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1   **Phylogenomics of piranhas and pacus (Serrasalmidae) uncovers how convergent diets obfuscate**
2   **traditional morphological taxonomy.**
3
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16 **ABSTRACT**
17   The Amazon and neighboring South American river basins harbor the world's most diverse
18 assemblages of freshwater fishes. One of the most prominent South American fish families are the
19 Serrasalmidae (pacus and piranhas), found in nearly every continental basin. Serrasalmids are keystone
20 ecological taxa, being some of the top riverine predators as well as the primary seed dispersers in the
21 flooded forest. Despite their widespread occurrence and notable ecologies, serrasalmid evolutionary
22 history and systematics are controversial. For example, the sister taxon to serrasalmids is contentious, the
23 relationships of major clades within the family are obfuscated by different methodologies, and half of the
24 extant serrasalmid genera are suggested to be non-monophyletic. We used exon capture to explore the
25 evolutionary relationships among 64 (of 99) species across all 16 serrasalmid genera and their nearest
26 outgroups, including multiple individuals per species in order to account for cryptic lineages. To
27 reconstruct the timeline of serrasalmid diversification, we time-calibrated this phylogeny using two
28 different fossil-calibration schemes to account for uncertainty in taxonomy with respect to fossil teeth.
29 Finally, we analyzed diet evolution across the family and comment on associated changes in dentition,
30 highlighting the ecomorphological diversity within serrasalmids. We document widespread non-
31 monophyly within Myleinae, as well as between *Serrasalmus* and *Pristobrycon,* and propose that reliance
32 on traits like teeth to distinguish among genera is confounded by ecological convergence, especially
33 among herbivorous and omnivorous taxa. We clarify the relationships among all serrasalmid genera,
34 propose new subfamily affiliations, and support hemiodontids as the sister taxon to Serrasalmidae.
35
36   **Key words: Characiformes, exon capture, ichthyochory, piscivory, molecular time-calibration**
37
38 **INTRODUCTION**
39   The family Serrasalmidae, piranhas and pacus (Fig. 1), is a diverse freshwater clade of
40 characiform fishes found throughout tropical and subtropical South America. Ninety-seven extant species
41 are primarily distributed east of the Andes, with just a single species found west in the Maracaibo Basin,
42 but the fossil record extends the historical distribution of the family further west into the Magdalena
43 region (Lundberg et al., 2010). While piranhas are generally carnivorous, their sister taxa, the pacus, are
44 herbivores that include some of the primary seed dispersers in flooded forests or varzea (Goulding, 1980;
45 Correa et al., 2007). Despite their keystone status and commercial significance throughout Amazonia, the
46 ecologies of piranhas and pacus are poorly understood and often misrepresented. The ferocious reputation
47 of piranhas stems largely from accounts of their feeding on corpses (Sazima & Guimarães, 1987) or
48 nipping bathers, the latter attributed to the protective nature of piranhas defending nests (Haddad &
49 Sazima, 2003, 2010; but see Kolmann et al., 2018b). We have only recently understood the role that pacus
50 play in structuring forests, as large frugivores that can disperse seeds over great distances (ichthyochory)
51 (Correa et al., 2015, 2016). Some rainforest trees appear particularly specialized for ichthyochory, with

52 seeds that have greater germination probability after digestion by fishes (Anderson et al., 2009) or by
53 bearing fleshy fruit with increased buoyancy (Horn et al., 2011; Correa et al., 2018). Large pacus like the
54 iconic tambaqui (*Colossoma*) and pirapitinga (*Piaractus*) are attracted to the sound of fruit falling in the
55 water, even 'staking out' fruiting trees for weeks at a time (Goulding, 1980).
56   From fruits and seeds, to fins and flesh, serrasalmids feed on a wide variety of prey and prey
57 materials (Correa et al., 2007), but many genera remain understudied regarding their evolutionary
58 ecology. Medium-sized pacus (Myleinae) feed heavily on the leaves, flowers, and stems of riparian and
59 aquatic plants (Correa & Winemiller, 2014), with some adapted to scraping river weed (Podostemaceae)
60 off rocks in a manner unique among otophysan fishes (Andrade et al., 2019a; Huie et al., 2019). Contrary
61 to the popular idea of piranhas as exclusively carnivorous, their diets vary greatly across seasons,
62 ontogeny, and species. Some piranhas are facultative frugivores (*Pristobrycon*; Nico & Taphorn, 1988;
63 Correa & Winemiller, 2014), while others feed on either fish scales (lepidophages, i.e. *Catoprion*;
64 Goulding, 1980; Sazima & Machado, 1990; Kolmann et al., 2018a) or fins (pterygophages, i.e.
65 *Serrasalmus elongatus*; Röpke et al., 2014). How such diverse diets have evolved in serrasalmids remains
66 uncertain, despite efforts by authors like Correa et al. (2007), because of the lack of a well resolved
67 phylogenetic framework for the family.
68   Early classifications (Eigenmann, 1915; Norman, 1929; Gosline, 1951; Géry, 1977) relied on
69 dentition to divide serrasalmids into two major groups, those with two rows of teeth on the upper jaw
70 ('herbivorous' pacus) vs. one row ('carnivorous' piranhas). A more comprehensive and cladistic analysis
71 of morphology by Machado-Allison (1982, 1983, 1985) also divided serrasalmids into two major groups,
72 but disagreed on their composition by transferring *Metynnis*, previously classified as a pacu, to the
73 piranha clade. The cladistic analysis of the newly discovered fossil †*Megapiranha* by Cione et al. (2009)
74 supported the placement of *Metynnis* sister to piranhas and provided the first morphological evidence for
75 the non-monophyly of the remaining pacus. Cione et al. (2009) and more recent studies have expanded
76 the breadth of ecological and morphological diversity among serrasalmids. For example, some species of
77 pacus have heterodont dentitions which vary along numerous phenotypic axes, from molariform to
78 incisiform, spatulate to crenulate (Huie et al., 2019; Kolmann et al., 2019). In fact, tooth characteristics
79 and arrangement remain the best characters for distinguishing pacu taxa (Andrade et al., 2013, 2016; Nico
80 et al., 2018).
81   Molecular phylogenies (Ortí et al., 1996, 2008) support the morphological phylogeny of Cione et
82 al. (2009) wherein the piranhas + *Metynnis* clade are sister to medium-sized taxa like *Acnodon* and
83 *Myleus* and are therefore nested within pacus (Supplements). However, molecular studies using
84 mitochondrial genes (Ortí et al., 1996, 2008; Hubert et al., 2007; Freeman et al., 2007) revealed problems
85 with generic monophyly and failed to reconcile genus-level relationships. Thompson et al. (2014)
86 constructed the most rigorous serrasalmid phylogeny to date and found rampant non-monophyly within
87 Myleinae and among *Serrasalmus* and *Pristobrycon* species (Supplements). These molecular analyses had
88 limited taxonomic sampling, including only two of the five *Pristobrycon* species, and also lacked
89 *Utiaritichthys*, which DNA barcoding suggests is nested within *Myloplus* (Machado et al. 2018).
90   Improving our understanding of the timeline of lineage, phenotypic, and ecological
91 diversification in serrasalmids thus requires broader species-level sampling. Resolution of recalcitrant
92 relationships or questionable monophyly (e.g. *Serrasalmus*) should benefit from phylogenomic
93 approaches that leverage much larger datasets than previously available. Thompson et al. (2014) dated
94 their serrasalmid phylogeny with two fossil calibrations and found the basal divergence between pacus
95 and piranhas (+ *Metynnis*) starting during the middle Paleocene (~ 60 mya) and the diversification of
96 Myleinae starting in late Eocene (45 mya). However, the age of other lineages within serrasalmids are
97 more uncertain, despite a rich fossil record that could improve our understanding of the timeline of
98 diversification within the family and their nearest characiform relatives (Dahdul, 2004, 2007).
99   We used exon-capture phylogenomic methods with greater taxon sampling than any previous
100 study to reassess relationships among serrasalmid genera. Using multiple fossil calibrations to estimate
101 relative divergence times among lineages, we explored how uncertainty about fossil choice shapes our
102 estimates of the timing of lineage diversification in serrasalmids. Finally, we used ancestral character state

