




RESEARCH ARTICLE

Lip service: Histological phenotypes correlate with diet and feeding ecology in herbivorous pacus

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Abstract

Complex prey processing requires the repositioning of food between the teeth, as modulated by a soft tissue appendage like a tongue or lips. In this study, we trace the evolution of lips and ligaments, which are used during prey capture and prey processing in an herbivorous group of fishes. Pacus (Serrasalminae) are Neotropical freshwater fishes that feed on leaves, fruits, and seeds. These prey are hard or tough, require high forces to fracture, contain abrasive or caustic elements, or deform considerably before failure. Pacus are gape-limited and do not have the pharyngeal jaws many bony fishes use to dismantle and/or transport prey. Despite their gape limitation, pacus feed on prey larger than their mouths, relying on robust teeth and a hypertrophied lower lip for manipulation and breakdown of food. We used histology to compare the lip morphology across 14 species of pacus and piranhas to better understand this soft tissue. We found that frugivorous pacus have larger, more complex lips which are 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, we found a novel connection to the primordial ligament which suggests that the lips are actuated by the jaw adductors. We propose that pacus combine hydraulic repositioning of prey inside the buccal cavity with direct oral manipulation, the latter using a combination of a morphologically heterodont dentition and compliant lips for reorienting food.

KEYWORDS

chewing, histology, prey manipulation, proboscis, tongues

1 | 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; Lumsden & Osborn, 1977; Ross et al., 2007). 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; Sasko et al., 2006; Ross et al., 2007; Kolmann et al., 2016; Schwarz et al., 2020). 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, soft tissue oral appendages have evolved many times (Lumsden & Osborn, 1977; Peterson et al., 2022; Shoshani, 1998).

Prominent lips have evolved in fish lineages like the astroblepids (climbing catfish), catostomids (suckers), gyrinocheilids (algae eaters), balitorids (hillstream loaches), prochilodontids (flannel-mouthed characins), curimatids (toothless characins), oxudercids (gobies), cichlids, and serrasalmids (pacus and piranhas) have independently evolved prominent lips (Lujan & Armbruster, 2012; Schaefer & Lauder, 1986; De Meyer & Geerinckx, 2014; Maie et al., 2011; Geerinckx et al., 2007; Sazima, 1986; Machado-Schiaffino et al., 2014; Tripathi & Mittal, 2010; Correa et al., 2007). Fish lips vary considerably with respect to their function and form. For instance, tubelip wrasses (*Labrichthys*) have robust, mucus-secreting lips that aid them in sucking polyps from coral and protect them from their stinging nematocysts (Huertas & Bellwood, 2017). In other fishes, such as the waterfall-climbing gobies, modified lips (alongside a pelvic suction disk) are used to scale slippery waterfalls on Pacific islands (Christy & Maie, 2019; Cullen et al., 2013). Lips in fishes are useful in a life without limbs—aiding in the manipulation of food or surrounding substrate. Such oral appendages are used to grip and gather food, but few fishes have the oral dexterity evident in tetrapods. There are, however, some that target prey that is hard, buoyant, and often protected by an inedible shell—in these instances, a deft oral appendage facilitates prey breakdown.

Pacus (Serrasalminae) are keystone riparian herbivore and seed dispersers that crush fruits and transport their seeds throughout the Amazon basin (Carvalho et al., 2021; Correa et al., 2007; Correa et al., 2015; Correa & Winemiller, 2014; Goulding, 1980; Goulding & Carvalho, 1982; Prudente et al., 2016). These fishes do not engulf prey whole but instead, reduce it through brute-force biting, excising small edible pieces each time the food item is repositioned (Alexander, 1967; Irish, 1987; Grubich et al., 2012). This feeding behavior emerges 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. Feeding on prey that is large and hard, tough and noxious, smooth and slippery, pacu lips must work in concert with the dental battery to effectively reposition and peel away at indigestible prey; like removing the skin of an orange. Under the skin and muscles, pacus have a robust, multicuspid dentition and a noticeable overbite which is opposed by an enlarged lower lip (Figure 1a–e; Irish, 1987; Kolmann et al., 2019; Shellis & Berkovitz, 1976).

Considering the role soft tissue appendages play in mammalian lineages, we can make two intuitive hypotheses of how pacu lips may function (Table 1): the lips

may act as an active participant in prey manipulation, as in rhinos where extensions of the upper lip aid in foraging and positioning (Kier & Smith, 1985; Leuthold, 1977; Owen-Smith, 1975). 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 (Aldous, 1941; Vander Wall et al., 1997). Passive lips might serve to trap the surface of vegetation, or they could protect the teeth from the gamut of plant defenses like phytoliths, and act 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, the muscle will be present (Kier & Smith, 1985; Witmer et al., 1999).

The well-resolved serrasalminid phylogeny that includes both the herbivorous pacus and the more carnivorous piranhas makes it possible to trace the evolutionary relationship between soft tissue characters and diet (Kolmann et al., 2021; Betancur et al., 2019; Kolmann et al., 2019). Some pacus specialize in 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; Witmer et al., 1999; Feilich et al., 2021). We also expect even greater variation in lip morphology between pacus and piranhas, except perhaps, for those piranha species that feed on plant materials (i.e., *Pygopristis*; Berkovitz, 1975; 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 (Figure 2). We might expect more dexterous lips in species for which prey handling is important, or more abrasion-resistant lips in species grazing on caustic or abrasive plant materials.

Using histology, CT scanning, and phylogenetic comparative methods we explore the evolution and morphology of lip phenotypes across pacus. Our goals are three-fold: (1) describe lip morphology at the tissue/cellular level among pacus and piranhas; (2) make functional predictions (i.e., active or passive control) of lips based on histology; and (3) map prey, dietary guild, and morphology on the serrasalminid phylogeny. Lips may provide herbivorous pacus and omnivorous piranhas with the soft tissue tools needed for feeding on diverse prey with relatively simple skulls. 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).

