* (E25), *E. planasaxatile* (E44), and *E. radiosum* (E49) had similar scale shapes and also similar ecologies (Figure 1). Divergence between close relatives and potential convergence between distant relatives in these instances suggests factors other than phylogeny contribute to scale shape variation.

Ecology and scale shape variation-All darters

Scale shape variation was observed among species from different water column positions (WCP; Figure 2A). Midwater and sub-benthic species showed complete separation in

morphospace along a combination of both PC1 and PC2 axes. Both of these groups showed some overlap in scale shape variation with the benthic darters (Figure 2A). Although sub-benthic darters occupy a similar region in morphospace (due to their unique, reduced scales), they were disparate in shape with variation along PC2. Benthic and midwater species also show considerable variation in scale shape, but along PC1 (Figure 2A).

Considerable scale shape variation with overlap among the four size categories was observed (Figure 2B). The least overlap occurred between the largest size category (extra-large darters between 96-200 mm TL) and all others. Members of the extra-large size class greater than 100 mm TL (Figure 2B) were particularly distinct, having elongated scales that loaded in the positive region of PC1. Almost all of those unique extra-large species were also categorized as midwater in WCP (Figure 2A). Species coded by spawning mode, environment type, substrate, or microhabitat had considerable overlap in variation along both axes.

We found a significant relationship between scale shape and ecological characteristics of darters (PLS: $r = 0.541$, $P = 0.001$). There was a distinct cluster of darters with long, thin scales that loaded negatively along the shape axis (Figure 3A; blue circle). These species were associated with the negative region of the ecological variable axis, where darters were usually midwater in WCP, environmental generalists, microhabitat generalists or pool-dwellers, burying or attaching spawners, living over fine substrates, and were primarily extra-large in size (Figure 3A). A large and diverse group of darters clustered in the positive space of both axes where species had scales that were shorter and wider, and lived in lotic environments, riffle or run microhabitats over course substrates, were sub-benthic or benthic in WCP, cluster-guarders in spawning mode, and small or large in maximum body size.

After accounting for shared ancestry using a PGLS, both size ($r^2 = 5.2\%$, $P = 0.077$) and

WCP ($r^2 = 10.9\%$, $P = 0.001$) were significantly related to scale shape. Microhabitat and

substrate, approached significance ($P = 0.15$ and 0.14 with $r^2=4.5\%$ and 1.5%, respectively),

suggesting a possible influence on scale shape.

Ecology and scale shape variation-Benthic darters

Despite considerable overlap in scale shape variation among benthic darters in the bentic-

focused ecological PCAs, riffle specialists had scales that loaded more positively along PC2

(Figure 4), with scales that are narrower at the anterior insertion with a widened and lengthened

posterior ctenial margin. Nearly complete separation was observed between scale shape variation

of riffle and run species, but these overlapped with scale shape variation of pool and generalist

species. Size, spawning mode, and substrate groups of benthic darters showed much variability

in scale shape and had considerable overlap in morphospace.

For benthic species, no distinct clusters were observed in the PLS (Figure 3 B), although

we detected a significant correlation between scale shape and ecological variables ($r = 0.561$, $P =$

0.001). Scale shape values that loaded positively had a shorter, wider scale body, and a wider

ctenial margin; those loading negatively along the shape axis had a narrower ctenial margin but a

wider anterior scale insertion (Figure 3 B). Along the ecological axis, positive values were

associated with darters that occupy lotic systems and specifically live in riffle microhabitats, are

clustering or burying spawners, are small, large, or extra-large in size and live over coarse

substrates. Darters loading negatively along this axis were those in pools, runs, or generalists in

microhabitat, attach their eggs to aquatic structures, are medium in size, and live over fine

substrates. However, despite possible trends with ecological factors found in the PLS results,

only body size was significantly associated with scale shape variation after accounting for phylogenetic signal (PGLS: $P = 0.094$, $r^2 = 6.9\%$). Microhabitat approaches significance and explains a comparable amount of shape variation as size, and thus, may also influence scale shape ($P = 0.13$, $r^2 = 6.6\%$). All other ecological variables were not significant in the PGLS.

CHAPTER IV: DISCUSSION

In this study, we quantified shape variation in darters to evaluate the relative influences of ecology, size, behavior, and evolutionary history on scale traits within this largely benthic group of fish species. In general, we found considerable scale shape variation both among and within clades of darters and accordingly, a strong and significant phylogenetic signal on scale shape variation. However, we also found potential evidence of divergence and convergence in scale shape, suggesting factors other than phylogeny alone have generated darter scale phenotypic diversity. These results were supported by significant associations between scale shape and ecological variables after accounting for phylogenetic signal, especially water column position and body size

Evolutionary history explains variation in darter body shapes among genera and many subgenera (Guill et al. 2003a), and is a strong predictor of phenotypic traits in many other aquatic and terrestrial taxa including Monogenean haptoral anchors (fish parasites Rodríguez-González et al. 2017) and Scarab beetle wings (Ospina-Garcés et al. 2018). While we found considerable variation in scale shape at all levels examined, our data provide examples of clades with a common scale shape among all examined members, and as expected, we found a significant effect of phylogeny on scale shape variation. This suggests that scale shape, similar to body shape, in many darters may be phylogenetically constrained. However, some of the noted discernable differences between close relatives (*i.e. E. swaini* and *E. caeruleum*) and similar scale shapes between more distant relatives (*i.e. Nothonotus* and *Neoetheostoma*) suggest some variation is driven by factors other than phylogeny, such as environment.

The results of our ecological PCA showed relatively little overlap in scale shape between darters occupying different water column positions (WCP). Water column position has been previously linked with variation in many aspects of darter morphological traits, as each position comes with different types and levels of selective pressures. For example, darters that bury in sandy runs share many similarities like slender bodies, small fins, a narrow inter-orbital width, and a loss or reduction in scale number, size, and ctenial spines. Given that they spend much of their time hidden below the substrate, these morphological features may reflect relaxed selection for protection and drag reduction (Kuehne and Barbour 1983; Page and Swofford 1984; Spinner et al. 2016). But, they may also be influenced by an increase in pressures related to burying efficiency (such as reduced skin friction) that has lead to convergence between distant relatives (Kuehne and Barbour 1983; Page and Swofford 1984; Spinner et al. 2016). Brighter coloration and a lack of fusiformity among benthic darters may result from lessened pressures of predation and flow, though their large pectoral fins may be important for increasing their grip on the bottom of a stream (Page and Swofford 1984). Midwater darters are the most exposed to flow and predators, and thus have smaller pectoral fins, fusiform body shapes, and cryptic coloration (Page and Swofford 1984). The differences in scale shape that we found between darters in different WCPs and the significant relationship between shape and WCP in the PGLS conform with patterns seen in overall morphological variation of darters and imply that selective pressures that have influenced the body and fins of these fishes also extends to the scales. Therefore, it is likely that the unique, reduced scales of sub-benthic darters aid in burying efficiency while the long, thin scales of the midwater darters aid in drag reduction.

Size was also found to be significantly associated with scale shape among darters in the PGLS, and the ecological PCA and PLS highlight the extra-large darters as primarily driving this result. Species with the most distinctive (long, thin) scale shape along PC1 were not only midwater, but also extra large-sized (particularly if over 100mm TL) darters. These darters (primarily genus *Percina*) are active roving predators with elongated, stream-lined bodies that swim in the water column, some even flip stones, to obtain macroinvertebrate prey, (Page 1983; Carlson and Wainwright 2010). A roving behavior contributes to most species that fit this description being considered generalist in habitat type (Page and Swofford 1984), because they regularly explore multiple habitats for food. Although these species are not specialists of the fastest flowing waters, because they spend considerable time moving above the substrates, out of the boundary layer, they experience regular unidirectional, viscous laminar flow (Krabbenhoft et al. 2009; Meyers and Belk 2014). Furthermore, body size is positively correlated with drag such that species with larger size experience increased drag forces relative to smaller species (Webb 1988). Larger fishes may also have less ability to fully shelter in the boundary layer (Carlson and Lauder 2011). Adaptations that reduce drag, or in particular, that increase steady-state swimming efficiency, such as a more fusiform body shape are predictable traits for such fishes that experience laminar flow (Langerhans 2008; Krabbenhoft et al. 2009; Meyers and Belk 2014). The observed scale morphology of the largest, midwater darter species examined seems to mirror these expectations: the long, thin scale shape, just like a long, streamlined body, may also reflect adaptations to laminar flows experienced while roving in the water column for food (Figures 1 & 5). Others have found flow type influences scale morphology in fishes. Wainwright (2019) found that pelagic damselfishes in laminar flow conditions had scales with smoother surfaces than benthic damselfishes in turbulent flows, which had rougher scale topographies,