103 estimation to explore how diet diversity, novelty, and convergence evolved across extant serrasalmid
104 lineages, and how major diet specializations have evolved in this clade. We document how ecological
105 convergence in diet may be shaping dental morphology and the effect this phenomenon has on taxonomic
106 character states used to distinguish among serrasalmid genera.
107
108 **METHODS**
109 *Taxonomic Sampling*
110　　　Exon sequence data were analyzed for 194 individuals (Table S1) including 44 previously
111 published in Arcila et al. (2017) and Betancur et al. (2019). Outgroups included 59 individuals
112 representing 50 species distributed among 38 genera in 12 families of Characiformes. The ingroup,
113 Serrasalmidae, included 135 individuals representing all 16 nominal valid genera and at least 65 of the 99
114 nominal valid species. Twelve specimens were excluded due to potential mislabeling or contamination
115 problems. A probe set for exon capture previously optimized for Otophysi by Arcila et al. (2017) was
116 used to capture 1051 exons for sequencing. Library preparation was performed at Arbor Biosciences
117 (www.arborbiosci.com), using a dual-round hybridization protocol (Li et al. 2013) to enrich the libraries
118 for the targeted exons on pools of eight species. Paired-end sequencing (100 bp) was performed at the
119 University of Chicago Genomics Facility on a HiSeq 4000. Up to 192 enriched libraries were combined
120 to form multiplex pools for sequencing in a single lane.
121
122 *DNA extraction and Exon Capture Protocols*
123　　　Genomic DNA was extracted from muscle biopsies or fin clips preserved in 90-99% ethanol
124 using either Qiagen DNEasy kits or the phenol-chloroform protocol in the Autogen platform available at
125 the Laboratory of Analytical Biology at the National Museum of Natural History (Smithsonian
126 Institution) in Washington, D.C. Laboratory protocols for library preparation and probe sets for exon
127 capture were optimized for Otophysi, and followed the procedures described in Arcila et al. (2017).
128 Library preparation and target enrichment was completed at Arbor Biosciences (www.arborbiosci.com).
129 Libraries were sequenced on an Illumina HiSeq 4000 at the University of Chicago Genomics Facility.
130
131 *Data Assembly and Alignment*
132　　　We used the bioinformatics pipeline optimized by Hughes et al. (2020) to obtain sequence
133 alignments for 951 exon markers from an initial set of 1051 (S1 appendix). Raw FASTQ files were
134 trimmed with Trimmomatic v0.36 (Bolger et al. 2014), to remove low quality sequences and adapter
135 contamination. Trimmed reads were then mapped with BWA-MEM (Li & Durbin, 2009) against a fasta
136 file containing all sequences used for bait design (see Arcila et al 2017). SAMtools v1.8 was used to
137 remove PCR duplicates and sort the reads that mapped to each of the exons (Li et al. 2009b). Sorted reads
138 (by species) were then assembled individually for each exon using Velvet (Zerbino & Birney, 2008), and
139 the longest contig produced by Velvet was used as the initial reference sequence for input to aTRAM v2.0
140 (Allen et al. 2017). aTRAM was run with Trinity v2.8.5 as the assembler to extend contigs iteratively.
141 Redundant contigs with 100% identity produced by aTRAM were removed with CD-HIT v4.8.1 using
142 CD-HIT-EST (Li & Godzik, 2006; Fu et al., 2012). Open reading frames for remaining contigs were
143 identified with Exonerate (Slater & Birney, 2005). Sequences for each exon were aligned with MACSE
144 v2.03 (Ranwez et al. 2018).
145　　　Resulting data matrices were combined with previously published exon sequences (Arcila et al.,
146 2017; Betancur-R. *et al.* 2019) (Table S1). For quality control and assessment, estimated gene trees were
147 visually assessed and flagged when Serrasalmidae was not monophyletic. These gene trees were visually
148 inspected to detect and remove putatively paralogous sequences, and samples with alarmingly long
149 branches. Alignments with fewer than 100 sequences also were removed from downstream analyses.
150
151 *Phylogenetic inference*
152　　　A species tree was estimated under the multi-species coalescent (MSC) using ASTRAL-III v5.6.3
153 (Mirarab et al., 2014; Zhang et al., 2018), with individual gene trees estimated under maximum likelihood