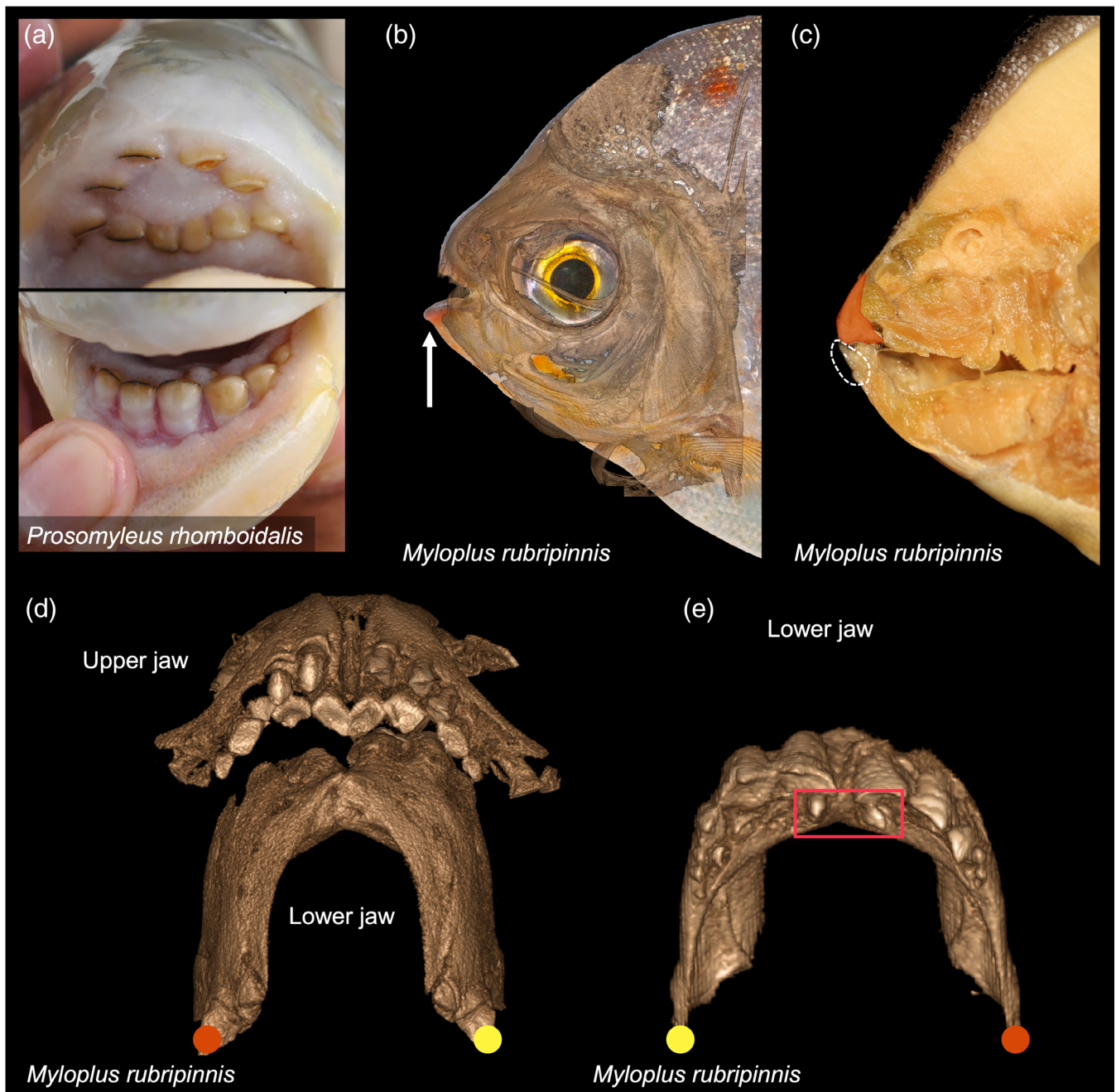


FIGURE 1 Overview of pacu hypertrophied lip. (a) Live photo of *Prosomyelus rhomboidalis* (adapted from Huie et al., 2019) showing robust lip morphology. (b) Live photo of *Myloplus rubripinnis* with ovalid uCT of the skull, arrow points to hypertrophied lip. (c) Sagittal section through middle of specimen showing the relationship of the jaws, teeth, and lip. Red overlay highlights the upper jaw, white dashed line encompasses hypertrophied lip. (d) uCT of the upper and lower jaws to better visualize the overlap of upper jaw teeth when no lip is present. Note asymmetrical replacement pattern of teeth (reader's left). (e) Dorsal view of lower jaws showcasing paired symphyseal teeth (red box)

2 | METHODS

2.1 | Specimen acquisition and dissection

This study includes 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 (Table 2; Correa et al., 2007; Kolmann et al., 2021). Specimens used for dissection, histology, and CT imaging were formalin-fixed, preserved in 70% ethanol, and are deposited at the University of Michigan Museum of Zoology (UMMZ; Ann Arbor, MI) and the Royal Ontario Museum (ROM; Toronto, ON)

TABLE 1 Hypothesis of pacu lip function based on histological composition

Hypotheses:	Function	Analogy	Histo-functional units					
			Keratin	Nerves	Muscle	Collagen	Mucosa	Immuno-type cells
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	

ichthyology collections (Table 3). 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 complex (adductor mandibulae division 1 = AM₁, adductor mandibulae division 2 = AM₂, etc.; Alexander, 1964; Datovo & Castro, 2012). Specimens were dissected on both sides, the infraorbital bones and opercular series were removed to observe the underlying muscle tissue.

2.2 | Histological sectioning & micro-computed tomographic (CT) imaging

We used histological sectioning to explore differences in tissue composition among pacu lips, as well as the upper and lower jaws. Our samples of *Pygocentrus* were frozen and then thawed prior to tissue sectioning and embedding, but we did not see tissue degradation. The lower jaw and associated soft tissues (lips, muscles, etc.) were excised from these specimens and fixed in 10% buffered formalin for 24 hr. All samples were rinsed in distilled water and decalcified in 10% EDTA following the protocol in Silva et al. (2011). After tissues were thoroughly decalcified, they were dehydrated in a stepwise series, starting from 25% ethanol. We embedded whole jaws in JB-4 embedding media (Electron Microscopy Science JB4 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 hours, and then stained with Lee's Basic Fuchsin and Methylene Blue stain and imaged using a Keyence VHX 500 microscope (Itasca, IL) and Nikon eclipse E600 compound microscope (Melville, NY). 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 micro-computed tomographic (microCT) imaging to visualize skeletal morphology in pacus and piranhas. Specimens were imaged using Friday Harbor Lab's Bruker 1,173 Skyscan (Bruker Corp, Billerica, MA).

Specimens were imaged at 65 kV and 123 μA 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.

We used a combination of gross dissection and diaphonization to visualize and explore the muscles and ligaments surrounding the hypertrophied lips in *Myloplus* cf. *rubripinnis*, *Tometes ancylorhynchus*, *Metynnis maculatus*, and *Piaractus brachypomus*. The goal of this method was to explore if there was any association between the lips and jaw adductor muscles. *Myloplus* and *Piaractus* have some of the most pronounced lips and feed on the toughest materials while *Metynnis*, a filter-feeding pacu, have a more slender lip. Using a modified clear and stain protocol presented in (Datovo & Vari, 2014), we double-stained cartilage and bone while leaving the muscles and ligaments intact in *Piaractus*, *Metynnis*, and *Tometes*. Using blunt dissection we then explored any ligamentous and/or muscle attachment to the lips in *Piaractus*, *Metynnis*, and *Myloplus* and imaged our results with a ZEISS SterEO v20 Discovery microscope (Zeiss Oberkochen, Germany).

