

Extreme Ecological Niche Differences Lead to Extreme Postzygotic Ecological Isolation: A Case of Hybridization Between Carnivorous and Herbivorous Cyprinidae Fishes

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Keywords: Hybrid fitness, foraging behavior, feeding habit, ecological niche, morphology

Posted Date: January 13th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-143733/v1>

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Version of Record: A version of this preprint was published at Frontiers in Zoology on April 21st, 2021. See the published version at <https://doi.org/10.1186/s12983-021-00401-4>.

1 Extreme ecological niche differences lead to extreme
2 postzygotic ecological isolation: a case of hybridization
3 between carnivorous and herbivorous Cyprinidae fishes

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19 **Abstract:**

20 **Background:** Postzygote isolation is an important part of species isolation,
21 especially for fish, and it can be divided into two aspects: genetic isolation and
22 ecological isolation. With the increase in parental genetic distance, the intensity
23 of genetic isolation between them also increases. Will the increase in parental
24 ecological niche differences also lead to the increase in ecological isolation
25 intensity between them? This question is difficult to answer based on the current
26 literature due to the lack of hybridization cases of extreme ecological niche
27 parents.

28 **Results:** Cyprinidae fish parents (*Schizothorax wangchiachii* and *Percocypris*
29 *pingi*) with extreme ecological niches (herbivorous and carnivorous) and their
30 F1 hybrids were used as research objects. Fish and periphytic algae were
31 selected as food corresponding to different parental resources. The foraging-
32 related traits of these hybrids are generally the same between parents; however,
33 the intermediate foraging traits of hybrids did not result in intermediate foraging
34 performance for parental resources, and these hybrids could hardly forage for
35 parental resources. The poor foraging performance of these hybrids for parental
36 resources was caused not only by the decline in the foraging ability of these
37 hybrids but, more importantly, by the decrease in foraging activity. Interestingly,
38 these hybrids initially showed a high interest in foraging small fishes; however,
39 after the first successful capture, these hybrids had difficulty ingesting fish and
40 spit them out, which led to the subsequent decrease in foraging activity. We

41 designed a series of experiments to explore the mechanism of the fish spitting
42 of these hybrids, excluding the taste and the size of prey, and found that the
43 decrease in their pharyngeal tooth puncture ability may be the reason.

44 **Conclusions:** This study was the first to demonstrate that these parents with
45 extreme niche differences will produce extreme postzygotic ecological isolation
46 for parental resources. The poor foraging performance of these hybrids for
47 parental resources is mainly due to the decrease in foraging activity.
48 Interestingly, these hybrids have obvious fish-spitting behaviour, which is a
49 typical example of the contradiction between intermediate traits and parental
50 resources.

51 Key-words: Hybrid fitness, foraging behavior, feeding habit, ecological niche,
52 morphology

53

54 **1. Introduction**

55 What are species? The concept of biology defines “species” as populations
56 that can mate with each other and have isolating barriers with other populations,
57 where isolation barriers can be divided into prezygote barriers and postzygote
58 barriers [1]. Many advances have been made in the evaluation of ecologically
59 associated prezygote barriers [2]. Prezygote barriers are often clearly
60 associated with ecological divergence and contribute to isolating barriers, for
61 example, via habitat isolation [3, 4], temporal isolation [5], sexual isolation [6],
62 and mechanical isolation [7]. Postzygote barriers can be divided into two

63 aspects [1]. First, intrinsic or genetic isolation reflects low hybrid fitness due to
64 general genetic incompatibilities between the genomes of divergent
65 populations [8, 9]. Second, extrinsic or ecologically dependent isolation
66 specifically refers to reduced hybrid fitness due to the maladaptive intermediacy
67 of their ecologically relevant genotypes and phenotypes in parental
68 environments [10]. Due to the primitive evolutionary status of fish and in vitro
69 fertilization, more cases of hybridization are observed in fish than in higher
70 vertebrates [11]. Thus, the postzygote isolation is particularly important for
71 fishes. Many studies have demonstrated intrinsic isolation, but the potential
72 ecological contributions to postzygote isolation are also very important [12-15].

