Homodonty and Heterodonty – and iconoclastic view of teeth

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Abstract

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Teeth are perhaps one of the most readily identifiable traits that categorize vertebrates. Indeed, much of what we know about human evolution is based on fossilized teeth. In a similar manner the teeth of other vertebrates can serve as an important clue in identifying the relationship between the morphology of the organism and its environment. Before this potential can be realized, we need a more integrative approach for examining the role of different tooth structures. Previous studies have explored the role of tooth shape largely divorced from the way teeth interact with each other, with food, and with different anatomical structures involved in feeding. My dissertation addresses how measuring dental mechanics can reveal nuances in shape, function, and evolution of teeth as well as how teeth function in combinations with lips, tongues, and jaw muscles.

Tooth function changes with shape, orientation (which way the tooth is pointing), position, and the dynamic forces produced by the jaw muscles. We refer to dentitions with all the same shaped teeth as homodont (‘homo’ = same, ’dont’ = tooth) and those with different shapes

and sizes as heterodont. Collapsing dentitions into such blunt and discrete categories, obscures both functional differences between teeth and insights into dental evolution. Consider that teeth that look the same may not function the same. Conical teeth are the most common tooth shape and prevalent in fishes which make up more than half of all vertebrate diversity, Conical teeth are superficially tasked with the simple job of puncture. However, there is a great deal of variation in the shape and placement of conical teeth. This variation suggests conical dentitions represent a single shape solution to different functional problems. To test this hypothesis I developed a statistical model – a 'functional homodonty' metric – that lets us explore how different combinations of teeth work together. The model incorporates parameters relevant to conical teeth, but there is no reason that the model cannot be expanded to investigate incisive, molariform, or triangular tooth shapes. Indeed, other groups have already applied the metric in very different conditions than I proposed. The evolutionary history and variation of dental morphologies creates opportunities to explore the bounds of the functional homodonty metric. By doing so, we increase our understanding of how and why morphological heterodonty evolves, and the importance that individual teeth serve along the jaw.

The latter half of my dissertation examines the coordinated actions of lips and teeth. Pacus are herbivorous cousins of piranhas and have to peel fruit and crack open seeds without the use of hands. My work showed how the morphology and behavior of the lips work in tangent with the teeth to hold, reposition, and break down complicated food items. In this published work I demonstrated the importance of a multidimensional approach to evolutionary questions and how quantifying variation along more than one axis (i.e space and time) provides the substrate for understanding dietary transitions.

Dedication

This dissertation is dedicated to my partner, Chris, who has been a constant source of support and encouragement during the challenges of graduate school and life. Who drove across the country to support me in my PhD, and who is willing to do it all again. I am truly thankful for having you in my life. This work is also dedicated to my parents, Michele and Brian, who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve. To my grandparents who fostered an early sense of exploration in nature and have always been my number one supporters.

Next, thank you to my mentors under whose guidance I have completed this dissertation. You have all enlightened me with academic knowledge, broadened my horizons, and provided me with valuable advice whenever I needed it most. Special thank you to Patricia Hernandez, without who I would have never found my way academia and to Adam Summers who taught me how to break things responsibly.

TABLE OF CONTENTS

2. INTRODUCTION

Teeth first evolved 450 million years ago as odontodes, the dermal plates and protective armor of large aquatic vertebrates. Odontodes are a synapomorphy (shared, derived trait) of vertebrates that include teeth, but also the denticles and spines of sharks, the armor plates of catfishes, and gar, and the toothy knob on the cranium of male chimeras (Ratfish). All teeth are odontodes, but not all odontodes are teeth. These earliest vertebrate traits represent incredible morphological diversity, yet in some vertebrate groups have remained unchanged for hundreds of millions of years. Because they fossilize so well and characterize almost all living clades, teeth are the best evidence for how organisms interact with their environment. From fossils we can understand how small cone-like teeth evolved into the broad, complex molars of modern day mammals. The relationship between tooth shape and function has been explicitly tested and modeled – flatter teeth with several cusps are used for grinding, and dagger-like cones work well for puncturing. There are more than 30,000 species of fishes and their combinations of tooth shape, replacement, and development match their diversity in diet, behavior, and ecology. However, tooth shape is only one axis of variation and while morphology often predicts function, in teeth the story is far more ambiguous. The aim of my dissertation is to reconcile biomechanical and ecological demands on tooth performance and determine how superficially similar teeth are used for significantly different tasks.

Fishes represent more than half of all vertebrate diversity, but for centuries descriptions of their teeth have remained generalized and categorical (i.e., heterodont versus homodont). This is in part due to the fact that most fishes have dentitions of simple, conical teeth. This is in stark contrast to the dental batteries of mammals where teeth have evolved complex shapes and specific arrangements to perform complicated tasks such as chewing. When observing a battery of teeth along a jaw, we make assumptions about how those teeth work based on their geometry and position. Conical teeth are assigned the simple job of puncture yet, there is so much variation even within conical teeth that these implications of tooth mechanics based on tooth shape fail to adequately capture their functional diversity.

In my dissertation I develop a quantitative and comparative framework of dental function by transforming key dental characteristics into continuous, biomechanical traits – this is the functional homodonty metric (published in the Journal of Anatomy, Cohen *et al.,* 2020). I demonstrate how combinations of conical teeth are linked to key feeding behaviors and that based on their *position*, teeth will vary significantly in their functional and mechanical implications. Historically, homodonty and heterodonty provide an anatomical framework for describing tooth shape in dental batteries. In reality, these terms represent a vague dichotomy and provide no quantitative metric of either tooth or dental function. Functional homodonty more accurately reflects how teeth work together in the dental battery to transmit stress and properly considers the full continuum of shape, force, and mechanical advantage (Cohen *et al.,* 2020 A, Cohen *et al.,* 2020B, Hulsey et al., 2020). Using fundamental lever mechanics, I generated a method for quantifying the function of dental batteries based on the estimated stress of each tooth (inferred using surface area) standardized for jaw out-lever (inferred using tooth position). This method reveals a homodonty–heterodonty functional continuum where small and large teeth work together to transmit forces to a prey item. We can then use this model to predict, on a tooth-by-tooth basis, where there is expected to be a separation of function. Functional homodonty is an integrative biomechanical metric of the teeth and jaws that, when incorporated with genetic networks, could provide further evidence in the evolution of specific dentitions or feeding strategies.

Binning dental morphology into discrete categories is limiting and obscures important phylogenetic and biomechanical information. Alternatively, continuous characters allow a finer scale comparison among environmental, ecological, and gene expression data than can be achieved with categorical morphological characters. The functional homodonty metric can transform anatomical measures of individual teeth into a quantitative metric of dental battery function. A morphological homodont has teeth of "similar" shape and size, but functionally homodont teeth exert similar stresses regardless of shape or size. Where morphological heterodonty captures shape regionalization across individual teeth, functional heterodonty highlights teeth with stresses that exceed a threshold across a dentition. In my second chapter, I use *Halichoeres* wrasses to explore how a functional homodonty continuum can be used in phylogenetic analyses. *Halichoeres* are a group of indo-pacific wrasses that all feed on similar invertebrates and corals. Here we show that functionally heterodont teeth have evolved at least three times in *Halichoeres* wrasse and in three different ways. Some species have a dentition dictated by a few exceptionally divergent teeth, where others have a highly regionalized dental battery. Teeth provide an information-rich opportunity to explore tendencies in developmental transitions, functional divergence, and even sexual selection. These data, on a small group of wrasses, suggest continuous dental variables can be a rich source of insight into the evolution of fish feeding mechanisms across a wider variety of species.

The latter half of my dissertation looks at the importance of soft tissue in prey manipulation and retention. Complex prey processing, like chewing, requires two essential tools: one to position the prey for processing and a second for crushing and shearing. In mammals, these tools are the lips and tongue for manipulation, and teeth for mastication (Hiiemae & Palmer, 2003; Ross *et al.,* 2007; Osborn *et al.,* 2020). In fishes the situation is more complicated because manipulation is not limited to lips and tongue – instead, fishes use kinetic skulls, accessory jaws (i.e., pharyngeal), or the flow of water for repositioning prey (Dean *et al.,* 2005; Ross *et al.,* 2007; Kolmann *et al.,* 2016; Schwarz *et al.,* 2020). Pacus are the herbivorous cousins of piranhas and are important seed dispersers and grazers in the Amazon River. Feeding on fruit, nuts, leaves, and stems, pacus must manipulate their prey, peeling inedible away from edible. This is a challenging feat for a creature without hands or feet to hold food in place. These fishes instead rely on a robust set of teeth and hypertrophied (enlarged) lower lip for the manipulation and breakdown of food. Using comparative morphological techniques (computed tomography, histology, clearing and staining) I compare the lip morphology across 14 species of pacus and piranhas to better understand this soft tissue adaptation. I then used this morphological data to trace the evolutionary relationship among soft tissue characters and diet across pacus and piranhas. If lips are tools that support herbivory in pacus we expect their morphology, like tooth shape, to vary with the particular plant type. I found that frugivorous pacus have larger, more complex lips, innervated and folded at their surface, while grazing species have callused, mucus-covered lips. Unlike mammalian lips or tongues, pacu lips lack any intrinsic skeletal or smooth muscle. This implies that pacu lips lack dexterity; however, I found a novel ligamentous connection to the lips from the jaw muscles that may instead actuate the lip during feeding.

We are only at the cusp of building a comparative framework that accounts for the morphological, functional, and behavioral diversity of dentitions. Teeth are a key vertebrate trait that can infer ancient ecologies and be used to understand modern biomechanical demands. The variety of teeth and dentitions hosts endless functional possibility. Take for instance the toothy armor of Pacific spiny lumpsuckers. These are rotund fishes that must protect themselves from bouncing around harsh intertidal waters. Their tooth-like armor provides a lightweight but harty solution as enamel, the top layer of teeth, is the hardest biological material (Woodruff et al 2021). The limited mechanical framework for dentitions in fishes and non-mammalian vertebrates is complicated by the fact that most vertebrates never hold onto their teeth for long. Some species, like the Pacific Lingcod (*Ophiodon elongatus)* replace 20 teeth every single day (Carr et al., 2021). My dissertation is inspired by the never-ending diversity of fishes and implements a multidisciplinary approach to explore the function and phenotypes fishes use to interact with their environment.

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Not your father's homodonty—stress, tooth shape, and the functional homodont

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Abstract

Teeth tell the tale of interactions between predator and prey. If a dental battery is made up of teeth that look similar, they are morphologically homodont, but if there is an unspecified amount of regional specialization in size or shape, they are morphologically heterodont. These are vague terms with no useful functional implication because morphological homodonty does not necessarily equal functional homodonty. Teeth that look the same may not function the same. Conical teeth are prevalent in fishes, superficially tasked with the simple job of puncture. There is a great deal of variation in the shape and placement of conical teeth. Anterior teeth may be larger than posterior ones, larger teeth may be surrounded by small ones, and patches of teeth may all have the same size and shape. Such variations suggest that conical dentitions might represent a single morphological solution for different functional problems. We are interested in the concept of homodonty and using the conical tooth as a model to differentiate between tooth shape and performance. We consider the stress that a tooth can exert on prey as stress is what causes damage. To create a statistical measure of functional homodonty, stress was calculated from measurements of surface area, position, and applied force. Functional homodonty is then defined as the degree to which teeth along the jaw all bear/exert similar stresses despite changes in shape. We find that morphologically heterodont teeth are often functionally homodont and that position is a better predictor of performance than shape. Furthermore, the arrangement of teeth affects their function, such that there is a functional advantage to having several smaller teeth surrounding a singular large tooth. We demonstrate that this arrangement of teeth is useful to grab, rather than tear, prey upon puncture, with the smaller teeth dissipating large stress forces around the larger tooth. We show that measurements of how shape affects stress distribution in response to loading give us a clearer picture of the evolution of conically shaped teeth.

KEYWORDS
Heterodonty, modeling, puncture, teeth

1 | INTRODUCTION

In the absence of detailed behavioral studies, teeth are often the best available evidence for what and how organisms eat. Some relationships between tooth shapes and function have been explicitly tested and modeled—flatter teeth with several cusps are used for grinding, and dagger-like cones work well to puncture (Evans and Sanson, 1998; Shergold & Fleck, 2005; Anderson and Labarbera, 2008; Ungar, 2014). Such generalized assumptions have been crucial in generating a direct relationship between form and function, and

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are used to tease apart trophic niches, phylogenetic relationships, and behavior (Anderson and Labarbera, 2008; Evans and Fortelius, 2008; Ungar, 2014; Corn *et al*., 2016; Deang *et al*., 2018). When observing a battery of teeth along a jaw, we make assumptions about how those teeth work based on their geometry and position. For instance, the shape regionalization in mammalian jaws corresponds to different functional guilds, that is, puncture, tearing, and chewing (Simpson, 1936; Keene, 1991; Ungar, 2014). Most vertebrates do not possess such extreme shape regionalization, and the function of the dental battery is considered uniform across all the teeth (Osborn and Crompton, 1973; Keene, 1991). But this is an untested assumption: If the shapes of the teeth are the same, then the job of each tooth is the same along the entire jaw. Among teleost fishes, conical dentitions are the most common, but conical teeth differ in shape across families, species, and within individuals. We propose that the position of teeth with respect to the jaw joint and to other teeth plays an important role in the variation we see even in supposedly simple conical teeth.

As a term, homodonty is an anatomical descriptor for similarly shaped and sized teeth within a dentition (i.e., morphological homodonty). Throughout the literature, however, morphological homodonty is used to infer function as a consequence of shape, that is, a field of small cones is used for prey retention (Schwartz, 1982; Keene, 1991; D'Amore, 2015). Any dentition with teeth that we cannot comfortably classify as homodont then becomes a heterodont dentition, from which we infer functional regionalization. But shape and function are not interchangeable, and these intuitive terms fail in two distinct ways. First, there is no metric to quantify how much variation in size or shape constitutes a departure from morphological homodonty. For example, is a sequence of right, circular cones of identical proportions but slightly decreasing in size a morphological homodont or heterodont dentition? Second, these dentition-centric concepts fail to capture the different stresses applied to the teeth by virtue of position along the jaw.

The ambiguity surrounding morphological homodonty is only worsened by its roots in mammalian anatomy. Defined through this lens, most teleosts are morphological homodonts; differences in their tooth morphologies cannot be binned into specific morphotypes such as canines, incisors, or molars. Moreover, the constraints on morphological homodonty in mammals are not necessarily the same in ray-finned fishes (Schwartz, 1982; Huysseune *et al*., 2002). Unlike most mammals, ray-finned fishes replace their teeth continuously. Replacement teeth are a problem for homodonty (Keene, 1991): Can an individual truly be homodont if new teeth are constantly being replaced, resulting in size differences across the jaw (i.e., heterodonty)? Development is also a problem for morphological homodonty as teeth may change in shape or size throughout ontogeny. Simpson (1936) began to address this issue by using the term "incipient heterodonty" to integrate developmental contingencies with the evolution of tooth shape. Other qualifiers for a heterodont dentition have included phylogenetic heterodonty, pseudoheterodonty, interfamilial heterodonty, and advanced heterodonty (Peyer, 1929; Simpson, 1936; Bellairs and Miles, 1965; Osborn, 1977; Ferguson, 1981; Keene, 1991). These definitions, which apply to specific cases of interest to particular authors, use morphology, development, and homology to interpret tooth function but do not measure it (Peyer, 1929; Simpson, 1936; Bellairs and Miles, 1965; Osborn, 1977; Ferguson, 1981; Keene, 1991).

Perhaps this hedging and lack of clarity in the literature means shape alone is a poor descriptor for dentition. Mihalitsis and Bellwood (2019) point out thatsimple cones have very different functions depending on their position along the jaw, their orientation, and their size relative to the teeth around them. This observation highlights that teeth that look alike may not function in the same way. The function of the oral jaws is dominated by lever mechanics, and by extension, so are their dentitions; the functional environment of a tooth changes depending on its jaw position. Morphological homodonty leads us to assume that similarly shaped and sized teeth at opposite ends of the jaw are functionally equivalent. But shape alone does not determine function, and the relationships among tooth morphology, function, and ecology are far more complex than previously thought (Whitenack and Motta, 2010; Corn *et al*., 2016; Bergman *et al*., 2017; Cullen and Marshall, 2019).

We propose functional homodonty, a stress-based metric describing a dental battery incorporating morphological measures that influence the function of individual teeth in a dentition. The stress exerted by a tooth on prey, and the reaction force of prey on teeth, is a function of the tooth shape and position relative to the jaw joint (Anderson and Labarbera, 2008; Whitenack and Motta, 2010; Neenan *et al*., 2014; Anderson *et al*., 2016; Galloway *et al*., 2016). Because conical teeth are simple shapes to model, we offer a simple computational framework for examining the functional heterodonty of conical teeth in the context of a fish's jaw. Our goals for this work are to (a) propose a stress-based model of tooth function that allows quantification of the stress for every tooth along the jaw; (b) use this model to quantify variation in stress in an idealized functional homodont and heterodont dentition; (c) calculate the stress distribution of a "functionally homodont" dentition; (d) measure the surface area and calculate stresses for teeth in the jaws of ten species of fishes; (e) use the mean stress and variation in stress to develop a metric for whether a particular tooth is functionally heterodont relative to the entire battery; and (f) determine whether clustering of teeth of different size and the same shape affects prey stress distributions.

2 | METHODS

2.1 | Specimen collection and Micro-source computed tomography scanning (μCT)

All specimens, on loan from museums or personal collections, were μCT-scanned using the Bruker 1173 SkyScan (Micro Photonics Inc.) at Friday Harbor Laboratories' Karl F. Liem Memorial Bioimaging Facility (Table 1), and the slice data were uploaded to MorphoSource.org (Table 1). We chose species with arrangements of conically shaped teeth that, on inspection, appeared either morphologically homodont or heterodont, as well as some species that did not fall easily into either category. Listed on the basis of appearance from morphological homodont to heterodont, we examined *Esox americanus*, *Ophiodon elongatus*, *Saurida tumbil*, *Lophius americanus*, *Labrus viridis*, *Hydrolycus armatus*, *Omosudis lowii*, and *Cynodon gibbus*. All specimens were scanned between 9.5 and 35 μm voxel resolutions at 65 kv and 123 uA.

Digital stereolithography models of the jaws and teeth from reconstructed μCT scans were created in Amira (v. 5.2.2, Visage Imaging, Inc.). Surfaces were then exported to MeshLab (Visual Computing Lab, ISTI-CNR) where jaw length, tooth height, radius, and positions were measured.

2.2 | Theoretical homodonty models

Using the R package "shapes" (Dryden and Mardia, 2016), we created conical tooth models and arranged several models serially to represent a battery of teeth. Several combinations of tooth morphologies were generated by altering the occlusal surface through changing cusp height (h) and cusp radius (r). The height of a tooth was determined to be from the tip to the jaw attachment. Radius was taken at 50% tooth height to standardize across all teeth. Standardization was important for our real tooth measurements and allowed us to ignore curvature and damage yielding an idealized tooth surface area, which we used to calculate stress under different conditions (Crofts and Summers, 2014) (Figure 1).

$$surfacearea = \pi r(r + h2 + r2) \tag{1}$$

The first treatment tested identical, evenly spaced teeth with different aspect ratios (slender to wide cones) in each trial. In the first iteration of the model, the individual teeth did not touch and were equally distributed along the jaw. As the aspect ratio increased, the bases of individual teeth moved closer together until they touched. No tests were conducted where teeth overlapped or where teeth hung off the edge of the defined jaw (See supplemental Figure S1). A point force from 5 to 10 N perpendicular to the jaw was applied to each tooth, and the resultant stress was calculated using equation

$$\sigma = forcesurfacearea \tag{2}$$

Teeth were modeled as cones so there is no effect of curvature or orientation.

After a baseline was established for how differently shaped teeth respond to stress, we then could incorporate anatomical constraints that accounted for the fact that (a) fish teeth are not typically under straight forces but rather situated along a lever arm and (b) there is not an unlimited space along the jaw to accommodate fluctuations in surface area.

$$Flever = (length * n)position \tag{3}$$

$$\sigma = Fleversurfacearea. \tag{4}$$

Our new stress calculation then included the position of the tooth, as teeth at different positions along the jaw will experience different forces (Equations 3 and 4, Figure 2).

Under these conditions, we predicted that more anterior teeth would experience less force than more posterior teeth (i.e., those near the jaw joint) based on basic lever mechanics. In our model, we defined functional homodonty as teeth bearing/exerting the same amount of stress while functionally heterodont dentitions consisted of teeth bearing/exerting different stresses regardless of size, shape, or position (Figure 2). We applied this model to the teeth in our CT-scanned fishes. For each specimen, we counted only the teeth ankylosed to the jaw to avoid any teeth that were undergoing replacement. In MeshLab, we calculated the surface area per tooth from the measure height and radius and measured the distance of each tooth from the jaw joint. From these measurements, we calculated stresses under an array of conditions by using Equation (4) with force ranging from 1 to 50 Newtons in 1 N increments.