2.3 | Phylogenetic reconstructions and statistical methods

We were interested in assessing the level of correspondence between lip histological characters, general diet guild (e.g., frugivore, folivore, etc.) or the presence of specific prey in the diets of pacus and piranhas. We used the serrasalmid molecular phylogeny from Kolmann et al., (2021) 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;

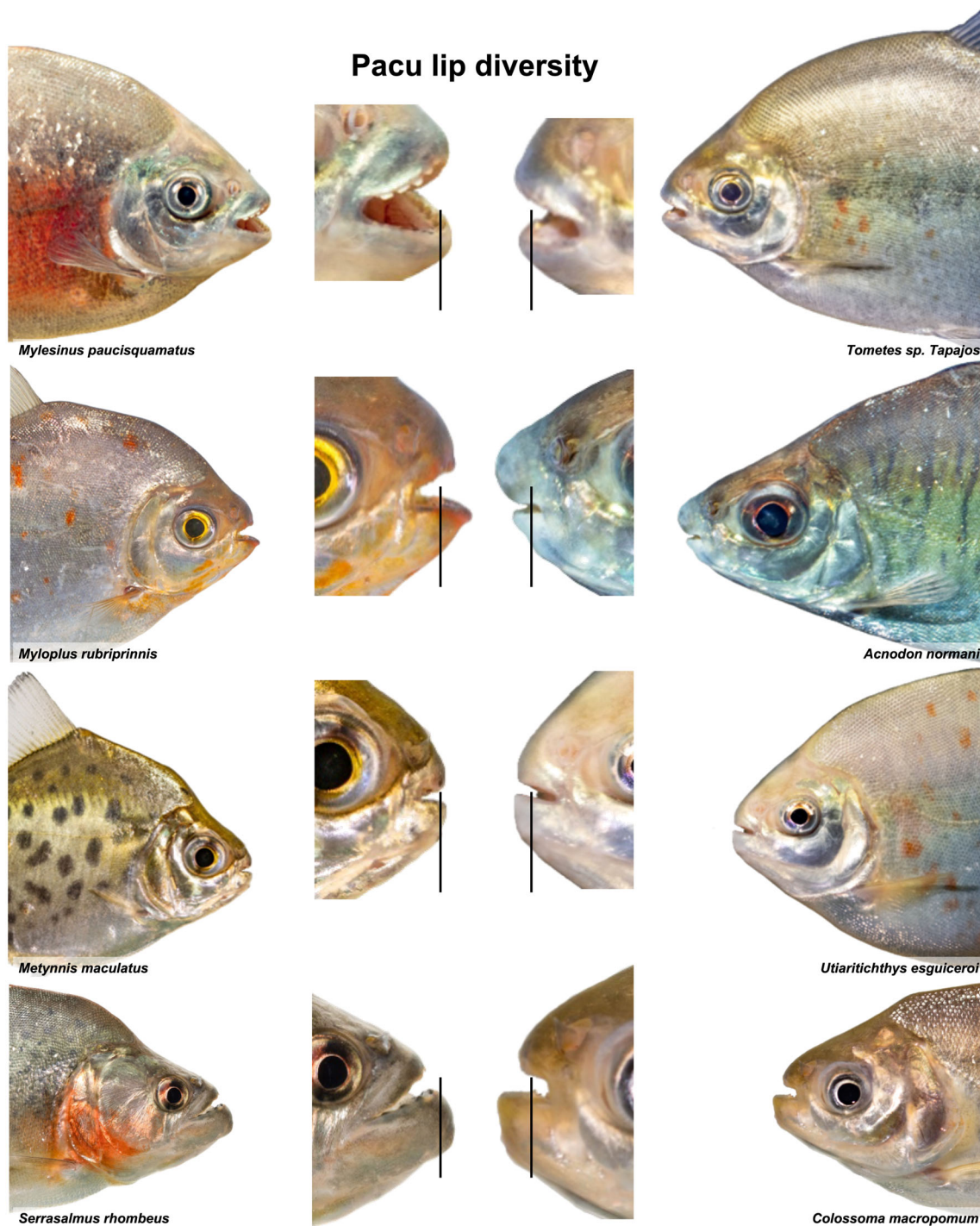


FIGURE 2 Live photos of pacu and piranhas (lower left; *Serrasalmus*) showing the diversity of facial phenotypes. Images represent the diversity of pacu and piranha facial diversity. Black line is placed at the junction between the lower jaw teeth and hypertrophied lip to give a sense of disparity across the clade. Photos by O. Lucanus

Mateussi et al., 2020). We also used the discrete diet categories assembled by Kolmann et al., (2021) to represent each species' generalized diet niche, in addition to considering the presence/absence of discrete prey items (e.g, flesh, fin rays and scales, insects, fruits and seeds, leaves and flowers, plankton, algae) 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 morphology (goblet cells, nerves, muscle, etc.), we used

TABLE 2 Species list noting clade, diet classification, and diet reference

Species	Clade	Pacu or piranha	Discrete diet guild	Flesh	Fins and Scales	Arthro-pods	Fruits and Seeds	Leaves and Flowers	Plankton	Algae	Diet references
<i>Piaractus brachyopomus</i>	Colossomatinae	pacu	Frugivore-Granivore	0	0	1	1	1	0	1	Honda, 1974; Goulding, 1980
<i>Colossoma macropomum</i>	Colossomatinae	pacu	Frugivore-Granivore	0	0	1	1	1	1	1	Goulding, 1980; Goulding & Carvalho, 1982; Lucas, 2008
<i>Acanodon normani</i>	Myleinae	pacu	Folivore-Omnivore	0	1	1	1	1	0	0	Leite & Jégu 1990; Andrade et al., 2019a, 2019b
<i>Mylopius schomburgkii</i>	Myleinae	pacu	Folivore-Omnivore	0	0	1	1	1	0	0	Nico, 1991; Dary et al., 2017
<i>Mylopius rubripinnis</i>	Myleinae	pacu	Folivore-Omnivore	0	0	1	1	1	0	0	Gonzalez & Vispo, 2003; Andrade et al., 2019b
<i>Mylopius torquatus</i>	Myleinae	pacu	Folivore-Omnivore	0	0	1	1	1	0	1	Nico, 1991; Pereira et al., 2007; Dary et al., 2017
<i>Tometes ancylothynechus</i>	Myleinae	pacu	Folivore-Omnivore	0	0	0	0	1	0	1	Andrade et al., 2016, 2019a, 2019b
<i>Myleus setiger</i>	Myleinae	pacu	Folivore-Omnivore	0	1	1	1	1	0	0	Dary et al., 2017; Andrade et al., 2019a
<i>Metynnis luna</i>	Serrasalminae	pacu	Planktivore-Algivore	0	0	0	0	0	1	0	Ramos et al., 2018; Andrade et al., 2019a, 2019b
<i>Metynnis maculatus</i>	Serrasalminae	pacu	Planktivore-Algivore	0	0	1	0	0	1	1	Pellice & Agostinho, 2006; Silva-Camacho et al., 2014
<i>Catoptrion mento</i>	Serrasalminae	piranha	Lepidophage	0	1	0	0	1	0	0	Vieira & Gery, 1979; Nico & Taphorn, 1988
<i>Serrasalmus rhombeus</i>	Serrasalminae	piranha	Piscivore	1	1	0	0	0	0	0	Nico & Taphorn, 1988; Winemiller, 1989; Mérona et al., 2001
<i>Pygocentrus nattereri</i>	Serrasalminae	piranha	Piscivore	1	1	1	0	0	0	0	Nico & Taphorn, 1988; Winemiller, 1989
<i>Pygopristis denticulata</i>	Serrasalminae	piranha	Frugivore-Granivore	0	1	1	1	1	0	0	Nico, 1991