154   (ML) using IQTREE v1.6.10 (Nguyen et al., 2014). Each locus was partitioned by codon position,
155   according to automatic model selection parameters obtained from ModelFinder using the 'TESTMERGE'
156   option (Kalyaanamoorthy et al., 2017), with ten independent Maximum Likelihood searches for each gene
157   alignment. Concatenated amino acid and nucleotide matrices also were analyzed with IQTREE, with
158   nucleotide sequences partitioned by codon position and the best substitution model was fitted using the
159   'TEST' option of ModelFinder. Protein sequences were translated from nucleotides using AliView v1.0
160   (Larsson, 2014) and the best model across all genes selected using ModelFinder. Ten independent
161   searches were run for each concatenated analysis. Branch support for the ML analyses of concatenated
162   matrices was assessed with 1,000 ultra-fast bootstrap (UFBoot) replicates (Minh et al., 2013) and 1,000
163   SH-like approximate likelihood ratio test (SH-aLRT) replicates (Guindon et al., 2010). Support for the
164   species tree topology obtained with ASTRAL-III was assessed with local posterior probabilities (PP,
165   Sayyari & Mirarab, 2016).
166
167   *Fossil calibration*
168   The oldest fossils associated with Serrasalmidae are isolated pacu-like teeth from the Bolivian El
169   Molino Formation (~73-60 mya; Gayet, 1991; Gayet & Meunier, 1998). Although often used to date the
170   origin of the family, these fossil teeth are unusual for serrasalmids for several reasons: (1) their small size
171   of < 0.75-1.0 mm (Gayet, 1991; Gayet & Meunier, 1998; Gayet et al., 2001) despite most serrasalmid
172   teeth being far larger (>1 cm; Shellis & Berkovitz, 1976; Kolmann et al., 2019); (2) pacu teeth, while
173   complex in shape, lack the lingual cusp visible in Gayet et al. (2001, Fig. 7d); (3) these fossil teeth lack
174   any of the interlocking morphologies typical of extant serrasalmid dentitions (Kolmann et al., 2019); (4)
175   the timeline of Bolivian serrasalmid fossil teeth leave a 25-32 mya gap in the fossil record until the first
176   confidently-identified serrasalmid tooth (~38 mya; DeCelles & Morton, 2002; Dahdul, 2007; Lundberg et
177   al., 2010).
178   The El Molino fossils may represent juvenile serrasalmid teeth, but this hypothesis seems
179   unlikely due to the absence of larger adult teeth and the well-known taphonomic bias towards larger-sized
180   skeletal elements. Furthermore, these fossil teeth strongly resemble dentitions from distantly related
181   alestids (Alestidae) discovered in various North African deposits (Murray et al., 2003, 2004a, 2004b). It is
182   possible that these Bolivian fossils are stem characoids, or simply indistinguishable at a more
183   circumscribed exclusive taxonomic level (as others have suggested, see Patterson, 1993; Otero et al.,
184   2008). This scenario is supported by the non-monophyly of South American characoids with respect to
185   the African Alestoidea (Alestidae+Hepsetidae), and its sister group relation to exclusively Neotropical
186   taxa in Neotropical Erythrinoidea + Curimatoidea (Arcila et al., 2017; Betancur et al., 2019). Given this
187   uncertainty, we excluded the putative pacu teeth from the El Molino formation in our first set of
188   calibrations (Scheme 1) but included them in our second set to assess its effect on divergence times within
189   the family (Scheme 2; Supplemental Materials). These calibration schemes included 11 outgroup fossils,
190   and three additional serrasalmid calibration points are based on Miocene fossils summarized in Lundberg
191   et al. (2010).
192   As running time-calibration analysis using a Bayesian framework for large phylogenomic
193   datasets can be computationally intensive, we randomly selected four 50-gene subsets of the 200 most
194   complete genes and pruned each subset to include one tip per taxon. We transformed the concatenated
195   ML DNA topology into a chronogram under penalized likelihood using the *chronos* function in R (ape
196   v5.3; Paradis et al., 2019). This chronogram was used as a starting tree for BEAST 2 v2.5.0 (Bouckaert et
197   al., 2014) to generate relaxed-clock divergence time estimates, the topology of the resulting trees was also
198   constrained to match the concatenated nucleotide phylogeny. Each subset of 50 genes was run
199   independently twice for 2.0×10^8 generations. Convergence was assessed in Tracer v1.7.1 (Rambaut et al.,
200   2018) by checking that ESS values were greater than 200 for all parameters. Independent runs from each
201   of the four different subsets were combined in LogCombiner if their 95% highest posterior densities for
202   divergence times overlapped, and a maximum clade credibility tree was generated in TreeAnnotator for
203   each of the two calibration schemes.
204

205   *Ancestral state reconstruction of diet evolution in piranhas and pacus*
206   Serrasalmid taxa were assigned to 11 categories based on diet (Supplemental Materials): (1)
207 omnivores (plant and fish feeders), (2) piscivores, (3) planktivores, (4) plankton & plant materials, (5)
208 plant materials & invertebrates, (6) plant materials, invertebrates & scales, (7) plant materials & seeds
209 (granivores), (8) plant materials, seeds & fruit (frugivores), (9) plant materials, seeds & invertebrates, (10)
210 fish & fish parts, and (11) phytophagous. Fish & fish parts applies to taxa that consume some
211 combination of scales, fin rays, and whole fishes (e.g., *Serrasalmus elongatus*, a fin-nipper or *Catoprion*,
212 a scale-feeder; Gonzalez & Vispo, 2003; Röpke et al., 2014; Nico & Morales, 1994). We used stochastic
213 character mapping (Huelsenbeck et al., 2003; Bollback, 2006) to reconstruct the evolution of these diet
214 modes across the dated phylogeny (Scheme 1) with the *make.simmap* function in the R package phytools
215 v. 0.6-99 (Revell, 2012). We used AICc to choose among different transition rate models (ER equal rates,
216 SYM, symmetrical rates, and ARD all rates different).
217
218 **RESULTS**
219 *Comparison of concatenation and species tree methods*
220   Concatenated analyses based on nucleotides or proteins placed hemiodontids as sister to
221 serrasalmids with strong support (99/98 and 94/93 UFBoot/SH-aLRT, respectively). In contrast, the MSC
222 approach produced a polytomy among three families, Serrasalmidae, Hemiodontidae, and Cynodontidae,
223 albeit with strong support for this trichotomy (PP >0.95). All other relationships among main characiform
224 lineages are consistent with higher-level phylogenetic studies of the order (Betancur-R. et al., 2019;
225 Supplements).
226   Concatenated and MSC approaches supported three major clades within Serrasalmidae (Fig. 2),
227 with some disagreement on relationships within these clades. Namely, concatenated amino acid and MSC
228 approaches support a different relationship among the three major lineages within the genus *Serrasalmus*,
229 relative to the concatenated nucleotide analysis (Fig. 2). These three major lineages are denoted in Figure
230 2 as the '*rhombeus*' clade (R), the short-snouted (brachycephalic) '*aureus*' clade (A), and the '*maculatus*'
231 clade (M). Both the concatenated and MSC approaches applied to the amino acid dataset resolved the
232 *Serrasalmus maculatus* clade (M) as sister to the remaining *Serrasalmus* taxa, the ['*rhombeus*'clade (R) +
233 *Pristobrycon calmoni*] and the '*aureus*' clade (A), with high support, but this relationship differs in the
234 concatenated nucleotide analysis (Fig. 2). All analyses resolve *Pygocentrus* as sister to *Serrasalmus* and
235 the associated *Pristobrycon* species nested therein, with high support (Fig. 2). (Supplements)
236   The concatenated amino acid analysis and the coalescent analysis resolve *Mylesinus*
237 *paucisquamatus* as sister to the clade comprised of *Tometes ancylorhynchus, T. kranponhah,* and
238 *Myloplus lucienae*, albeit with low support in the amino acid dataset (Fig. 2). Alternatively, the
239 concatenated nucleotide dataset resolves *Mylesinus paucisquamatus* as sister to the *Myleus + Myloplus*
240 *planquettei* clade with moderately high support. The placement of *Utiaritichthys* differed between the
241 concatenation and MSC analyses, though it was always nested within *Myloplus* (Fig. 2). For the first time,
242 we can report that the newly described *Myloplus taphorni* (Andrade et al., 2019b), an endemic to the
243 Mazaruni Basin in Guyana, is sister to *Myloplus ternetzi*, a northern Guiana Shield endemic. Our study
244 also firmly resolves *Acnodon* as monophyletic and sister to all remaining members of Myleinae,
245 something that all previous studies had found difficult to resolve with certainty (Fig. 2).
246
247 *Time calibration*
248   Bayesian analyses in BEAST2 converged on estimates of the posterior distributions, as indicated
249 by ESS values > 200 for all parameters. For each fossil calibration scheme, independent runs based on
250 each of the four 50-gene subsets produced very similar mean-age estimates for all nodes. In contrast, we
251 note marked differences between the two fossil calibration schemes with most obvious discrepancies in
252 the estimated age for the MRCA of Serrasalmidae (HPD = 38-42.9 mya *vs*. 61-64.6 mya) and the split
253 between serrasalmids and their hemiodontid sister clade (HPD = 55.5-76 mya *vs*. 68-81), although the
254 latter estimates overlap considerably (Fig. 3). However, the ages of more inclusive clades overlap
255 considerably between both dating schemes. Piranhas (Serrasalminae) are Miocene in age, in agreement