2.3 | Statistical measurements of homodonty

Our goal was to generate a stress-based model of tooth function by defining functional homodonty in a statistically meaningful way. We define functional homodonty as all of the teeth along a jaw experiencing similar stress values for a given force; in an idealized functional homodont, all of the teeth would experience the same stress,

TABLE 1 List of specimens with scanning parameters and MorphoSource DOIs

Species	Voxel size	kV	μA	Filter	Scanning facility	MorphoSource DOI
Anoplogaster cornuta	28.2	60	110	1 mm Al	Karel F. Liem Bioimaging Center	10.17602/M2/M29328
Cynodon gibbus	35.0	65	123	1 mm Al	Karel F. Liem Bioimaging Center	10.17602/M2/M30061
Esox americanus	35.5	60	110	1 mm Al	Karel F. Liem Bioimaging Center	10.17602/M2/M97833
Hydrolycus armatus	35.0	65	123	1 mm Al	Karel F. Liem Bioimaging Center	10.17602/M2/M97833
Labrus viridis	35.5	65	123	1 mm Al	Karel F. Liem Bioimaging Center	10.17602/M2/M57652
Lophious americanus	25.9	65	123	1 mm Al	Karel F. Liem Bioimaging Center	10.17602/M2/M97822
Omosudis lowii	25.9	65	123	1 mm Al	Karel F. Liem Bioimaging Center	10.17602/M2/M97851
Ophiodon elongatus	9.5	65	133	1 mm Al	Karel F. Liem Bioimaging Center	10.17602/M2/M29013
Saurida tumbil	35.1	65	123	1 mm Al	Karel F. Liem Bioimaging Center	10.17602/M2/M76671

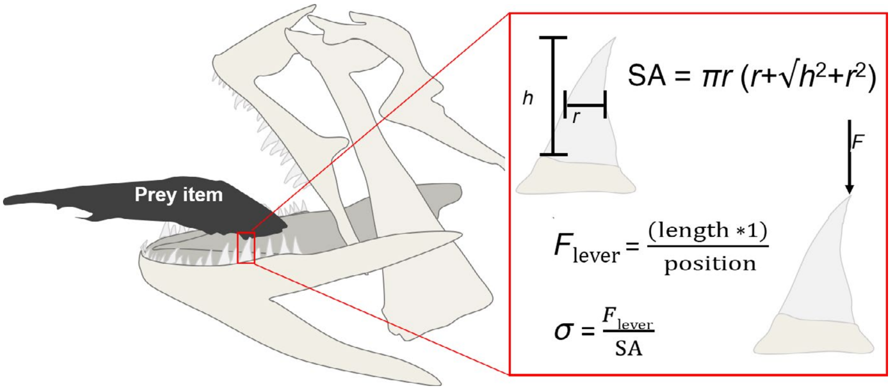

FIGURE 1 Measuring stress: The height and radius of individual teeth were measured to calculate surface area (SA). This was then used to calculate stress under different applied forces simulating tooth–prey interactions

regardless of size or position. This can be measured by subtracting the mean stress of the dentition from the stress experienced by each tooth to calculate residual (observed minus expected) stress. In a perfect functional homodont, each tooth would experience the mean stress, resulting in a residual stress of zero. In a functionally heterodont dentition, some teeth would experience significantly larger or smaller stresses than the mean, indicating a different function for those teeth.

There are two challenges in doing this with real fish dentitions. First, we need to examine patterns across multiple dentitions, which require normalizing stresses within dentitions to compare them across species. One standard method of normalizing a continuous variable is to subtract the mean value from each observation to center the data and then divide by the root-mean square or standard deviation to scale it. We chose to normalize to the median stress, rather than the mean stress, because we expect many of the dentitions to include some teeth with much higher or lower stresses than the majority of teeth in the dentition, which would skew the mean. To calculate the normalized stress, we (a) divided the raw stress by the squared length of the jaw to control for size variation; (b) divided the size-corrected stress of each tooth by the median stress for that dentition; and (c) subtracted the median stress from each value to calculate the residual stress, resulting in a distribution with a median stress of 0 and residual stresses measured in units of median stress for each dentition. This centers and scales the data without changing the relative magnitude of stress experienced by each tooth compared to other teeth in the dentition.

Second, even for functionally homodont dentitions in which all of the teeth experience very similar stresses, we are unlikely to calculate *identical* stresses (and consequent zero residuals) for each tooth. This is due to a combination of technical variance (i.e., surface area uncertainty due to the resolution of the CT scan), biological variance, and mechanical tolerance—tooth shape may not need to completely negate variation in stress due to position along the jaw, as long as it compensates for it sufficiently. In other words, we expect that in real dentitions, even functional homodonts do not have uniform residual stresses of zero. Instead, we define tolerance limits for the variation in stress and consider that teeth within these limits serve the same function in the jaw.

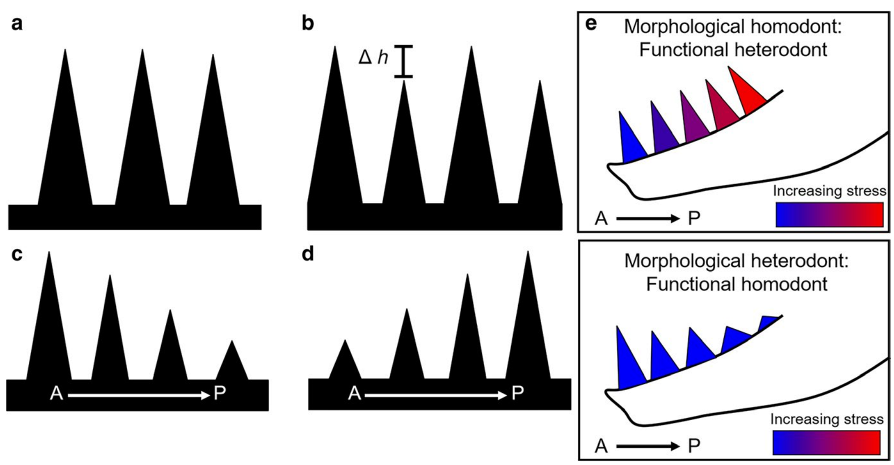

FIGURE 2 Problems with morphological homodonty. (a) All teeth are the same shape and size along the jaw; (b) some variation in shape of size is permitted and still can be considered morphologically homodont; (c) decrease in size or shape toward the jaw joint does not indicate heterodonty but a morphological homodont under constrained space. (d) Same pattern as panel (c) but in reverse. This is because morphological homodonty discounts the functional constraints of tooth position. (e) Definition of functional heterodonty in the context of morphological homodont

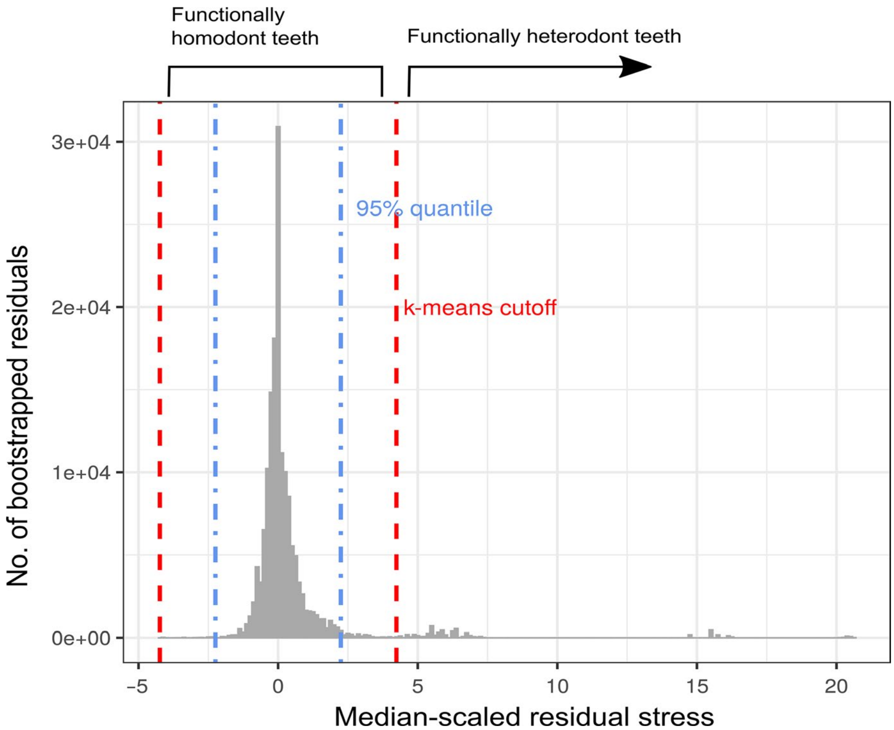

FIGURE 3 Bootstrap residual stress analysis: the distribution of residuals used to generate biologically relevant cutoffs

To generate reasonable tolerance limits for functional homodonty in real dentitions, we chose to bootstrap a residual stress distribution from the species whose dentitions we measured for this study in order to generate cutoffs based on biological data. To bootstrap the stress distributions, we did the following:

1. Randomly subsampled (without replacement) 50% of the teeth from a median-normalized dentition.
2. Found the differences between the actual and the median stress (residual stress) for each tooth in that subsampled dentition.
3. Repeated the above two steps until we generated 10,000 residual stress values for each dentition in the data set.

The resulting distribution of residuals was centered around 0, with small clusters of extreme values representing teeth that experienced significantly more stress than the median for a given dentition.

2.4 | Choosing a threshold for heterodonty

We generated two kinds of thresholds to determine which teeth should be categorized as functionally homodont (near-zero residual stress) and functionally heterodont (large residual stress). In each case, we examined the absolute values for the residuals, because teeth that experience much more or much less stress than the median would be functionally heterodont.

First, we found the quantiles that contained 95% of all the bootstrapped residuals (Figure 3, blue line); teeth with residuals outside of this interval were as or more extreme than 95% of the values in the distribution, indicating an extreme deviation from median stress. The 95% quantile thresholds for our bootstrapped distribution were ±2.25, meaning any tooth that was experiencing a 2.25-fold difference from median stress would be classified as functionally heterodont.

However, the distribution (in Figure 3) is clearly not normally distributed (Shapiro–Wilk normality test: $p < 2e\text{-}16$) and shows small, distinct peaks of high residuals, a pattern that quantile intervals fail to capture. To address this feature of the distribution, we also used *k*-means clustering to identify clusters of high and low residuals. Briefly, the *k*-means clustering algorithm identifies a user-specified number of clusters within a continuous distribution that minimizes the total sum of squared errors (Diday and Brito, 2007). We partitioned the data into two clusters and recovered a high (residual stress = 8.1) and a low (residual stress = 0.1) cluster. K-means assigns each observation in a distribution to one of the clusters, meaning each tooth was assigned to the low (homodont) cluster if its residual stress was closer to 0.1, and to the high (heterodont) cluster if its residual stress was closer to 8.1. The average of the two clusters therefore determines the threshold (±4.3). We recovered similar clusters using k-medoids clustering, a related algorithm that is slower and more robust than k-means.

Most cutoffs are arbitrary, but some cutoffs are more arbitrary than others. In our case, k-means has two advantages over using quantiles: First, quantiles are more sensitive to the proportion of homodont and heterodont teeth used to generate the bootstrap distribution. If we included a >5% proportion of teeth with high-residual stresses, then even if the teeth still fell into distinct clusters, a 95% quantile would need to extend to that high-residual cluster to capture 95% of the data, even though the underlying pattern has not changed. Second, we expect that if functionally heterodont teeth are under different selective pressures than the rest of the teeth in a jaw, they would cluster elsewhere on the residual stress distribution, rather than making up the thin tails of the extreme ends of a unimodal distribution. K-means clustering better reflects our underlying biological hypothesis of a multimodal stress distribution.

2.5 | Tooth loading experiments

We used a material testing system (MTS, *Synergie 100 tensile tester with 500N load cell*) to investigate the implications of functionally homodont teeth. We constructed idealized models to measure how load and draw change with tooth orientation after puncture. In doing this experiment, we challenged the idea that conical teeth are best shaped for and influenced by their roles in puncturing prey. The first model consisted of a single cone represented by a 30-mm-long, 11-gauge needle (~1 mm diameter) adhered to an acrylic block. The second model consisted of multiple 11-gauge needles adhered to a second block. The center needle was 30 mm in height and surrounded by six 11-gauge needles 5 mm in height with the surface area of the combined smaller needles equal to the center needle. The first model represented a singular and isolated conical tooth and the second model represented an individual that has a larger fang surrounded by smaller teeth.

Using the MTS, we inserted each model perpendicularly into a block of ballistic gelatin (10% w/v) to represent teeth tearing through flesh. Moving vertically, the models were pulled by the MTS through the gelatin for 10 cm at 0.30 cm/s, while force measurements were recorded. Each model was tested five times, each time in a new gel block. The maximum load was calculated in Newtons and analyzed in R.

3 | RESULTS

3.1 | Diversity of conical teeth

Our sample of fishes includes morphologically diverse conical teeth ranging from evenly spaced right cones, as in the lower jaw of *E. americanus* (Figure 4b), to the curved, saber-like teeth in *Anoplogaster cornuta* (Figure 6b). In most species, the shapes and sizes of conical teeth vary along and across the upper and lower jaws, that is, they are morphologically heterodont. Additionally, many teeth curve lingually, as in *Op. elongatus*, *Lo. americanus* (Figure 5), and *A. cornuta* (Figure 6b). The teeth of *La. viridis*, *S. tumbil*, *E. americanus* (Figure 4), and *Om. lowii* (Figure 5a) have no curvature and are either oriented normal to the jaw or angled slightly anteroposteriorly. Within our samples, most individuals have teeth that decrease in height toward the articulating surface of the premaxilla and dentary. The only exception was *Om. lowii* (Figure 6a), which has two sets of larger saber

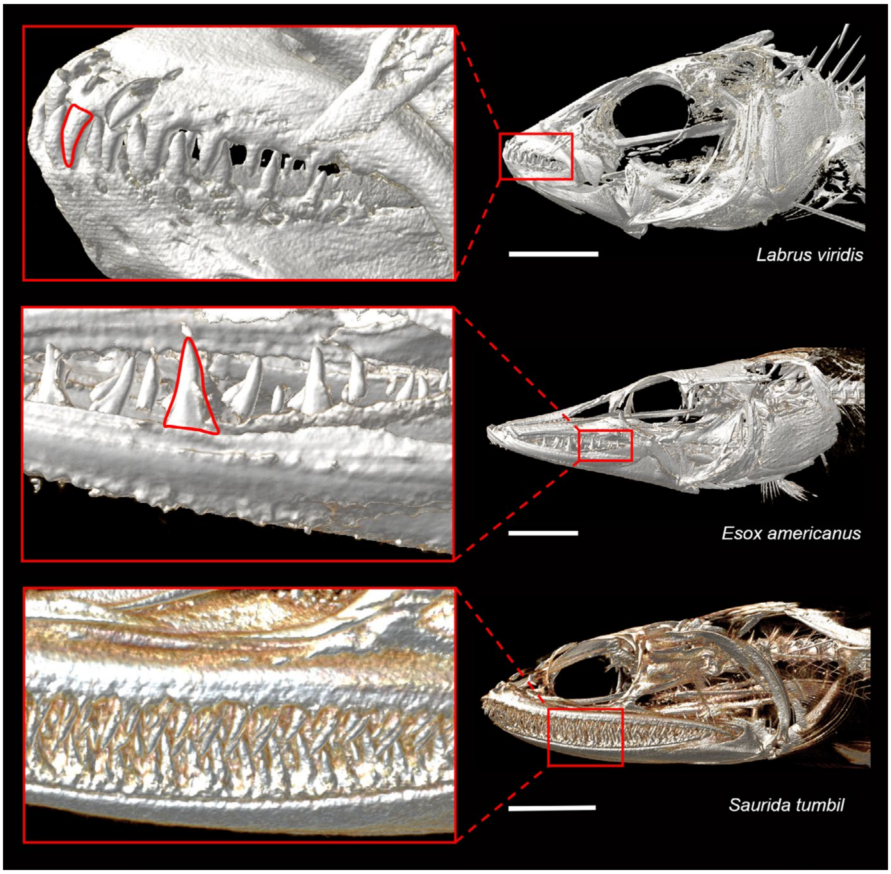

FIGURE 4 CT scans of morphological homodonts. Teeth are more or less the same size and shape along the jaw. This excludes *Saurida tumbil* that has clusterings of larger fangs at the symphysis of the premaxilla. Scale bar set to 10 mm

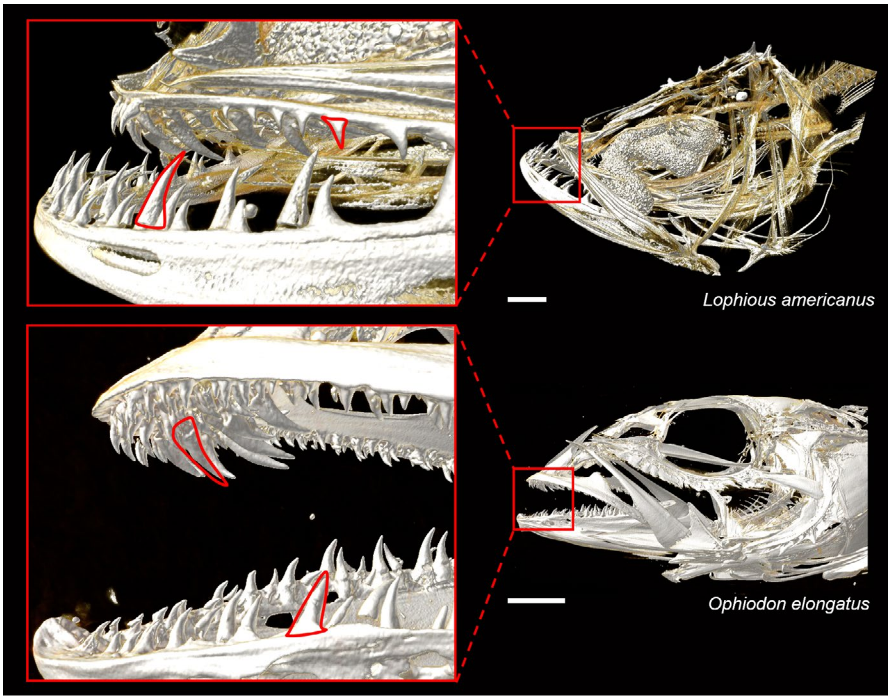

FIGURE 5 CT scans of morphological heterodonts. Both *Lophius americanus* and *Ophiodon elongatus* have variably sized cones along the jaws with the greatest variation at the symphysis of the dentary and premaxilla. Scale bar set to 5 mm

teeth followed by smaller conically shaped teeth that increase in height posteriorly.

3.2 | Functional homodonty

3.2.1 | Theoretical models

When equal force is applied to the tips of an array of morphologically homodont teeth, the stress in the teeth is the same across the array. When the array of teeth is considered as a jaw, with a jaw joint (fulcrum), an input, and an output lever, a single jaw closing force leads to an uneven distribution of forces at the tip of each tooth (Figure 2e). This uneven distribution of forces leads to unequal stresses in the teeth. In this same jaw-jointed model, if the teeth are allowed to change shapes to equalize the stresses, they become broader and flatter toward the jaw joint. The teeth are functionally homodont but morphologically heterodont. Because the teeth need are broader nearer the jaw joint, it is possible to specify a load and tooth density where the rear teeth will not fit in the confines of the jaw (Galloway *et al*., 2016).

3.2.2 | Statistical models

Within the context of our model, all individuals were classified as functionally homodont except for *S. tumbil*, *E. americanus*, and *C. gibbus* (Figure 7, see supplementary Figures S2-S5 for individual homodonty graphs). Similar to our predictive model, there is a strong correlation between absolute stress and position of teeth within the jaw. Unlike the theoretical model, measurements from natural specimens show that the relative position of teeth had a large effect on stress. Large teeth at the front of the jaw experience similar stresses to smaller teeth closer to the jaw joint. But if large teeth are surrounded by small teeth, then their absolute stress goes down. Our model can identify the bounds of functional homodonty by differentiating dentitions with extreme stress disparity from those with low enough disparity that the teeth are not functionally distinct.

For example, the dentitions of *E. americanus* and *La. viridis* both had strongly supported stress gradients ($R^2 > .99$, $p < 2*10^{-16}$ for log-transformed stress-position models), but our residual cutoff only classified *Esox* as a functional heterodont (Figure 8). This is because of the magnitude of the stress gradient: In *Labrus*, the tooth that experienced maximum stress was bearing ≥2X the median stress (a residual of about 1). In *Esox*, several teeth experienced >5X the median stress of the dentition, putting them above the functional heterodonty cutoff level of 4.3.

3.3 | Tooth loading: functional homodonty results in increased load

Using a MTS, we tested idealized models to further investigate why smaller teeth are grouped around much larger teeth. The first model represented a single fang puncturing and pulling prey, while the second model represented the grouping of small and large conical teeth (Figure 9a). In the single cone model, $\bar{x}_{load} = 0.645 \pm 0.121$ N, while the multi-cone model $\bar{x}_{load} = 1.721 \pm 0.443$ N (Figure 9b). On average, the multi-cone model was able to withstand 4X the load of the single cone model. We then visualized puncture and drag through the gel with polarized light and found similarly that the multi-cone model produced fewer stress rings in the gel compared to the single cone model.