Note: Discrete diet guild data from categories outlined in Kolmann et al., 2021.

TABLE 3 Histological traits listed by species

Species	Mucus cells lingual	Mucus cells labial	Folds	Fat	Disorganized CT tissue	Organized CT tissue	Macrophages	Heavy vascularization	Muscle	Sensory cells	Channels	Keratin
<i>Piaractus brachypomus</i>	1	0	1	0	1	1	1	0	0	1	0	1
<i>Colossoma macropomum</i>	1	0	1	1	0	1	1	0	0	1	1	1
<i>Acnodon normani</i>	1	0	1	0	1	1	0	1	0	0	0	1
<i>Mylopus setiger</i>	0	1	1	0	1	0	1	1	0	0	0	1
<i>Tometes ancylorhynchus</i>	0	1	0	0	1	0	0	1	0	0	0	1
<i>Myloptus rubripinnis</i>	1	0	1	0	1	1	1	1	0	1	0	1
<i>Myloptus cf torquatus</i>	1	0	1	0	1	1	1	1	0	1	0	1
<i>Myloptus schomburgkii</i>	1	0	1	0	1	0	0	1	0	0	0	1
<i>Metynnis cf luna</i>	1	1	0	1	1	0	0	0	0	1	0	1
<i>Metynnis maculatus</i>	1	1	0	1	1	0	0	0	0	1	0	1
<i>Catoptrion mento</i>	0	0	0	0	0	1	0	0	0	0	0	1
<i>Serrasalmus rhombeus</i>	0	0	0	1	1	0	0	1	0	1	0	1
<i>Pygocentrus nattereri</i>	0	0	0	1	1	0	0	1	0	0	0	1
<i>Pygopristis denticulata</i>	1	0	1	0	1	0	0	0	0	0	0	1

distance-based redundancy analysis (dbRDA; Legendre & Anderson, 1999). Distance-based RDA (dbRDA) submits a dissimilarity matrix to a principal coordinates analysis (PCoA). 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 might mask dietary complexity. 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 phylogenetic space for serrasalmids: a projection of each species' trait values into a two-dimensional space, connected by the branches of a phylogeny. This allows us to explore how similar sister taxa and dietary guilds are to one another in a shared morphospace. We plotted this phylogenetic space using the *phylogenetic space* 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 (diet guild). 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 *pvcust* functions (*vegan* and *pvcust* packages; Suzuki & Shimodaira, 2006). We then used the *cophylo* functions in *phytools* (Revell, 2012) to render the topological diagrams.

3 | RESULTS

3.1 | 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 3a, Table 3). In general, pacu lips are composites of collagen, putative sensory nerves, and macrophages while only some lips are covered with goblet cells. Folivorous species tend to have more mucus-producing cells embedded in the outer, epidermal layer than other herbivorous pacus like *Myloplus*, which lack goblet cells on the outer surface of the lip (Figure 3b and pink box). *Colossoma* and *Piaractus* had more densely innervated lips than all other pacus (Figure 3a, c; Table 3). All serrasalmids have keratinized lips, but folivores in particular, and other herbivores in general, have thicker layers than piranhas.

The epidermis, dermis, and hypodermis are clearly differentiated in pacus, but not in piranhas (Figure 3a–d, Table 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 3d, inset; Table 3). This histological generalization holds true for the typical carnivorous piranhas *Pygocentrus* and *Serrasalmus* (Figure 4a,b, Ferreira et al., 2014), the 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. However, the keratinized epidermis is thicker in *Pygopristis* lips than in other piranhas, and unlike *Pygocentrus*, *Serrasalmus*, or *Catoprion*, in *Pygopristis* we found an abundance of goblet cells near the medial mandibular symphysis (Figure 4 A). This region of the lip in *Pygopristis* is also conspicuously folded, with goblet cells lining the inner surface (Figure 4a). The primordial membrane of these fishes keeps the maxilla and premaxilla rigid, with little to no obvious movement (Datovo & Castro, 2012).

3.2 | Lip diversity of frugivorous, granivorous, and omnivorous pacus

All pacus discussed in this section feed primarily on nuts, seeds and/or fruits (Dary et al., 2017; González & Vispo, 2003; Nico, 1991). *Myloplus schomburgkii*, *Myloplus* cf. *torquatus*, and *Myloplus rubripinnis* all have lips with an epidermal layer formed of tightly packed cells that is also heavily folded, making the lip much thicker than those of other herbivorous pacus (Figure 3a; Table 3). The outer surfaces of the lip are also covered in keratinized epithelium. There are no mucus-producing (goblet) cells on these outer 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 inner surface of the lip, nearest to the teeth, is studded with goblet cells and is folded. In these *Myloplus* 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 lip region of these *Myloplus* species as well (Figure 3a,b).

The hypertrophied lips of fruit and seed-feeding *Piaractus* (Goulding, 1980; Honda, 1974) bear many similarities to the lips of *Myloplus* with the dermal region filled with blood vessels, nerves, and collagen (Figure 3b, pink box & Figure 3c). Unlike *Myloplus*, the dermal region adjacent to the lip epidermis is composed mainly of loose collagen fibers interspersed with elastin (Figure 3a; Table 3).