256 with Thompson et al. (2014), with the modern radiation of *Serrasalmus* (and *Pristobrycon*) stemming
257 from Messinian time periods (5.3-7.2 mya). The split between *Pygocentrus* and *Serrasalmus* being only
258 slightly older, Tortonian-Messinian (combined HPD = 6-9 mya; Fig. 3). The split between piranhas and
259 their more herbivorous cousins, *Metynnis* (both Serrasalminae), occurred on a long branch during the
260 mid-Oligocene to mid-Miocene (combined HPD = 14-24 mya). While the radiation of carnivorous
261 piranhas happened rapidly, the diversification of medium-sized pacus (Myleinae) was more gradual,
262 occurring sometime within the same timeframe as *Metynnis* from piranhas (mid-Oligocene to mid-
263 Miocene) (combined HPD = 11-30 mya; Fig. 3). Conversely, the largest pacu species (Colossominae)
264 diverged from one another (*Piaractus* from *Mylossoma* + *Colossoma*) early on in their history, late
265 Eocene to mid-Oligocene (combined HPD = 27-41 mya; Fig 3) according to Scheme 1 or earlier in
266 Scheme 2, from late Paleocene to late Eocene (combined HPD = 39-63; Fig. 3).

267
268 *Diet reconstruction*
269   For the more exclusive diet categories, the transition model with the lowest AICc (-102.5) was
270 the 'ARD' or All Rates Different model, which allows transitions and reversions between every state to
271 vary independently. Using this model, we found that shifts from piscivory or partial piscivorous feeding
272 modes (i.e. fin- and scale-feeding) to omnivorous feeding modes where plants are consumed alongside
273 flesh, are common in piranhas (Fig. 4). We also found equally probable support for obligate piscivory,
274 omnivorous, or partial piscivorous ancestor for all piranhas. Strong support for obligate piscivory is
275 inferred only at the nodes uniting *Pygocentrus* and the '*rhombeus*' clade of *Serrasalmus*, respectively
276 (Fig. 4). This is due to the generally omnivorous to outright herbivorous feeding habits of the sister taxa
277 to all other piranhas, i.e. *Pygopristis denticulata* + *Pristobrycon striolatus,* which predominantly feed on
278 plant materials as adults and their close relative, *Catoprion mento*, a scale-feeder (Nico & Taphorn, 1988;
279 Nico & Morales, 1988).
280   Also noteworthy is that reversals to more plant-based diets are characteristic of the
281 brachycephalic '*aureus*' (A) clade of piranhas, notably *Serrasalmus gouldingi* and the majority of
282 *Pristobrycon* taxa, which feed as adults on fruits, seeds, leaves, insects and only occasional fish or fish
283 parts (Nico & Taphorn, 1988; Prudente et al., 2016) (Fig. 4). Although several groups show increasing
284 specialization for piscivory (e.g. *Pygocentrus*), this feeding mode does not appear to be an evolutionary
285 dead-end for piranhas, with frequent transitions from piscivory to scale- and fin- feeding, or omnivorous
286 and herbivorous diets (Supplements).
287   Our more comprehensive diet reconstructions document nuance among the diets of herbivorous
288 taxa (Correa et al., 2007). Large-bodied pacus (*Colossoma, Piaractus*) are more frugivorous than smaller-
289 bodied pacus. Also notable are the multiple instances of specialized 'phytophagy' in select taxa like
290 *Tometes, Mylesinus, Ossubtus,* and *Utiaritichtys*, which specialize on the flowers, leaves, and stems of
291 particular river weed plants found only in fast-flowing rapids (Pereira & Castro, 2014; Andrade et al.,
292 2019) (Fig. 4). The paraphyly of *Tometes* in our phylogeny may suggest the sharp incisiform teeth critical
293 in distinguishing *Tometes* from other myleines are misleading. Finally, several species of *Metynnis* are
294 partially to entirely planktivorous, a novelty for serrasalmids and a hallmark for the *Metynnis* clade in
295 general (Canan & Gurgel, 2002) (Fig. 4). However, feeding on plankton appears restrictive, with
296 transitions out of obligate or facultative planktivory being exceedingly rare (Table 2; Fig. 4).

297
298
299 **DISCUSSION**
300 ***An inconvenient tooth: homoplasy, uncertainty, & taxonomic characters***
301   From taxonomic identification to fossil calibrations, our understanding of serrasalmid evolution is
302 substantially influenced by the selective regimes acting on tooth form and function. Authors like Géry
303 (1977) used tooth morphotypes to distinguish between serrasalmid subfamilies and more recent studies
304 have used these same tooth morphologies to make assumptions about diet (Huby et al., 2019), the latter
305 classifying species as either herbivorous or carnivorous depending entirely on whether that taxon has
306 either molariform or triangular teeth (respectively). Our findings reinforce the notion that serrasalmid