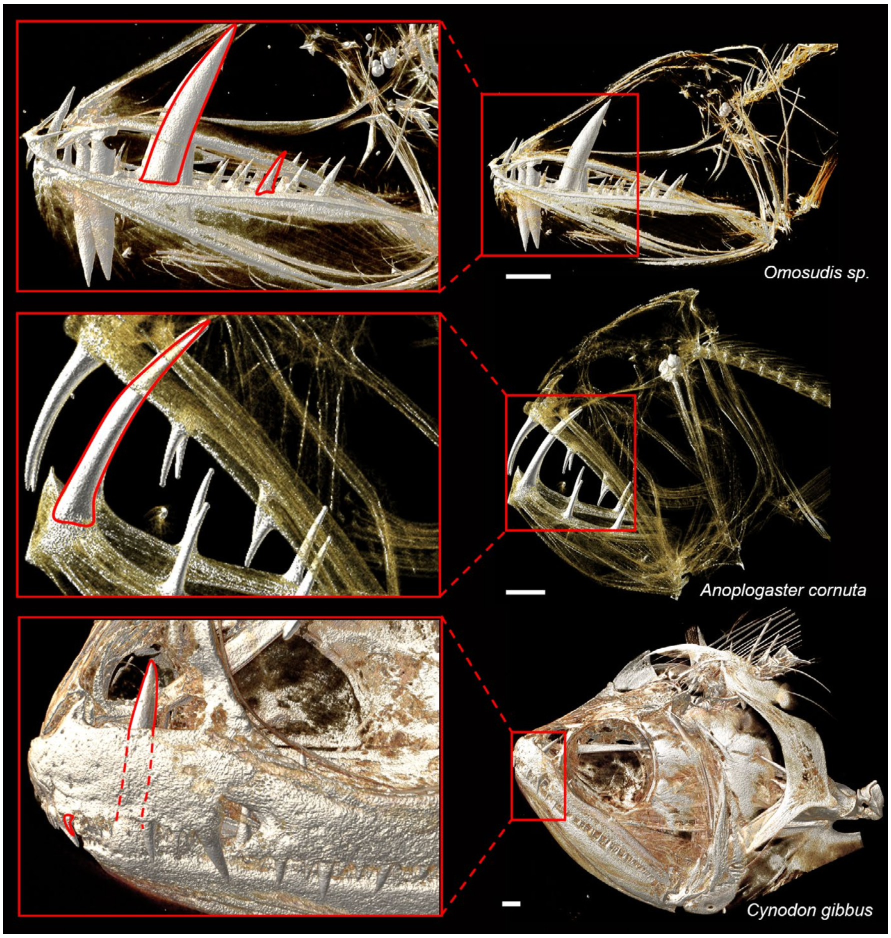

FIGURE 6 CT scans of fangs and saber teeth. Very different from the conical teeth shown before, the large fangs of these three species highlight the possibility of functionally heterodont dentition. Scale bar set to 10 mm

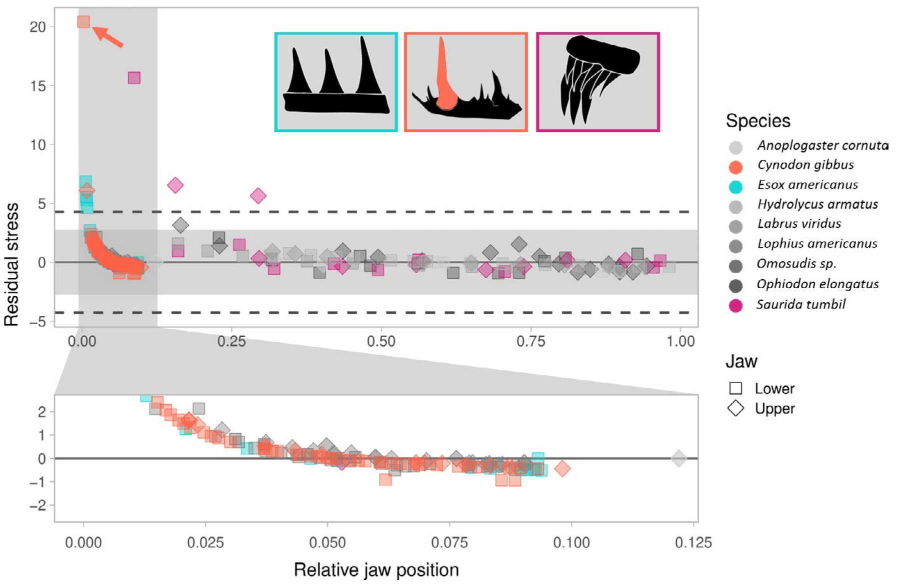

FIGURE 7 Functional homodonty: Graph displays residual stress calculated for each tooth along the jaws in all nine individuals. Solid gray line indicates ideal functional homodont, where all teeth express the same residual stress value. Individual points are the distance (in units of median stress per dentition) away from an ideal functional homodont. Dashed gray lines indicate k-means cutoff. From this measure, we can quantify how functionally heterodont an individual tooth is within the context of the jaw. Lower panel is a zoomed-in inset of data collapsed on the far, left-hand side of the graph

4 | DISCUSSION

There are very few true morphological homodonts because wear, replacement, and ontogeny all contribute to tooth shape. A worn tooth will necessarily function differently than a recently replaced tooth, and even similarly shaped teeth at the front and back of the jaw do not function the same because of simple lever mechanics. Despite our intuition, morphological homodonty is not a substitute for functional homodonty. Shape gives us a realm of possibility for what the tooth can do. A conical tooth, depending on where it is

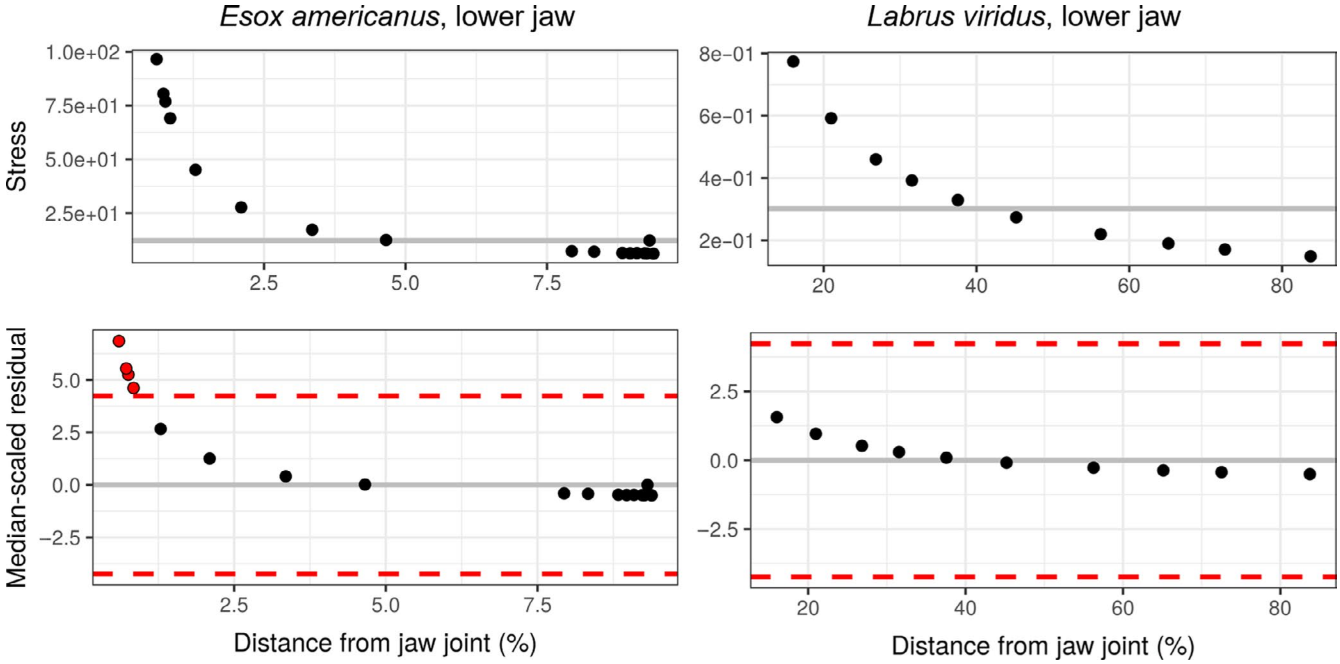

FIGURE 8 Comparison of heterodont teeth *Esox americanus* and *Labrus viridis*. Solid gray line represents the idealized functional homodont, as in Figure 7, and the dashed red lines represent the k-means cutoffs. Points in red highlight teeth that are functionally heterodont

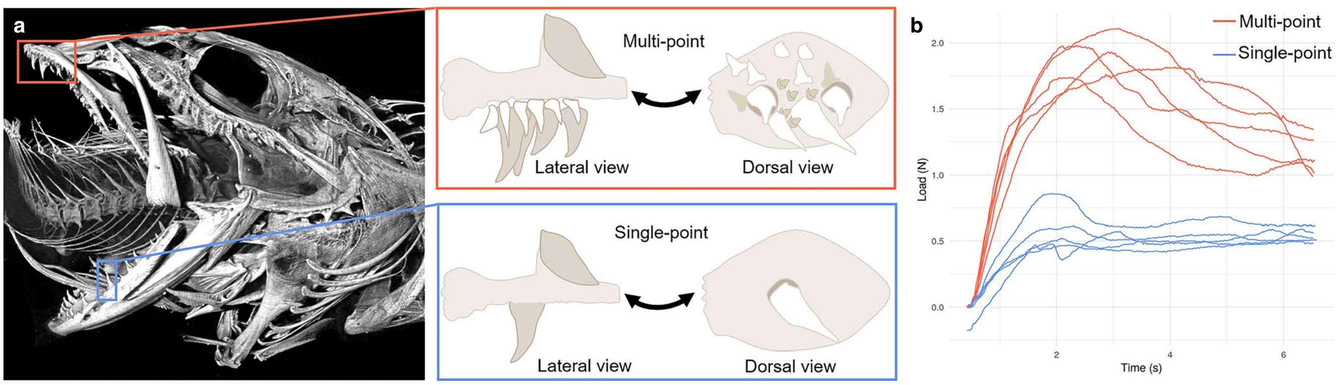

FIGURE 9 Usage of functionally homodont teeth. (a) CT scan of *Ophiodon elongatus* consuming a *Myoxocephalus polyacanthocepahlus* with insets diagramming dental morphologies. (b) Results of gel-puncturing experiments show that a clustering of small teeth around larger fangs allows for increase in load. A single cone tears flesh with far less force rather than puncturing and pulling flesh into the mouth

in the jaw, how many neighbors it has, and how tall it is relative to those neighbors, could be for killing, retaining prey, doing tissue damage, or caging prey in the mouth. For instance, the positioning, and mix of larger and smaller cones in the dental battery of the Pacific lingcod (*Op. elongatus*), reflects the role of their battery of conical teeth as prey retention devices (Figure 10). Likely, both large and small conical teeth work to disperse stresses during prey capture. A single tooth cannot ensure prey capture or retention rather, all teeth are involved. In contrast, the dogtooth characin (*C. gibbus*) anterior fangs are both morphologically and functionally heterodont, and are used for a killing stab while the rest of the teeth are used for prey retention (Van der Sleen and Albert, 2017). It is important for evocative terms such as functional heterodont and homodont to have both a functional implication and a metric for quantifying where a particular dentition sits on the continuum between similarity and difference.

We can then use this model to predict, on a tooth-by-tooth basis, where there is expected to be a separation of function. The need for understanding tooth function at this level of detail is demonstrated in an investigation of coral-reef piscivores by Mihalitsis and Bellwood (2019). They identified three dental morphotypes in a sample of 29 species—macrodont, viliform, and edentulus. Each morphotype was associated with particular functional characters, but still relied on a categorical classification system. This classification is considerably more meaningful than typical morphological homodonty and heterodonty, but not all conical dentitions will fall into these categories. Our model offers a metric that can categorize teeth and dental batteries on a continuous scale that is useful for evolutionary and ecological studies. For instance, we could test specific hypotheses of regionalization, such as whether functional heterodonty is more likely to evolve in the anterior than in the posterior region of the maxilla. Within a large sample of fishes, we could address modularity, answering questions such as "are there regions of the jaws more likely to develop heterodont teeth?" The functional heterodonty metric also highlights the importance of some patterns that have been overlooked. What could the well-spaced regions of groups of blade-like teeth mean for *Alepisaurus*? *Esox* has a strikingly morphologically homodont dentition, yet our analyses found four functionally heterodont teeth (Figure 8). What do these three teeth do differently from the rest of the jaw? Is this pattern found across Esocidae? Is there a developmental contingency? Our model changes the assumptions we make when first looking at a battery of teeth, forcing us to think in terms of their deployment rather than just their shape.

Transitions from morphological homodonty to morphological heterodonty and gestalt tooth shape are used in constructing phylogenies (Keene, 1991; Bertrand, 2014; Conway *et al*., 2015) to indicate functional shifts in how prey is retained or manipulated. However, molecular phylogenies reveal that dentition data create phylogenetic noise, not signal (Ruber *et al*., 1999; Shimada, 2002; Sansom *et al*., 2016). We suspect that collapsing ambiguous dentitions into discrete categories (i.e., homodont or heterodont) erases important phenotypic information. Phylogenetic comparative methods can be applied to discrete or continuous characters, but continuous characters offer more analytical flexibility (Garamszegi, 2014; Adams and Collyer, 2017; Harmon, 2018; Adams and Collyer, 2019). Unlike morphological homodonty, functional homodonty can act as a continuous trait by measuring the deviation of each tooth from its expected performance under idealized functional homodonty. A continuous measure of functional homodonty, applied to individual teeth, also opens up the possibility of QTL mapping for loci involved in the process of tooth differentiation (Albertson *et al*., 2005, Fraser *et al*., 2009). Albertson *et al*. (2005) used development and genetic networks to describe the variation in the cichlid jaw to understand different feeding modalities. Yet, their study could not incorporate biomechanical parameters that likely play a role in how these skeletal elements are shaped (Alberston *et al*., 2005). Other studies use QTL analysis to understand the genetic and developmental underpinnings of tooth origination (Fraser *et al*., 2009). Functional homodonty is a biomechanical metric of the teeth and jaws that, when incorporated with genetic networks, could give further evidence in the evolution of specific dentitions or feeding strategies.

Consider the seven piscivores in our data set—*A. cornuta*, *Omosudis* sp., *C. gibbus*, *H. armatus*, *Op. elongatus*, *S. tumbil*, and *Lo. americanus*. Five are certainly piscivorous, and while there is limited diet data available, most ichthyologists would include *Anoplogaster* and *Omosudis* as piscivores. Of these species, the simple, conical teeth of *Ophiodon*, *Saurida*, and *Lophius* fit the morphological homodont model, while the rest are morphological heterodonts. Our data show a very different pattern. First, the fangs of *Anoplogaster*, perhaps because there are just two other teeth to compare them to, are functionally homodont to the rest

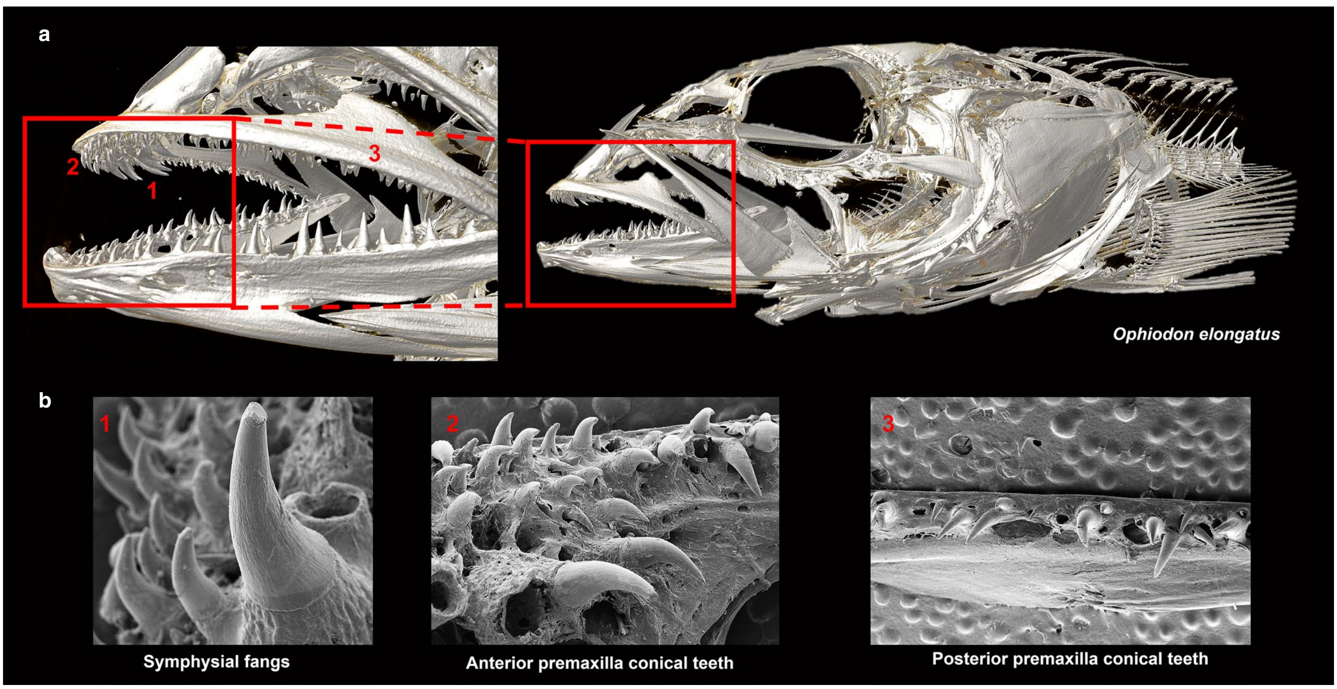

FIGURE 10 Highlighting the morphology of *Ophiodon elongatus*, a morphological heterodont that is functionally homodont. (a) CT scan highlights the different regions of the jaw that correspond to different tooth morphologies and clustering of teeth. (b) Scanning electron microscopy (SEM) of teeth along the upper jaw of *O. elongatus*

of the dentition. Perhaps those fangs, as obtrusive as they are, are not doing anything special. Instead, they are functioning in the same way as the few other, much smaller, teeth in these deep-sea fishes. With a much larger number of teeth in its battery, *Omosudis* is also functionally homodont. This dentition may be uniformly selected to pierce the soft bodies of the bathypelagic fishes and cephalopods that have been reported as their prey. The huge fangs of *Cynodon* are functionally heterodont, because we know these teeth are used for impaling prey and the smaller teeth are used for retention. Yet, the functional homodonty of *Hydrolycus* is a surprise that may lead to insights into how they catch and process their piscine prey. Of the three morphological homodonts, only *Lophius* is functionally homodont, likely because all of their teeth uniformly play a role in restraining the edges of prey they gulp down. In contrast, we *Saurida* holds struggling prey with anterior teeth, while those near the jaw joint crush prey. The most interesting morphological homodont we recovered as a functional heterodont is *Ophiodon*, where small conical teeth do not function in the same ways as larger conical teeth. This observation led to our loading experiments, which revealed that the teeth of *Ophiodon* act to spread the load so that prey cannot simply tear free of the larger fangs.

Functional homodonty is a statistical metric that lets us explore how different combinations of teeth work together. Peculiar patterns, previously subsumed in the over-broad category of morphological heterodonty, suddenly stand out. Our model incorporates parameters relevant to conical teeth, but there is no reason that the model cannot be expanded to include incisive, molariform, or triangular dentitions. The evolutionary history and variation of dental morphologies create excellent opportunities to explore the bounds of the functional homodonty metric. By doing so, we increase our understanding of how and why morphological heterodonty evolves, and the importance that individual teeth serve along the jaw.

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AUTHOR CONTRIBUTIONS

KEC and APS conceived the study and analyzed data. KEC and HIW derived and finalized the statistical model. KEC wrote the initial draft, and produced the figures. KEC, HIW, and APS were involved in revision and final approval of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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SYMPOSIUM

The Evolutionary Continuum of Functional Homodonty to Heterodonty in the Dentition of *Halichoeres* Wrasses

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Synopsis Vertebrate dentitions are often collapsed into a few discrete categories, obscuring both potentially important functional differences between them and insight into their evolution. The terms homodonty and heterodonty typically conflate tooth morphology with tooth function, and require context-dependent subcategories to take on any specific meaning. Qualifiers like incipient, transient, or phylogenetic homodonty attempt to provide a more rigorous definition but instead highlight the difficulties in categorizing dentitions. To address these issues, we recently proposed a method for quantifying the function of dental batteries based on the estimated stress of each tooth (inferred using surface area) standardized for jaw out-lever (inferred using tooth position). This method reveals a homodonty–heterodonty functional continuum where small and large teeth work together to transmit forces to a prey item. Morphological homodonty or heterodonty refers to morphology, whereas functional homodonty or heterodonty refers to transmission of stress. In this study, we use *Halichoeres* wrasses to explore how a functional continuum can be used in phylogenetic analyses by generating two continuous metrics from the functional homodonty–heterodonty continuum. Here we show that functionally heterodont teeth have evolved at least 3 times in *Halichoeres* wrasses. There are more functionally heterodont teeth on upper jaws than on lower jaws, but functionally heterodont teeth on the lower jaws bear significantly more stress. These nuances, which have functional consequences, would be missed by binning entire dentitions into discrete categories. This analysis points out areas worth taking a closer look at from a mechanical and developmental point of view with respect to the distribution and type of heterodonty seen in different jaws and different areas of jaws. These data, on a small group of wrasses, suggest continuous dental variables can be a rich source of insight into the evolution of fish feeding mechanisms across a wider variety of species.

Introduction

Vertebrates are spectacularly diverse in their ecology, behavior, and morphology, yet most vertebrates rely on the same structures to capture food: jaws and teeth (Estes and Williams 1984; Massare 1987; Davit-Beal et al. 2007; Jones 2009). The constraints and opportunities generated by, and from these structures, have been generated with both physical and mathematical modeling (Lucas and Luke 1984; Evans and Sanson 1998; Shergold and Fleck 2005; Anderson and LaBarbera 2008; Ungar 2014). Predictions based on tooth shape alone produce incomplete anecdotes of functional diversity in the same dentition. The dentition of ambush predators such as *Ophiodon elongatus* are a great example; these fishes rely on a large tooth surrounded by many smaller teeth to maximize the damage delivered to a prey item (Galloway et al. 2016; Mihalitsis and Bellwood 2019). The conical shape of their teeth does not matter as much for function as their

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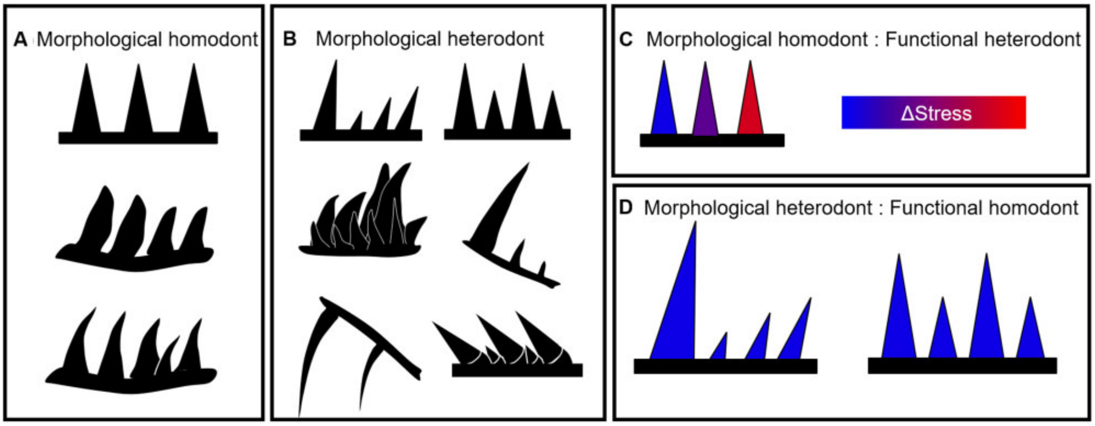

Fig. 1 Morphological versus functional homodonty. (**A**) Morphologically homodont dentition; all teeth are similar in shape or size compared to (**B**) morphologically heterodont dentition have some sort of regionalization in shape or size. A morphological cut off is hard to come by as significant changes in tooth shape are biased by our interpretation of dentitions. Functional homodonty and heterodonty (**C and D**) uses changes in stress to draw an unambiguous line between tooth shape and tooth function.

position. Generating a biomechanical framework for how particular tooth shapes and dentitions function is essential to understanding how they evolve (Evans and Sanson 1998; Lucas 2004; Evans and Sanson 2005; Anderson and Labarbera 2008).