suggesting modifiations to scale morphology based on flow types occupied. A few extra-large, midwater darter species departed from these trends such as *P. macrocephala* (P78) and *P. maculata* (P80), which (though they are both >100 mmTL and midwater) did not have long, thin scales (Figure 1). Based on size and water column position, we expected these species to have the long, thin scale shape of other large, midwater darters. *Percina macrocephala* often are found hovering above the substrate in pools or sheltering in brush, water willows, or large rocks (Etnier and Starnes 1993). *Percina maculata* reside mostly in pools (Etnier and Starnes 1993) and also hide under banks and in woody debris (Etnier and Starnes 1993). In general, the lower level of roving activity and increased use of shelter in these examples may contribute to a reduction in laminar drag forces experienced. *Percina shumardi* (P88), a primarily benthic and medium size darter, shared the long, thin scale type of the larger bodied, midwater darters (Figure 1). This species is usually found in large, flowing river environments with finer substrates that likely provide reduced boundary layer shelter from flow. Thus, this species may experience similar flow-related selection pressures of the large bodied, midwater darters. However, darters likely occupy a spectrum between the benthic and midwater zones of streams and therefore, the scale shape of *P. shumardi* may alternatively indicate that this species spends more time above the benthos than previously thought. Importantly, we observed extra-large, benthic species (e.g., *P. evides, P. palmaris*) and alternatively smaller, midwater species (e.g., *P. stictogaster*, *P. smithvanizi*) that did not have long, thin scales characteristic of fishes that are both midwater and large in size. This indicates that for many darters, scale shape, in part, reflects selection imposed through a combination of both WCP and Size.

Body size was the only variable significantly associated with scale shape among benthic darters after accounting for phylogenetic signal, but there was little variation among size categories in the PCA and no specific clusters of darters were observed in the PLS. The extreme ends of the PLS ecological axis separated medium size darters from primarily large and extra-large darters; medium benthic darters also usually occupied pools, while the larger of the benthic darters usually occupied riffles. Though microhabitat was not significant in the PGLS, both the PLS and the PCA found the most visible differences in scale shape occurred between pool and riffle microhabitat groups. Riffle darters face turbulent flows in their benthic environment (Meyers and Belk 2014), and those riffle darters that are large may have reduced opportunities to fully shelter in the substrate boundary layer. Darters that occupy riffles and experience turbulent flow are characterized by short, wide bodies, that are thought to be the result of selective pressure of living in a riffle environment (Page and Swofford 1984). Again scale shape in this group of darters seems to mirror body shape in that larger riffle species had short, wide scales (Figures 3B, 4, 5) that may similarly reflect adapatation to turbulent flows. On the other end of the PGLS ecological axis, the medium pool darters (like *E. parvipinne*), which face laminar flows in their environment similar to the extra-large, midwater darters had scale shapes similar to them as well (Figures 3B, 4, 5). These observations indicate a likely important relationship between benthic darter scale shape, body size, and microhabitat occupied, including predominant flow type (turbulent vs. laminar) and that this relationship is likely mediated by the amount of shelter (like the substrate boundary layer) available.

Although our analyses indicate that scale shape is influenced by phylogeny (Kmult tests: P=0.001), WCP (PGLS: P=0.001), and size (all species PGLS: P=0.077, benthic species PGLS:

P=0.094), there was considerable variation in many of our categorical groups (taxonomic or

ecological). Some of this was likely an artifact of placing species in coarsely defined ecological

categories, especially considering measurable body shape variation in some darters has been

documented across highly nuanced environmental gradients and narrow geographic areas

(Hopper et al. 2015). Additionally, shape is likely a product of interacations among

environmental variables like those noted in this study (WCP and size). Many closely related

species of darters, such as those in a single terminal clade, are often ecologically and

behaviorally similar, and these traits are also known to be constrained by evolutionary history

(Kuehne and Barbour 1983; Page 1983; Page and Swofford 1984; Guill et al. 2003a,b; Geheber

and Frenette 2016). Thus, phylogenetic constraint on scale shape may be indirect in some cases,

resulting from constraint on habitat requirements in general within a clade. Clarifying the relative

influences of phylogeny (direct and/or indirect) and ecology on specific morphological traits in

such clades that also have evolutionarily constrained ecologies may be difficult. However, the

similarity in patterns of scale shape variation with variation in other morphological traits like

those documented by Page and Swofford (1984), Meyers and Belk (2014), and Wainwright

(2019) suggests fish scale morphology plays an important role in drag reduction and facilitating

movements through different flow types, even among benthic species that utilize the boundary

layer of streams.