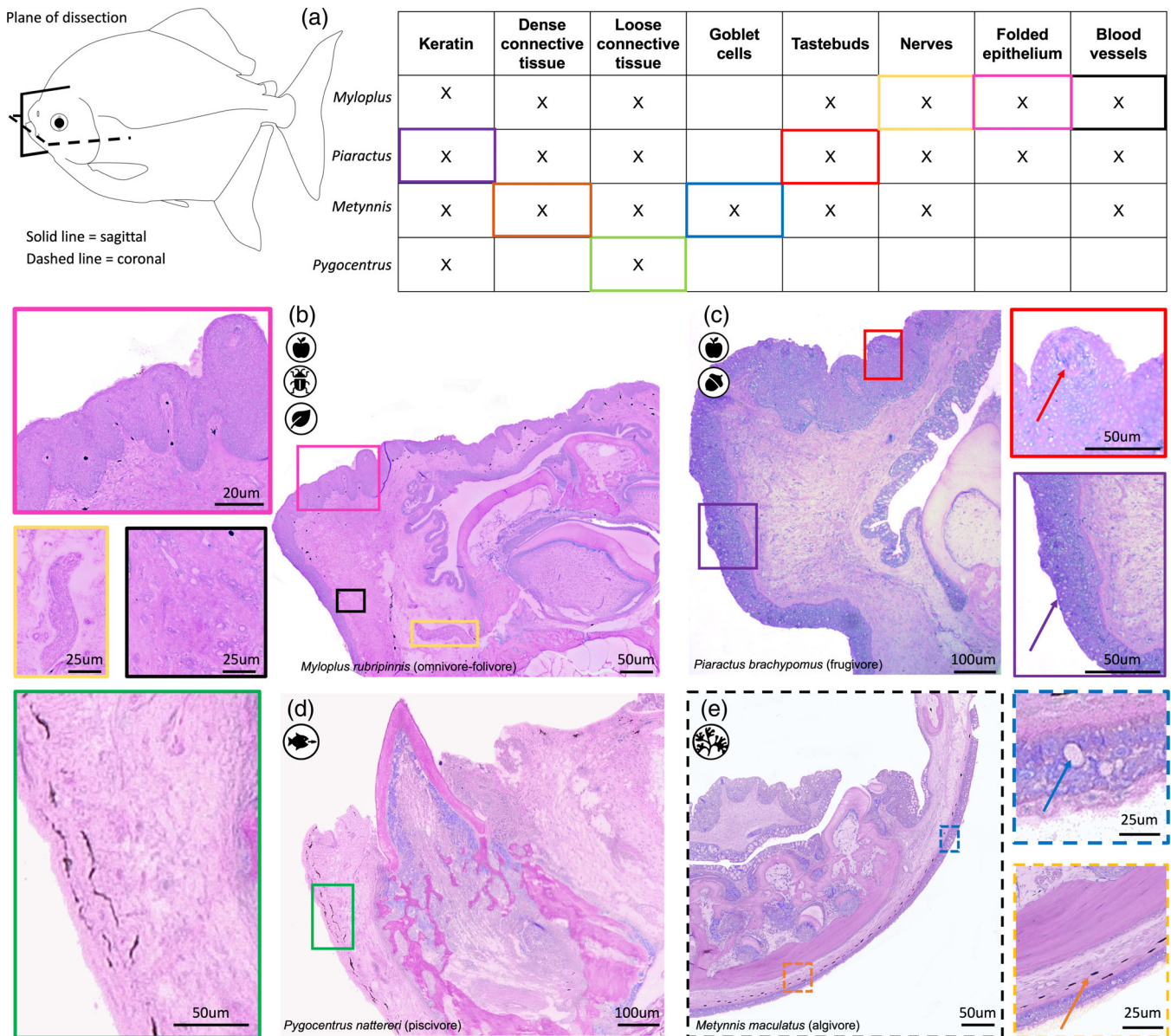


FIGURE 3 Histological overview of the lower lips in pacus and piranhas. (a) Table of reference for histological sections showing a sampling of the morphology observed in this subset of species and schematic demonstration the direction of histological images. (b) Sagittal section through the lip of *Myloplus rubripinnis* showing the distribution of a folded epithelium (pink box), nerves (yellow box), channels, and connective tissues (black box). (c) Sagittal section through the lip of *Piaractus brachypomus* showing large taste buds (red box & arrow) and keratinized epithelium (purple box & arrow). (d) Sagittal section through the lips of *Pygocentrus nattereri* showing a fairly simple and squishy lip (green box). (e) Coronal section through the lip of *Metynniss maculatus* showing goblet cells (blue box) and dense connective tissues (orange box & arrow). This figure represents overall trends in morphology, while a full account of all histological traits can be found in Table 3

Additionally, the epithelium in *Piaractus* is more folded than in the three *Myloplus* species, lacks mucus-secreting cells, and studded with macrophages and taste buds (Figure 3c, blue box). *Colossoma*, another fruit- and seed-eating pacu, has wide channels distributed throughout the keratinized epidermis of the lip (Figure 4c). Directly below the keratin is a thin layer of longitudinal epidermal cells. The dermis and hypodermis are very spongy and composed

of organized collagen bundles and adipose tissue (Figure 4c). *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 & Jégu, 1990). Their lips share a similar 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