73 Regarding ecologically dependent isolation, important examples include an
74 investigation of hybrids between the benthic and the limnetic forms of three-
75 spine stickleback [10, 15]. Both F1 and F2 hybrids grew more poorly in the
76 parental environments than each parent. There are many other similar
77 examples [12, 16-19]. Why are those hybrids at a disadvantage in the parental
78 environments than each parent? Currently, the main explanation is the
79 contradiction between intermediate morphology [10, 15] or kinematics [19] of
80 hybrids between parents and foraging of parent food resources.

81 Regardless of the presence of prezygote barriers or postzygote barriers,
82 their isolation intensity increases with the increase in genetic distance between
83 populations, and the postzygote isolation intensity can be measured by the
84 survival and fertility of hybrids [13, 20]. Here mainly emphasizes the genetic

85 isolation. Then, will the increase in ecological niche differences also lead to the
86 increase in ecological isolation intensity? The current research cannot easily
87 answer this question due to the lack of hybridization cases of extreme
88 ecological niche parents. Therefore, we put forward two hypotheses.
89 Hypothesis 1 is that extreme ecological isolation exists between extreme
90 ecological niche parents. Hypothesis 2, the opposite hypothesis, is that
91 intermediate morphology of hybrids produces intermediate foraging
92 performance for parent resources, regardless of differences in ecological niche
93 between parents.

94 Fortunately, we have recently obtained healthy, morphologically and
95 genetically stable F1 hybrid crosses of carnivorous and herbivorous Cyprinidae
96 fishes (Fig. 1) [21]. Both parents used in this study were cold-water Cyprinidae
97 fishes from the upper Yangtze River basin in the south-eastern Tibetan Plateau,
98 and they have similar breeding periods. *Schizothorax wangchiachii* (SW) has a
99 sharp horny front jaw and mainly scrapes and eats periphytic algae from rocks
100 (Fig. 1 and Supplementary Movie 1). *Percocypris pingi* (PP) is a typical
101 carnivorous fish with a sub-superior mouth (Fig. 1 and Supplementary Movie
102 2). Morphologically, the *Schizothorax* genus and *Percocypris* genus were once
103 thought to belong to two different subfamilies [22]. However, molecularly, they
104 were shown to be sister genera in a recent study [23, 24].

105 To answer the above question, carnivorous fish, herbivorous fish and their
106 F1 hybrids were used to explore the ecological adaptability of the F1 hybrids

107 through comparative behavioural and morphological studies.

108 **2. Materials and methods**

109 2.1 Experimental fish acquisition

110 In March 2017 and 2019, a hybridization experiment and parental
111 reproduction were performed; details on the methods can be found in the
112 literature [21]. Age-two fishes (PP (122.03±1.78 mm, 25.2±1.05 g), SW
113 (106.78±1.41 mm, 18.43±0.74 g) and PS (125.84±2.71 mm, 29.22±1.85 g))
114 were used to quantify both external and skeletal characteristics, and age-one
115 fishes (PP (9.08±0.34 mm, 12.07±0.90 g), SW (9.23±0.14 mm, 13.03±1.50 g)
116 and PS (9.17±0.48 mm, 14.02±3.76 g)) were used to quantify foraging and
117 behavioural features.

118 2.2 Food types

119 We used two types of food corresponding to parent resources. The first type
120 consisted of small fishes, *Sinibrama taeniatus* (0.0507±0.0043) or *Carassius*
121 *auratus* (0.0748±0.0023), the former of which is mainly distributed in the upper
122 Yangtze River and the latter of which is widely distributed. The second type
123 consisted of periphytic algae (Spirogyra, tough population on the pool wall or
124 tender population on stone. Unfortunately, we did not produce the most
125 palatable diatoms for SW in the pond; however, *Schizothorax* fishes, such as
126 SW, still eat a certain amount of spirogyra under natural conditions [25]), it is
127 widely distributed and abundant in China's water system.