307   diets are considerably more diverse than commonly believed, although these trends had been well-
308   documented long ago (Goulding, 1980; Correa et al., 2007). Similarly, 'herbivory' and 'carnivory' are not
309   ecological monoliths. For example, silver dollars or pacucitos (*Metynnis*) feed on plankton, whereas
310   others medium-sized pacus consume insects (e.g. *Mylesinus*; Santos et al., 1997), or even scales (*Acnodon*
311   *normani*; Leite & Jégu, 1990). Similarly, piranhas are not all piscivorous - many have reverted to more
312   herbivorous feeding modes and some lineages (e.g. *Pygopristis, Pristobrycon striolatus*; Nico & Taphorn,
313   1988) are not ancestrally piscivorous. In fact, unlike other fishes, piscivory does not appear to limit niche
314   evolution in piranhas, with closely related lineages displaying variable degrees of omnivory and
315   piscivory, as well as 'mutilating' diet modes like fin- or scale- feeding (Collar et al., 2009; Collar et al.,
316   2014; Fig. 4). Instead, the evolution of serrasalmid feeding structures and diets is far more diverse and
317   complex than widely appreciated.
318   　　　　Tooth shape is not predictive of diet among most herbivorous and carnivorous piranhas. The
319   specialized scale-feeding wimple piranha, *Catoprion mento*, has teeth unlike any of its relatives (Kolmann
320   et al., 2018a, 2019), while *Catoprion*'s sister taxon *Pygopristis*, the only serrasalmid with pentacuspid
321   teeth, feeds on plants and insects as an adult (Fink, 1989; Nico, 1991). But there are several notable cases
322   of morphological convergence. Species assigned to the non-monophyletic genus *Tometes* share
323   remarkably similar sharp, incisiform teeth, which we propose relates to convergence among species
324   adapted to feeding on rheophilic river weed (Andrade et al., 2016; Huie et al., 2019). Similarly, all
325   *Pristobrycon* species are noted for having short, deep skulls whereas most *Serrasalmus* have more
326   elongate faces (Machado-Allison, 1985). Our data demonstrate that most short-snouted *Serrasalmus* (like
327   *S. manueli* or *S. gouldingi*) and short-snouted *Pristobrycon* ('*aureus*' clade) are omnivorous. Shorter jaws
328   are more effective at transferring jaw muscle forces to hard prey like seeds (Goulding, 1980), so dietary
329   convergence among brachycephalic fishes may have misled morphological taxonomy again. These
330   examples highlight the difficulty in translating diagnostic characters useful for identifying species in the
331   field to phylogenetically informative characters that reflect shared evolutionary history. They also
332   highlight how incorporating natural history observations (diet) and functional considerations can help
333   taxonomists steer clear of homoplasy.
334
335   ***Disagreement over the origin (time) of serrasalmids, but not their diversification timeline***
336   　　　　We propose that a dating scheme which casts doubt on the taxonomic affinity of putative pacu
337   teeth from Cretaceous deposits in Bolivia, is the more conservative approach to estimating the timeline of
338   serrasalmid diversification. Isolated fossil material, particularly unarticulated teeth, are difficult to assign
339   to specific taxonomic groups. Paleontologists studying heterodont elasmobranchs have a long-established
340   history of skepticism when designating extinct species or evaluating taxonomic affinity based solely on
341   isolated teeth (Shimada, 2005; Whitenack & Godfried, 2010; Marrama & Kriwet, 2017). Similarly,
342   convergent and heterodont tooth morphologies are quite prevalent among serrasalmids, but also most
343   characiforms in general (Murray et al., 2004a; Kolmann et al., 2019), and this phenomenon may lead
344   taxonomic classification of fossils astray. Without a quantitative evaluation of tooth characters across
345   pacus and relevant outgroups, we argue that assignment of isolated teeth to certain lineages or taxa is
346   fraught with uncertainty.
347   　　　　The common practice of recycling fossil calibrations from study to study may lead to
348   compounding issues with uncertainty and imprecision in our fossil calibration estimates. Although pacus
349   have a well-documented fossil record (Lundberg et al., 1998, 2009; Gayet et al., 2001; Dahdul, 2007), the
350   assumption that isolated teeth are the product of evolutionary stasis (Lundberg et al., 1986) may be
351   premature without (1) broader consideration of outgroups, (2) evaluations of convergence across the
352   phylogeny, or (3) discovery of more articulated skeletons. Given the notable teeth in serrasalmids, it is
353   ironic that their sister taxon, unambiguously identified here as the hemiodontids, have miniscule teeth or
354   are entirely edentulous (Roberts, 1971, 1974). At first glance, serrasalmids would appear to have more in
355   common with their toothier distant relatives, the payaras (Cynodontidae) than hemiodontids; however, we
356   note that our estimates for the divergence among these three clades is Paleocene-Eocene (55-81 mya),

357   enough time for edentulism and near-edentulism to have evolved independently in both aardvarks
358   (Afrotheria) and anteaters (Xenartha) (Upham et al., 2019).
359   The diversification of serrasalmids, particularly large-bodied, fruit-eating pacus, has been
360   associated in the literature with the coincident diversification of fruiting plants (Correa et al., 2015).
361   However, this shared co-evolutionary timeline between frugivorous fishes and their prey plants have
362   primarily relied on stem ages of the asterids, rosids, and other plant groups like the spurges
363   (Euphorbiaceae) (Horn et al., 2011; Correa et al., 2015). However, modern Amazonian plant communities
364   are thought to have established themselves roughly ~40-50 mya during the Eocene, and particularly those
365   plants relying on ichthochory (fish-based seed dispersal; Jaramillo et al., 2010). Our proposed timeline for
366   serrasalmid diversification (Fig. 3) is also Eocene (56-23 mya) in age, rather than Paleocene (66-56 mya),
367   and corresponds better with the crown ages of particularly the most recent common ancestors of plant
368   genera consumed by pacus today. *Colossoma macropomum* consumes fruits and seeds from rubber trees
369   (*Hevea spruceana*), tucumã or jauari palms (*Astrocaryum* sp.), pouteria trees, and even the hallucinogenic
370   iporuru plant (*Alchornea* sp.) (Goulding, 1980). Similarly, pirapitinga (*Piaractus*) also consume tucumã
371   palm fruit, as well as fava (*Vicia faba*), and even luffa (Cucurbitaceae) (Goulding, 1980). The ages of
372   these plant genera are all late Eocene to Oligocene in age (~41-22 mya; Wojciechowski, 2003; Schaefer
373   et al., 2009; Bartish et al., 2011; Roncal et al., 2012), intriguingly corresponding with the diversification
374   timeline of frugivorous, large-bodied pacus (*Mylossoma, Colossoma,* and *Piaractus*) and most other
375   serrasalmid genera. These estimates are also in line with the Eocene timeline for fruit-eating vertebrates
376   like birds and mammals (Fleming & Kress, 2011).
377
378   ***Suggestions for serrasalmid taxonomy, moving forward***
379   The taxonomy and systematics of Serrasalmidae have a long-checkered past "fraught with
380   confusion and instability" (Nico et al., 2018:172). Recent morphological studies have helped distinguish
381   and diagnose a variety of valid genera and species (e.g., Pereira and Castro, 2014; Andrade et al., 2016a;
382   2016b; 2016c; 2017; 2018; 2019b; Ota et al., 2016; Mateussi et al., 2018; Nico et al., 2018; Escobar et al.,
383   2019). The current study provides robust molecular support for recognizing three major lineages of
384   Serrasalmidae at the subfamilial rank: Colossominae (pacus common to lowland, white water habitats),
385   Myleinae (pacus common to upland clear- and black water habitats), and Serrasalminae (Metynnis and
386   piranhas, cosmopolitan). Furthermore, there is strong support for the sister group relationship between
387   Myleinae and Serrasalminae. Those results are consistent with previous phylogenies based on
388   morphological (Cione et al., 2009) and molecular (Ortí et al., 2008; Thompson et al., 2014) data.
389   Based on our phylogenomic analysis of the family, we recommend the following (with more
390   detail in the Supplements): (1) the establishment of a new subfamily delineating large-bodied pacus from
391   myleine pacus, the Colossominae (see Supplements for proposed morphological synapomorphies); (2)
392   that *Pristobrycon* Eigenmann 1915 should be synonymized (as has been repeatedly suggested) with
393   *Serrasalmus* Lacepède 1803. Despite disagreement among our tree-building methods regarding the exact
394   placement of *P. calmoni*, the type species for *Pristobrycon*, the taxon is repeatedly found nested within
395   *Serrasalmus* (Fig. 2; Thompson et al., 2014). Moreover, the distinguishing characteristic of *P. calmoni*,
396   the presence of a pre-anal serra, is shared with *Serrasalmus* but not with half of other *Pristobrycon*
397   species (Machado-Allison, 2002). (3) As such, *Pristobrycon striolatus* (and the cryptic *P. scapularis*;
398   Andrade et al., 2019) should also be elevated to its own generic rank. Finally, *Myloplus* should be broken
399   into several genera, particularly *Myloplus rhomboidalis*, which was formerly known as *Prosomyleus*. Our
400   phylogenetic framework can serve as a guide for future systematic reappraisals of the family, particularly
401   at the genus-level, although the group still requires significant morphological reassessment going forward.
402
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FIGURES