CHAPTER V: REFERENCES

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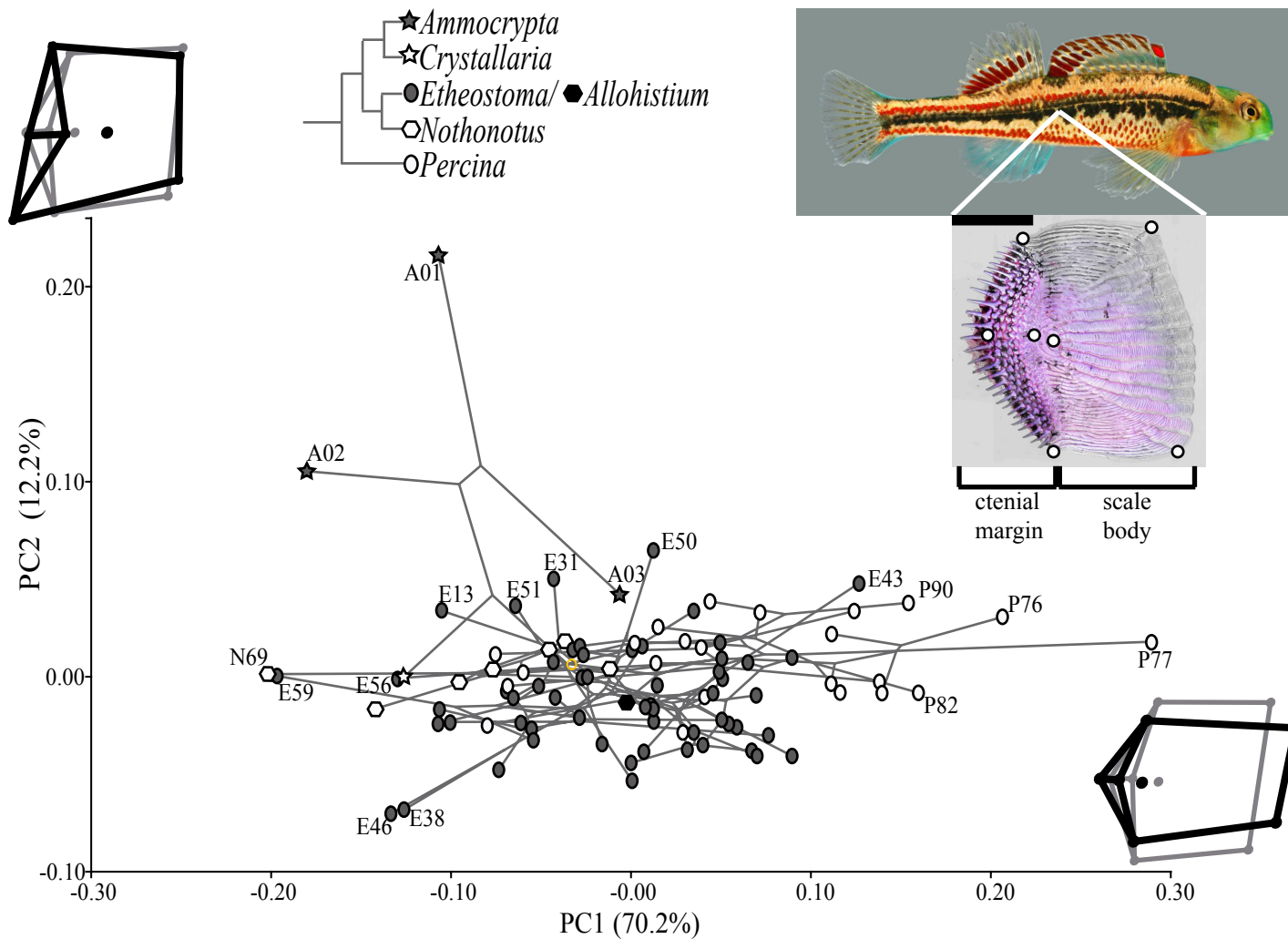
Figure 1. The phyloPCA of average scale shape for all darter species examined. Symbols identify genus and species identifier codes and correspond to those in Appendix 1. Transformation grids on PC axes 1 and 2 depict shape changes (black) from the overall average scale shape (gray) of all darters along each respective axis. Lines represent phylogenetic relationships of examined taxa following Near et al (2011). Upper right images showing scale extraction location and with enlarged scale showing the seven landmarks (white dots) used to assess scale shape variation in Etheostomatinae darters.
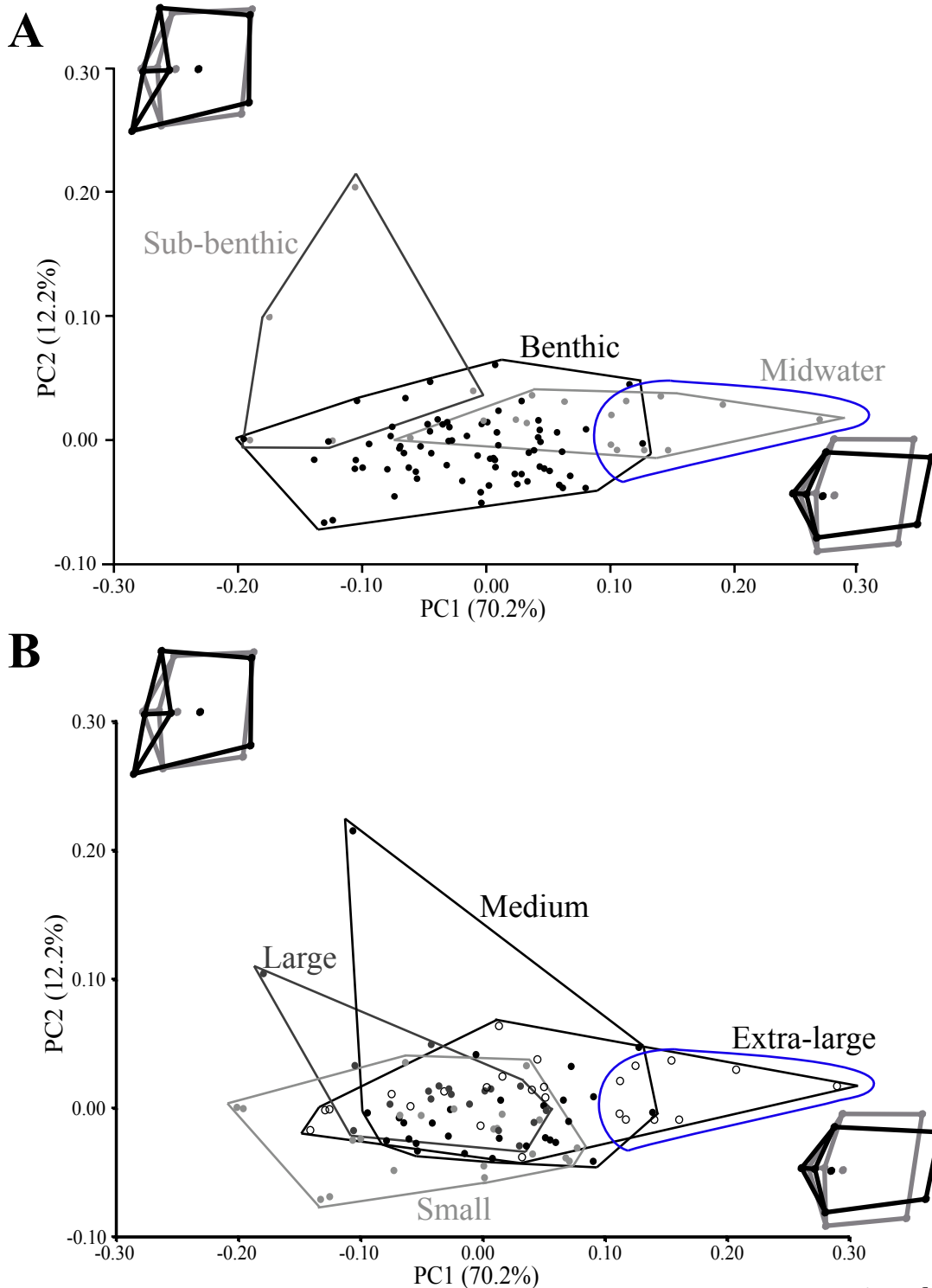
Figure 2. Principle component analyses of scale shape variation for all Etheostomatinae darters by ecological and behavioral variables measured. Minimum convex polygons summarize: (A) variation by water column position (WCP) and (B) variation by maximum body size (Size), with each point on the plot representing the average shape of one species. Transformation grids associated with PC axis 1 and PC axis 2 depict shape changes (black) from the overall average scale shape (gray) of all darters along each respective axis. Other variables examined showed complete overlap in morphospace (not shown). Species circled in blue are of note as both extra-large in size and midwater in WCP.
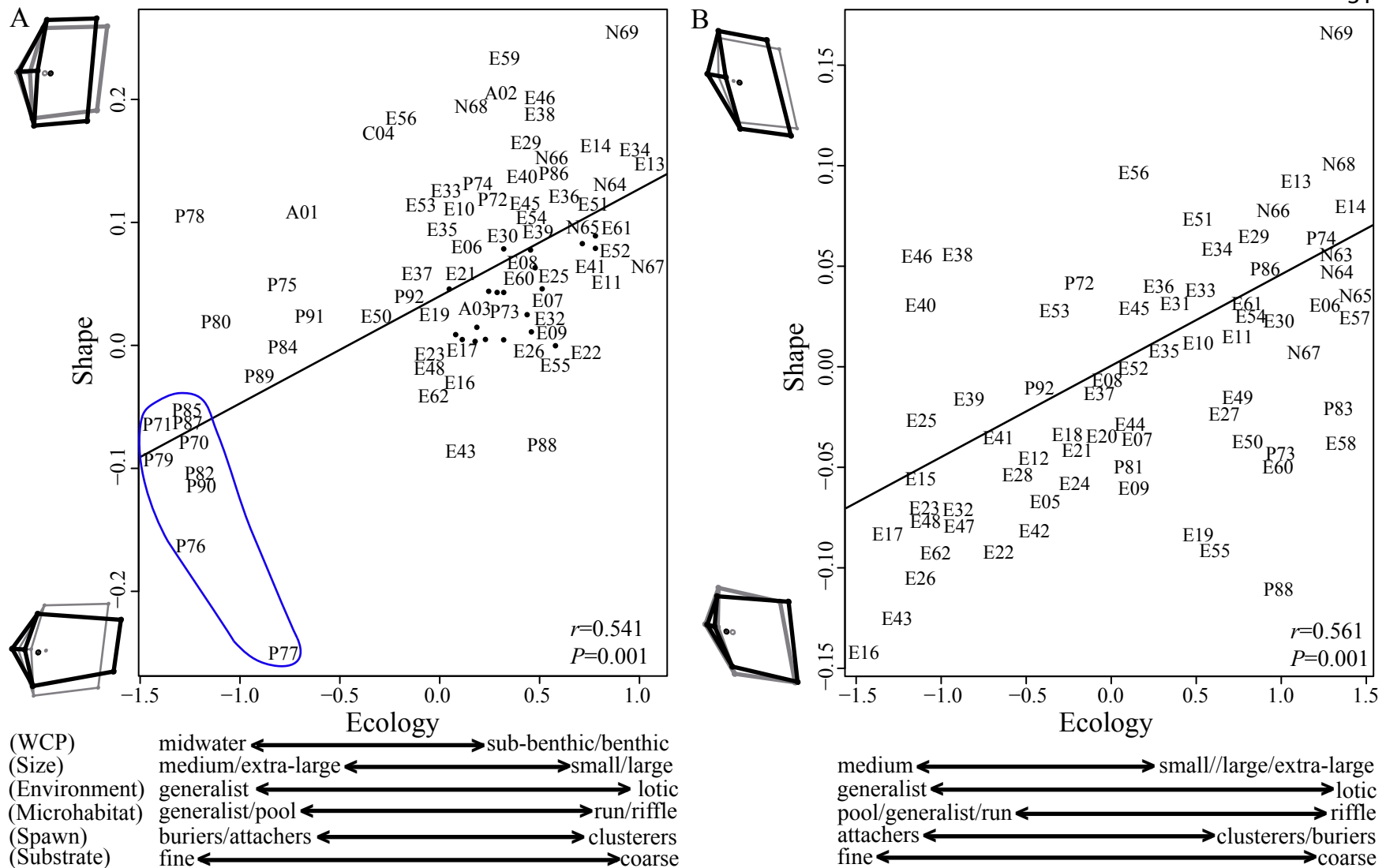
Figure 3. Partial least square regression analyses of (a) all Etheostomatinae darter species examined and (b) benthic species. X-axes show the positve or negative loadings of each ecological variable based on the species points in morphospace. Species are represented by points or codes used in Appendix 1. Transformation grids associated with the ends of each shape axis depict shape changes (black) from the overall average scale shape (gray) of all darters included in each analysis. Species circled in blue on (a) are species discussed in the text with a long, thin scale shape.
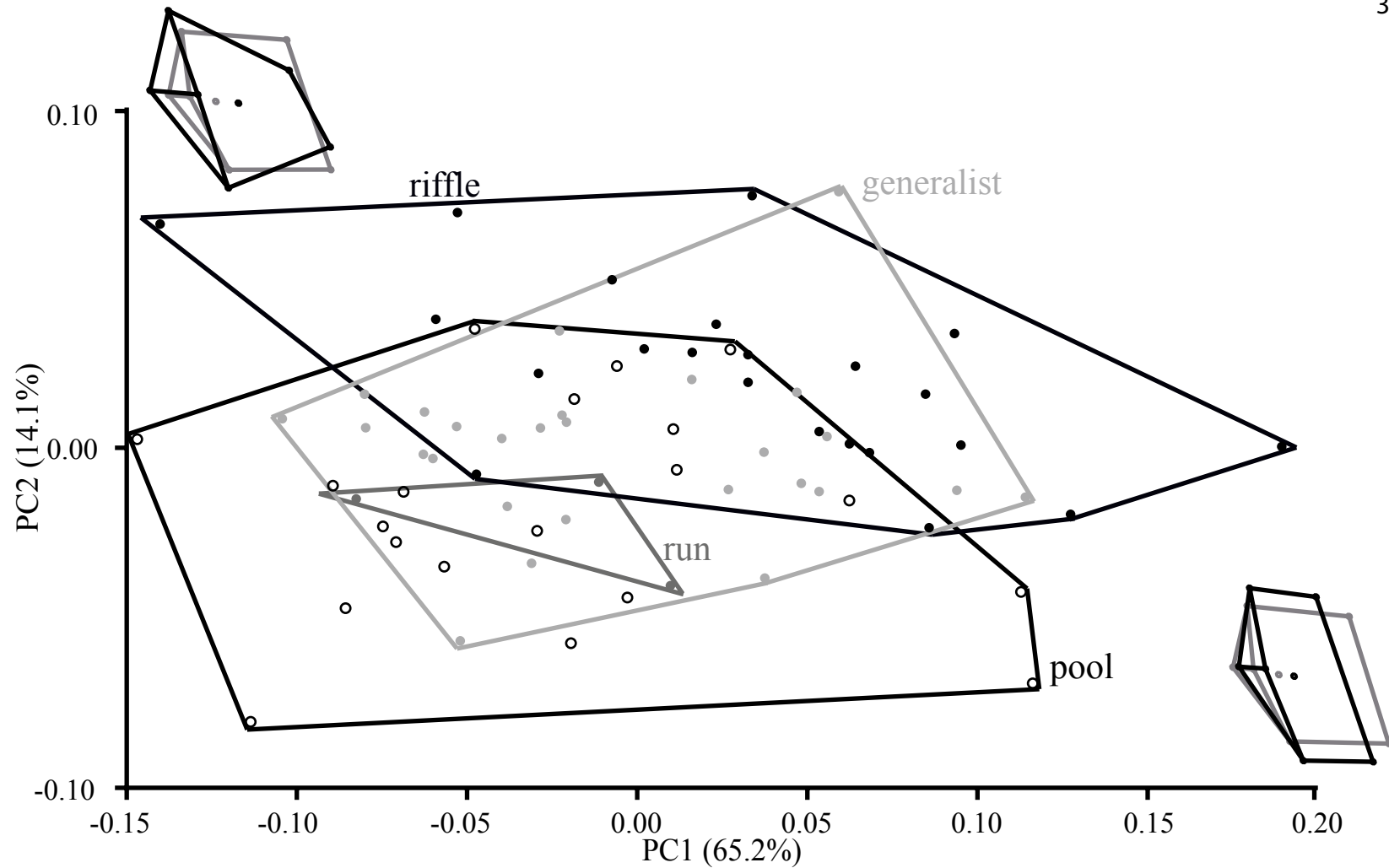
Figure 4. Principle component analysis of scale shape variation for benthic only Etheostomatinae darters examined in the context of microhabitat type. Minimum convex polygons summarize variation by microhabitat type with each point on the plot representing one species. Transformation grids associated with PC axis 1 and PC axis 2 depict shape changes (black) from the overall average scale shape (gray) of all benthic darters along each respective axis. Other variables examined showed considerable variation and overlap among all categories in morphospace (not shown).

