Internal vertebral morphology of bony fishes matches the mechanical demands of different environments

Dana Baxter
BIO-199
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PI: Dr. Eric Tytell
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Abstract:

Bony fishes reside in different habitats. Such habitats can be classified broadly into the pelagic, demersal, and benthic habitats. Pelagic fishes mainly swim in open water, benthic fishes spend most of their time on the floor of the body of water, and demersal fishes stay close to but do not rest for long times on the floor of the body of water. These different habitat categories also likely necessitate distinct swimming styles. Pelagic fishes often swim continuously, benthic fishes often remain mostly stationary with occasional bursts of acceleration, and demersal fishes swim up and down in the water column. These swimming styles probably depend on the mechanical properties of the vertebrae and notochord. Bony fishes have hourglass shaped vertebrae, called amphicoelous vertebrae, which contain a hole, or foramen, through which the notochord (an important developmental structure) is threaded. Therefore, we hypothesize that the morphology of the vertebrae and notochord will be different to support the swimming styles that are required in the habitat in which a species resides. We predict that pelagic fishes will have narrower notochordal foramina and shallower vertebral cones because this will leave less space for flexible notochordal material, meaning that the vertebral columns of pelagic fishes will be stiffer and thus more conducive to the open water swimming required for pelagic fishes. We examined differences in vertebral morphology in 55 species from the three habitat categories by measuring vertebrae along the length of the body of each fish based on micro-computed tomography scans. From these scans, six vertebral features were measured: notochordal foramen diameter, centrum body length, and the cone angles and diameters for the anterior and posterior vertebral cones. Using a phylogenetic generalized least squares (PGLS) analysis to control for phylogeny, we found that notochordal foramen diameter, anterior cone angle, and posterior cone angle differed significantly among species from benthic, demersal, and pelagic habitats. Benthic

species had significantly larger posterior cone angles than the other habitat groups, while pelagic species had significantly smaller posterior cone angles than the other habitat groups and significantly smaller notochordal foramen diameters than demersal species. We also ran a linear discriminant analysis (LDA), which showed distinct groupings for morphologies from the benthic and pelagic categories. Overall, we found significant differences in internal vertebral morphology between species in different habitat categories, differences which could accurately discriminate benthic and pelagic based on vertebrae. This might help us infer habitat differences for species where behavior and habitat may be difficult to monitor, and for fossil fishes as well. If vertebral measurements can be used to understand differences between species in multiple habitats, this work provides a window into exploring the potential habitats in which extinct species resided.

Introduction:

Teleost fishes live in different habitats within the water column. Three broad habitat categories that have an effect on fish body structure are benthic, demersal, and pelagic. Benthic species are those that spend most of the time in contact with the substrate of the body of water, demersal species are those that are close to the substrate of the body of water but not directly in contact, and pelagic species are open-water swimmers (Friedman et al. 2020). Species tend to diversify when there are open niches along the habitat axis, whether that be due to habitat shifts (Hollingsworth et al. 2013), the aftermath of mass extinctions (Ribeiro et al. 2018), or general adaptive radiation (Cooper et al. 2010). Therefore, habitat diversification often leads to morphological diversification, due to the new niches to fill.

Diversification along the benthic-pelagic habitat axis has occurred in all groups of fishes. In freshwater fishes, this increase in biodiversity in the benthic-pelagic habitat axis has been documented during the diversification of cyprinids, highlighting the impact of this habitat axis on biodiversity (Hollingsworth et al. 2013). This habitat diversification can also be seen in the cichlid fishes. The evolution and diversification of cichlids (as seen through the lens of the evolution of their feeding mechanics) were found to have a vast effect on the biomechanics, and this increase in diversity allows fish with differing features to fit into different niches. These different niches along the benthic-pelagic habitat axis require different swimming modes, which may contribute to shaping diversification and biodiversity in this habitat axis (Cooper et al. 2010).

Marine fishes have also diversified along this habitat axis. For example, the Carangaria clade of marine fishes has a wide array of morphological differences, and a lot of the increase in biodiversity occurred across the Cretaceous-Palaeogene extinction. This increase in biodiversity occurred due to the diversification of previously unspecialized fishes in filling newly available habitat niches that had been emptied as a result of the extinction (Ribeiro et al. 2018).