Teeth represent an information-rich opportunity to explore tendencies in developmental transitions, functional divergence, and even sexual selection. For example, the unilateral tooth replacement in pacus and piranhas is a synapomorphy for the clade and represents a constructional constraint in maintaining an interlocked dentition (Berkovitz 1975, 1980; Kolmann et al. 2019). The morphology of the dentine in fossils of shark teeth provides new synapomorphies across orders and even pushes us to reconsider the origin of particular groups (Jambura et al. 2020). Characterizing the entire dental battery as either homodont or heterodont has been used as a tool to understand dental evolution for nearly a century (Cope 1888; Simpson 1936 ; Keene 1991; Schwartz 2013; Bertrand 2014; Conway et al. 2015; D'Amore 2015; Cullen and Marshall 2019). Most bony fishes are considered morphologically homodont and have a battery of similarly shaped and sized teeth (i.e., Fig. 1A; Kenne 1991; Ungar 2014; Berkovitz and Shellis 2016). Morphological heterodonty is reserved for dentitions that have noticeable differences in shape or size (i.e., Fig. 1B; Kenne 1991). For example, pacu, the herbivorous relatives of piranhas, have both incisiform and molariform teeth that are used to distinguish genera (Berkovitz 1980; Kolmann et al. 2019). The evolution of morphological heterodonty is consistently used in systematics often to infer differences in prey processing (Kenne 1991; Becerra et al. 2018). We explicitly do not assume that size and shape are the same as function—instead, we pose the question: do teeth that look alike actually function in the same way (Mihalitsis and Bellwood 2019; Cohen et al. 2020; Hulsey et al. 2020)?

A lack of size or shape morphological diversity in a dental battery does not necessarily indicate a lack of functional diversity (Gregory 1933; Evans and Sanson 1998; Whitenack and Gottfried 2010; Anderson and Rayfeild 2012; Schwartz 2013; Mihalitsis and Bellwood 2019; Cohen et al. 2020). The morphologically homodont dentition of piscivorous coral-reef fishes does not appear functionally diverse, but there is room for important functional decoupling in the jaws (Mihalitsis and Bellwood 2019). A fang at the front of the jaw moves at high velocity and ensures prey capture, and the large forces at the posterior fangs do the most damage (Mihalitsis and Bellwood 2019). Here, two similarly shaped teeth are functionally very different, owing to their position along the jaw. Morphological homodonty conceals phenotypic variation by only considering the dental battery in terms of shape and size. But if you care about dental function then stress is the important parameter, because stress predicts how much damage a tooth can do to a prey item (Frazzetta 1988; Freeman and Weins 1997; Shimizu et al. 2005; Dean et al. 2008; Clark and Summers 2012; Smits and Evans 2012; Schofield et al. 2016; Bergman et al. 2017; Marcus et al. 2017; Mihalitsis and Bellwood 2019; Cohen et al. 2020). If stress is critical for understanding dental function, then it should also be critical for understanding the evolution of dental batteries. To address this problem, we need to understand that broad dental characterization misses critical information in evolutionary comparisons, and we need a tooth by tooth metric that

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Table 1 Specimen table: list of species represented in this study

Species	Museum	Cat. number	Morphosource DOI	Scanning facility
Halichoeres argus	FMNH	FMNH 124452	doi : 10.17602/M2/M57770	Karel F. Liem Bioimaging center
Halichoeres binotopsis	FMNH	FMNH 75982	doi : 10.17602/M2/M48820	Karel F. Liem Bioimaging center
Halichoeres dispilus	FMNH	FMNH 72294	doi : 10.17602/M2/M56335	Karel F. Liem Bioimaging center
Halichoeres hartzfeldii	FMNH	FMNH 110701	doi : 10.17602/M2/M57428	Karel F. Liem Bioimaging center
Halichoeres hortulanus	FMNH	FMNH 126864	doi : 10.17602/M2/M58197	Karel F. Liem Bioimaging center
Halichoeres leucurus	FMNH	FMNH 126976	doi : 10.17602/M2/M58195	Karel F. Liem Bioimaging center
Halichoeres maculipinna	FMNH	FMNH 65217	doi : 10.17602/M2/M56339	Karel F. Liem Bioimaging center
Halichoeres melanochir	FMNH	FMNH 126991	doi : 10.17602/M2/M58200	Karel F. Liem Bioimaging center
Halichoeres podistigma	FMNH	FMNH 110709	doi : 10.17602/M2/M57006	Karel F. Liem Bioimaging center
Halichoeres prosepeion	FMNH	FMNH 120160	doi : 10.17602/M2/M57775	Karel F. Liem Bioimaging center
Halichoeres richmondi	FMNH	FMNH 124120	doi : 10.17602/M2/M57772	Karel F. Liem Bioimaging center

All CT scans are free to download from morphosource.org and represented here by DOI. FMNH (Field Museum of Natural History, Chicago, IL, USA).

incorporates stress into our quantification of dentitions.

Qualitative categorizations of dental characters, such as morphological homodont, macrodont, or edentulate, do not capture functional variation in dentitions (Ruber et al. 1999; Shimada 2002; Sansom 2016). Discrete categorizations summarize and discard information in an effort to simplify dentitions for comparative analysis. Continuous characters allow a finer scale comparison among continuous environmental, ecological, and gene expression data than can be achieved with categorical functional characters (Garamszegi 2014; Adams and Collyer 2018). The concept of functional homodonty can transform anatomical measures of individual teeth into a quantitative metric of dental battery function. A morphological homodont has teeth of "similar" shape and size, but functionally homodont teeth exert similar stresses regardless of shape or size. Where morphological heterodonty captures shape regionalization across individual teeth, functional heterodonty highlights teeth with stresses that exceed a threshold across a dentition (Cohen et al. 2020). Functional homodonty is a biomechanical description of the dentition that has the potential to be combined with phylogenetic information to reveal evolution of specific dentitions or feeding strategies.

To demonstrate that functional homodonty–heterodonty continuum provides meaningful insight into the evolution of dentitions, we applied the method to 11 of the ∼80 species of *Halichoeres* wrasses (family Labridae). In this study, we use three metrics to summarize the functional homodonty method. We calculate (1) the proportion of functionally homodont to functionally heterodont teeth to identify individuals with the most potential for function diversity across their dental battery, (2) the average squared residual of functional homodonty as a measure of functional diversity in the dental battery, and (3) we compare the average squared residual of functional homodonty to the proportion of functionally heterodont teeth to highlight different types of heterodonty. Our goal is to lay the groundwork for the further exploration of a biomechanical metric that can be extended to other taxa and detect functional diversity that is otherwise lost in discrete categorizations of dentitions.

Methods

Study species

The genus *Halichoeres* is common on rocky or soft reefs in the Indian, Atlantic, and Pacific oceans where they feed primarily on small benthic invertebrates (Randall 1967; Clifton and Motta 1998). They have a full dentition on both upper and lower jaws ranging from 10 to 15 teeth, including upper and lower anteriorly pointing fangs, and a pair on the posterior process of the maxilla. The modest size of the group, a well resolved phylogeny, similar habitat, and purported generalist diet make *Halichoeres* a useful group to explore the functional homodonty metric in an evolutionary context.

Measurements and scans

All CT scans were downloaded from the Scan All Fishes project on Morphosource.org (Table 1) these scans were originally presented in Evans et al. (2019). Each specimen was scanned at Friday Harbor Laboratories' Karel F. Liem Memorial Bio-Imaging Facility using a Bruker 1173 SkyScan (Micro Photonics Inc., Allentown, PA, USA). All specimens

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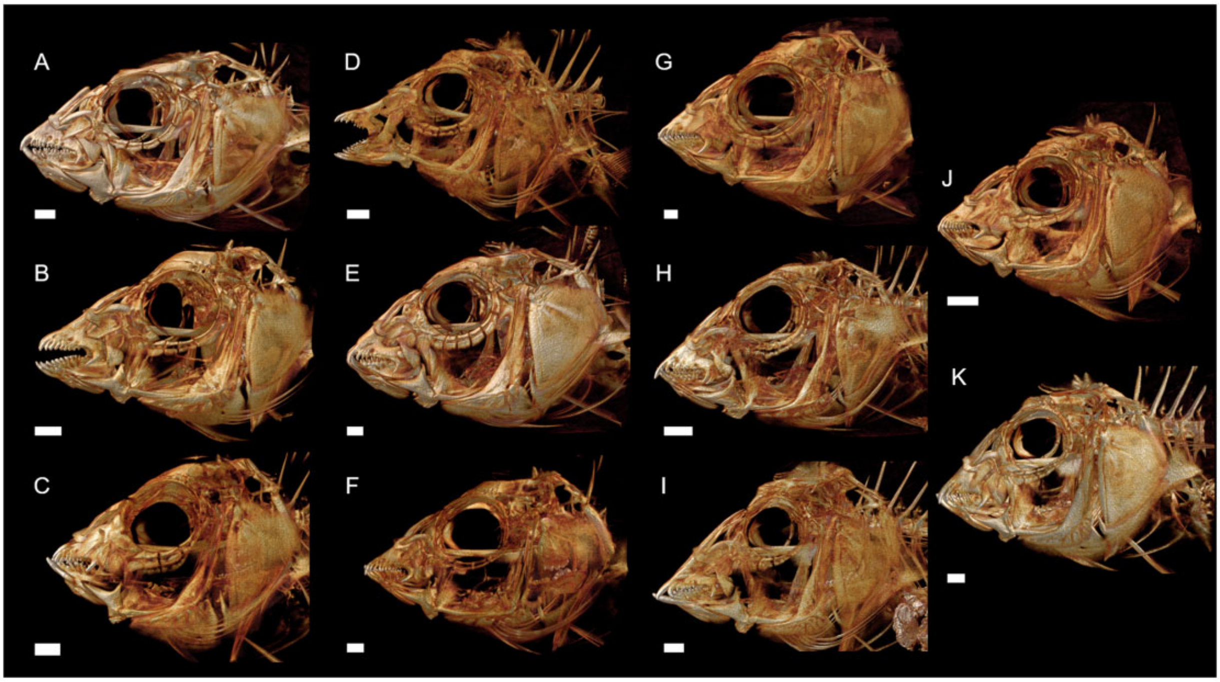

Fig. 2 CT scan renderings of 11 species of *Halichoeres* wrasses representing different tooth morphologies. (**A**) *H. hartzfeldii*, (**B**) *H. richmondi*, (**C**) *H. podostigma*, (**D**) *H. melanochir*, (**E**) *H. dispilus*, (**F**) *H. argus*, (**G**) *H. prosopeion*, (**H**) *H. maculipinna*, (**I**) *H. hortulanus*, (**J**) *H.leucurus*, and (**K**) *H. binotopsis*. Scale bar is set to 1000 μm.

were scanned between 34 and 35.5 μm voxel size at 65 kV and 123 uA. A total of 11 *Halichoeres* wrasse species were chosen for this study representing individuals with varying tooth morphologies across the three *Halichoeres* radiations (Fig. 2). Digital surface models of the jaws and teeth were created in Amira version 5.2.2 (Visage Imaging, Inc., Richmond, VIC, Australia). Surfaces were then exported to Meshlab (Visual Computing Lab, ISTI-CNR, Pisa, Italy) where jaw length, tooth height, radius, and tooth positions were measured.

The stress of each tooth was calculated following the principles of simple lever mechanics. Using the surface area of a cone we approximate the surface area of each tooth by measuring tooth height—defined by the total length from tooth tip until the tooth meets the jawbone—and tooth radius (Fig. 3):

$$\text{Surface area} = \pi r \left(r + \left(\sqrt{h^2 + r^2}\right)\right) \quad (1)$$

To establish a basic relationship between surface area and tooth stress we use the entire height of the tooth. Following the guide of puncturing mechanics, stresses should be concentrated at the tip of the tooth; however, our metric is not meant to establish where an individual tooth bears the greatest stress but instead to identify patterns in stress across teeth in the same dentition. Therefore, we ignore expected changes in stress from the tip to the base of each tooth in order to establish a relationship between the variation of tooth morphologies in a dental battery and stresses along the jaw. The standardization of tooth morphology allowed us to ignore curvature and damage yielding an idealized tooth surface area, which we used to calculate stress under different conditions (Crofts and Summers 2014).

Considering that teeth are on a lever, we had to first establish the amount of force a tooth is predicted to exert based on its position.

$$F_{lever} = F_{in} * \sin(a) * \left(\frac{Inlever}{Outlever}\right) \quad (2)$$

Our model assumes that the mechanical advantage, and force at the input lever is the same across jaws and that the input muscle force and input lever arm are the same for all teeth on the jaw. We modeled the upper jaw (premaxilla) with a static bite point assuming that it was in the most retracted position of its sliding position in the anterior jaws linkage (Westneat 2004). The model also assumes a single angle of muscle input force, with jaws in closed position, and no changes in gape were modeled. We simplified our system by assuming that F_{in}

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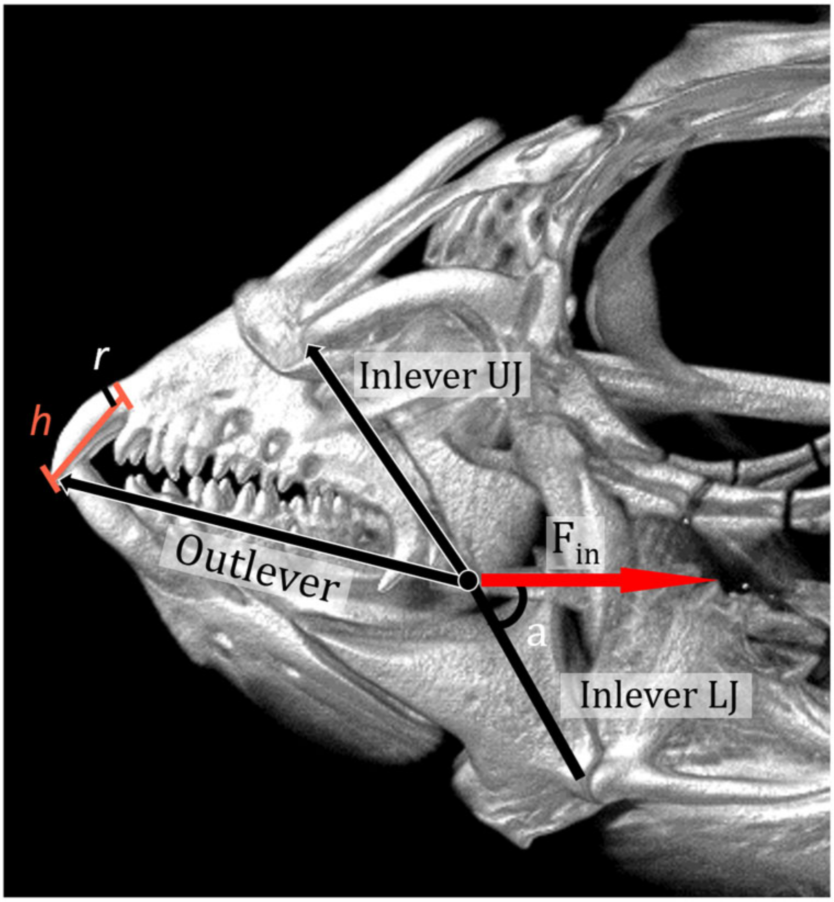

Fig. 3 Measuring stress: Skull of *H. hartzfeldii*, showing tooth morphology and FH calculation protocol. Using tooth surface area derived from linear measurements, measured from CT scans in MeshLab we calculate the stress of each tooth in the dental battery considering the force and jaw position. The force equations are derived from lever mechanics.

and F_{lever} ratios were constant in all calculations, generating F_{tooth} equation where F_{tooth} is equivalent to F_{lever} (Equation 3).

$$F_{tooth} = \frac{F_{in} \ * \ P_{tooth}}{L_{jaw}} \tag{3}$$

where F_{tooth} is the force at a particular tooth, F_{in} is the input force (set to 1N in our model), P_{Tooth} is the position of the tooth in question, and L_{jaw} is the length of the jaw. We calculated the stress at the surface of each tooth by dividing the calculated force on a tooth (F_{tooth}) by the surface area (Equation 4).

$$\sigma = \frac{F_{\text{tooth}}}{SA_{\text{tooth}}} \tag{4}$$

where σ is stress and SA_{tooth} is the tooth surface area.

We expressed the position of each tooth and the height of each tooth as a percentage of jaw length. We compared the stress at each tooth across jaws, individuals, and species and compared the stress residuals across teeth. Normalized stresses were inputs for a bootstrap analysis for determining a functional homodonty threshold.

Generating a functional homodonty threshold

In an ideal functional homodont, all teeth experience the exact same stress, regardless of size or position (Cohen et al. 2020; Table 2). This is an unrealistic expectation for real specimens: we are unlikely to calculate identical stress values for each tooth even in a functional homodont, given biological and technical variance. Instead, for functionally homodont dentitions, we expect stress values along the jaw to not differ significantly. Our challenge with testing functional homodonty in real organisms, therefore, is defining reasonable tolerance limits for significant variations in stress.

To do this, we calculated stress residuals for each dentition by subtracting the median stress for a

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Table 2 Important definitions for the functional homodonty method

Term	Definition
Functional homodonty method	Method detailed in Cohen et al. (2020) for (1) using tooth surface area and position to calculate tooth stresses through the transmission of force and (2) estimating a threshold for functionally heterodont teeth by bootstrapping stress values from multiple dentitions.
Functionally homodont teeth	All of the teeth in a dentition have statistically similar stresses that do not exceed a set threshold of stress
Functionally heterodont teeth	One or more of the teeth in a dentition has statistically different stresses that exceed a set threshold of stress from the majority of the dentition
Idealized functional homodont	All of the teeth in a dentition bear the exact same median stress
Idealized functional heterodont	All of the teeth in a dentition bear stresses that exceed the functional heterodonty threshold
Functional homodonty–heterodonty continuum	A continuum ranging from the idealized functional homodont to the idealized functional heterodont onto which all dentitions can be mapped using the functional homodonty method

dentition from each stress value and dividing by median stress (i.e., centering and scaling to median stress), allowing for comparison across dentitions. An idealized functional homodont would experience the same stress on each tooth regardless of size or position, resulting in residuals of 0 for each tooth in that dentition. To determine a threshold for functional homodonty, we bootstrapped a residual stress distribution per Cohen et al. (2020). Half of the teeth were randomly subsampled without replacement from a dentition and normalized residuals calculated; this procedure was repeated 10,000 times for each dentition. The resulting distribution of residuals was centered around 0, with small clusters of extreme values representing teeth that experienced ~1.8 times or more stress than the median for a given dentition (Fig. 4). The multimodal structure of bootstrapped residuals pointed us to clustering techniques to determine threshold values for functionally homodont/heterodont teeth. Our first implementation of this method (Cohen et al. 2020), used *k*-means to distinguished between high and low residual peaks (Maechler et al. 2019). In *Halichoeres* the wider range of bootstrapped residuals with extreme values resulted in poor fits using k-means. Instead, we used the more robust *k*-medoids clustering algorithm which uses data points as cluster centers (Maechler et al. 2019). Because *k*-medoids is considerably slower than *k*-means and could not be run on our entire sample of bootstrapped residuals at once, we performed the *k*-medoids clustering (with $n = 2$ clusters) on a random subsample of 5000 residuals 100 times, defining the threshold as the mean of the two resulting cluster centers. The resulting threshold ranged from 1.64 to 1.88 times median stress, with an average of 1.8 times median stress (Fig. 4, inset).

Summary statistics and phylogeny comparison

We generated two metrics from the normalized stress calculations, each designed to emphasize a specific aspect of the dentition: the average squared residual and proportion of functional heterodont teeth. To measure the degree of functional difference among teeth from the same battery, we calculated the average squared residual (where residuals = stress − median stress) for stress across a jaw. We also calculated the proportion of individual teeth in each battery that differed in function from the majority; unlike the variation in stress, this metric ignores the fold-difference of stress but emphasizes regionalization. We visualized these continuous traits on a time-calibrated phylogeny (Aiello et al. 2017; Supplementary Fig. S1) using the contMap function from the phytools R package contMap (Revell 2012). The phylogeny of 340 labrid species was time-calibrated using fossil data (Aiello et al. 2017), pruned down to the local clade containing the set of taxa studied here (Supplementary Fig. S1) and then pruned to the set of species studied here for comparative analysis using the R package ape (Paradis et al. 2004).