Figure 1 – Morphological diversity of various serrasalmid species and genera. Piranhas divided from pacus by vertical and horizontal lines. Photo of *Ossubtus* by L. Sousa, others by M. Sabaj.

Figure 2 - Comparison of concatenated nucleotide and amino acid trees and clade support in Serrasalmidae. Black circles represent nodes with > 99% UFBoot and aLRT support. Proposed morphological synapomorphies for Serrasalmidae and Colossominae discussed in Supplemental Materials.

Figure 3 - Comparison of HPD results for both time-calibration schemes. Blue bars represent HPD range from Scheme 1 and red bars represent HPD ranges from Scheme 2. Ranges are almost younger for Scheme 1, relative to Scheme 2. Raw mean estimated ages for the most divergent estimates are presented above their respective bars. Fruit icon is the rubber tree, *Hevea brasiliensis*, a preferred prey item of large-bodied pacus (Goulding, 1980).

Figure 4 - Discrete diet trait evolution in Serrasalmidae. (1) *FishFishParts*: Scales, fins, whole or partial fishes found in diet; (2) *Omnivory*: Whole or partial fishes & plants found in stomachs; (3) *Phytophagy*: fishes feeding either obligately or facultatively on riverweed (Podostemaceae); (4) *Piscivory*: feeding on whole or partial fishes; (5) *Planktivory*: obligate plankton feeders; (6) *Plankton & Plant Materials*: fishes feeding on plankton, algae, and other plant parts; (7) *Plant Materials & Seeds*: granivores feeding on seeds and other plant parts; (8) *Plant Materials, Seeds & Fruits*: frugivores, feeding on fruits, seeds, and other plant parts; (9) *Plant Materials, Seeds & Insects*: those species feeding on a combination of plant parts and invertebrates.

SUPPLEMENTS

S1: Figure of previous evolutionary hypotheses among serrasalmid genera.
S2: Fossil dating & time calibration.
S3: Fossil dating & time calibration references.
S4: Serrasalmid diet table.
S5: Diet transition matrix from SIMMAP simulations.
S6: Serrasalmid diet references.
S7: Taxonomic recommendations & morphological synapomorphies.
S8: Table of museum specimens, tissue #s, cat #s, locale.