Thus, in general, fish species diversify along the benthic-pelagic habitat axis due to the diversity of niches to fill, and this pattern holds for many groups (Hollingsworth et al. 2013; Ribeiro et al. 2018; Cooper et al. 2010). These open niches may permit new morphologies, as shown in the diversity of morphology between niches along the benthic-pelagic habitat axis.

Specifically, one recent analysis found that body shape of marine fishes differs between benthic, demersal, and pelagic habitats (Friedman et al. 2020). The body shapes of these fishes were found to depend on the habitat in which the fish resided, possibly because of varying locomotor requirements in each habitat. The highest morphological diversification was found in

benthic fishes, because they encounter many different types of substrates while swimming, rather than just water or a uniform substrate (Friedman et al. 2020).

Not only do fish in these habitats differ in body shape but living in these three different habitat categories also may necessitate different swimming styles. By definition, pelagic fishes live in open water, which means that many of them swim constantly. Demersal and benthic fishes interact with the substrate to varying degrees, which may permit different styles of movement. For example, some benthic fishes such as the round goby (Kornis, Mercado-Silva, and Zanden 2012) require less constant swimming, only using brief thrusts for swimming when necessary (Pennuto and Rupprecht 2016). In contrast, many marine pelagic fishes need to travel far distances, which forces them to have higher-functioning cruising and sprinting gaits, as well as high-speed swimming in order to escape from predators in open water (Webb 1994; Aleyev 1977) . Therefore, fish in different habitats require different swimming styles to cope with the requirements of living in that habitat.

We hypothesize that the differences among species in body shape and swimming mode in these habitats may contribute to internal morphological differences, specifically in the vertebral column. Fish are known to have similar components making up their backbones, but due to body shape and swimming style differences exhibited between habitats, there may be variation in the vertebrae of fish that span these three habitat categories: benthic, demersal, and pelagic.

In general, teleost fishes have characteristically hourglass shaped vertebral centra, called amphicoelous centra (Laerm 1976). Each vertebral centrum has a hole through the middle called the notochordal foramen, through which the notochord passes (Symmons 1979). The notochord is an important element in the extensive vertebral column development of teleost fishes, along with sclerotome and myotome formation (Ramanujam 1929).

The notochord fills the joint between vertebrae, and these intervertebral joints may be crucial in fish locomotion. The joints act like hinges between each vertebra. The mechanical properties of the joints contribute to the stiffness of the vertebral column (Porter and Long 2010; Long 1992; 1995; Long, Koob-Edmunds, and Koob 2004; Nowroozi and Brainerd 2012), although others have questioned whether fish bend enough during normal swimming for the intervertebral joints to have any effect (Nowroozi and Brainerd 2013). Nevertheless, there are small variations to the notochordal and vertebral structure based on the swimming style of the fish in question (Symmons 1979).

We therefore examined the variation in internal vertebral morphology in benthic, demersal, and pelagic fishes across the actinopterygian fishes. To identify differences in vertebral morphology of bony fishes due to habitat, we controlled for phylogeny using the Betancur 2017 phylogenetic tree (Betancur-R et al. 2017). This 2017 tree is a modified version of the Betancur 2013 phylogenetic tree detailing the phylogeny of bony fishes (Betancur-R. et al. 2013). The 2017 tree contains a larger number of phylogenetic orders than the 2013 version, but there are still some uncertain divisions in the newer tree (Betancur-R et al. 2017).

Because actinopterygian fishes have different swimming styles that can depend upon their habitat category (Webb 1994), fish body shape depends on this habitat category as well (Friedman et al. 2020). We hypothesize then that actinopterygian vertebral structure may be different depending upon the habitat category in which the fish lives. Based on the physical necessities for each habitat (pelagic, demersal, and benthic), we hypothesize that pelagic fishes would require a stiffer backbone than fishes from other habitats, due to the demands of constant swimming in open water. Thus, we predict that pelagic fishes should have smaller foramina and

shallower vertebral cones, leaving less space for flexible notochordal material, and ultimately leading to a stiffer backbone.