Results

Functional homodonty method

In 11 species and more than 440 teeth, 17 teeth bear stresses that exceed the homodonty threshold (Fig. 4, dashed line). While all of the 11 species of *Halichoeres* represented in this dataset have at least one tooth that exceeds the functional homodonty threshold, three species had especially high values for at least one of the metrics we calculated: *Halichoeres dispilus*, *Halichoeres melanochir*, and *Halichoeres maculipinna* (Fig. 6) . Each have a single

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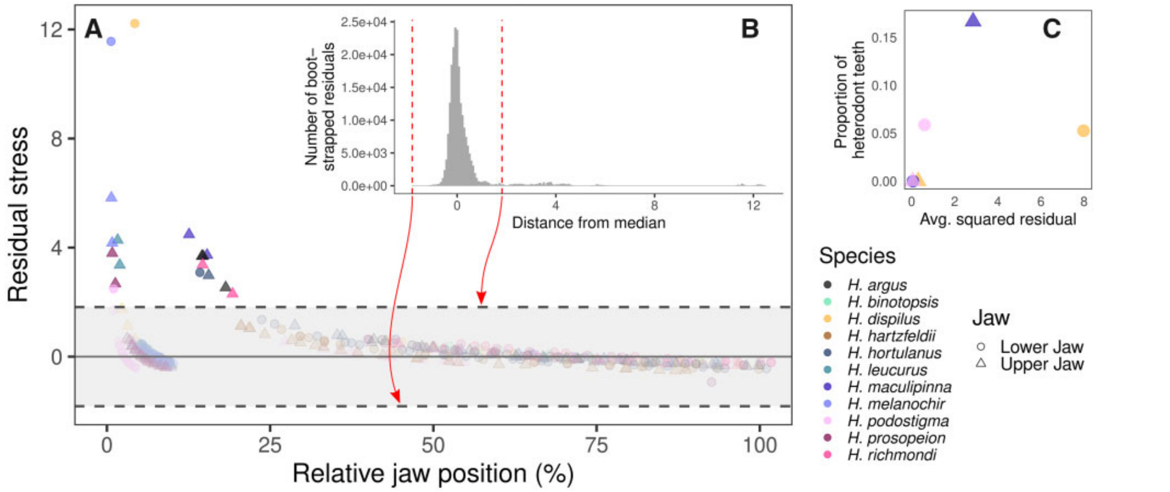

Fig. 4 Functional homodonty metric. (**A**) teeth from the jaws of 11 *Halichoeres*. Teeth above the dashed line exceed the functional homodonty threshold (**B**) defined by the bootstrapping of residuals. We then use k-mediods to generate a biologically relevant threshold (red dashed line). (**C**) Comparison of the proportion of functionally heterodont teeth to the average squared residual in four species of *Halichoeres* wrasse to highlight the tendencies emerging from this comparison. (1) High proportion functionally heterodont teeth: low residual, (2) Low proportion of heterodont teeth: high residual, and (3) High proportion of functionally heterodont teeth: high residual.

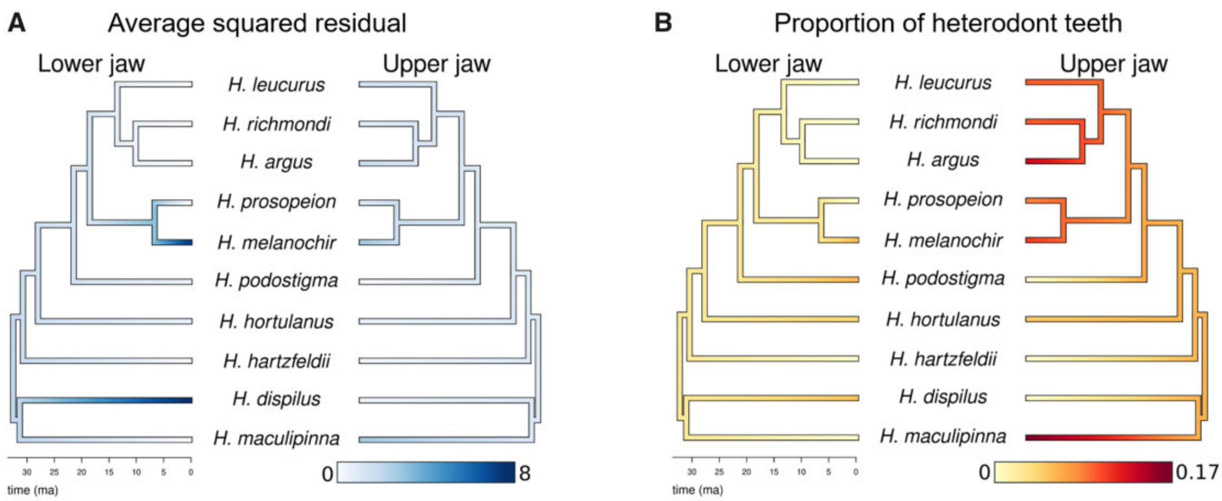

Fig. 5 Phylogeny comparing the upper and lower jaws in 11 species of *Halichoeres*. There are more functionally heterodont teeth in the upper jaws than the lower jaws, represented by a greater number of heterodont teeth (**A**). But functionally heterodont teeth in the lower jaws have a bigger impact and are represented by a larger residual (**B**). Note, *H. binoptisis* is removed from the phylogenetic comparisons as stresses were only calculated in the lower jaw.

tooth on the lower jaw that bears 12 times the median stress of that dentition, far exceeding the functional homodonty threshold of 1.8 times median stress. While *H. maculipinna* had no teeth bearing stresses that high, the specimen we analyzed had 2 of 12 teeth on the upper jaw exceed the functional homodonty threshold, the highest proportion of any of the dentitions we measured (Fig. 4A). The upper and lower jaw fangs of *H. macculipinna* occlude far beyond the anterior end of the jaws with hardly any curvature. The species *H. melanochir* most closely resembles a morphological homodont and *H. dispilus* has two sets of large canines that extend far beyond the end of the premaxilla or dentary while the rest of their teeth are similar in size (Fig. 2C–E).

Across all 11 species, functionally heterodont teeth were more frequent on the upper jaws. While fewer teeth on the lower jaws exceeded our functional heterodonty threshold, those that did typically had much higher residuals, in some cases bearing up to

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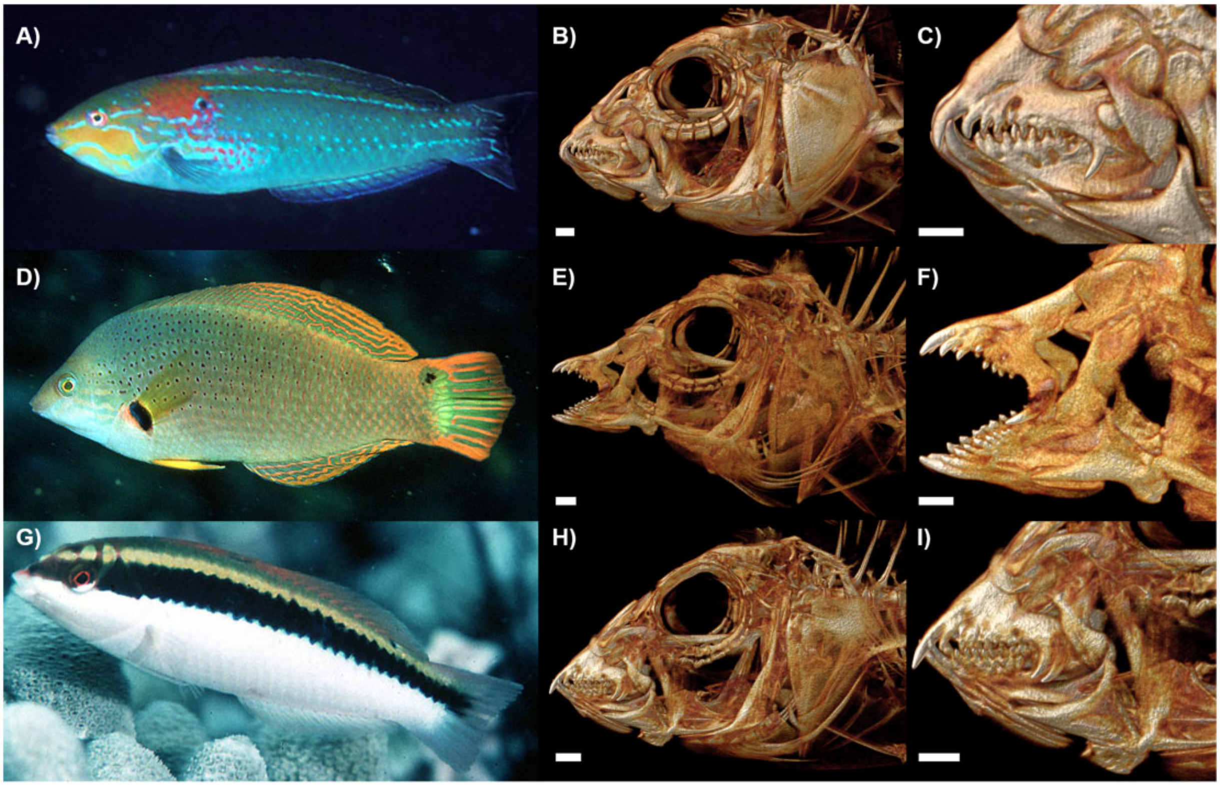

Fig. 6 Functionally heterodont species of *Halichoeres*. (**A–C**) *H. dispilus*, (**D–F**) *H. malenochir*, (**G–I**) *H. podostigma*. Live photos for all three species downloaded from FishBase.org and provided by J.E. Randall. Scale bar is set to 1000 μm.

twice the stress of the teeth on the upper jaw of the same fish (Figs. 4C and 5, especially *H. dispilus* and *H. melanochir*).

There are differences in the magnitude of functionally heterodont teeth across all 11 species. For instance, *H. podostigma* has a singular functionally heterodont tooth on its lower jaw that barely exceeds the heterodonty threshold. This tooth bears 2.4 times the median stress of the rest of the dentition. By comparison, *H. melanochir* has several teeth that bear between 4 and 12 times the stress of functionally homodont teeth in the same dentition.

Proportion versus average squared residual of functionally heterodont teeth

Ancestral state reconstruction suggests the ancestral *Halichoeres* had a small number of functionally heterodont teeth, and these were more likely on the upper jaw than the lower. The three species with high numbers of heterodont teeth or high average squared residuals were in two of the three *Halichoeres* clades. Along the continuum of functional homodonty to heterodonty, we find three tendencies (Fig. 5). First, some taxa departed from functional homodonty because of one or two exceptional teeth in a battery of otherwise functionally homodont teeth. This is represented by large residuals and a small proportion of functionally heterodont teeth (*H. dispilus*). Next, are fishes with regionalization of function, these have small residuals but a high proportion of functionally heterodont teeth (*H. maculippina*). Finally, a dental battery could have large and small teeth interspersed evenly across the jaws as in *H. melanochir* (Fig. 4Aand C) which leads to a high proportion of functionally heterodont teeth combined with high residuals.

Discussion

The biomechanical function of teeth is largely dependent on tooth morphology, orientation, position along the jaw lever from front to back, and the dynamic forces of the jaw muscles driving them into a prey item (Barel 1982; Westneat 2003; Anderson et al. 2016). Here we present a way to calculate the biomechanical function of teeth in jaws of labrid fishes from the perspective of their geometry and the relative bite stresses they exert. Our central conclusion is that *Halichoeres* wrasses have a wide diversity of tooth arrangements that lend to functional homodonty. However, some species have strikingly functionally heterodont teeth—either a few teeth or

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regions of teeth with disparate function. There will always be some level of heterodonty at the tips of the teeth across any dentition, but our metric draws a clear line between functional homodonty and functional heterodonty.

There are dentitions that have distinct regionalization, where many teeth are quite similar to one another, but patches are performing very different tasks (Cohen et al. 2020; Mihalitsis and Bellwood 2019). In contrast, there are dentitions where one or a few teeth are radically different from the rest of the dental battery. In the first case, there will be a large number of functionally heterodont teeth, but a low average squared residual, and in the second case there will be a few functionally heterodont teeth, but they will have a very high squared residual. In *Halichoeres* wrasses two species have extremely functionally heterodont teeth, and they arrive at heterodonty by different means. In *H. melanochir*, the teeth are strongly regionalized with a high proportion of functionally heterodont teeth, whereas in *H. dispilus* just two large canines in the upper jaw dictate functional heterodonty leading to high residuals (Figs. 4A and 5).

Our method converts a categorical trait, morphological homodonty, into a continuous one, functional homodonty, creating opportunities for more nuanced analyses and directing phylogenetic and biomechanical hypotheses in new directions (Shimada 2002; Westneat and Alfaro 2005; Kolmann et al. 2019; Hulsey et al. 2020). Adding the perspective of phylogeny bears two different fruits. First, we can identify three apparently independent evolutions of functional heterodonty: *H. dispilus*, *H. melanochir*, and *H. maculipinna* (Fig. 6). Second, our ancestral state reconstruction implies an ancestral *Halichoeres* wrasse had a small number of functionally heterodont teeth, and the proportion of functionally heterodont teeth is variable over evolutionary time. A metric for functional heterodonty also allows us to generate computed derivatives that reveal trends and potentially important information about selective pressures (Linde et al. 2004; Kolmann et al. 2019). The higher proportion of functionally heterodont teeth on upper jaws than lower jaws may be due to the upper jaw being supported by the cranium, while the lower jaw is a cantilever beam (Powlik 1995; Linde et al. 2004; Westneat 2004; Westneat and Alfaro 2005; Grubich et al. 2008; Smits and Evans 2012; Olsen and Westneat 2016). Also, the magnitude of functionally heterodont teeth is larger in the lower jaw than upper, perhaps because of mobility in the lower jaw relative to the entire body of the fish (Figs. 4 and 5). How a species arrives at functional heterodonty should point biomechanists in very different directions when asking about the functional consequences of teeth.

We were excited to find such disparate dentitions, and three independent derivations of heterodonty, in just 11 species of a genus of wrasse that all occupy similar nearshore, shallow water, coral reef habitats, and have all been assigned the same broad dietary niche (Randall and Böhlke 1965; Clifton and Motta 1998; Fulton and Bellwood 2002; Jones 2007). Implementing this analysis across a broader phylogenetic and ecological range of wrasses will lead to discovery of further, heretofore cryptic variation in dental function that we expect will inform natural history and diet studies. The *Halichoeres* radiation may be an intriguing area of the labrid phylogeny to explore in more detail, as the genus is not monophyletic, with 80 species spread across three clades, with other genera such as *Macropharyngodon*, *Thalassoma*, and *Coris* interspersed among them (Westneat and Alfaro 2005). The hogfishes and tuskfishes (*Bodianus*, *Choerodon*, and relatives) often have extraordinarily large, recurved canines and regionally specialized teeth, yet their close relatives such as *Pseudodax* and *Clepticus* possess jaws specialized for browsing or planktivory, suggesting an interesting trajectory of tooth evolution. The cheiline wrasses are also diverse in tooth morphology, jaw mechanics and dietary preferences (Westneat 1995) and are the sister-clade to the parrotfishes, offering another area in which to explore the evolution along the functional homdonty–heterodonty continuum.

Even in this small, and not particularly diverse clade of wrasses, there are some examples of dentitions that warrant further examinations. *Halichoeres leucurus* is morphologically homodont, but the teeth in the upper and lower jaw occlude with a degree of precision that is not common in fishes (Kolmann et al. 2019). Physical models of teeth, made from high-resolution CT scans of actual dentitions, could highlight advantages of this unusual tooth arrangement (Evans and Sanson 2003; Qian et al. 2013; Crofts and Summers 2014). There are visually arresting fanged dentitions, such as *H. hortulanus*, which do not cross the threshold for functional heterodonty, but nevertheless have teeth that suggest some differences in function (Fig. 2A). Careful natural history observations of differences in behavior of species with prominent fangs may reveal how and when these fangs are deployed. Other biomechanical models, such as Mandiblever, take into account tooth angle and its interaction with gape angle across a realistic bite (Westneat 2003). Teeth are, in some

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sense, an endpoint in a series of modules which determine function (Evans and Sanson 2003; Lucas 2004; Anderson and Rayfeild 2012; Kolmann et al. 2019)—the powerful, subdivided adductor musculature, through tendons and ligaments connecting jaw elements, to the levers and linkages of the jaws, and even the seemingly insignificant dental ligaments securing tooth to jaw combine to ensure that the tip of the tooth transmits sufficient stress to penetrate prey.

Acknowledgments

Special thanks to Dr Kory Evans who CT scanned all specimens represented in this study and uploaded them as free to download in morphosource.org. Special thanks to the facilities provided by the Karl F. Liem imaging center at Friday Harbor Labs, WA, and oVert for open access to CT scans. Thank you to Dr Darrin Hulsey and Dr Gareth Fraser for organizing the associated symposium.

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Data availability statement

All CT data used in this study are publicly available and free to download from morphosource.org in the Scan All Fishes project.

Supplementary data

Supplementary data available at *ICB* online.

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Histological phenotypes and complex prey processing behaviors in pacus

Running title: Lip phenotypes in fishes

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ABSTRACT

Coordinated actions of soft and hard tissues make complex prey processing, like chewing or winnowing, possible. While complex processes like chewing were described in mammals, these behaviors are in fact widespread among vertebrates, with various lineages evolving the multicuspid teeth, robust jaws, and shearing kinematics beneficial for the efficient breakdown of food. In this study, we demonstrate how soft tissues such as lips, ligaments, and muscles interact with the mineralized skeleton during prey capture and prey processing in fishes. Pacus (Serrasalmidae) are Neotropical freshwater fishes that feed on leaves, fruits, and seeds, and insects. These prey items are hard or tough, require high forces to fracture (nuts or seeds), contain abrasive or caustic elements (phytoliths and druse), or deform considerably before failure (fruit rind). Pacus are gape-limited and lack pharyngeal jaws, a tool that many bony fishes use for dismantling and/or transporting prey. Instead, we show how pacus couple hydraulic repositioning of prey with direct oral manipulation, using a sharp, morphologically heterodont dentition and squishy lips to reorient food. We find that frugivorous pacus tend to have larger, more complex lip morphologies, innervated and folded at their surface, while grazing species have callused, mucus-covered lips. Unlike mammalian lips, trunks, or tongues which are inherently muscular, pacu lips lack any skeletal or smooth muscle. While this implies that pacu lips lack dexterity, we describe how these lips are actuated by the jaw adductors *via* a ligamentous intermediate.

Keywords: chewing, histology, prey manipulation, proboscis, tongues

INTRODUCTION

Chewing requires two essential tools: one to position the prey for processing and a second for crushing and shearing. In mammals, these tools are the lips and tongue for manipulation, and teeth for mastication (Hiiemae & Palmer, 2003; Ross et al., 2007; Osborn et al., 2020). So, soft tissues are responsible for positioning, while hard tissues fracture and break down food. In fishes the situation is more complicated because manipulation is not limited to lips and tongue – instead, fishes use kinetic skulls, accessory jaws (i.e., pharyngeal), or the flow of water for repositioning prey (Dean et al., 2005; Ross et al., 2007; Kolmann et al., 2016; Schwarz et al., 2020). Chewing in fishes remains the domain of hard tissues. Emphasis on teeth and jaws for prey processing in fishes has distracted from the prominence of soft tissues in some lineages. In mammals, prey manipulation driven by the tongue and lips has evolved once, but in fishes these sorts of cranial, soft tissue appendages have evolved many times (Lumsden and Osborn 1977, Grossnickle et al.,. 2021; Peterson et al., 2022). Herbivous linegease of fish such as astroblepids, catostomids, gyrinocheilids, balitorids, prochilodontids, curimatids, oxudercids, cichlids, and serrasalmids, have independently evolved prominent lips (Lujan & Armbruster, 2012; Schaefer & Lauder, 1986; De Meyer & Geerinckx, 2014; Benjamin, 1986; Maie et al., 2011; Geerinckx et al., 2007; Sazima, 1986; Machado-Schiaffino et al., 2014; Correa et al., 2007). Within serrasalmids there are two lineages – the carnivorous piranhas with sharp, interlocking teeth, and the pacus, herbivores with high-cusped molars and a hypertrophied lower lip.

In general, prey processing and manipulation in, like plucking fruits, husking seeds, and chopping leaves is made easier by having a tongue or digits for manipulation. Fishes typically lack these tools, just consider pacus (Serrasalmidae): keystone riparian grazers and seed dispersers that crush fruits and transport their seeds throughout the Amazon basin (Goulding, 1980; Correa et al., 2007; Correa & Winemiller, 2014). Pacus are not able to engulf an entire prey item and reduce it through brute force. Instead, they grasp and excise edible pieces, then reposition it for further reduction (Alexander, 1964; Irish, 1987; Grubich et al., 2012; Figure 1). This is because pacus are gape-limited and cannot protrude their jaws to suction prey towards their mouths (Goulding, 1980; Irish, 1987) – instead they are bobbing for fruit from below. However, pacus have a hidden anatomical tool which may aid in prey-processing: under the skin and muscles, pacus have a noticeable overbite, that is filled by an enlarged lower lip that conceals their robust, multicuspid teeth (Figure X; Irish, 1987; Kolmann et al., 2019). Pacus feed on prey that is large and hard, tough and noxious, smooth and slippery, their lips must work in concert with the dental battery to effectively reposition and breakdown prey.

How soft tissues tools like lips play a role during feeding in fishes is less understood compared to the acumen of studies on teeth and jaws (Crofts & Summers, 2014; Crofts, 2015; Cohen et al., 2022). Looking at the role these appendages play in mammalian lineages we can make two intuitive hypotheses of how lips may function in pacus. Pacu lips may act as an active participant in prey manipulation as in the trunks of elephants and lips of rhinos – extensions of the upper lip that aid in foraging and manipulation (Owen-Smith, 1975; Kier & Smith, 1985). Alternatively, pacu lips are possible passive participants in prey processing procedures, as seen in the cheek chambers of chipmunks and other rodents that store food prior to reduction. Passive lips might serve to trap the surface of vegetation, or they could protect the teeth from the gamut of plant defenses like phytoliths, acting as a callus. If the former - active role - is the case, there should be musculature around the circumference of the lip, while the latter hypothesis - a passive role - implies that little, if any, muscle will be present (Burne, 1917; Kier & Smith, 1985; Witmer et al., 1999). Any similarities in prey-handling and soft tissue manipulation of food would extend already documented patterns of analogy among herbivorous pacus and mammals (Huie et al., 2019).