Sub-benthic
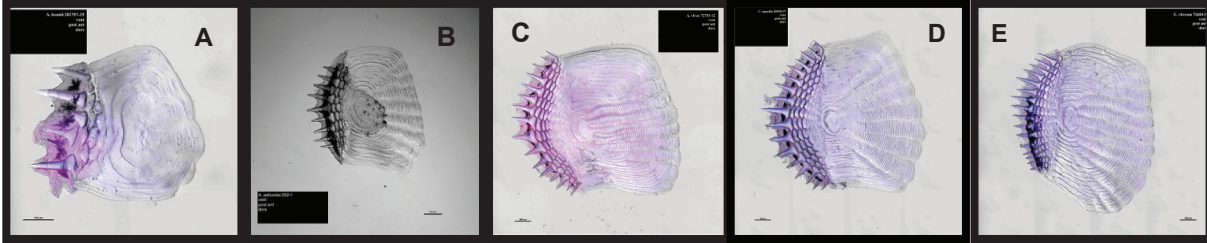
Pool
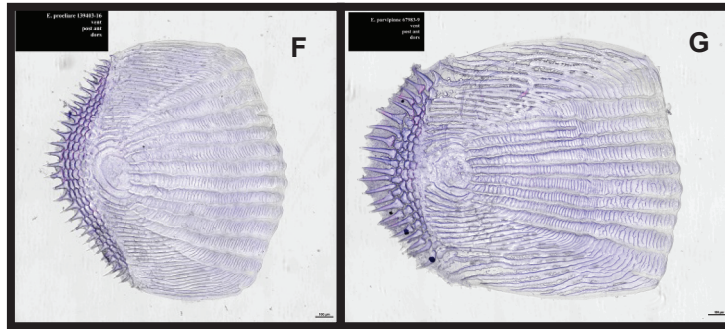
Benthic, riffle
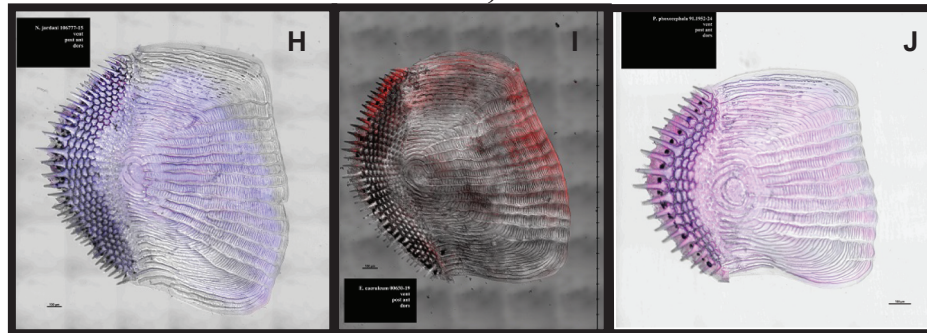
Midwater, extra-large
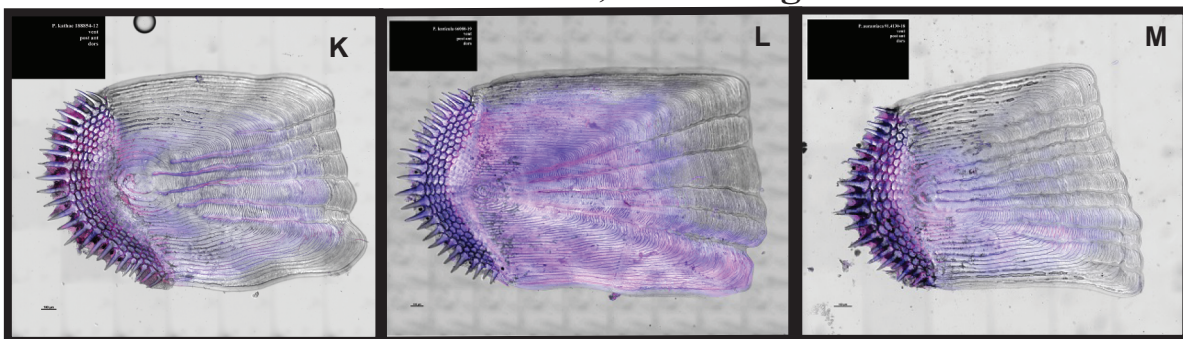
Figure 5. Darter species scale photos. Represented by each photo are: (A) *A. beani*, (B) *A. pellucida*, (C) *A. vivax*, (D) *C. asprella*, (E) *E. vitreum*, (F) *E. proeliare*, (G) *E. parvipinne*, (H) *N. jordani*, (I) *E. caeruleum*, (J) *P. phoxocephala*, (K) *P. kathae*, (L) *P. lenticula*, and (M) *P. aurantiaca*.