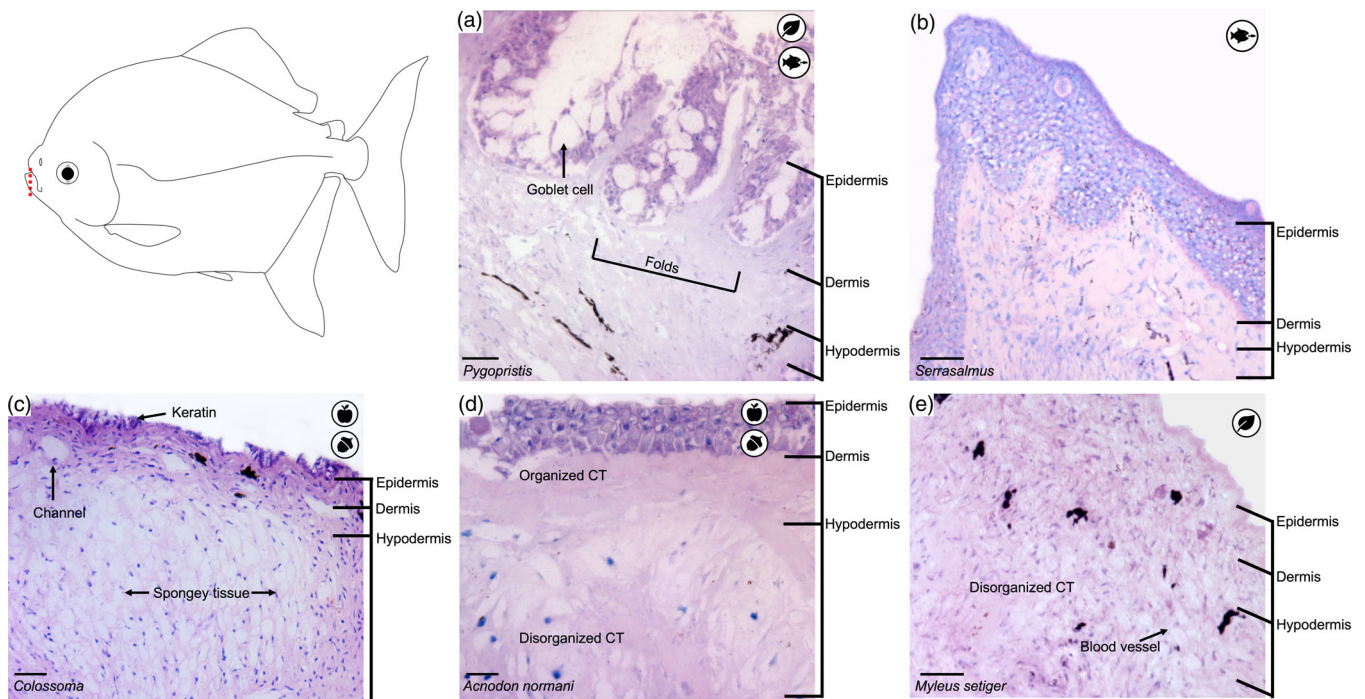


FIGURE 4 Comparative serrasalmid lip histology. (a) Sagittal section through a portion of the lip in *Pygopristis* highlighting the folded epithelium and abundance of goblet cells. (b) Sagittal section through the lip of *Serrasalmus*. (c) Sagittal section through a portion of the lip in *Colossoma* highlighting large channels and distinct tissue regionalization. (d) Sagittal section through the lip of *Acnodon normani* and (e) section through the lip of *Myleus setiger* highlighting the lack of tissue regionalization and abundance of blood vessels. Schematic fish with a red, dashed line represents where samples were taken from. CT, connective tissue. Scale bar set to 1,000 μm

alternating layers of organized and disorganized collagen (Figure 4d; Table 3). There are no large nerves or macrophages in *Acnodon's* lip, relative to other pacus, but there are mucus-producing goblet cells on the inner surface of their lips, as in frugivorous pacus like *Colossoma* and *Piaractus* (Table 3).

In both *Myloplus* and *Piaractus*, an extension of the primordial ligament originates from the adductor mandibulae muscle divisions (specifically, A_1) and inserts on the distal edge of the lip (Figure 5a,b). In all serrasalmids, the primordial ligament is distinct from the primordial membrane and tightly links the upper and lower jaws (Datovo and Vari, 2014). In both *Myloplus* and *Piaractus* the primordial ligament still extends from the membrane attaching the two jaws, but a third segment forks out from the membrane-ligament attachment to the adductor muscles. This third segmentation inserts onto the posterior end of the hypertrophied lip. We found that contraction of the A_1 pulls on the edges of the hypertrophied lip and that pulling on the lip in turn flexes A_1 . The ligamentous attachment is more robust and tendon-like in *Myloplus* than in *Piaractus* but both are distinct from the original ligament (Figure 5b). Contrary to our predictions about lip morphology, no muscle tissue was found in any lips, whether hypertrophied or not.

3.3 | Lip diversity of folivorous, phytophagous, algivorous, and planktivorous pacus

Lips of folivorous pacus such as *Tometes* and *Myleus* (Andrade, Fitzgerald, et al., 2019a, Andrade, Winemiller, et al., 2019b), as well as the lips of planktivorous and algivorous species like *Metynnis luna* and *Metynnis maculatus* (Ramos et al., 2018; Andrade, Fitzgerald, et al., 2019a, Andrade, Winemiller, et al., 2019b), are thinner and composed of three layers. The epidermal layer is relatively thin and studded liberally with goblet cells and large taste buds (Figure 3a,e, Figure 4e; Table 3). The dermis is made up of densely-packed, regular connective tissue, and is followed by a third layer consisting primarily of disorganized connective tissue.

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 superficial part of the dermis, with more organized and densely packed collagen along the circumference of the lip. The hypodermis is 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 3e, inset;

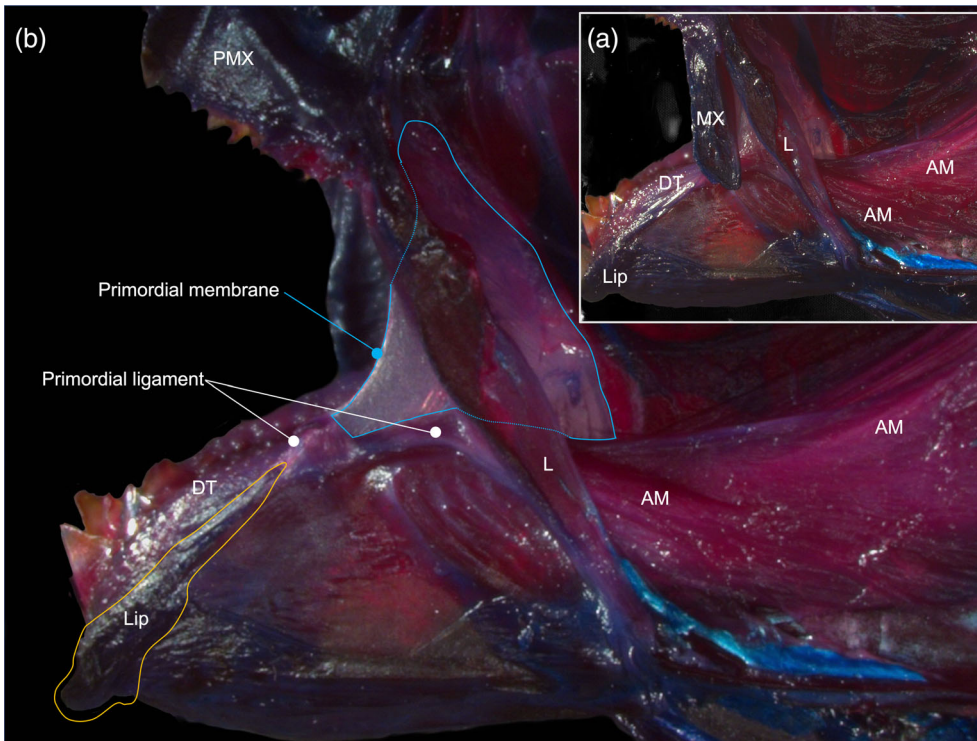


FIGURE 5 Primordial ligament attachment to hypertrophied lip in stained *Piaractus*. (a) Stained specimen with maxilla in place to show anatomical condition. (b) Maxilla is removed, and the primordial membrane and ligament are preserved. Outlines show connection between the primordial membrane, ligament, and lip. AM, adductor mandibulae; DT, dentary; L, preangular ligament; MX, maxilla; PM, primordial ligament; PMX, premaxilla. Solid lines represent anatomy in the foreground, dashed lines represent anatomy in the background

Table 3). Dissected and diaphonized *Metynnis* specimens did not show any additional connection between the lip and the primordial ligament. Rather, the ligament and its associated membrane were in the positions described by Datovo and Vari (2014), and there was no apparent connection between the lip and the adductor musculature.