The well resolved serrasalmid phylogeny that includes both the herbivorous pacus and the more carnivorous piranhas (Kolmann et al., 2020) makes it possible to trace the evolutionary relationship among soft tissue characters and diet. The slick skin of fruits, hardness of seeds, and abrading phytoliths in leaves makes being a herbivore challenging. Some pacus specialize on one type of herbivorous prey while others deal with many. If lips are tools that support herbivory we expect that lip morphology, like tooth shape, varies with the particular plant tissue type. Degree of hypertrophy, muscularity, vascularization, and fibrous composition are all aspects of lip morphology that could vary with the demands of handling different types of prey (Owen-Smith, 1975; Burne, 1917; Witmer et al., 1999; Feilich et al., 2020). We also expect even greater variation in lip morphology between pacus and piranhas, except for those piranha species that feed on plant materials (i.e, *Pygopristis*; Machado-Allison, 1985; Nico, 1991). The presence or absence of a lip may not be as important as what that lip is made of, in which case the evolution of lips in pacus should reflect adaptations to particular dietary challenges: folivory, frugivory, granivory, or carnivory.

Using histology, CT scanning, and videography we explore the evolution and morphology of lip phenotypes across pacus. Our goals are four-fold: (1) describe lip cellular morphology among pacus and piranhas; (2) make functional predictions (i.e, active or passive control) of lips based on histology; (3) use videography to characterize lip use during prey capture and processing; and (4) map prey, dietary guild, and morphology on the phylogenetic history of serrasalmid fishes. In fishes teeth and jaws are responsible for the breakdown and fracturing of prey but, there is likely an equal diversity of soft tissue adaptations that help with repositioning and manipulation. Lips may provide herbivorous pacus and omnivorous piranhas with the soft tissue tool needed for feeding on diverse prey with relatively simple skulls. The ecological significance of prey-handling in pacus should not be overlooked as these giant fishes are among South America’s most important seed dispersers (Correa et al., 2015).

METHODS

Specimen acquisition and dissection

This study included 25 wild-caught specimens from 14 species, comprising 68% of total serrasalmid generic richness (Froese & Pauly, 2014), and incorporating representatives from all major clades and diet guilds (Correa et al., 2007; Kolmann et al., 2020). Specimens were previously formalin-fixed, preserved in 70% ethanol and then used for dissection, histology, and CT imaging were from University of Michigan Museum of Zoology (UMMZ; Ann Arbor, MI) and Royal Ontario Museum Ichthyology (ROM; Toronto, ON) collections (Supplemental Table 1). Live, wild-caught specimens recorded in videos were filmed in a commercial aquarium holding facility (Below Water, Inc, Montreal, Quebec). We dissected museum specimens to observe lip, muscle, and tendon morphology, with particular attention paid to the primordial ligament and its association with the adductor mandibulae muscle series (adductor mandibulae division 1 = AM_1, adductor mandibulae division 2 = AM_2, etc.; Alexander, 1964; Datovo & Castro, 2012). The primordial ligament aids in adduction of the lower jaw in association with the adductor mandibulae complex (Alexander, 1964). Specimens were dissected on both sides, the infraorbital bones and opercular series removed to observe the underlying muscle tissue. Photographs were made with a handheld cellular camera phone for later reference and tracing.

Histological sectioning & micro-computed tomographic (CT) imaging

We used histological sectioning to explore differences in tissue composition among pacu lips, upper and lower jaws. Our samples of *Pygocentrus* were frozen and then thawed prior to tissue section and embedding, but we did not see tissue degradation (Figure 3). The lower jaw and associated soft tissues (lips, muscles, etc.) were excised from the specimens and fixed in 10% buffered formalin for 24 hours. Samples were rinsed in distilled water and decalcified in 10% EDTA following the protocol in Histological Processing of Teeth and Periodontal Tissues for Light Microscopy Analysis (Silva, Moreira, & Alves, 2011). After tissues were thoroughly decalcalcified, they were dehydrated in a stepwise series, starting from 25% ethanol. We embedded whole jaws in JB-4 embedding media (Electron Microscopy Science JB-4 embedding media protocol). Jaws and associated tissue were sectioned laterally and axially at 3-5 μm. Sections were placed on glass slides, dried for 24 hrs, and then stained with Lee's Basic Fuchsin and Methylene Blue stain and imaged using a Keyence VHX 500 microscope (Itasca, IL, USA) and Nikon eclipse E600 compound microscope (Melvile, NY, USA). At times of low dissolved oxygen content in water, lips in some characiform species can become hypertrophied and over-vascularized, useful for absorbing oxygen at the water's surface (Winemiller, 1989) - we did not observe these characteristics in any of our samples.

We also used contrast-enhanced micro-computed tomographic (microCT) imaging to visualize skeletal morphology in pacus and piranhas. Specimens were imaged using Friday Harbor Lab's Bruker 1173 Skyscan (Bruker Corp, Billerica, MA). Specimens were imaged at 65kV and 123uA with a 1.0 mm aluminum filter. In preparation for CT imaging, specimens were either wrapped in ethanol-moistened cheesecloth or slightly hydrated with 70% ethanol and heat-sealed within a plastic bag, then placed in a plastic tube, and stabilized with foam. Jaw anatomy was then visualized using volume rendering in the program 3DSlicer (www.slicer.org), following the Buser et al. (2020) CT segmentation and visualization workflow.

Observations on feeding behavior

We were interested in how pacus were using their lips and jaws to obtain and process prey. Pacus (n = 5 species) were filmed feeding on pellet food and vegetable chunks in the Below Water Inc. wholesale aquarium facility. Videos were taken opportunistically while the fishes fed, using a Canon R5 and EF f/2.8L 100mm Macro lens and iPhone 12 Pro Apple inc. Fish were housed in groups in aquariums of 600-1600 liters, centrally filtered, and complete with natural sand, rock, and wood mimicking their natural habitats. Laminar current pumps added current from 4000-16000 liters per hour to closely mimic the conditions in nature for the rheophilic species. The species filmed feeding were *Tometes ancylorhynchus* (Rio Xingu, Brazil), *Tometes* sp. (Rio Tapajos, Brazil), *Utiaritichthys esguiceroi* (Rio Papagaio, Brazil), all grazers on vascular riparian plants, *Myloplus cf. torquatus* (Rio Orinoco, Colombia), a generalist lowland herbivore, and *Mylesinus paucisquamatus* (Rio Tocantins, Brazil), a specialist feeder on submerged bryophytes (Vitorino et al., 2016). We also examined videos of frugivorous *Colossoma macropomum* and *Piaractus brachypomus* feeding (Goulding, 1980), obtained from YouTube and from videos donated by the Seattle Aquarium. We chose not to consider the feeding behaviors of piranhas, given their lack of hypertrophied lips (described below).

Phylogenetic reconstructions & statistical methods

We were interested in assessing the level of correspondence between lip histological characters, general diet niche (e.g., frugivore, folivore, etc.) or the presence of specific prey in the diets of pacus and piranhas. We used the serrasalmid phylogeny from Kolmann et al., (2020) for all analyses, pruned to include only the taxa in our sampling scheme. This tree is the most comprehensive dated phylogeny for serrasalmids, includes sampling of all extant genera, and is in broad agreement with other current phylogenetic hypotheses (Thompson et al., 2014; Mateussi et al., 2020). We also used the discrete diet categories obtained by Kolmann et al. (2020) to represent each species' generalized diet niche, in addition considering the presence/absence of discrete prey items (e.g., seeds, fin rays, leaves, etc.) from gut contents as a more nuanced approach to capturing diet diversity across serrasalmids.

To explore any relationships between the combination of different prey items (e.g., fruits, flowers, fish flesh, etc.) found in gut contents and different species' lip histological configurations (goblet cells, nerves, muscle, for example), we used distance-based redundancy analysis (dbRDA; Legendre and Anderson, 1999). Distance-based RDA submits a dissimilarity matrix to a principal coordinates analysis (PCoA), the results of which are suitable for generalized redundancy analysis. This method allows for a finer-scale investigation into diet nuances according to what prey items are present, rather than grouping predators into broad categories that gloss over differences among diets. We used dbRDA on discrete histological traits (presence/absence of histological characters) and diet data (presence/absence of prey items) to generate a lip histological phylomorphospace for serrasalmids - a projection of each species' trait values into a two-dimensional space and connected by the branches of a phylogeny. This allows us to explore how different or similar sister taxa, subclade members, and dietary guilds are to one another in a morphospace framework. We plotted this phylomorphospace using the *phylomorphospace* function in phytools (Revell, 2012).

We also used co-phylogenetic tree networks to visualize how histological patterns align with historical patterns (phylogeny) and/or ecology (dietary convergence or lability). We clustered species together according to their lip histological similarity using a UPGMA heuristic ('average' method in *hclust*; Murtagh and Legendre, 2014) on a binary distance matrix, as implemented by the *hclust* and *pvclust* functions (vegan and pvclust packages; Suzuki & Shimodaira, 2006). We then used the *cophylo* and *phylosig* functions in phytools (Revell, 2012) to render the topological diagrams and measure phylogenetic signal, respectively.

RESULTS

Histological form-function relationships of pacu and piranha lips

We found several histological trends in lip composition; namely, differing patterns of soft tissue regionalization among pacu species (Figure 3). In general, pacu lips are composites of collagen, putative sensory nerves, and macrophages and only some lips are covered with goblet cells. Folivorous species tended to have more mucus-producing cells embedded in the outer, epidermal layer than other herbivorous pacus, like *Myloplus,* which lack goblet cells on the labial side of the lip. Almost all pacus had highly innervated lips and frugivorous species like *Colossoma* and *Piaractus* had more densely innervated lips (Figure 3; Supplemental Figure). All serrasalmids had keratinized lips, but folivores in particular, and other herbivores in general, had thicker layers than piranhas; however, our ability to say this definitively is limited by preservation artifacts at the lips' surface from EDTA.

The epidermis, dermis, and hypodermis are clearly differentiated in pacus, but not in piranhas (Figure 3). The lips of piranhas are built of large collagen bundles, interwoven through the volume of the lip with large putative sensory nerves. Composing the epidermis of piranha lips is a thin, keratinized epithelium with few or absent macrophages or mucus-secreting cells (Figure 3; Supplemental Figure). This histological generalization holds true for the typical carnivorous piranhas *Pygocentrus* and *Serrasalmus* (Ferreira et al., 2014), the ectoparasitic, scale-feeding wimple piranha, *Catoprion mento* (Nico & Taphorn, 1988) and for *Pygopristis* - an omnivorous piranha that feeds on fins, scales, insects, as well as fruits and seeds (Nico, 1991). Despite having a diet more similar to pacus than other piranhas, the lip of *Pygopristis* is solely composed of disorganized collagen. The keratinized epidermis is thicker in *Pygopristis* lips than in other piranhas, but unlike *Pygocentrus, Serrasalmus,* or *Catoprion,* in *Pygopristis* we found an abundance of goblet cells near the medial mandibular symphysis (Figure 3; Supplemental

Figure). This region of the lip in *Pygopristis* is also conspicuously folded, with goblet cells lining the lingual edge.

Lip diversity according to diet: frugivores, granivores, & omnivores

All pacus considered in this section feed primarily on nuts, seeds and/or fruits, i.e., prey that are either difficult to crack, deform before fracture, and yet all require considerable oral manipulation prior to ingestion. *Myloplus schomburgkii, M. torquatus,* and *M. rubripinnis* (which consume leaves, stems, flowers, seeds, and insects; Nico, 1991; Gonzalez & Vispo, 2003; Dary et al., 2017) both have enlarged or hypertrophied lips (Figure 3; Supplemental Figure). The epidermal layer of these *Myloplus* lips is formed of tightly packed cells and is heavily folded, making the lip much thicker than those of other herbivorous pacus. The labial surfaces of the lip are also covered in a keratinized epithelium. There are no mucus producing (goblet) cells on these labial surfaces; however, there are enlarged taste buds and immune-function macrophages densely distributed in the epidermis (except in *M. schomburgkii*; Figure 3). The epidermal layer on the lingual surface of the lip, nearest to the teeth, is studded with goblet cells and is folded. In these two species, the lip's dermal layer is made up of both organized and disorganized connective tissue with collagen and elastin distributed throughout. There is an abundance of nerves and blood vessels within the dermal region of these two *Myloplus* species as well (Figure 3; Supplemental Figure).

The hypertrophied lips of fruit and seed feeding *Piaractus* (Honda, 1974; Goulding, 1980) bear many similarities to the lips of *Myloplus.* The dermal region is filled with blood vessels, nerves, and collagen (Figure 3; Supplemental Figure). Unlike *Myloplus*, the dermal region adjacent to the lip epidermis is composed mainly of loose collagen fibers interspersed with elastin (Figure 3; Supplemental Figure). Additionally, the epithelium in *Piaractus* is more folded than in the three *Myloplus* species, lacks mucus-secreting cells, is tightly packed with epithelial cells, and studded with macrophages and taste buds. *Colossoma*, another fruit- and seed- eating pacu, has the spongiest lip. Interstitial channels are distributed throughout the keratinized epidermis of the lip. Directly below the keratin is a thin layer of longitudinal epidermal cells. The dermis and hypodermis is composed of organized collagen bundles and adipose tissue (Figure 3; Supplemental Figure).

Acnodon is not a frugivorous pacu but an omnivore with one of the most varied diets among pacus, including fish scales, seeds, and flowers, as well as insects (Leite & Jegu, 1990). Their lips share a similar lip regionalization strategy with that of frugivores like *Colossoma* and *Piaractus*. The outer epidermal layer is covered by keratin and the dermal and hypodermal regions are composed of varying layers of collagen (Figure 3; Supplemental Figure). There are no substantially larger nerves or macrophages in *Acnodon*'s lip but there are mucus-producing goblet cells seen on the lingual surface of their lips like in frugivorous pacus like *Colossoma* or *Piaractus* (Figure 3; Supplemental Figure).

In both *Myloplus* and *Piaractus*, an extension of the primordial ligament originating from the adductor mandibulae muscle divisions (specifically, A1) inserts on the distal edge of the lip (Figure 4). We found that contraction of the A1 pulls on the edges of the hypertrophied lip and that pulling or flexing of the lip in turn moves A1. The ligamentous attachment is more robust and tendon-like in *Myloplus* than in *Piaractus* but both are distinct from the original ligament (Figure 4). Contrary to our predictions about lip morphology, no muscle tissue was found in any hypertrophied pacu lips (or any other lips).

Lip diversity according to diet: folivores, phytophages, algivorous, and planktivores

These pacu species feed on prey that requires shear stress to fracture cellulose and plant walls prior to chemical digestion. Lips of folivorous pacus such as *Tometes* and *Myleus* (Andrade et al., 2016, 2019a,b), as well as the lips of planktivorous and algivorous species like *Metynnis luna* and *Metynnis maculatus* (Ramos et al., 2018; Andrade et al., 2019a,b), are thinner and composed of three layers; the epidermal layer is relatively thin and studded liberally with goblet cells and large taste buds (Figure 3; Supplemental Figure). The dermis is made up of densely packed, regular connective tissue followed by a third layer consisting primarily of disorganized connective tissue.

Tometes' lips are made of disorganized connective tissue, with no evidence of muscles or different layers of collagen (Figure 3; Supplemental Figure). The lips of *Myleus* are composed primarily of disorganized collagen tissue with some organized tissues just beneath the dermis in the stratum compactum and have small blood vessels running throughout. Deeper in the dermal layers tubes of organized collagen that run the circumference of the lip. The epidermis is wavy and composed of long columnar cells intermittently studded with macrophages. In both species of *Metynnis,* the lips' epidermal layer is thick and studded with many goblet cells. In *Metynnis*, layers of circumferentially oriented collagen fibers comprise the more superficial part of the dermis, with more organized and densely packed collagen along the circumference of the lip. The hypodermis is composed of disorganized and loosely packed collagen fibers. Small blood vessels pass through the lip nearest the teeth and small nerves are seen adjacent to the dentary (Figure 3; Supplemental Figure). Dissection of *Metynnis and Myleus* did not show any additional connection between the lip and the primordial ligament.

Observations on feeding behavior

For a typical feeding event in pacus, prey is suctioned towards the mouth, from within a head length's distance away, after which prey manipulation begins (Ferry et al., 2015; Figure 5). Prey manipulation strategies fall into two general categories: (1) intraoral manipulation with the mouth typically closed and involving only the teeth, or (2) a combination of intraoral and perioral (i.e., between the lips) processing, where prey is passed back and forth between buccal, dental, and lip regions repetitively (Figure 5; Supplemental Videos). During feeding, *Tometes* and *Metynnis* relied primarily on suction production for prey capture and reorientation, with prey manipulation limited to interactions with the teeth. We saw no lip actuation in any of the videos nor did these species use their lower lip for leverage when approaching food (Supplemental Videos). *Mylesinus paucisquamatus* relies on suction for prey capture and is only observed using lip manipulation once prey is inside the mouth and the jaws are closed. In contrast, *Piaractus, Colossoma,* and *Myloplus* actively employ their lips to manipulate and stabilize prey, prior to and during, occlusion of prey between the teeth (Figure 5; Supplemental Videos). All *Myloplus* species were seen contracting their lips around captured prey, rotating and gripping it (Figure 5; Supplemental Videos). During several encounters with prey, both *Myloplus* species and *Utiaritichthys* use their lower lips to scoop substrate-bound food into their mouths. *Myloplus* conspicuously protrude their lower lip when approaching food on the substrate, in preparation for freeing the food from the benthos. This 'scooping' behavior serves as an intermediary behavior between prey capture and prey processing (Figure 5; Supplemental Videos).

Additionally, folivorous and omnivorous *Myloplus* and *Utiaritichthys* (Pereira & Castro, 2014) hold the distal-most portion of their food in place with the lower lip, while the symphyseal teeth chew on the margins of the food item (Figure 5; Supplemental Videos). During several encounters with prey, we see the lip of *Myloplus* deforming around a prey item and eventually rotating or pulling the food into its mouth. Once the prey is in the mouth, the lips are sealed completely while intra-oral manipulation takes place, as evidenced by cyclical movement, contraction and relaxation, of the pharyngeal chamber. *Myloplus* and *Utiaritichthys* were observed holding food against the lip (and perhaps the teeth as well) while rapidly closing the lower jaw, slamming prey into the double rows of upper jaw teeth. Intraoral manipulation was observed in almost all pacus regardless of lip thickness (Figure 5; Supplemental Videos). All pacus possess two elongated symphyseal teeth on the dentary that sit just behind the oral dentition. Our video analysis shows widespread intraoral manipulation of prey likely assisted by these two teeth.

Trait-Diet Correlations and Phylomorphospaces

In general, we find that there is only weak-moderate correspondence between the phylogeny and histological characters, except where folivorous and planktivorous/algivorous species are concerned (Figure 6a). *Catoprion mento* has the least complex lip relative to other serrasalmids, and so in some ways the most divergent phenotype - fitting its odd ecological role as a scale-feeder or lepidophage (Nico & Taphorn, 1988; Kolmann et al., 2018; Figure 6a; Supplementary Appendices). Serrasalmines (piranhas like *Serrasalmus* and *Pygocentrus*, as well as *Metynnis*) cluster together with folivorous taxa like *Myleus* and *Tometes,* except for *Pygopristis,* which is more similar to omnivores like *Acnodon* and *Myloplus schomburgkii* (Andrade et al., 2016, 2019a, b; Dary et al., 2017). Two *Myloplus* species cluster with *Colossoma* and *Piaractus*, our most obligate frugivorous taxa, with *Colossoma* being more distinctive from these other species (Figure 6a).

From the discrete character based phylomorphospace, pacus occupy an overall larger position of trait space along all of the first three dbRDA axes, where RDA axis 1 accounts for 44.8% of total variability, and axis 2 and axis 3 account for 25.5% and 13.3%, respectively for a total of 83.7%. Along the first RDA component axis is where the major separation of histological traits occurs as well, with fattier lips loading negatively and lips characterized by every other trait (mucus cell presence, folds, macrophages, etc.) load positively (Figure 6b). This is expected as pacus have more types of tissues incorporated into their lower lips than any of the piranha species (Figure 6a). Omnivorous *Pygopristis* falls along the periphery of this morphospace, along the middle of dbRDA axis 1, but strongly negative on axis 2. This places the omnivorous piranha closer to other omnivorous taxa like *Acnodon,* and further from other piranhas like *Pygocentrus* or *Serrasalmus* (Leite & Jegu, 1992; Ferreira et al., 2006). Our two planktivorous and algivorous *Metynnis* species fall along the upper margins of the morphospace (Figure 6b). Interestingly, the outlying positions of *Metynnis* and *Pygopristis*, relative to their piranha cousins, makes it so that serrasalmines have more diverse lip phenotypes than myleine and colossomatine pacus.

DISCUSSION

The hypertrophied lips of pacus are composite structures — built of alternating layers of collagen, fat, elastin, and keratin. We hypothesize that the multilayered organization of collagen fibers in pacus (Figure 6b) lends versatile functions that monopolize on the biomechanical behaviors of composite materials. *Colossoma, Piaractus,* and *Myloplus* have the largest lips and feature the most complex arrangement of soft tissues (Figures 3, 6b). Lip complexity is signified by the number of tissue types (goblet cells, macrophages, etc.), how the tissues are arranged (epidermis, hypodermis, etc.); and by the attachment of the primordial ligament to the internal structure of the lip. Another axis of variation is the organization of collagen bundles in the lip - whether they are layered, bundled, or remain amorphous.