PIRANHAS

Serrasalmus   *Pygopristis*   *Pygocentrus*
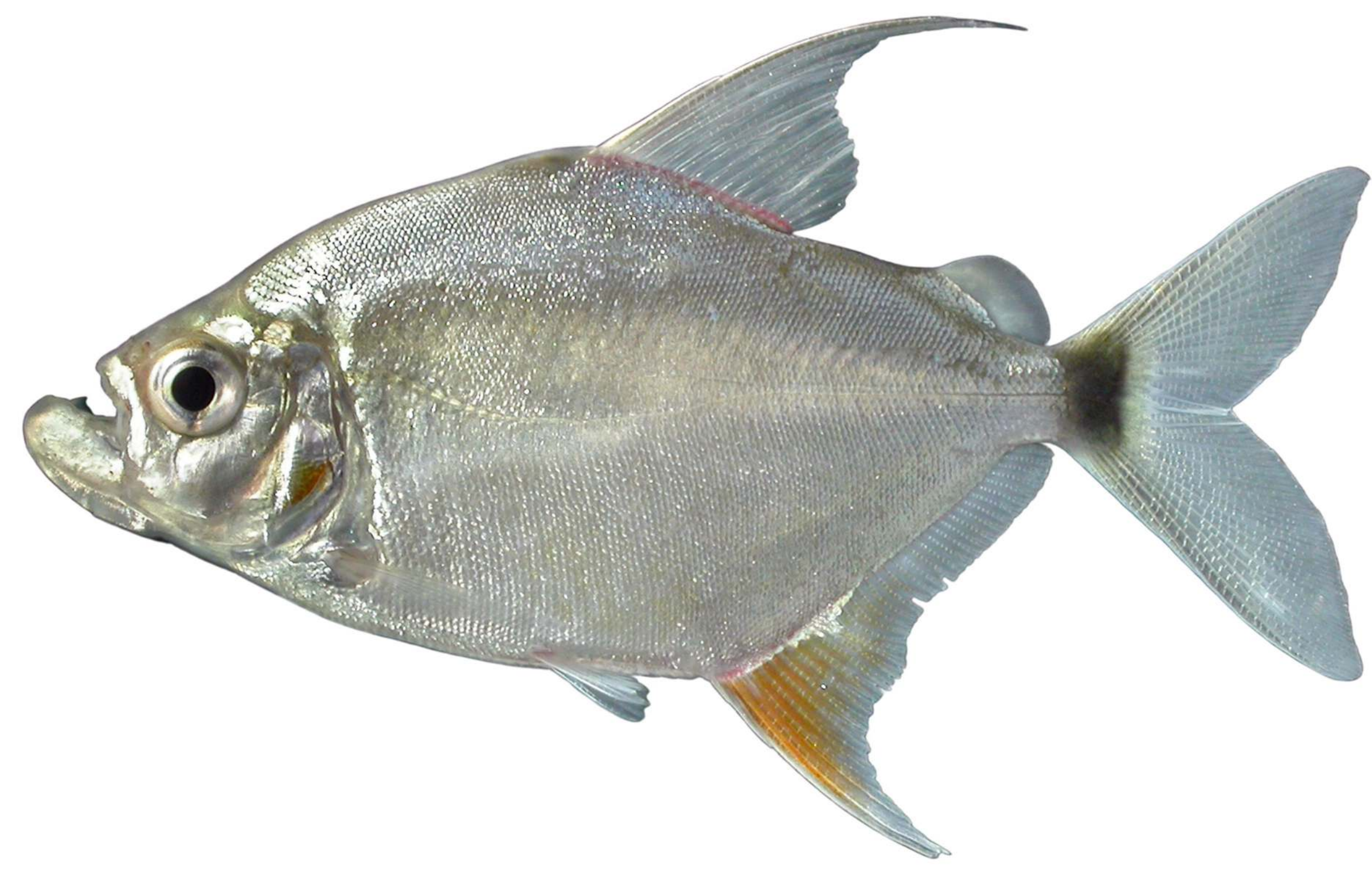
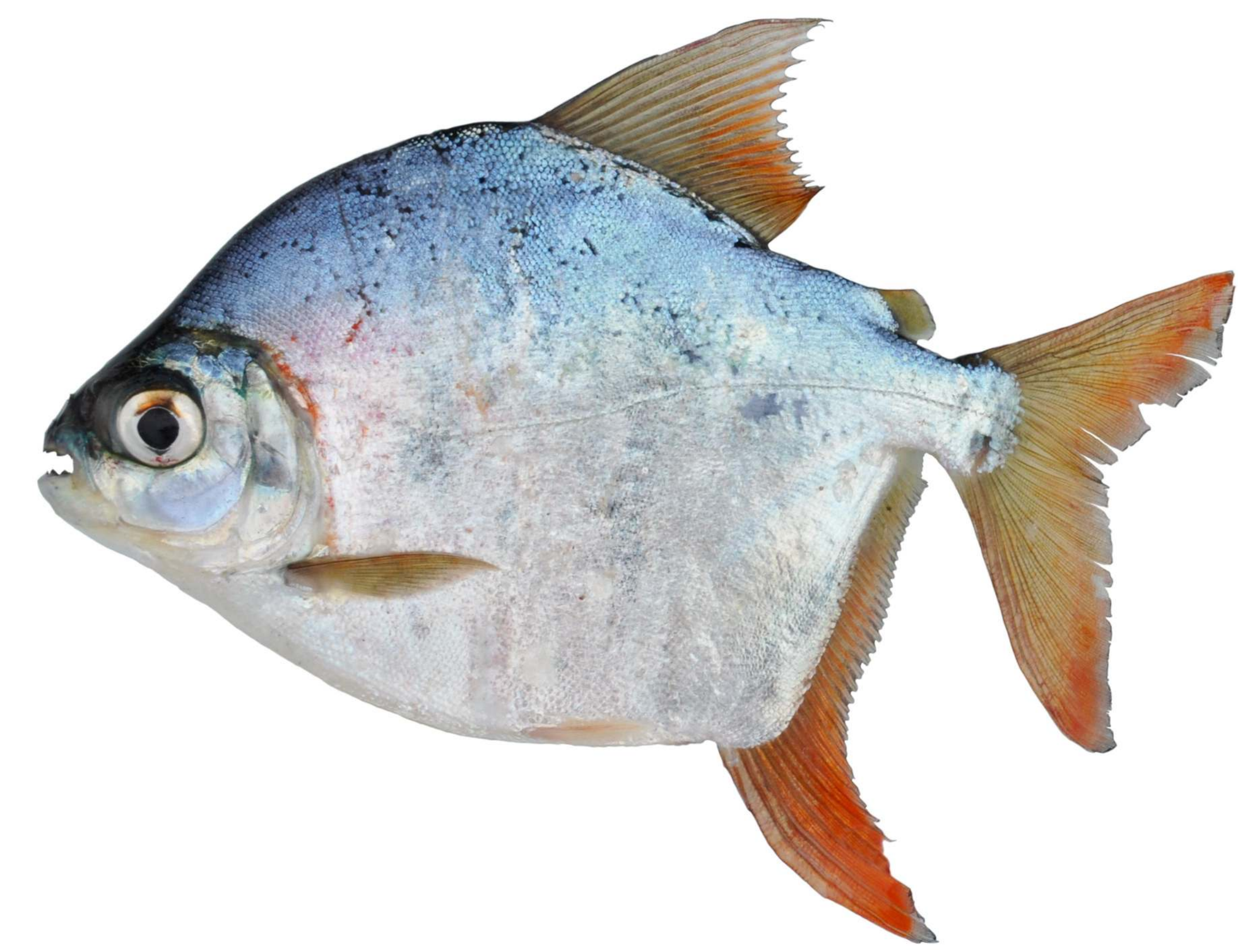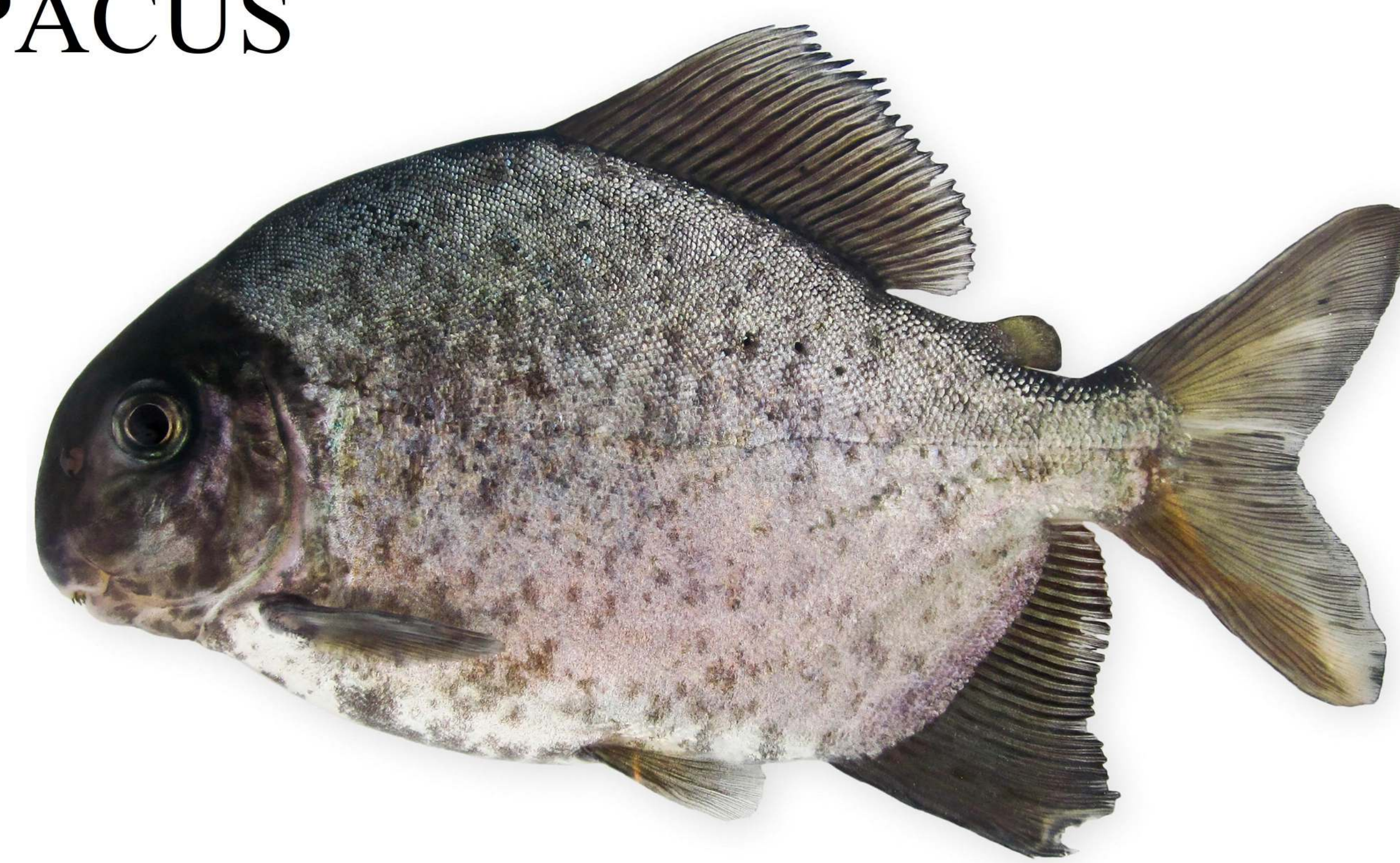*Catoprion*   *Pristobrycon*   *Metynnis*