Methods:

We used micro-computed tomography scans to measure six different vertebral features from species across the phylogenetic tree (Betancur-R et al. 2017). Figure 1 shows a phylogenetic tree of the species used. We classified these species into benthic (species in contact with the substrate most of the time), pelagic (open water swimmers), and demersal (species close to the substrate but not resting on it all of the time) habitats. For all of the species we examined that overlapped those from Friedman et. al. (2020), we used their classification. For other species, we classified habitat based on descriptions of the species from guidebooks or relevant journal articles (Gilbert and Williams 2002; Page 1991; van der Sleen and Albert 2018; Lowry et al. 2005; Allen 2002; Matsui and Rosenblatt 1987; Fine, Horn, and Cox 1987; McGinnis and Alcorn 2006; Pearcy, Stein, and Carney 1982; Mérigoux, Ponton, and de Mérona 1998; Magid 1967; Jaafar et al. 2004; Rodríguez-Olarte, Mojica Corzo, and Taphorn Baechle 2011; Bailey 1994; Proctor and Lynch 2011; Phomikong et al. 2015; Vašek et al. 2008; Basolo 1990; Pietsch and Orr 2015).

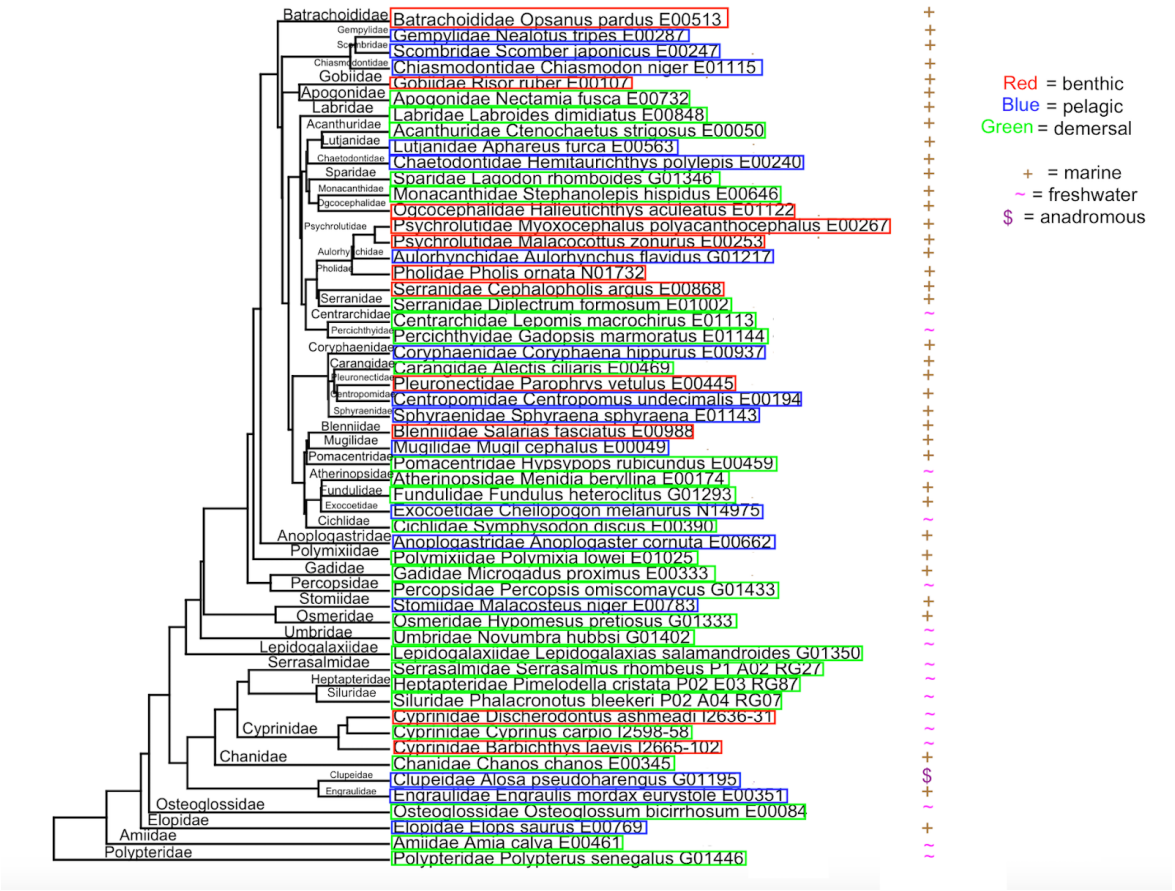

Figure 1: **Phylogenetic Tree Organized by Habitat**. This figure depicts the phylogeny (based on the Betancur 2017 phylogenetic tree) of all species included in this study, colored by habitat category. The species in red are benthic, the species in blue are pelagic, and the species in green are demersal.