Appendices

Appendix 1. Species used to assess scale shape variation, ecological classifications and references for classifications. The number of individuals examined in parentheses. Terminal clades are from Near et al. (2011) with the addition of genus *Allohistium* following MacGuigan and Near (2018). Ecological, behavioral and size variable assignments used for each species were drawn from several sources, which are provided in the References section below the table. References for Environment and Microhabitat columns are the same.

| Species Code	Species (# specimens)	Water column position	Environment	Microhabitat	Spawning mode	Body size	Substrate type
A01	*A. beani* (30)	Sub-benthic[26]	Lotic [1; 26; 43]	Run	Burier[35]	Small[30]	Fine[30]
A02	*A. pellucida* (30)	Sub-benthic[26]	Lotic [22; 26; 30]	Run	Burier[35]	Medium[30]	Fine[30]
A03	*A. vivax* (30)	Sub-benthic[26]	Lotic [1; 26; 43]	Run	Burier[24; 46]	Medium[30]	Fine[30]
C04	*C. asprella* (30)	Sub-benthic[26]	Lotic[22; 41; 43]	Run	Burier[35]	Large[30]	Fine[30]
E05	*E. asprigene* (30)	Benthic[3]	Generalist[22; 30; 43]	Generalist	Attacher[9; 35]	Small[30]	Coarse[30]
E06	*E. autumnale* (30)	Benthic[3]	Lotic[30; 41]	Riffle	Burier[41]	Large[30]	Coarse[30]
E07	*E. baileyi* (30)	Benthic[3]	Lotic[12; 22; 30]	Generalist	Attacher[35; 38; 49]	Small[12]	Coarse[30]
E08	*E. barbouri* (30)	Benthic[3]	Lotic[12; 22; 30]	Pool	Clusterer[12; 32; 35]	Small[12]	Coarse[30]
E09	*E. barrenense* (30)	Benthic[3]	Lotic[12; 30]	Generalist	Attacher[12; 49; 56]	Small[30]	Coarse[30]
E10	*E. bison* (30)	Benthic[3]	Lotic[12; 30]	Generalist	Burier[12]	Medium[12; 30]	Coarse[30]
E11	*E. blennius* (29)	Benthic[3]	Lotic[1; 22; 30]	Riffle	Attacher[35]	Medium[30]	Coarse[30]
E12	*E. boschungi* (29)	Benthic[3]	Lotic[1; 22; 30]	Pool	Attacher[1; 2; 35]	Medium[30]	Coarse[30]
E13	*E. brevispinum* (30)	Benthic[3]	Lotic[30; 42]	Riffle	Clusterer[42]	Medium[30]	Coarse[30]
E14	*E. caeruleum* (26)	Benthic[3]	Lotic[12; 22; 30]	Riffle	Burier[22; 56; 57]	Medium[12]	Coarse[30]
E15	*E. chlorosoma* (29)	Benthic[3]	Generalist[22; 30; 43]	Pool	Attacher[33; 43]	Small[30]	Fine[30]
E16	*E. collis* (30)	Benthic[3]	Generalist[16; 30; 42]	Pool	Attacher[21; 35]	Small[16; 30; 42]	Fine[30]
E17	*E. colorosum* (30)	Benthic[3]	Lotic[1; 30]	Pool	Attacher[1; 18]	Small[30]	Fine[30]
E18	*E. cragini* (30)	Benthic[3]	Lotic[22; 30; 41]	Pool	Burier[10; 22; 30]	Small[30]	Fine[30]
E19	*E. crossopterum* (28)	Benthic[3]	Lotic[12; 30]	Generalist	Clusterer[27; 35]	Large[12; 30]	Coarse[30]
E20	*E. derivativum* (30)	Benthic[3]	Lotic[12; 30]	Pool	Clusterer[12; 27; 32]	Medium[30]	Coarse[30]

Species Code	Species (# specimens)	Water column position	Environment	Microhabitat	Spawning mode	Body size	Substrate type
E21	*E. duryi* (**30**)	Benthic[3]	Lotic[12]	Generalist	Attacher[12; 33; 39]	Small[12]	Coarse[30]
E22	*E. edwini* (**30**)	Benthic[3]	Lotic[1; 30; 40]	Run	Attacher[1; 22; 40]	Small[30]	Fine[30]
E23	*E. etnieri* (**30**)	Benthic[3]	Lotic[12]	Generalist	Attacher[12; 35; 38]	Medium[12]	Fine[30]
E24	*E. flavum* (**29**)	Benthic[3]	Lotic[12]	Generalist	Attacher[20; 35; 39]	Small[12]	Coarse[30]
E25	*E. fusiforme* (**29**)	Benthic[3]	Generalist[22; 30; 40]	Pool	Attacher[14; 22; 26]	Small[30]	Fine[30]
E26	*E. gracile* (**27**)	Benthic[3]	Generalist[22; 30; 40]	Pool	Attacher[4; 35; 43]	Small[30]	Fine[30]
E27	*E. gutselli* (**30**)	Benthic[3]	Lotic[12; 30]	Riffle	Attacher[35]	Large[30]	Coarse[30]
E28	*E. histrio* (**30**)	Benthic[3]	Lotic[1; 22; 43]	Riffle	Attacher[35; 49]	Medium[30]	Fine[30]
E29	*E. jessiae* (**29**)	Benthic[3]	Lotic[1; 22; 30]	Generalist	Burier[12; 26; 47]	Small[30]	Coarse[30]
E30	*E. kanawhae* (**30**)	Benthic[3]	Lotic[16; 22; 30]	Generalist	Burier[35]	Medium[30]	Coarse[30]
E31	*E. kennicotti* (**30**)	Benthic[3]	Lotic[12; 22; 30]	Pool	Clusterer[28; 31; 35]	Medium[12]	Coarse[30]
E32	*E. lachneri* (**30**)	Benthic[3]	Lotic[1; 30]	Pool	Attacher[35]	Small[30]	Fine[30]
E33	*E. lawrencei* (**30**)	Benthic[3]	Lotic[12; 30]	Generalist	Burier[12]	Medium[12; 30]	Coarse[30]
E34	*E. lepidum* (**29**)	Benthic[3]	Lotic[12; 22; 30]	Riffle	Attacher[12; 30; 50]	Small[12]	Coarse[30]
E35	*E. luteovinctum* (**30**)	Benthic[3]	Generalist[12; 30]	Generalist	Burier[19]	Medium[12; 30]	Coarse[30]
E36	*E. lynceum* (**30**)	Benthic[3]	Lotic[12; 30]	Riffle	Attacher[35]	Small[30]	Coarse[30]
E37	*A. maydeni* (**22**)	Benthic[3]	Lotic[12; 30; 51]	Pool	Attacher[16; 35]	Large[30]	Coarse[30]
E38	*E. microperca* (**30**)	Benthic[3]	Lotic[22; 30; 37]	Pool	Attacher[37; 56; 57]	Small[30]	Fine[30]
E39	*E. nigrum* (**30**)	Benthic[3]	Generalist[12]	Run	Clusterer[26; 56; 57]	Medium[30]	Fine[30]
E40	*E. nuchale* (**23**)	Benthic[3]	Generalist[1; 22; 30]	Pool	Attacher[11]	Small[30]	Fine[30]
E41	*E. okaloosae* (**30**)	Benthic[3]	Lotic[22; 30; 40]	Run	Attacher[8; 22; 35]	Small[30]	Fine[30]
E42	*E. oophylax* (**29**)	Benthic[3]	Lotic[12; 30]	Pool	Clusterer[12; 27; 35]	Large[30]	Fine[30]
E43	*E. parvipinne* (**30**)	Benthic[3]	Lotic[1; 30; 43]	Pool	Attacher[1; 17; 43]	Small[30]	Fine[30]
E44	*E. planasaxatile* (**29**)	Benthic[3]	Lotic[12; 30]	Generalist	Attacher[12]	Small[30]	Coarse[30]
E45	*E. podostemone* (**30**)	Benthic[3]	Lotic[16; 22; 30]	Generalist	Clusterer[15; 16; 35]	Medium[30]	Coarse[30]
E46	*E. proeliare* (**29**)	Benthic[3]	Generalist[22; 26; 30]	Pool	Attacher[5; 22; 43]	Small[30]	Fine[30]
E47	*E. pseudovulatum* (**32**)	Benthic[3]	Lotic[12; 30]	Pool	Clusterer[12]	Medium[30]	Fine[30]
E48	*E. pyrrhogaster* (**30**)	Benthic[3]	Lotic[12; 30]	Generalist	Attacher[7; 12; 35]	Small[12; 30]	Fine[30]