PACUS

Myleus   *Tometes*   *Ossubtus*

Myloplus schomburgkii   *Myloplus rhomboidalis*   *Acnodon*
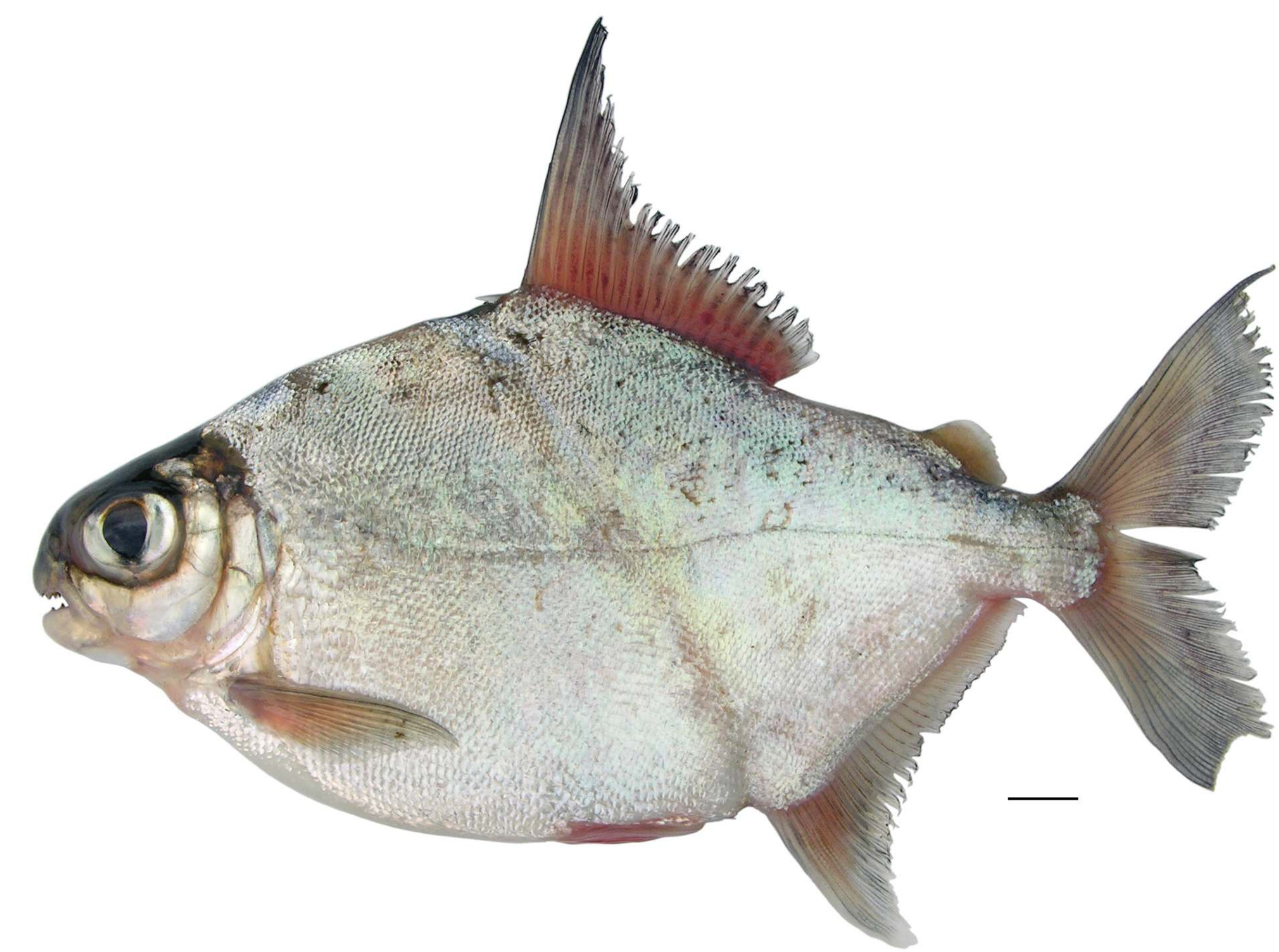
Mylossoma   *Colossoma*   *Piaractus*

Colossominae

Myleinae

Serrasalminae

increasing herbivory

- Fish & Fish Parts (scales, fins & mucus)
- Piscivory (whole fish)
- Omnivory (plants & fish)
- Planktivory
- Plankton & Plant Materials
- Plant Materials & Seeds (granivory)
- Plant Materials, Seeds & Fruit (frugivory)
- Plant Materials, Seeds, & Insects
- Phytophagy

Piaractus mesopotamicus
Piaractus brachypomus
Colossoma macropomum
Mylossoma aureum
Mylossoma albiscopum
Acnodon oligacanthus
Acnodon normani
Myloplus rhomboidalis
Myloplus ternetzi
Utiaritichthys cf longidorsalis
Myloplus cf torquatus
Myloplus rubripinnis
Myloplus asterias
Ossubtus xinguense
Myloplus schomburgkii
Tometes trilobatus
Tometes lebaili
Myloplus arnoldi
Tometes kranponhah
Tometes ancylorhynchus
Mylesinus paucisquamatus
Myleus setiger
Myloplus planquettei
Metynnis lippincottianus
Metynnis guaporensis
Metynnis fasciatus
Metynnis luna
Metynnis altidorsalis
Metynnis cf argenteus
Metynnis maculatus
Metynnis hypsauchen
Metynnis cf longipinnis
Pristobrycon striolatus
Pygopristis denticulata
Catoprion mento
Pygocentrus cariba
Pygocentrus piraya
Pygocentrus nattereri
Serrasalmus manueli
Serrasalmus gouldingi
Pristobrycon careospinus
Serrasalmus eigenmanni
Serrasalmus medinai
Pristobrycon aureus
Pristobrycon calmoni
Serrasalmus brandtii
Serrasalmus cf gibbus
Serrasalmus spilopleura
Serrasalmus maculatus
Serrasalmus irritans
Serrasalmus elongatus
Serrasalmus compressus
Serrasalmus geryi
Serrasalmus altispinis
Serrasalmus rhombeus
Serrasalmus altuvei