Vertebral Measurements:

The micro-computed tomography (CT) scans were downloaded as image stacks off of Morphosource (Duke University 2012) or Open Science Framework (Summers 2015) databases, with the exception of a few scans. The scan for *Chaetostoma lineopunctatum* was scanned by Matthew Kolmann from the AUM collection, specimen number 51201. The scan for *Phalacronotus bleekeri* was scanned by Milton Tan and Matthew Kolmann (Tan and Kolmann 2017). The scan for *Cyprinus carpio* was scanned by Kevin Conway from Texas A&M University.

We measured vertebral features for vertebrae at 30% to 90% of the fish's body length, in increments of 10%. We used four reference points along the length of the body to calculate which points were at 10%-90% of the respective species' body length and measured the vertebrae at those points if there was a vertebral centra present at that percentage of the body length. These four reference points along the body length were the snout, the first vertebra behind the head, the vertebra where the hemal arches first connect into a hemal spine, and the final vertebra before the tail. Once these points were used to calculate the 10%-90% points along the body at which to measure the vertebrae, seven points were placed on sagittal sections through the center of vertebrae at those points measured along the length of the body in order to measure six different aspects of the vertebrae along the body (Figure 2B).

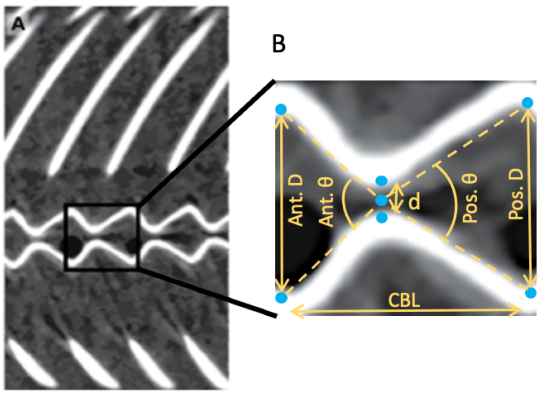

Figure 2: **Vertebral Centra Measurements**. A depicts the amphicoelous nature of the centra. B depicts the points on each vertebra used to collect vertebral measurements (Panel A made by Dr. Cassandra Donatelli).

Using 3D Slicer (version 4.8.1), we measured the notochordal foramen diameter, the anterior and posterior cone angles, the centrum body length, and the anterior and posterior cone diameters based on seven points at each vertebral centrum (Figure 2B) present at the equidistant body points along the length of the fish. These measurements were converted to .fcsv files.

Statistical Analysis:

To examine differences in vertebral morphological measurements as they relate to pelagic, demersal, or benthic habitat categories, as well as in order to control for differences across the phylogeny, we performed phylogenetic generalized least squares tests, using lectures and exercises from the Comparative Methods in R conference for reference to understand the process of PGLS analysis in R (L.L.J. Harmon et al. 2015). The R packages used in these PGLS

analyses were “geiger” (Pennell et al. 2014), “ape version 5.3” (Paradis and Schliep 2018), “nlme version 3.1.145” (Pinheiro et al. 2020), “emmeans” (Lenth 2020), and “car” (Fox and Weisberg 2019). Other important R packages were “tidyverse” (Wickham et al. 2019), “phytools” (Revell 2012), “here” (Müller 2017), “multcompView” (Graves, Piepho, and Dorai-Raj 2019), “ggplot2” (Wickham 2016), “Hmisc” (Jr, Dupont, and others 2020), “readxl” (Wickham and Bryan 2019), “plotly” (Sievert 2020), “ggthemes” (Arnold 2019), “naniar” (Tierney et al. 2020), “dplyr” (Wickham et al. 2020), “ggbeeswarm” (Clarke and Sherrill-Mix 2017), and “patchwork” (Pedersen 2019).

Linear Discriminant Analysis:

To examine each vertebral feature measurement taken and to align the measurements by habitat on the axes that best group the measurements by habitat, we ran a linear discriminant analysis (LDA). This LDA allowed for the vertebral measurements taken for each feature (centrum body length, notochordal foramen diameter, anterior/posterior cone diameters, and anterior/posterior cone angles) to be grouped by habitat and aligned on axes in a way that best separated the habitat groups. The R packages used for the LDA were “FactoMineR” (Lê, Josse, and Husson 2008), “factoextra” (Kassambara and Mundt 2020), and “MASS” (Venables and Ripley 2002). Other important R packages were “patchwork” (Pedersen 2019), “GGally” (Schloerke et al. 2020), “scico” (Pedersen and Crameri 2020), “tidyverse” (Wickham et al. 2019), and “ggrepel” (Slowikowski 2020). To examine the classification accuracy, we performed a cross validation, examining whether the LDA was able to accurately predict the actual habitat in which a species resides based on the vertebral measurements. We then tested reduced data sets with fewer vertebrae to identify the vertebrae needed for best classification.