Collagen is a robust material and its organization can maximize its strength under tension and efficiency under deformation (Fratzl, 2008). For example, the organization of long collagen bundles in the remora adhesive disc keeps the structure stable under tension, allowing strong, efficient, passive adhesion to a host (Wang et al., 2019, Cohen et al., 2020). If the layering of collagen tissues in the pacu lip is similar to what is observed in the remora disk, then when the collagen is slack, the lip is compliant and deforms around prey, forming a seal. But when stiffened, collagen turns the lip into a point of hard contact like an oral digit (Owen-Smith, 1975). This may have arisen twice in the serrasalmids, because the omnivorous piranha *Pygopristis*, has a folded and mucus-laden lip similar to that of the pacus *Myloplus* and *Piaractus.* An extension of this hypothesis to a predictive framework suggests that in fleshy lipped species from other lineages, it is worth looking for a linkage between collagen organization and the ability to grip and manipulate prey larger than the mouth.

We found no intrinsic musculature in pacu lips, despite the lip's obvious mobility during prey processing in *Myloplus, Piaractus,* and *Utiaritichys* (Supl. videos) and other recorded instances in *Piaractus* (Irish, 1987; Lomax & Brainerd, 2020). We propose that the lips are actuated indirectly by the jaw adductors *via* the primordial ligament (Figure 4). In fishes, the primordial ligament is a fibroelastic band that encircles the mouth (pharyngeal arch 1). This ligament is difficult to isolate in most adult fishes, and presumably has little functional significance (Alexander, 1967; Osse, 1969; Anker, 1974; Gosline 1986; Datovo and Vari, 2012). However, in serrasalmids the primordial ligament is distinct, albeit embedded in the primordial membrane, and inserts along the postero-medial edge of the maxilla, spanning both upper and lower jaw (Datovo and Castro, 2012; Datovo and Vari, 2013). The hypothesized function of the ligament is to keep the upper and lower jaws in tension during opening and closing. We found that in species of pacus with mobile lips, an extension of the primordial ligament attaches to the distal edge of the lip (Figure 4). Dissections show the adductor mandibulae muscles attach to this extension rendering this part of the primordial ligament a *de facto* tendon. Activation of the adductor complex during feeding would pull on the primordial ligament, tensioning the proximal lip, allowing it to deform around food, and increasing the surface area in contact between lip and prey. We propose that in characiform fishes, the morphology of the primordial ligament has been selected for different functional roles depending on the size, shape, and surface texture of the prey.

Actuated lip control is not seen in all pacu species we surveyed, *Metynnis maculatus* and *Myleus setiger* for example, have no ligamentous connection – their lips are truly passive. This does not suggest they are without function, but rather that the function may lie in defense rather than manipulation. Plants invest in myriad strategies for reducing or discouraging grazing by a diverse cast of herbivores: structural or mechanical deterrents like spines or druse, digestion-inhibiting chemicals, toxic cocktails, and by limiting nutritional content to above-ground foliage (Belovsky & Schmitz, 1994; Hanley et al., 2007). Many herbivores must deal with the short-term issue of poison, and the long-term effects of plant material that can slice, stab, abrade, and dissolve oral tissues. The abrasion-resistant nature of keratinized epithelium and stiff lips suggest that pacus like *Mylesinus* and *Tometes,* which ingest leaves and stems, are adapted to structural plant defenses such as sclerophylly (Lucas et al., 2000; Hanley et al., 2007; Figure 6). These lip structures offset the mechanical wear incurred by tough, hard, or rough plant materials, and are correlated with dental adaptations like high-crowned teeth in grazing pacus (Huie et al., 2019).

The unique tooth replacement strategy in pacus and piranhas, whereby all the teeth on one side of the head are replaced all at once by a fully formed dental battery growing in the jaws (Berkovitz, 1975; Kolmann et al., 2019), seem advantageous given the high wear rates pacu dentitions incur (Shellis & Berkovitz, 1976; Irish, 1987). The variety of plant mechanical defenses offers an explanation for the diversity of tissue composition in grazing pacus. These fishes may be capitalizing on plants with defenses or different plant anatomies with their own defensive peculiarities, not unlike what is seen in reef fishes that tackle stinging prey (Hanley et al., 2007; Huertas & Bellwood, 2017; Figure 6).

The importance of soft tissues for prey handling cannot be overstated – without a tongue to reposition the bolus, mastication would not be nearly as efficient for mammals (Hiiemae & Palmer, 2003; Gintof et al., 2010; Olson et al., 2021). Pacu lips participate in both prey capture and prey processing, by performing the following: (1) a scooping function, where the lip lifts prey from the substrate, (2) repositioning prey, perhaps to orient food to take advantage of localized prey material properties (i.e., crack propagation), and (3) by gripping prey while the fish is on the move (Figure 4a-c, Supl. video). Perhaps this interaction of ductile lip opposite hard dentition appears optimal for gripping slippery objects (Barlow & Munsey, 1976); contrast this scenario to one where a steel pincer is used to seize soap, or where foam digits are used to grasp a heavy object. Where ductile meets rigid is perhaps where the most dexterous digit is demonstrated. Pacus gnaw at prey, peeling away an indigestible outer coating, like removing the skin of an orange. It would be instructive to learn whether the intraoral processing we observed in pacus involves the two lingual symphyseal teeth located on the dentary, directly behind the primary lower jaw dentition (Kolmann et al., 2019). Are they performing some manner of prey husking, without forelimbs or tongue to aid in food stabilization (Figure 2).

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FIGURES

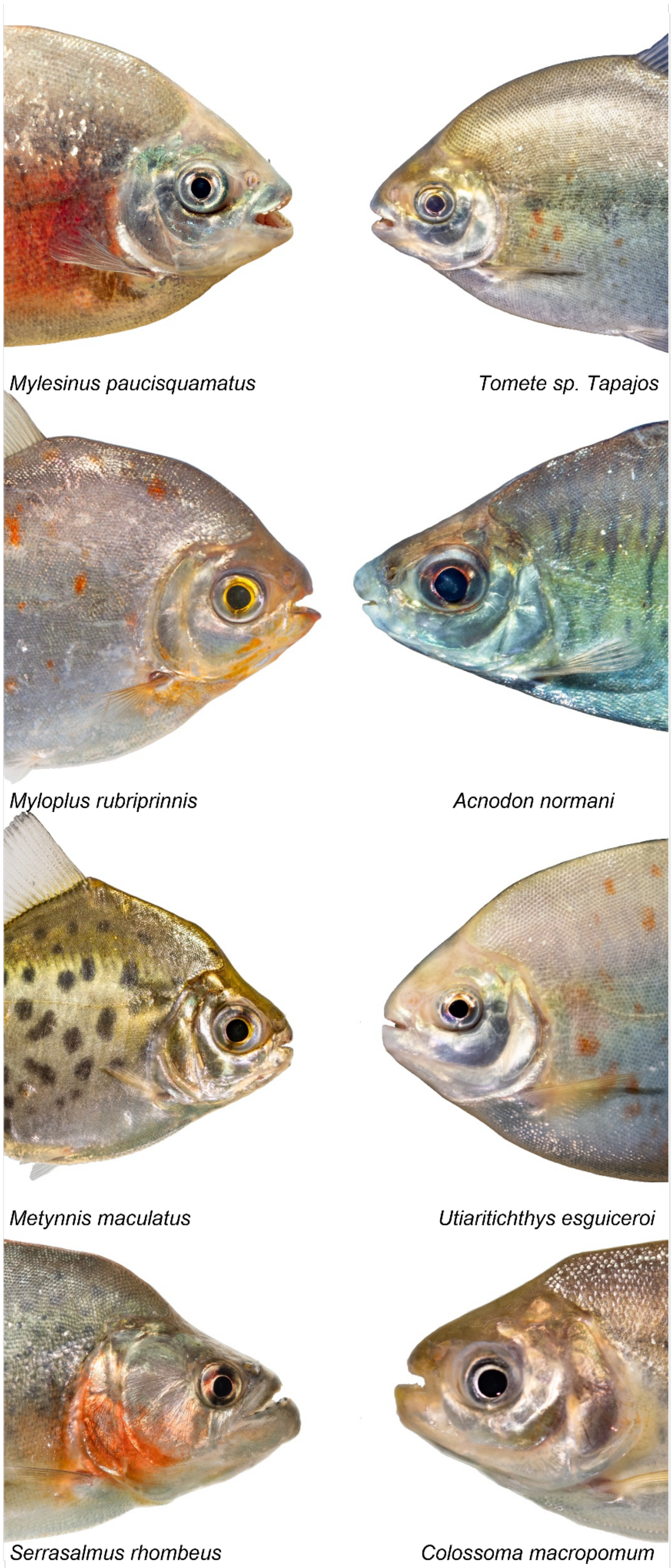

Mylesinus paucisquamatus
Tomete sp. Tapajos
Myloplus rubripinnis
Acnodon normani
Metynnis maculatus
Utiaritichthys esguiceroi
Serrasalmus rhombeus
Colossoma macropomum

Figure 1. Live photos of pacus and piranhas (lower left; *Serrasalmus*) showing the diversity of facial phenotypes. Photos by O. Lucanus.

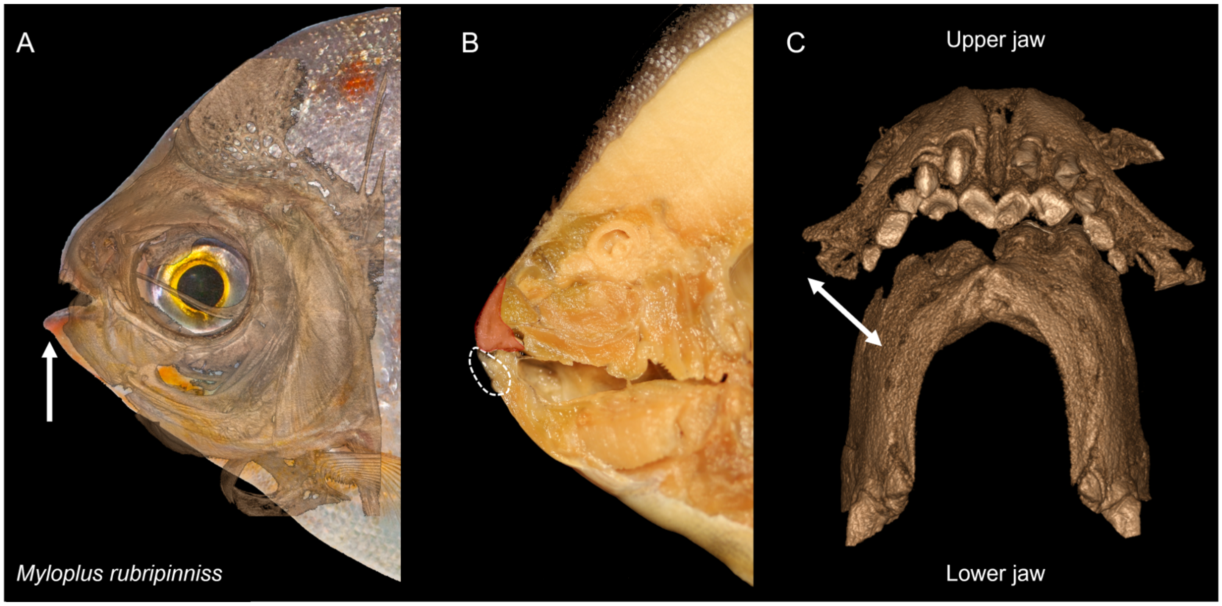
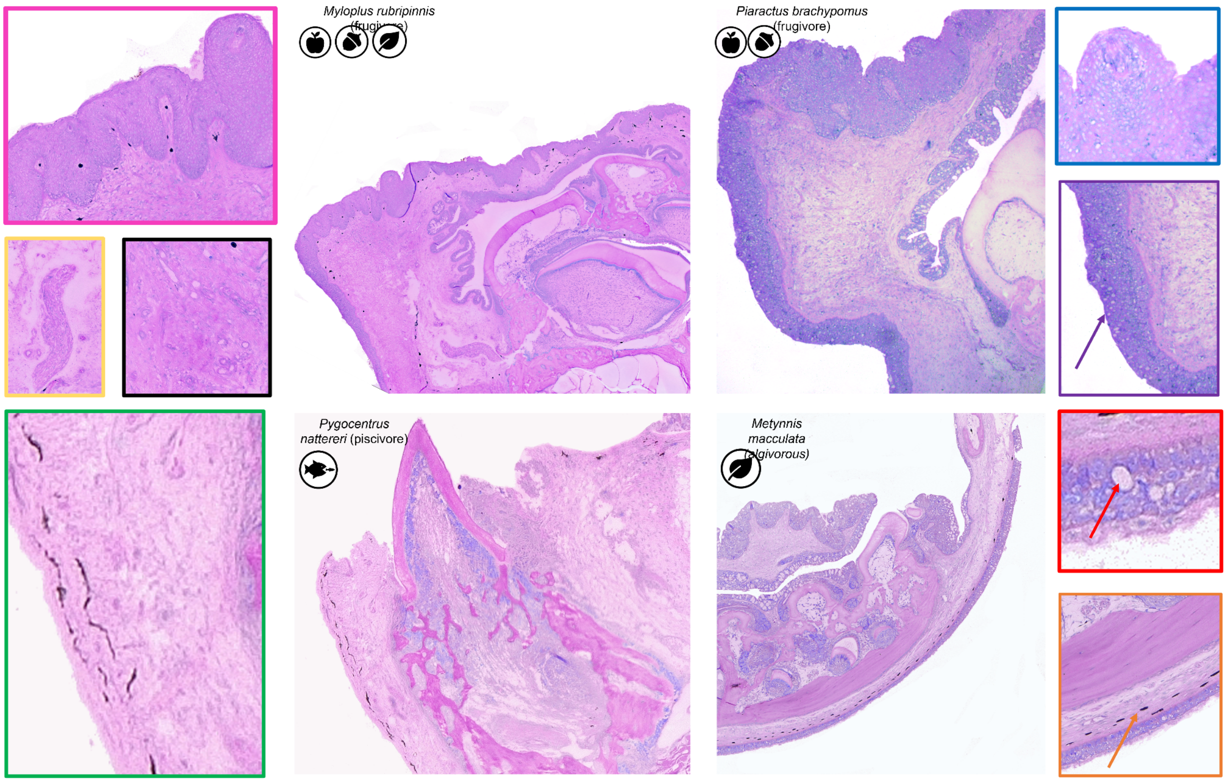


Figure 2. Hypertrophied lip and overbite. (A) Live photo of *Myloplus rubripinnis* with uCT of skull overlaid, arrow points to hypertrophied lip. (B) Sagittal section through middle of specimen showing the relationship of the jaw, red overlay identified the upper jaw, white dashed line encompasses soft tissue (hypertrophied lip) that fill the space between the upper and lower jaws. (C) Ventral view of the jaws clearly demonstrating the extent that the upper and lower jaws are displaced (double sided arrow). Note asymmetrical replacement pattern of teeth (reader's left).



	Keratin	Dense connective tissue	Loose connective tissue	Goblet cells	Tastebuds	Nerves	Folded epithelium	Blood vessels
Myloplus	X	X	X		X	X	X	X
Piaractus	X	X	X		X	X	X	X
Metynnis	X	X	X	X	X	X		X
Pygocentrus	X		X					

Fig. 3. Histological overview of the lower lips in pacus and piranhas. Sagittal sections through the lips of *Myloplus, Piaractus, and Pygocentrus* and a coronal section through the lip of *Metynnis.* Each species represents a diet guild examined in this study, small boxes showcase morphology of histological traits used in phylo-histospace. Full presence/absence chart can be found in the supplements.

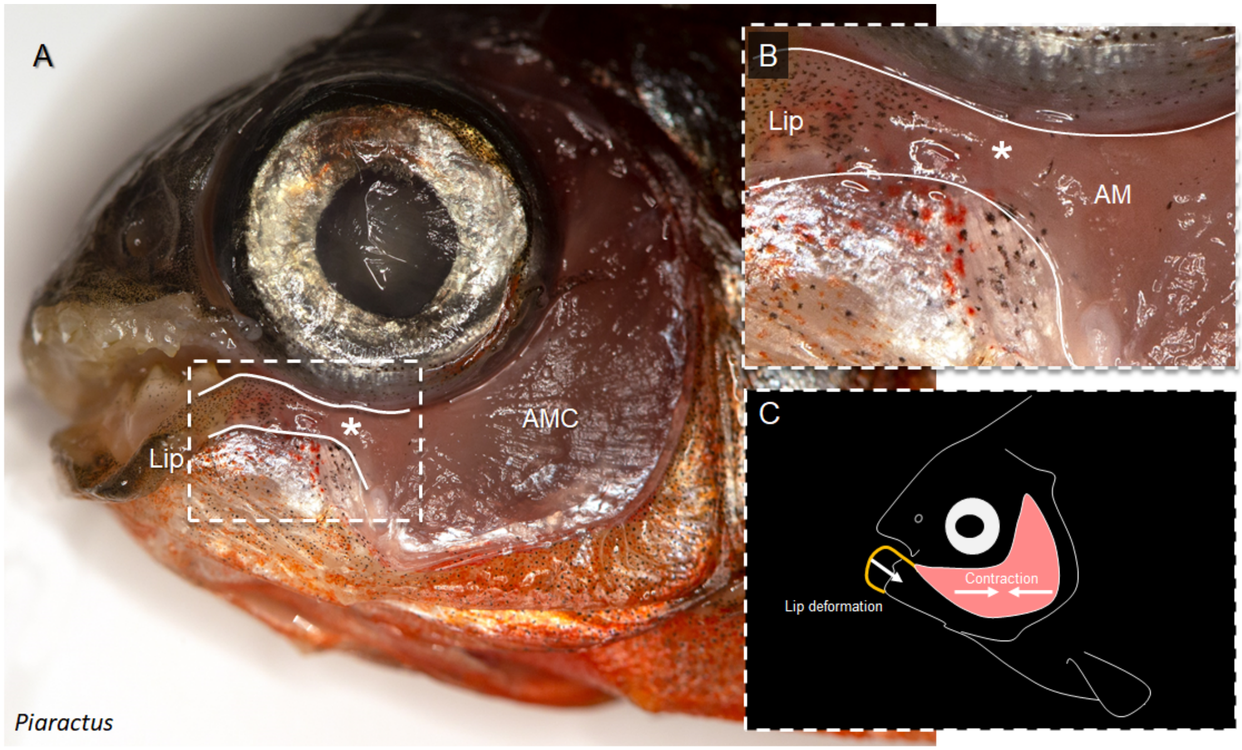


Fig. 4 - Primordial ligament attachment to hypertrophied lip in dissected *Piaractus*. Primordial ligament denoted by (*). Inset shows the dashed portion of the anatomy magnified. The main body of the ligament which originates from the AM and attaches the upper and lower jaws along the maxilla has been dissected away. Sharp tip probe used to pin down the lip has been digitally removed. AM: adductor mandibulae, AM_C adductor mandibulae complex.

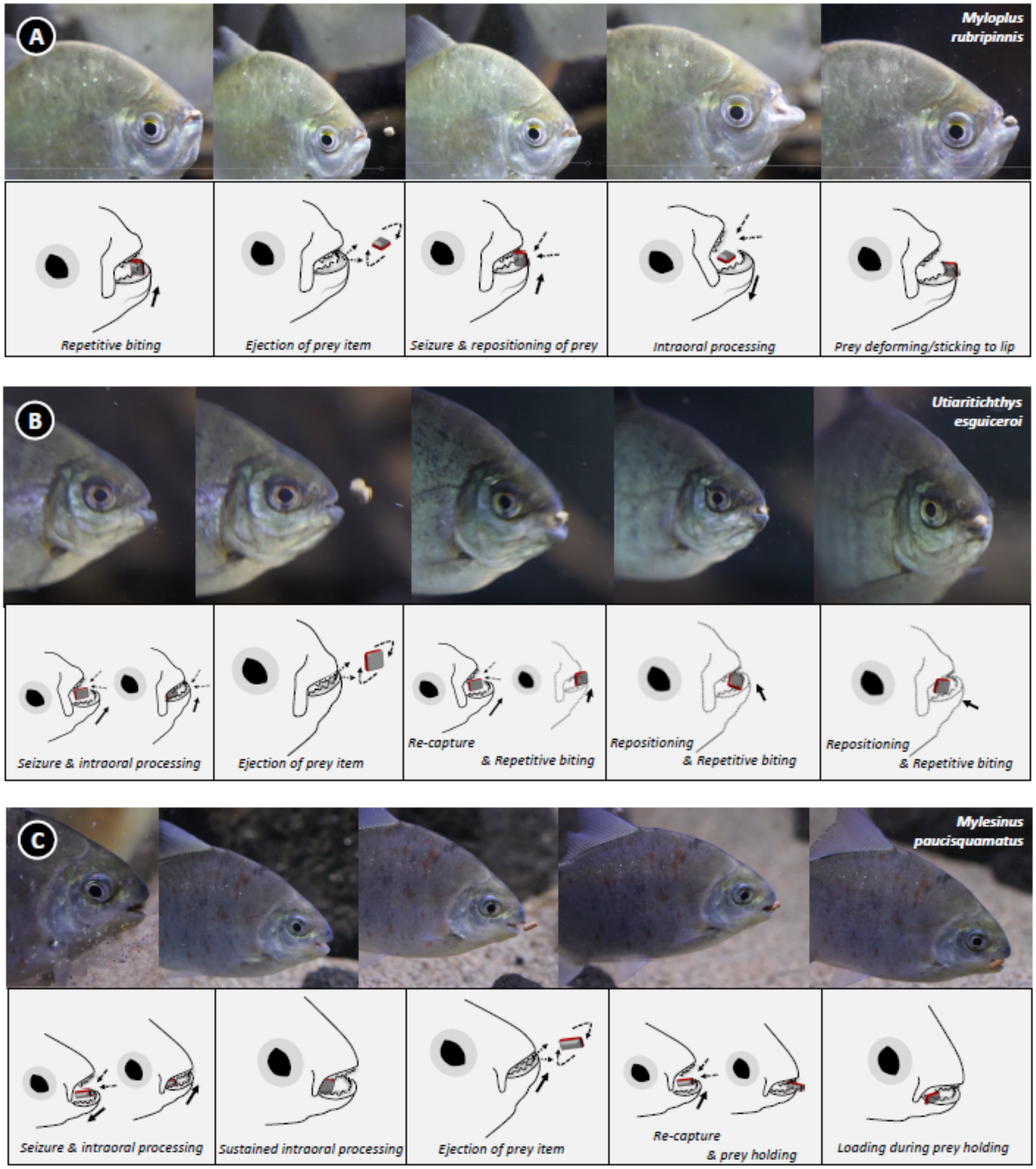
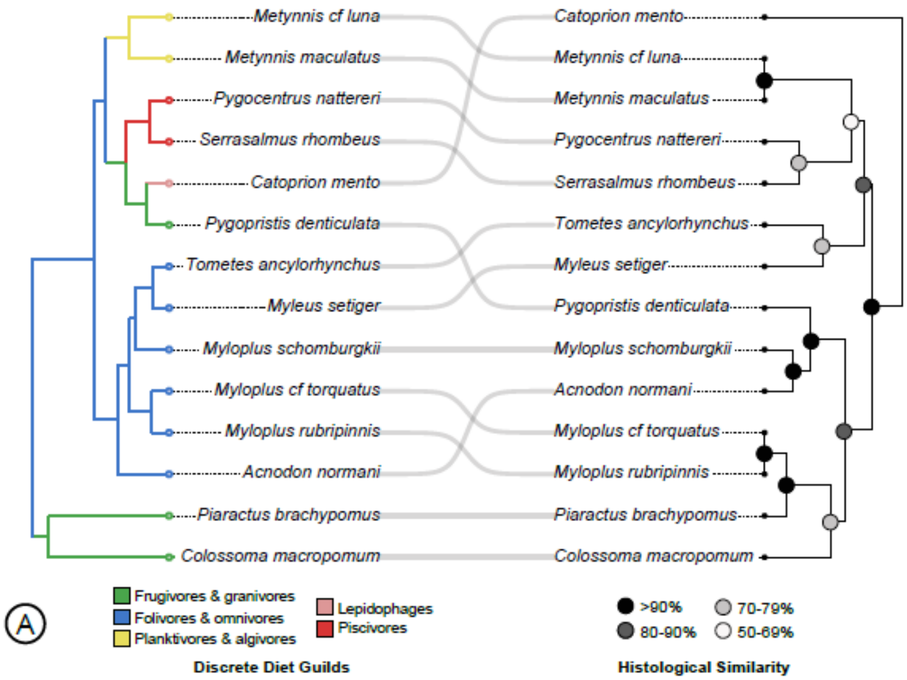


Fig. 5 - Pacu feeding behaviors using the lower lip. Still-frame images and simple schematics contrast how pacus with large, complex lips (A, *Myloplus*; B, *Utiaritichthys*) vary in the behavioral retinue relative to small-lipped species like (C) *Mylesinus*. All species used some

suction to gain initial purchase on prey (aquarium food wafers). Pacus then use their lips to stabilize food, reposition food prior to biting, and to lift food from the substrate ('scoop').