The lips of *Tometes* are composed of disorganized connective tissue, with no evidence of muscles or different layers of collagen (Table 3). In *Tometes* the primordial ligament is taut and robust compared to that of *Myloplus* and *Piaractus*, and while clearly differentiated from the primordial membrane, does not insert onto the lip in any way. Instead, the ligament attaches directly to the upper jaw, following the path of the primordial membrane (Datovo and Castro, 2012). The lips of *Myleus* are composed primarily of disorganized collagen tissue with some organized tissues just beneath the dermis in the stratum compactum, and they have small blood vessels running throughout (Figure 4e). Deeper in the dermal layers are tubes of organized collagen that run along the circumference of the lip. The epidermis is wavy and composed of long columnar cells intermittently studded with macrophages.

3.4 | 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 (Data S3)). 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; Andrade, Fitzgerald, et al., 2019a, Andrade, Winemiller, et al., 2019b; Dary et al., 2017). Two *Myloplus* species cluster with *Colossoma* and *Piaractus*, our most obligate frugivorous taxa, with *Colossoma* being more distinct 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.) loading positively (Figure 6b). This is expected as pacus have more tissue types incorporated into their lower lips than any of the piranha species (Figure 6a).

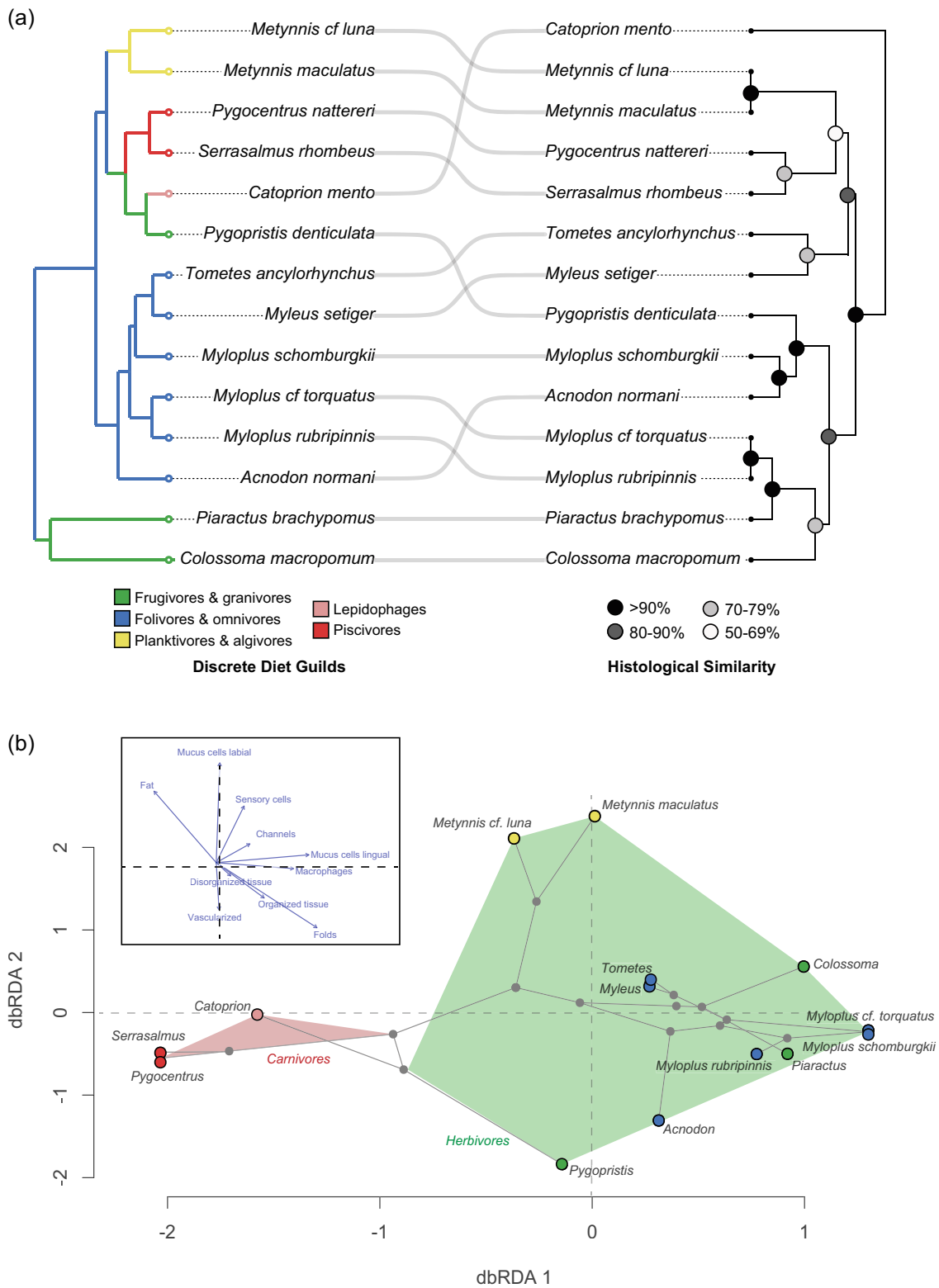


FIGURE 6 Evolutionary ecology of pacu & piranha lips. (a) There is significant mismatch between phylogeny and trait similarity among pacu—convergence in lip morphologies demonstrates ecological similarity among species. Diet guilds mapped onto phylogeny (left) from Kolmann et al., (2021); histological similarity among lips according to presence/absence of histological characters (right). Percentages reported on the right-hand cluster algorithm are the AU (approximately unbiased) p-values for each node. (b) Ordination demonstrating differences in disparity among herbivorous and carnivorous diet guilds. Distance-based redundancy analysis (dbRDA) was used to visualize lip histological diversity among taxa. Note how more complex lips, with more histological characters, cluster on the lower right of the histo-space

Omnivorous *Pygopristis* falls along the periphery of this morphospace, near the middle of dBRDA axis 1, but strongly negative on axis 2. This places the omnivorous *Pygopristis* (a piranha) closer to more omnivorous pacus like *Acnodon*, and further from carnivorous piranhas like *Pygocentrus* or *Serrasalmus* (Leite & Jegu, 1990; Ferreira et al., 2014). 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, make it so that serrasalmines have more diverse lip phenotypes than myleine and colossomatine pacus. The Serrasalminae (*Metynnis* and all piranhas), given their broad distribution in morphospace, are a particularly disparate group with respect to lip morphologies and ecologies.