Species Code	Species (# specimens)	Water column position	Environment	Microhabitat	Spawning mode	Body size	Substrate type
E49	*E. radiosum* (**30**)	Benthic[3]	Generalist[22; 30; 41]	Generalist	Burier[22; 41; 45]	Medium[30]	Coarse[30]
E50	*E. sagitta* (**30**)	Benthic[3]	Lotic[12; 22; 30]	Generalist	Burier[7; 12; 35]	Large[30]	Coarse[30]
E51	*E. sitikuense* (**30**)	Benthic[3]	Lotic[12; 30]	Generalist	Clusterer[12]	Small[30]	Coarse[30]
E52	*E. smithi* (**30**)	Benthic[3]	Generalist[12; 22; 30]	Pool	Clusterer[12; 31; 35]	Small[12]	Coarse[30]
E53	*E. spectabile* (**30**)	Benthic[3]	Lotic[37; 41]	Generalist	Burier[21; 23; 41]	Medium[30]	Coarse[30]
E54	*E. stigmaeum* (**30**)	Benthic[3]	Lotic[1; 22; 30]	Generalist	Burier[1; 26; 47]	Small[30]	Fine[30]
E55	*E. swaini* (**30**)	Benthic[3]	Lotic[22; 26; 43]	Pool	Burier[26; 43; 44]	Small[43]	Coarse[30]
E56	*E. swannanoa* (**30**)	Benthic[3]	Lotic[12; 22; 30]	Generalist	Attacher[35; 46]	Large[12]	Coarse[30]
E57	*E. tetrazonum* (**30**)	Benthic[3]	Lotic[30; 37]	Riffle	Burier[26; 37; 35]	Medium[30]	Coarse[30]
E58	*E. variatum* (**30**)	Benthic[3]	Lotic[16; 30; 52]	Riffle	Burier[22; 35; 46]	Large[30]	Coarse[30]
E59	*E. vitreum* (**30**)	Sub-benthic[26]	Lotic[16; 30; 58]	Generalist	Clusterer[35; 58]	Small[30]	Fine[30]
E60	*E. whipplei* (**30**)	Benthic[3]	Lotic[22; 30; 41]	Generalist	Burier[35]	Large[30]	Coarse[30]
E61	*E. zonale* (**30**)	Benthic[3]	Lotic[12; 30]	Riffle	Attacher[12; 37; 53]	Large[12]	Coarse[30]
E62	*E. zonistium* (**30**)	Benthic[3]	Lotic[12; 26]	Generalist	Attacher[7; 12; 35]	Medium[12]	Fine[30]
N63	*N. acuticeps* (**30**)	Benthic[3]	Lotic[12; 22; 30]	Riffle	Burier[12; 35]	Medium[30]	Coarse[30]
N64	*N. bellus* (**27**)	Benthic[3]	Lotic[12; 22; 30]	Riffle	Burier[13; 35]	Medium[12; 30]	Coarse[30]
N65	*N. camurus* (**28**)	Benthic[3]	Lotic[12; 22; 26]	Riffle	Burier[12; 22; 35]	Large[12]	Coarse[30]
N66	*N. jordani* (**30**)	Benthic[3]	Lotic[12; 22; 30]	Riffle	Burier[12; 25; 35]	Medium[30]	Coarse[30]
N67	*N. microlepidus* (**27**)	Benthic[3]	Lotic[12; 22; 26]	Riffle	Clusterer[12; 33; 35]	Large[12]	Coarse[30]
N68	*N. rufilineatus* (**28**)	Benthic[3]	Lotic[12; 22; 30]	Riffle	Burier[12; 35; 55]	Large[12]	Coarse[30]
N69	*N. tippecanoe* (**30**)	Benthic[3]	Lotic[12; 22; 30]	Riffle	Burier[12; 35; 54]	Small[12]	Coarse[30]
P70	*P. aurantiaca* (**30**)	Hyperbenthic[3]	Lotic[12; 22; 30]	Generalist	Burier[12; 22; 35]	Extra large[30]	Coarse[30]
P71	*P. caprodes* (**30**)	Hyperbenthic[3]	Generalist[22; 26; 41]	Generalist	Burier[35; 41]	Extra large[30]	Fine[30]
P72	*P. copelandi* (**29**)	Benthic[3]	Generalist[22; 30; 41]	Generalist	Burier[22; 35; 41]	Small[30]	Fine[30]
P73	*P. crassa* (**29**)	Benthic[3]	Lotic[22; 26;30]	Generalist	Burier[35]	Large[30]	Coarse[30]
P74	*P. evides* (**26**)	Benthic[3]	Lotic[12; 30; 43]	Riffle	Burier[35; 43]	Large[30]	Coarse[30]
P75	*P. gymnocephala* (**31**)	Hyperbenthic[3]	Lotic[16; 30]	Riffle	Burier[35]	Large[30]	Coarse[30]
P76	*P. kathae* (**30**)	Hyperbenthic[3]	Lotic[12; 30]	Generalist	Burier[35]	Extra large[30]	Coarse[30]

Species Code	Species (# specimens)	Water column position	Environment	Microhabitat	Spawning mode	Body size	Substrate type
P77	*P. lenticula* (30)	Hyperbenthic[3]	Lotic[1; 30; 43]	Riffle	Burier[35]	Extra large[30]	Coarse[30]
P78	*P. macrocephala* (13)	Hyperbenthic[3]	Lotic[12; 22; 30]	Pool	Burier[35]	Extra large[30]	Coarse[30]
P79	*P. macrolepida* (30)	Hyperbenthic[3]	Generalist[22; 26; 30]	Generalist	Burier[35]	Extra large[30]	Fine[30]
P80	*P. maculata* (30)	Hyperbenthic[3]	Lotic[30; 48]	Pool	Burier[35; 36]	Extra large[30]	Fine[30]
P81	*P. nevisense* (30)	Benthic[3]	Lotic[22; 26; 30]	Generalist	Burier[16; 22]	Large[30]	Fine[30]
P82	*P. nigrofasciata* (30)	Hyperbenthic[3]	Lotic[22; 30; 43]	Pool	Burier[35]	Extra large[30]	Fine[30]
P83	*P. palmaris* (30)	Benthic[3]	Lotic[1; 12; 26]	Riffle	Burier[35]	Large[12]	Coarse[30]
P84	*P. phoxocephala* (30)	Hyperbenthic[3]	Lotic[22; 30; 48]	Riffle	Burier[34; 35]	Large[30]	Coarse[30]
P85	*P. rex* (19)	Hyperbenthic[3]	Lotic[16; 22; 30]	Generalist	Burier[16]	Extra large[30]	Coarse[30]
P86	*P. roanoka* (30)	Benthic[3]	Lotic[16; 22; 30]	Riffle	Burier[16; 35]	Medium[30]	Coarse[30]
P87	*P. sciera* (30)	Hyperbenthic[3]	Lotic[22; 26; 30]	Generalist	Burier[35]	Extra large[30]	Coarse[30]
P88	*P. shumardi* (30)	Benthic[3]	Lotic[22; 30; 43]	Riffle	Burier[35]	Medium[30]	Coarse[30]
P89	*P. smithvanizi* (28)	Hyperbenthic[3]	Lotic[1; 30]	Generalist	Burier[35]	Medium[30]	Coarse[30]
P90	*P. squamata* (30)	Hyperbenthic[3]	Lotic[12; 22; 30]	Generalist	Burier[35]	Extra large[30]	Coarse[30]
P91	*P. stictogaster* (30)	Hyperbenthic[3]	Lotic[12; 22; 30]	Pool	Burier[6, 35]	Medium[30]	Fine[30]
P92	*P. vigil* (30)	Benthic[3]	Lotic[30; 43]	Generalist	Burier[35]	Medium[30]	Fine[30]

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Appendix 2. Materials examined. Institutional abbreviations followed those listed at https://asih.org/standard-symbolic-codes. Materials are grouped by genera and listed alphabetically by species, state, drainage, and institution with collection lot numbers in numerical order. Numbers in parentheses are number of specimens with scales extracted and imaged from collection lot.