Results:

Morphology of vertebrae differ in fishes from different habitats

We compared the vertebral measurements for fishes in different habitats, while controlling for phylogenetic effects using a phylogenetic generalized least squares (PGLS) analysis (L.L.J. Harmon et al. 2015). We first took the maximum, minimum, and mean of the vertebral feature along the length of the body of the fish. The overall pattern of variation across habitats was similar for maximum, minimum, and mean values, and so we focus here on the maximum values. Overall, the maximum notochordal foramen diameter and the maximum angle of the anterior and posterior vertebral cones were significantly different for fishes in the three habitats.

The maximum angle of posterior and anterior vertebral cones along the length of the body was significantly different between habitat categories (Figure 3A, B; ANOVA, $df = 2$, $p<0.001$). Specifically, the maximum posterior vertebral cone angle of the benthic species was found to be significantly larger than both demersal (emmeans, $df = 52$, $t = 2.814$, $p = 0.0186$) and pelagic species (emmeans, $df = 52$, $t = 4.518$, $p = 0.0001$). Similarly, the maximum anterior cone angles were significantly different between habitat categories (Figure 3B; ANOVA, $df = 2$, $p<0.001$). The maximum anterior cone angles of the pelagic species were significantly smaller than those of the benthic (emmeans, $df = 52$, $t = 4.317$, $p = 0.0002$) or demersal species (emmeans, $df = 52$, $t = 2.436$, $p = 0.0474$).

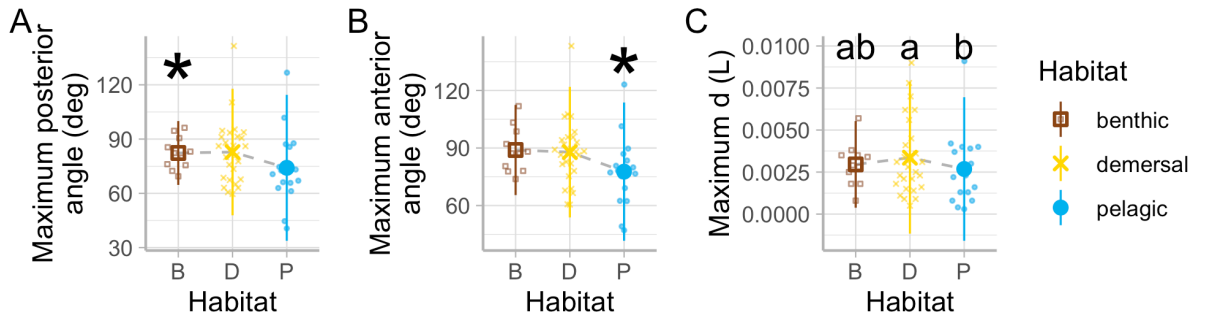

Figure 3: **PGLS for the maximum measurements of notochordal foramen diameter and anterior/posterior cone angles**. A depicts the maximum posterior cone angle for each species along the length of the body, grouped by habitat, where benthic has a significantly larger cone angle. B depicts the maximum anterior cone angle for each species along the length of the body, grouped by habitat, where pelagic has a significantly smaller cone angle. C depicts the maximum notochordal foramen diameter, where the pairwise comparison shows pelagic fishes have a smaller foramen diameter than demersal.

The maximum notochordal foramen diameter along the length of the body was significantly different between species in different habitat parameters (Figure 3C; ANOVA, df = 2, p = 0.0055). Pelagic species had significantly smaller foramina than demersal species (emmeans, df = 52, t = 3.073, p = 0.0093).

Across the habitats, we did not find significant differences in the posterior cone diameter (Figure 4A; ANOVA, df = 2, p = 0.0621), anterior cone diameter (Figure 4B; ANOVA, df = 2, p=0.2723), or maximum centrum body length (Figure 4C; ANOVA, df = 2, p = 0.769).