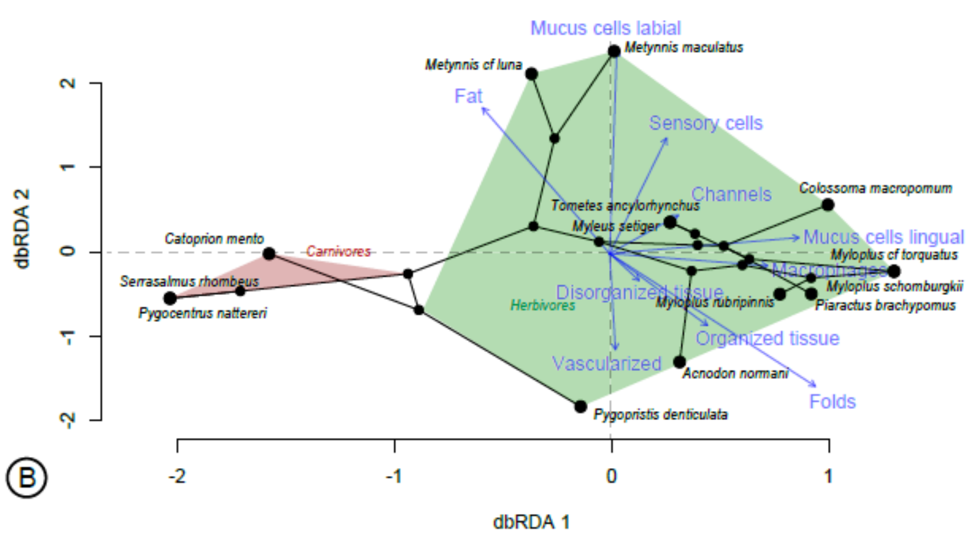


Fig. 6 - Evolutionary ecology of pacu & piranha lips. (A) Co-phylogenetic network showing correspondence between diet, phylogeny, and histological trait clustering. Diet guilds mapped onto phylogeny (left) from Kolmann et al., (2020); histological similarity among lips according to presence/absence of characters (right). Percentages reported on the right hand cluster algorithm are the AU (approximately unbiased) p-values for each node. (B) distance-based redundancy analysis (dbRDA) exploring the presence/absence of prey items and histological traits among serrasalmids. Note how more complex lips, with more histological characters, cluster on the lower right of the histo-space.

TABLES

			Histology functional units					
Hypotheses:	Function	Analogy	keratin	nerves	muscle	collagen	mucosa	immuno-
passive lip kinesis	protection from wear	callus	X			X	X	X
	better grip	friction ridges	X	X		X	X	
active lip kinesis	sensory feedback	vibrissae	X	X		X		
	prey manipulation	trunk, tongue	X	X	X	X	X	

Table 1. Hypothesis of pacu lip function based on histological composition

Species	**Clade**	**Pacu or Piranha**	**Discrete Diet Guild**	**Flesh**	**Fins & Scales**	**Arthro-pods**	**Fruits & Seeds**	**Leaves & Flowers**	**Plankton**	**Algae**	**Diet Reference**

Piaractus brachypomus	Colossomatinae	pacu	Frugivore-Granivore	0	0	1	1	1	0	1	Honda, 1974; Goulding, 1980
Colossoma macropomum	Colossomatinae	pacu	Frugivore-Granivore	0	0	1	1	1	1	1	Goulding, 1980; Goulding & Carvalho, 1982; Lucas, 2008
Acnodon normani	Myleinae	pacu	Folivore-Omnivore	0	1	1	1	1	0	0	Leite & Jégu 1990; Andrade et al., 2018, 2019
Myloplus schomburgkii	Myleinae	pacu	Folivore-Omnivore	0	0	1	1	1	0	0	Nico, 1991; Dary et al., 2017
Myloplus rubripinnis	Myleinae	pacu	Folivore-Omnivore	0	0	1	1	1	0	0	Gonzalez & Vispo, 2002; Andrade et al., 2019
Myloplus torquatus	Myleinae	pacu	Folivore-Omnivore	0	0	1	1	1	0	1	Nico, 1991; Pereira et al., 2007; Dary et al., 2017
Tometes ancylorhynchus	Myleinae	pacu	Folivore-Omnivore	0	0	0	0	1	0	1	Andrade et al., 2016, 2018, 2019
Myleus setiger	Myleinae	pacu	Folivore-Omnivore	0	1	1	1	1	0	0	Dary et al., 2017; Andrade et al., 2018
Metynnis luna	Serrasalminae	pacu	Planktivore-Algivore	0	0	0	0	0	1	0	Ramos et al., 2018; Andrade et al., 2018, 2019
Metynnis maculatus	Serrasalminae	pacu	Planktivore-Algivore	0	0	1	0	0	1	1	Pelicice & Agostinho, 2006; Silva-Camacho et al., 2014
Catoprion mento	Serrasalminae	piranha	Lepidophage	0	1	0	0	1	0	0	Vieira & Gery, 1979; Nico & Taphorn, 1988
Serrasalmus	Serrasalminae	piranha	Piscivore	1	1	0	0	0	0	0	Nico & Taphorn, 1988; Winemiller, 1989; Merona et al, 2001

rhombeus											
Pygocentrus nattereri	Serrasalminae	piranha	Piscivore	1	1	1	0	0	0	0	Nico & Taphorn, 1988; Winemiller, 1989
Pygopristis denticulata	Serrasalminae	piranha	Frugivore-Granivore	0	1	1	1	1	0	0	Nico, 1991

discrete diet guild data from categories outlined in Kolmann et al., 2020

Table 2. Species list noting clade, diet classification, and diet reference.

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Supplementary Data for:

Supplementary Methods:

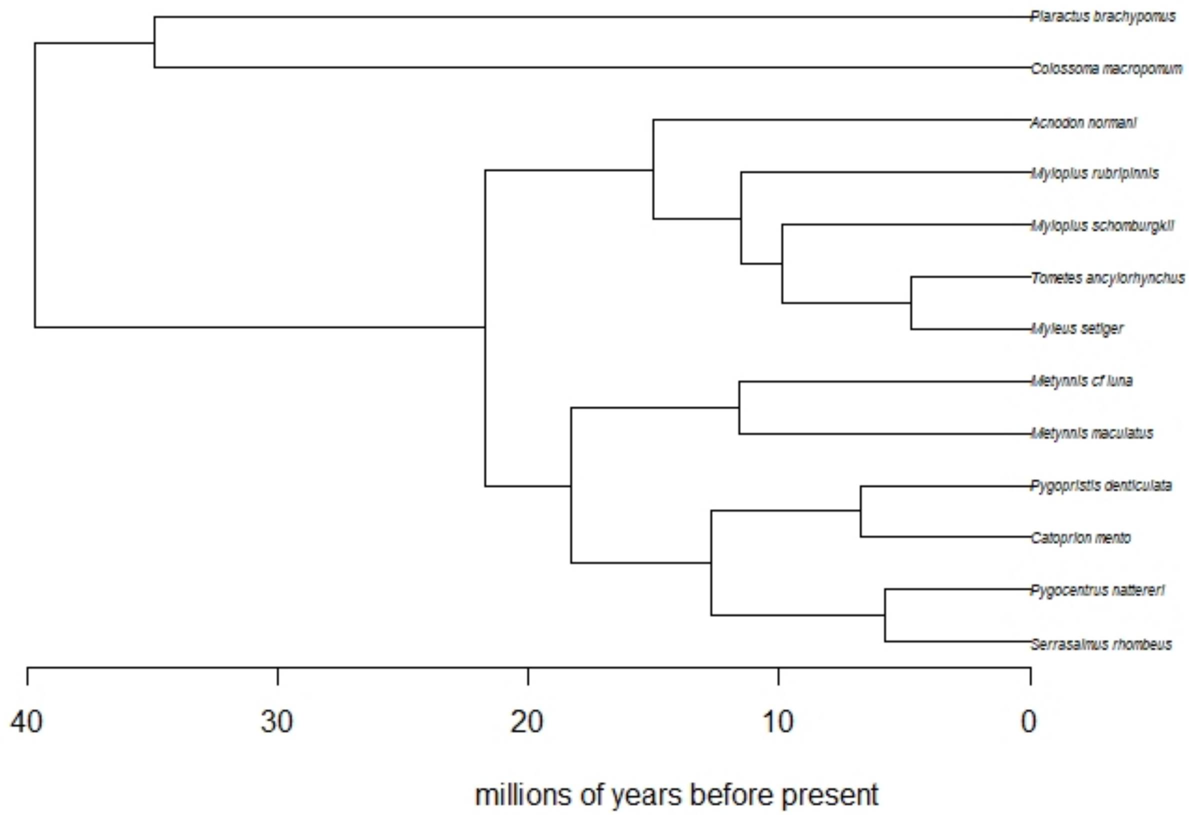
We dissected museum specimens to observe lip, muscle, and tendon morphology, with particular attention paid to the primordial ligament and its association with the adductor mandibulae muscle series (adductor mandibulae division 1 = AM_1, adductor mandibulae division 2 = AM_2, etc.; Alexander, 1964; Datovo & Castro, 2012). The primordial ligament aids in adduction of the lower jaw in association with the adductor mandibulae complex (Alexander, 1964). Specimens were dissected on both sides, the infraorbital bones and opercular series removed to observe the underlying muscle tissue. Photographs were made with a handheld cellular camera phone for later reference and tracing.

We also used contrast-enhanced micro-computed tomographic (microCT) imaging to visualize skeletal morphology in pacus and piranhas. Specimens were imaged using Friday Harbor Lab's Bruker 1173 Skyscan (Bruker Corp, Billerica, MA). Specimens were imaged at 65kV and 123uA with a 1.0 mm aluminum filter. In preparation for CT imaging, specimens were either wrapped in ethanol-moistened cheesecloth or slightly hydrated with 70% ethanol and heat-sealed within a plastic bag, then placed in a plastic tube, and stabilized with foam. Jaw anatomy was then visualized using volume rendering in the program 3DSlicer (www.slicer.org), following the Buser et al. (2020) CT segmentation and visualization work-flow.

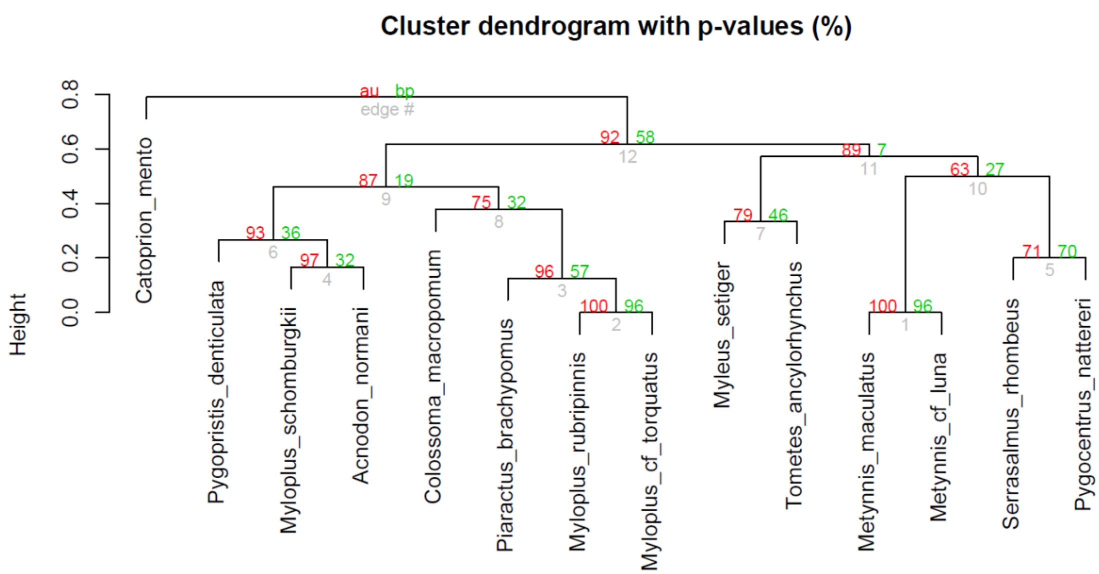
Appendix 1: Table of specimen sizes and histological methodology

Specimen	Size (SL, mm)	Museum #	Section Thickness (um)	Stain
Piaractus brachypomus	37.8	UMMZ tbd	3.5	Lee's Basic Fuchsin and Methylene Blue
Colossoma macropomum		UMMZ tbd	3.5	Lee's Basic Fuchsin and Methylene Blue
Acnodon normani	108.3	ROM tbd	3.0	Lee's Basic Fuchsin and Methylene Blue
Myloplus schomburgkii	47.2	ROM tbd	3.0	Lee's Basic Fuchsin and Methylene Blue
Tometes ancylorhynchus	91.0	ROM tbd	3.5	Lee's Basic Fuchsin and Methylene Blue
Myleus setiger	96.8	ROM tbd	3.5	Lee's Basic Fuchsin and Methylene Blue
Myloplus rubripinnis	86.7	ROM tbd	3.5	Lee's Basic Fuchsin and Methylene Blue
Myloplus cf torquatus	67	ROM tbd		Lee's Basic Fuchsin and Methylene Blue
Metynnis maculatus	60	ROM tbd	3.0	Lee's Basic Fuchsin and Methylene Blue
Metynnis cf luna	64.8	ROM tbd	3.5	Lee's Basic Fuchsin and Methylene Blue
Catoprion mento		UMMZ tbd	3.0	Lee's Basic Fuchsin and Methylene Blue
Pygopristis denticulata		UMMZ tbd	3.5	Lee's Basic Fuchsin and Methylene Blue
Pygocentrus nattereri	32.0	ROM tbd	3.5	Lee's Basic Fuchsin and Methylene Blue
Serrasalmus rhombeus		UMMZ tbd	3.5	Lee's Basic Fuchsin and Methylene Blue

Appendix 2: Trimmed phylogeny used for Comparative Methods (From Kolmann et al., 2020)



Appendix 3: Histological similarity clustering based on UPGMA estimation
(red values are approximately unbiased (AU) p-values and while green are bootstrap probability (BP) values, where AU is preferred)



Appendix 4: Diet Data References

CONCLUSION

Odontodes are a synapomorphy of vertebrates that includes teeth, but also the denticles and spines of sharks, the armor plates of catfishes, and gar, and the toothy knob on the cranium of male chimeras (Berio and Debiais-Thibaud, 2021; Debiais-Thiabaud *et al.,* 2011; Donoghue, 2021; Donoghue & Rucklin, 2016; Fraser *et al.,* 2009; Hulsey *et al.,* 2020). Odontodes are simply described as any mineralized structure consisting of dentine found outside the body or inside the mouth (Fraser *et al.,* 2009, Fraser *et al.,* 2010). They are sometimes capped with enamel and are highly variable in form. This variation in capping material and shape have been particularly informative in interpreting the fossil record. Teeth are a special kind of odontode: they are mineralized structures always made of dentine, usually capped with enamel, and restricted to the mouth.

All odontodes and teeth develop from the coordination of the same gene regulatory network and only differ in the punctuated expression of those genes (Fraser *et al.,* 2009, Fraser *et al.,* 2010, Ellis *et al.,* 2015, Jernvall & Thesleff, 2012). To build an odontode you first need a placode - a collection of embryonic tissues that act as the signaling center. The placode forms as a thickening of epithelium stimulated by mesenchymal and cranial neural crest cells (Huysseune & Sire 1998; Ohazama *et al.,* 2010; Smith & coates, 1998). Despite a shared and incredibly conserved genetic toolbox deployed for the initiation and development of odontes, denticles and teeth there is substantial diversity of shape, natural history and function.

Tooth shape, function, development, and material properties have been extremely well studied across vertebrate lineages (Crofts & Summers, 2014; Crofts *et al.,* 2020; Anderson & LaBarbera, 2008; Evans & Sanson, 1998; Freeman & Lemman, 2007; Smits & Evans 2012; Crofts *et al.,* 2017; Corn *et al.,* 2016). Less understood is replacement, displacement, and attachment of teeth and tooth like structures. Teeth, for humans and most mammals, are replaced just once, but that is not necessarily the case for odontodes in a more inclusive sense. Odontodes and teeth of polyphyodont vertebrates are replaced continuously throughout the lifetime of the animal. For teeth, this patterned replacement is encoded in the dental lamina – an odontogenic signaling center that maintains a population of stem cells (Buchtova *et al.,* 2012; Reif, 1982; Smith *et al.,* 2009). Whether teeth are built along a continuous lamina, as in sharks and snakes, or within specific signaling centers, as in alligators, salmon, and lizards, this population of cells holds the temporal and spatial architecture for dental replacement (Huysseune *et al.,* 2012; Tsai *et al.,* 2016; Wu *et al.,* 2013). In some cases, the dental lamina can even determine a new tooth shape, replacement pattern, or instructions for returning to a previous morphology. Such examples can be found in the seasonal teeth of the Atlantic stingray that change during mating season or the juvenile teeth of puffer fish that are completely lost to make way for a fused beak (Fraser *et al.,* 2012; Kajiura & Tricas, 1996). How replacement is initiated or maintained without a clear boundary or stem cell population remains an open question.

The denticulated skin of sharks has shaped dental hypotheses for decades and yet, we have no good sense if and how these outer teeth are replaced. We can reasonably assume that any maintenance or replacement is likely controlled by the same candidate genes governing the oral teeth and other odontodes. Recent work has shown that denticle development happens along zones of inhibition (Cooper *et al.,* 2018; Smith, 2003; Fraser & Smith, 2010). These areas demarcate different morphologies and patterns crucial to the performance of these fields of teeth. Importantly, sharks are not the only species with odontodes, in fact the re-emergence of teeth outside the mouth in teleosts has become more profound over the past decade (Rivera-Rivera, 2021). It may well be that like sharks, bony fishes are too still covered in teeth and understanding how odontodes are replaced without a set location of stem cells could provide greater insight into why some organisms lose the ability for continuous replacement. .

There are obvious advantages to a polyphyodont dentition. Continuous replacement allows easy maintenance of a sharp edge and the flexibility or plasticity for changes that fit environmental or developmental needs. On the contrary, diphyodont dentitions – those that replace only once – have obvious downsides of permanent wear or damage. In return for a shorter lifespan diphyodont teeth may be bigger, able to transmit large forces, or have more complex topography for the efficient breakdown of prey. Historically, polyphyodont dentitions have been considered metabolically expensive, however, this is an unlikely selective pressure against continuous replacement. Sharks and cartilaginous fishes have been losing their teeth for almost 500 million years and with one of the lowest metabolic rates among vertebrates. It is unlikely that these animals would be as successful if every day they were throwing away a solid percentage of their metabolic investment. Even more recently we have an understanding at the cellular level of tooth initiations – it does not take many cells to build a tooth, just two (Larionova *et al.,* 2022). There is also evidence that in primates' restriction to food does not affect tooth growth or size. So why do we lose our edge? In searching for plausible selective pressures against polyphyodonty I hypothesize that the evolution of diphyodont dentitions is driven by constructional constraints of the jaws and that constant remodeling required of polyphyodont dentition cannot be maintained given the forces and stresses produced by chewing.

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