Allohistium maydeni: Tennessee: Ohio River Drainage: UT 91.2174 (3), 91.4859 (1), 91.4881 (3), 91.4886 (7), 91.4972 (1), 91.4995 (2), 91.5003 (1), 91.5019 (1), 91.7531 (2), 91.8462 (1).

Ammocrypta beanii: Louisiana: Lake Pontchartrain Drainage: TU 202757 (12); Pearl River Drainage: TU 195856 (18). *A. pellucida*: Kentucky: Ohio River Drainage: MOSU 552 (4), 603 (12), 1001 (1), 2351 (3), 2761 (10). *A. vivax*: Texas: Neches River Drainage: 72753 (30).

Crystallaria asprella: Louisiana: Pearl River Drainage: TU 68924 (15); Mississippi: Pearl River Drainage: TU 28530 (15).

Etheostoma asprigene: Texas: Neches River Drainage: TU 112163 (11), 116149 (19). *E. autumnale*: Missouri: White River Drainage: TU 191354 (30). *E. baileyi*: Kentucky: Cumberland River Drainage: UT 91.7240 (20), 91.5076 (10). *E. barbouri*: Kentucky: Ohio River Drainage: MOSU 1300 (3), 2608 (2), UT 91.1805 (25). *E. barrenense*: Tennessee: Green-Ohio River Drainage: APSU 00678 (14), 002374 (2), 02376 (14). *E. bison*: Tennessee: Ohio River Drainage: APSU 00629 (1), 01730 (3), 01734 (1), 01766 (8), 01780 (2), 01811 (2), 01849 (1), 01851 (1), 01874 (3), 01977 (1), 02029 (2), 59598 (5). *E. blennius*: Tennessee: Tennessee-Ohio River Drainage: APSU 00647 (1), 00698 (4), 00729 (3), 00807 (1), 00977 (1), 01266 (1), 01942 (1), 01961 (1), UT 91.6118 (17). *E. boschungi*: Alabama: Ohio River Drainage: TU 79718 (21); Tennessee: Ohio River Drainage: INHS 36293 (1), 79437 (6); UT 91.4292 (2). *E. brevispinum*: North Carolina: Yadkin River Drainage: NCSM 33827 (18), 64683 (12). *E. caeruleum*: Tennessee: Cumberland-Ohio River Drainage: APSU 00722 (2), 00800 (2); Duck-Tennessee-Ohio River Drainage: APSU 00630 (3), 00972 (15), 01175 (2), 02011 (1), 02032 (1). *E. chlorosoma*: Louisiana: Pearl River Drainage: TU 43831 (30). *E. collis*: North Carolina: Deep River Drainage: NCSM 18191 (20), 53166 (2); Haw River Drainage: NCSM 48521 (8). *E. colorosum*: Florida: Blackwater River Drainage: 154458 (30). *E. cragini*: Missouri: Spring-Neosho-Arkansas River Drainage: INHS 75481 (7); Arkansas River Drainage: TU 191344 (13), 191351 (10). *E. crossopterum*: Kentucky: Ohio River Drainage: APSU 00064 (11), 00090 (10), 00102 (9). *E. derivativum*: Tennessee: Cumberland River Drainage: TU 191447 (30). *E. duryi*: Tennessee: Tennessee-Ohio River Drainage: NCSM 83925 (30). *E. edwini*: Alabama: Yellow River Drainage: TU 73179 (24); Florida: Blackwater River Drainage: TU 124296 (6). *E. etnieri*: Tennessee: Cumberland-Ohio River Drainage: APSU 00572 (5), UT 91.6593 (25). *E. flavum*: Tennessee: Cumberland-Ohio River Drainage: APSU 00719 (24); Tennessee-Ohio River Drainage: APSU 01976 (2), 01932 (1), 01989 (1), 02010 (2). *E. fusiforme*: Florida: Choctawatchee: TU 105558 (30). *E. gracile*: Kentucky: Black Slough-Mayfield Creek-Mississippi River Drainage: MOSU 2126 (18); Cumberland-Ohio River Drainage: MOSU 2626 (4); Obion River Drainage: MOSU 2162 (2), 2205 (6); Mississippi: Big Black River Drainage: TU 133700 (27). *E. gutselli*: North Carolina: Little Tennessee-Tennessee River Drainage: TU 26217 (6), 29502 (1); 91.3577 (23). *E. histrio*: Mississippi: Bayou Pierre Drainage: TU 55690 (30). *E. jessiae*: Georgia: Tennessee River Drainage: UT 91.4507 (6); Tennessee: Nolichucky-French Broad-Tennessee-Ohio River Drainage: UT 91.3309 (13), 91.4190 (10). *E. kanawhae*: Virginia: New River Drainage: TU 70479 (17), 70058 (8), 19575 (5). *E. kennicotti*: Tennessee: Tennessee-Ohio River Drainage: TU 88688 (15). *E. lachneri*: Alabama: Sipsey River Drainage: UT 91.2194 (11); Tombigbee River Drainage: UT 91.3562 (19). *E. lawrencei*: Kentucky: Green-Ohio River Drainage: UT 91.7336 (30). *E. lepidum*: Texas: Colorado River Drainage: TU 97425 (30). *E. luteovinctum*: Tennessee:

Duck-Tennessee-Ohio River Drainage: APSU 04356 (30). *E. lynceum*: Tennessee: Obion River Drainage: APSU 01473 (24), 01457 (6). *E. microperca*: Missouri: Gasconade River Drainage: INHS 75828 (30). *E. nigrum*: Tennessee: Obion River Drainage: APSU 00449 (8), 01116 (14), 01574 (8). *E. nuchale*: Alabama: Alabama River Drainage: TU 56514 (24). *E. okaloosae*: Florida: Choctawhatchae Bay Drainage: TU 31859 (14), 56795 (6), 102742 (10). *E. oophylax*: Tennessee: Cumberland-Ohio River Drainage: APSU 02652 (2); Tennessee-Ohio River Drainage: APSU 01224 (1), 01754 (14), 01778 (2), 02646 (1), 02647 (2), 02648 (2), 02649 (1), 02654 (2), 02657 (3). *E. parvipinne*: Alabama: Alabama River Drainage: TU 35057 (6); Choctawhatchee-Alabama River Drainage: TU 183319 (16); Mississippi: Homochitto-Mississippi River Drainage: TU 67983 (8). *E. planasaxatile*: Tennessee: Duck-Tennessee-Ohio River Drainage: APSU 00641 (19), 01178 (1), 01713 (1), 01838 (3), 01928 (2), 01965 (4). *E. podostemone*: Virginia: Roanoke River Drainage: TU 69183 (4), 71030 (10), 72318 (3), 73198 (5), UT 91.3026 (8). *E. proeliare*: Mississippi: Big Black River Drainage: TU 139403 (30). *E. pseudovulatum*: Tennessee: Duck-Tennessee-Ohio River Drainage: APSU 04661 (6); Tennessee-Ohio River Drainage: APSU 01707 (1), 01719 (1), 01727 (2), 01731 (1), 01808 (1), 01822 (1), 01835 (1), 01847 (1), 01850 (1), 01870 (1), 01886 (1), 01894 (1), 01912 (1), 01922 (1), 01931 (1), 01944 (1), 01959 (1), 01973 (1), 01974 (1), 02002 (1), 02006 (1), 02026 (1), 02030 (1), 02047 (1), 02665 (1). *E. pyrrhogaster*: Tennessee: Obion River Drainage: APSU 02668 (18), 02679 (4), UT 91.2757 (8). *E. radiosum*: Arkansas: Saline-Little River Drainage: TU 97689 (30). *E. sagitta*: Kentucky: Cumberland-Ohio River Drainage: MOSU 872 (1), 888 (1), 1954 (1), 2021 (3), 2148 (2), 2082 (1), 2729 (1); Tennessee: Cumberland-Ohio River Drainage: UT 91.3522 (8), 91.3549 (9), 91.4015 (2), 91.4941 (1). *E. sitikuense*: Tennessee: Tennessee-Ohio River Drainage: UT 91.6373 (30). *E. smithi*: Kentucky: Cumberland-Ohio River Drainage: INHS 61173 (12), 61182 (6), 75017 (10), 84190 (2). *E. spectabile*: Missouri: Arkansas River Drainage: TU 191355 (21); Oklahoma: Arkansas River Drainage: TU 188642 (9). *E. stigmaeum*: Alabama: Coosa-Alabama River Drainage: TU 68212 (30). *E. swaini*: Mississippi: Pearl River Drainage: TU 128766 (30). *E. swannanoa*: North Carolina: French Broad-Tennessee-Ohio River Drainage: TU 29639 (12); Tennessee: French Broad-Tennessee-Ohio River Drainage: UT 91.4530 (18). *E. tetrazonum*: Missouri: Missouri-Mississippi River Drainage: TU 38033 (16), 118111 (8), 188902 (6). *E. variatum*: Ohio: Scioto-Ohio River Drainage: UT 91.5466 (9); West Virginia: Elk-Kanawha-Ohio River Drainage: UT 91.3011 (15), 91.5415 (6). *E. vitreum*: Virginia: Roanoke River Drainage: TU 72681 (30). *E. whipplei*: Mississippi: Mississippi River Drainage: TU 3614 (30). *E. zonale*: Tennessee: Hiawassee-Tennessee-Ohio River Drainage: UT 91.4050 (30). *E. zonistium*: Tennessee: Big Sandy-Tennessee-Ohio River Drainage: APSU 01226 (9), 01229 (21).