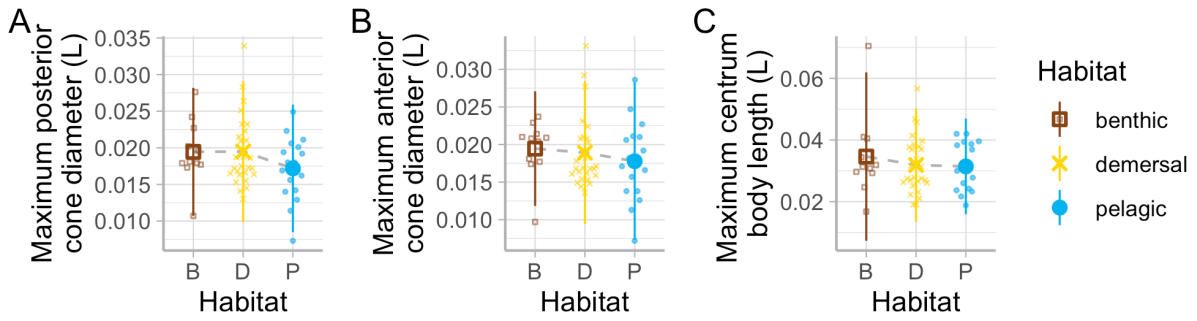

Figure 4: **PGLS for the maximum measurements of centrum body length and anterior/posterior cone diameters**. A depicts the maximum posterior cone diameter for each species along the length of the body, grouped by habitat. B depicts the maximum anterior cone diameter for each species along the length of the body, grouped by habitat. C depicts the maximum centrum body length for each species along the length of the body, grouped by habitat.

Internal vertebral morphology accurately distinguishes benthic and pelagic species

Having shown that various aspects of vertebral morphology are correlated with habitat, we performed a linear discriminant analysis (LDA) to determine how well the vertebral features for each species predicted the habitats.

We first examined which combinations of vertebrae might be most predictive of the habitat categories. The most accurate combination of vertebral measurements were the vertebrae at the 40%, 80%, and 90% points along the length of the body of each fish. Figure 5A shows that the combination of the measurements from vertebrae at 40%, 80%, and 90% of the body length accurately predicted a species' habitat 47% of the time. The benthic and pelagic groupings have rather distinct separation, but the demersal category overlaps both of the others. Incorrect habitat predictions are shown on Figure 5A with red crosses, and most of them lay within this demersal group. When the LDA and cross validation was run exclusively to predict benthic versus pelagic species, the predictions were accurate 57% of the time using the 40%, 80%, and 90% points.

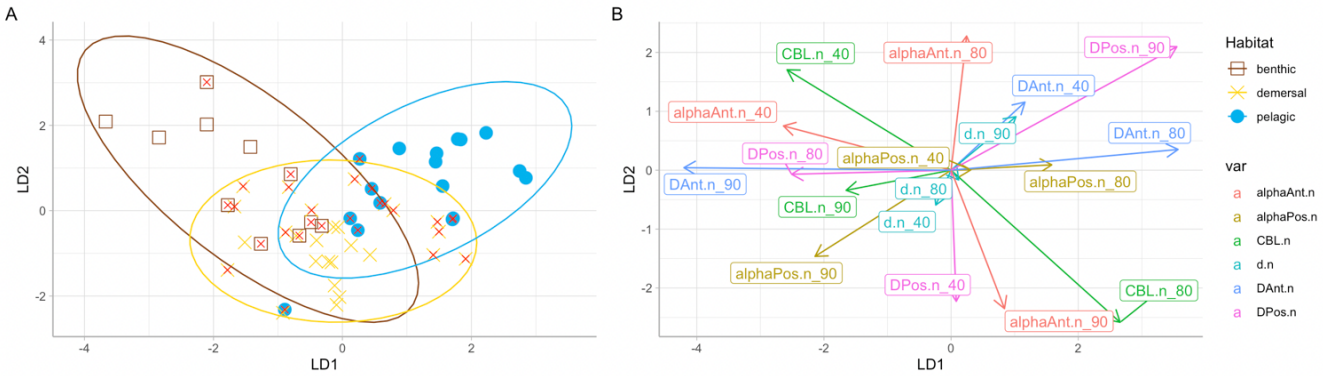

Figure 5: **LDA by habitat with best cross validation predictions and LDA biplot**. A depicts linear discriminant analysis separated by habitat (benthic, demersal, and pelagic), using just the 40%, 80%, and 90% body points. The red x's indicate the species used in this study that were not predicted correctly in the cross validation. B depicts a linear discriminant analysis biplot, defining the LD axes using the measurements for each vertebral feature at 40%, 80%, and 90% of the body length of the species.