4 | DISCUSSION

We found no intrinsic musculature in pacu lips, despite the lip's obvious mobility during prey processing in *Myloplus*, *Piaractus*, *Colossoma*, *Utiaritchthys* (Supl. videos (Data S1)), and previously recorded instances in *Piaractus* (Irish, 1987; Lomax & Brainerd, 2020, Supplemental videos 1–6 (Data S1, S2)). We propose that the lips are actuated indirectly by the jaw adductors via the primordial ligament (Figure 5b). In fishes, the primordial ligament is a fibroelastic band that encircles the mouth (Alexander, 1967; Osse, 1969; Anker, 1974; Gosline, 1986; Datovo & Vari, 2014). 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 & Vari, 2014). However, in serrasalminids, the primordial ligament is distinct, albeit embedded in the primordial membrane, and inserts along the postero-medial edge of the maxilla. This ligament spans both the upper and lower jaws and keeps them in tension (Datovo and Castro, 2012; Datovo & Vari, 2014). We found that in some species of pacus with mobile lips, an extension of the primordial ligament attaches to the edge of the lip (Figure 5b). Dissections show that the *adductor mandibulae* muscles attach to this extension, rendering this part of the primordial ligament a de facto tendon (Figure 5b).

We hypothesize that activation of the adductor complex during feeding would put the primordial ligament in tension, thereby pulling on the lip, deforming it around prey, and increasing the contact area between lip and food item. We propose that in other characiform fishes like pacus, bryconids, and alestids (Datovo & Castro, 2012), the morphology of the primordial ligament has been selected for different functional roles depending on the size, shape, and surface texture of prey. The

importance of soft tissues for prey handling cannot be overstated—without a tongue to reposition the bolus, mammalian mastication would not be nearly as efficient as it is (Gintof et al., 2010; Hiiemae & Palmer, 2003; Olson et al., 2021). Likewise, the interaction of a ductile lip opposite a hard dentition is a useful arrangement for gripping slippery objects (Barlow & Munsey, 1976). It may be that the pairing of dissimilar modulus materials, like teeth against a lip, is a generalizable solution to the slippery problem of grabbing wet fruit and holding onto it. Pacu lips may participate in both prey capture and prey processing, and high-resolution kinematic data is required to understand the full extent of lip actuation and function.

All pacu lips are not necessarily actuated however, *Metynnis maculatus* for example, has no ligamentous connection—their lips are truly passive. This does not suggest they are without function, but rather that they function in defense rather than manipulation. Plants invest in myriad strategies for reducing or discouraging grazing such as 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 poisoning, and the long-term effects of plant materials that can slice, stab, abrade, and dissolve oral tissues. The abrasion-resistant nature of a keratinized epithelium and callused 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 3). 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 (Figure 1a, d,e; Huie et al., 2019). The variety of plant mechanical defenses offers an explanation for the diversity of tissue compositions in grazing pacus. These fishes may be capitalizing on plants with particular 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).

Our results suggest that the hypertrophied lips of pacus — built of alternating layers of collagen, fat, elastin, and keratin, are compliant and capable of large deformations, allowing them to conform to, and grab onto, a wide variety of prey. *Colossoma*, *Piaractus*, and *Myloplus* have the largest lips and feature the most complex arrangement of soft tissues, allowing for all three taxa to handle food larger than their gape (Figures 3 and 6b). 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., 2020, Cohen et al., 2020). The layering of collagen tissues in the pacu lip is similar to the remora disk, so when the collagen is slack, the lip is compliant and deforms around the 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 at least 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* (Figure 5)—lineages, which diverged at least 15–25 million years ago (Kolmann et al., 2021). An extension of this hypothesis to other lineages suggests that a linkage between collagen organization and the ability to grip and manipulate prey larger than the mouth might be common for fleshy-lipped species.

Large, protruding lips are not unknown in fishes. Hypertrophied lips in cichlids are associated with foraging along rocky faces or cobbled substrates (Baumgarten et al., 2015; Burrell, 2015; Machado-Schiaffino et al., 2014), while enlarged lips in corallivorous wrasses protect their mouths from nematocysts (Huertas & Bellwood, 2017). Alternatively, keratinized lips in suckers (Catostomidae), sucker loaches (Gyrinocheilidae), or armored catfishes (Loricariidae) aid in scraping substrates and station-holding (Benjamin, 1986; Doosey & Bart Jr., 2011; Geerinckx et al., 2007). In all of these cases, lips aid in prey capture, either by abrading the substrate to remove attached prey or by forming a gasket-like seal over crevices to suck up prey. Likewise, differences between black and white rhinoceros species' lips reflect browsing in the former and grazing in the latter (Leuthold, 1977; Owen-Smith, 1975). The elephant's trunk or the tapir's snout, both extensions of the upper lip, are archetypal in the foraging behavior of these taxa. Even in aquatic mammals, sea cows and dugongs use their lips to gather aquatic vegetation (Heinsohn & Spain, 1974). Pacu lips are used beyond prey capture and serve a complementary role to the hard dentition during prey processing. In this manner, the soft tissues comprising pacu lips are functionally analogous to tongues or snouts in mammals.

In conclusion, we find that pacu lips vary in cellular composition based on diet and presumed prey handling behaviors. While no lips had intrinsic musculature, some may still be active participants in prey manipulation via the primordial ligament. Not all herbivores deal with the challenges of the plant materials in the same way. Some pacu lips are best suited against the myriad of chemical defenses in stems and leaves while others are best at helping to break down larger fruits and seeds. Unlike the lips of other fishes, their hypertrophied oral appendage in pacus aid in both prey capture and prey processing. We suspect that intraoral processing relies on the hypertrophied lips which aid in food stabilization while the

dentition (specifically the two symphyseal teeth located on the dentary) deals with hard prey.

AUTHOR CONTRIBUTIONS


Karly E. Cohen: Conceptualization (lead); data curation (lead); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); software (equal); validation (equal); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Oliver Lucanus:** Data curation (supporting); resources (supporting); visualization (equal); writing – review and editing (equal). **Adam P. Summers:** Funding acquisition (equal); supervision (equal); validation (equal); writing – review and editing (equal). **Matthew A. Kolmann:** Data curation (equal); funding acquisition (equal); investigation (equal); project administration (equal); supervision (equal); validation (equal); writing – original draft (equal); writing – review and editing (equal).

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