Nothonotus acuticeps: North Carolina: Nolichucky-French Broad-Tennessee-Ohio River Drainage: 91.4018 (11); Tennessee: Nolichucky-French Broad-Tennessee-Ohio River Drainage: UT 91.1875 (5), 91.1876 (3), 91.4036 (11). *N. bellus*: Kentucky: Barren-Green-Ohio River Drainage: APSU 01307 (4), UT 91.1811 (6); Tennessee: Barren-Green-Ohio River Drainage: APSU 00676 (2), 00766 (1), 02387 (2), UT 91.3772 (15). *N. camurus*: Tennessee: Big South Fork Cumberland-Cumberland-Ohio River Drainage: UT 91.5435 (10); Clinch-Tennessee-Ohio River Drainage: UT 91.5829 (9); Nolichucky-French Broad-Tennessee-Ohio River Drainage: UT 91.8213-6548 (11). *N. jordani*: Alabama: Alabama River Drainage: TU 106777 (15); Georgia: Alabama River Drainage: UT 91.5161 (8), 91.5875 (7). *N. microlepidus*: Kentucky: Little-Cumberland-Ohio River Drainage: APSU 00391 (5); Tennessee: East Fork Stones-Cumberland-Ohio River Drainage: UT 91.3030 (6); Harpeth-Cumberland-Ohio River Drainage: UT 91.711 (6); Red-Cumberland-Ohio River Drainage: UT 91.3739 (13). *N. rufilineatus*: Tennessee: Cumberland-Ohio River Drainage: APSU 00797 (5), 01205 (8), 01261 (12); Buffalo-Tennessee-Ohio River Drainage: APSU 00694 (3); Piney-Duck-Tennessee-Ohio River Drainage: APSU

02008 (2). _N. tippecanoe_: Kentucky: Cumberland-Ohio River Drainage: UT 91.7305 (20); Tennessee: Cumberland-Ohio River Drainage: UT 91.4296 (10).

Percina aurantiaca: Tennessee: Clinch-Tennessee River Drainage: UT 91.4130 (24), 91.3652 (6). _P. caprodes_: Arkansas: White River Drainage: TU 50422 (10), 50428 (20). _P. copelandi_: Arkansas: Fourch la Fave Drainage: TU 97099 (4); Little River Drainage: TU 93159 (6); Ouachita River Drainage: TU 188842 (2), 202555 (4); Red River Drainage: TU 103417 (1), 103418 (3); Oklahoma: Kiamichi-Red River Drainage: UT 91.1896 (4); Little River Drainage: TU 147542 (6). _P. crassa_: North Carolina: Cape Fear River Drainage: 75482 (30). _P. evides_: Georgia: Hiawassee-Tennessee-Ohio River Drainage: UT 91.5184 (9); Tennessee: Tennessee-Ohio River Drainage: APSU 03420 (1), 04525 (2), 04529 (1), 04531 (12); Duck-Tennessee-Ohio River Drainage: APSU 01067 (1), 01841 (1); Nolichucky-French Broad-Tennessee-Ohio River Drainage: APSU 04526 (1), 04528 (2). _P. gymnocephala_: North Carolina: New-Kanawha-Ohio River Drainage: NCSM 2928 (8); South Fork New-New-Kanawha-Ohio River Drainage: NCSM 36669 (6), 36670 (9), 53397 (9). _P. kathae_: Alabama: Alabama River Drainage: TU 167172 (1), 60463 (1); Cahaba-Alabama River Drainage: TU 188854 (1), 202291 (2); Coosa-Alabama River Drainage: TU 152202 (2); Black Warrior River Drainage: TU 167905 (7); Tombigbee-Mobile Bay Drainage: TU 60060 (4), 77069 (4), 127789 (2), 184019 (4); Georgia; Conasauga-Alabama River Drainage: TU 121094 (1); Mobile Bay Drainage: TU 30463 (1). _P. lenticula_: Mississippi: Pascagoula River Drainage: TU 30074 (10), 53715 (8), 58772 (1), 58807 (4), 59560 (2), 60556 (2), 66080 (1); Pearl River Drainage: TU 189925 (2). _P. macrocephala_: Kentucky: Ohio River Drainage: MOSU 2369 (1), 2373 (1), 2385 (1); Green-Ohio River Drainage: APSU 05045 (3), INHS 76989 (1), 76990 (1), 76991 (1), 76992 (1), 76995 (2), 77000 (1), MOSU 2291 (3); Pennsylvania: Allegheny-Ohio River Drainage: MOSU 920 (1); Tennessee: Tennessee-Ohio River Drainage: UT 91.737 (1), 91.1544 (3). _P. macrolepida_: Louisiana: Calcasieu River Drainage: TU 120560 (1); Texas: Neches River Drainage: TU 111060 (7), 111805 (12), 116113 (6), 116151 (4). _P. maculata_: Illinois: Mill Creek-Wabash-Ohio River Drainage: UT 91.1709 (21), 91.5751 (4); Kentucky: Kinniconick Creek-Ohio River Drainage: UT 91.2596 (4). _P. nevisense_: North Carolina: Contentnea Creek-Neuse River Drainage: NCSM 519 (20), 24549 (4), 52881 (6). _P. nigrofasciata_: Alabama: Alabama River Drainage: TU 40780 (2); Louisiana: Pearl River Drainage: TU 203645 (28). _P. palmaris_: Alabama: Tallapoosa-Mobile River Drainage: UT 91.3449 (13), 91.8285 (5); Georgia: Etowah-Coosa-Mobile River Drainage: UT 91.4371 (7); Tallapoosa-Mobile River Drainage: UT 91.2497 (5). _P. phoxocephala_: Indiana: Wabash-Ohio River Drainage: UT 91.1952 (29); Ohio: Ohio River Drainage: UT 91.2971 (1). _P. rex_: Virginia: Roanoke River Drainage: INHS 27297 (1), 83454 (2), 83526 (1), 83900 (3), TU 71033 (12). _P. roanoka_: Virginia: Roanoke River Drainage: TU 71032 (30). _P. sciera_: Tennessee: Hatchie River Drainage: APSU 03406 (2), 03446 (3), 03448 (1); Mississippi River Drainage: APSU 03441 (1); North Fork Obion-Obion River Drainage: APSU 03442 (15); Obion River Drainage: APSU 01660 (1), 01671 (1); Wolf River Drainage: APSU 03444 (4), 03447 (2). _P. shumardi_: Louisiana: Pearl River Drainage: TU 100922 (2), 111574 (13), 124459 (15). _P. smithvanizi_: Alabama: Tallapoosa-Alabama River Drainage: UAIC 8515.12 (17), UT 91.8286 (6); Georgia: Tallapoosa-Alabama River Drainage: UT 91.7813. _P. squamata_: North Carolina: French Broad-Tennessee-Ohio River Drainage: NCSM 7312 (4), 7328 (3), 25045 (2), 52375 (1), 52468 (1), 55302 (1), 70560 (2); Hiawassee-Tennessee-Ohio River Drainage: NCSM 3947 (2); Tennessee: Emory-Tennessee River Drainage: UT 91.556 (1); Obed River Drainage: UT 91.559 (13). _P. stictogaster_: Kentucky: Red-Kentucky River Drainage: MOSU 10 (3), 241 (4), 1827 (3); Kentucky River Drainage: MOSU 908 (4), 2497 (2), 2599 (10), 2601 (4). _P. vigil_: Mississippi: Pascagoula River Drainage: TU 182025 (30).