Figure 5B shows the LD axis loadings (n loadings). Because notochordal foramen diameter (in BL) is a very small value in comparison to vertebral cone angle (in degrees), each variable was divided by its standard deviation over the whole data set in order to plot all of the

variables over a range of ± 1-2 standard deviations. Calculating the axis loadings in this way did not have an effect on the results of the LDA. None of the vertebral measurements contribute significantly to one axis over the other. This is shown in the biplot by the vectors for each variable at a particular vertebra pointing in opposite directions. Because the axis loadings are in opposite directions for different vertebrae but the same feature, this indicates that variation along the length of the body of the fish may be important for distinguishing habitat groups and predicting in which habitat a species resides. An LDA on data from all six vertebrae produces similar results, distinctly separating the benthic, demersal, and pelagic groups within the LD axes (data not shown). Cross-validation analysis on this model showed that it predicted the correct habitat 29% of the time.

Discussion:

We have shown that fish species that reside in different habitats have vertebrae with different morphology. Maximum notochordal foramen diameter, maximum anterior cone angle, and maximum posterior cone angle were significantly different for fishes from pelagic, demersal, and benthic habitats. In general, fish from pelagic habitats tended to have shallower cones and narrower foramina than fish from the other habitats (Figure 3). We suggest that these differences result in pelagic fishes having overall stiffer backbones, which may help them to swim continuously.

These differences may have also arisen due to adaptive radiation allowing for the filling of open niches in the changing benthic-pelagic habitat axis (Cooper et al. 2010; Hollingsworth et al. 2013; Ribeiro et al. 2018). Overall, the body morphology differs in fishes along the benthic-pelagic habitat axis in both freshwater (Hollingsworth et al. 2013) and marine fish (Ribeiro et al.

2018), and these differences also occur in different bodily structures of the fish, such as feeding mechanisms (Cooper et al. 2010) or body shape (Friedman et al. 2020). Differences in vertebral morphology may also be part of similar diversification processes.

The vertebrae, the notochord, and their segmentation hold importance for the way that each fish swims (Symmons 1979). Through the PGLS analysis, as hypothesized, pelagic fishes were found to have more bone and less notochord than demersal or benthic species. For example, both the anterior and posterior cones had smaller angles, which mean that the cones were shallower, leaving less space within the vertebrae of pelagic species for flexible notochordal material. Thus, the pelagic vertebrae may contribute to a stiffer vertebral column that would be more conducive to the open water swimming that pelagic fishes need to endure.

Based on a linear discriminant analysis, we found that the morphology of three vertebrae were sufficient to classify benthic and pelagic species, and the predictions were accurate 57% of the time. When including demersal species as well, this accuracy was lower at 47%, and many of the misclassified species were on the LD axes where all three habitat categories overlapped. The inaccuracies being present where demersal overlaps with benthic and pelagic could be due to the differences in the demersal habitat (as physically between benthic and pelagic in depth) being less distinct than the differences between benthic and pelagic habitats. Therefore, because the demersal habitat holds this overlap with the other two habitats, solely predicting the habitat between benthic and pelagic is more accurate.

Despite the significant differences between notochordal foramen diameter and vertebral cone angles based on habitat, as well as the predictability of classifying benthic and pelagic species based on vertebral measurements, aspects of this hypothesis still need to be tested. First,

are vertebral columns in pelagic fishes stiffer as a result of having less notochordal material? Second, are stiffer backbones better for the more constant swimming required of pelagic fishes?

Conclusion:

The internal morphology of the vertebrae of actinopterygian fishes differs significantly among fishes in benthic, demersal, and pelagic habitats. These morphological differences were especially distinct between benthic and pelagic habitat groups, which could be classified accurately 57% of the time based on just three vertebrae. This predictive ability of vertebral measurements by habitat could shed light on species that have been particularly difficult to study, because they live in inaccessible habitats or are difficult to track. It may also be useful to help classify the habitats of fossil fishes. Having a larger understanding of the impact of habitat on vertebral structure can assist in predicting the habitats in which many fishes belong where a way to study which habitats those fishes belong to may no longer be possible.

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