128 2.3 Morphology

129 The external morphology of age-two SW (n=30), PP (n=30) and PS (n=30)
130 was studied, and the examination standards are shown in Supplementary Table
131 1. Then, we selected 10 fish individuals for quantification of skeletal morphology.
132 Their opercular bone, pharyngeal bone, dentary bone and skull were obtained
133 by boiling, and the examination standards are described in Supplementary Fig.
134 1. Finally, 19 external morphological indicators and 19 skeletal morphological
135 indicators were quantified in this study, as shown in Supplementary Tables 1-2.
136 To visually show the comprehensive morphological differences between the
137 three fishes, we conducted principal component analysis (PCA) of the Z-scores
138 of three categories of indicators using IBM SPSS Statistics (version 21.0,
139 Armonk, New York, United States) (Supplementary Tables 3-6).

140 The body shapes were photographed using an SLR camera (Canon EOS
141 100D, Japan). The details of the heads fixed by Bouin's fixative and bones were
142 photographed (Fig. 1) by a stereomicroscope (Nikon SMZ25). Age-two PP, SW
143 and PS were scanned (Fig. 2) using a MicroCT Skyscan 1176 (Bruker, Belgium)
144 to obtain the holistic bone structure; specific methods are described in [26], and
145 it were slightly modified in this study.

146 2.4 Comparison of foraging habit

147 We fed PP, SW and PS with small fishes (*S. taeniatus*) and tough periphytic
148 algae on the pool wall (Fig. 3). Specific experimental methods can be found in
149 Supplementary method 1. We compared each fish species' foraging level (FL)
150 using the following formula:

151
$$FL = M2/(M1 - M2)$$

152 where $M1$ represents body weight, and $M2$ represents chyme weight.

153 2.5 Hybrid vs *P. pingi* in foraging fish

154 We compared the foraging capacity of PP (n=15) and PS (n=18) for small
155 fishes (*S. taeniatus*) (Fig. 4a). Specific experimental methods are described in
156 Supplementary method 2. We observed experimental fishes by video and
157 quickly replayed the video and counted the following indicators: first attack time
158 (FAT), first success time (FST), the success rate of the first attack (SRFA), first
159 attack time after the first successful capture (FAT2), attack frequency (AF), the
160 success rate of the total attacks (SRTA), and the spitting rate (SR). Details of
161 these indicators are as follows:

162 FAT: The time when an experimental fish first attacked the small fishes. To
163 exclude the influence of irritability, only the experimental fishes that launched
164 the first attack within 5 min were included in all statistical comparisons.

165 FST: The time when an experimental fish first successfully caught a small
166 fish. If it did not succeed within 30 min, a value of 30 min was used as its first
167 success time.

168 SRFA: The success rate when an experimental fish first attacked the small
169 fishes.

170 FAT2: The time when an experimental fish first attacked after the first
171 successful capture.

172 AF: The average number of attacks per minute of an experimental fish; this

173 value was calculated using the following formula:

$$174 \quad AF = N/T$$

175 SRTA: The success rate of the total attacks; this value was calculated using
176 the following formula:

$$177 \quad SRTA = N'/N$$

178 SR: The spitting rate of fish; some individuals catch fish and then spit them
179 out; this value was calculated using the following formula:

$$180 \quad SR = N''/N'$$

181 where N represents the total number of attacks; T represents the time at the
182 end of the experiment; N' represents the total catch before the end of the
183 experiment (not intake); and N'' represents the number of fish spitted.

184 2.6 Hybrid vs *S. wangchiachii* in foraging periphytic algae

185 We compared the abilities of SW (n=16) and PS (n=20) to forage tender
186 periphytic algae (Fig. 4b). Specific experimental methods are described in
187 Supplementary method 3. We quickly replayed the video and evaluated the
188 following indicators: FAT, AF, FL and foraging efficiency (FE). The details of
189 these indicators are as follows:

190 FAT: The time when an experimental fish first scraped periphytic algae from the
191 rocks.

192 AF: The average number of scrapings per hour of experimental fish; this value
193 was calculated using the following formula:

$$194 \quad TAF = (N2 + N5 + N8)/3$$

195 FE: The average weight of a single scrape of periphytic algae per unit weight
196 of experimental fish; this value was calculated using the following formula:

$$197 \quad EF = M2 / (TAF \times 8 \times (M1 - M2))$$

198 where N_2 , N_5 , and N_8 represent the number of attacks in the second, fifth
199 and eighth hours, respectively, M_1 represents the body weight of the
200 experimental fish; and M_2 represents the chyme weight of the experimental
201 fish.

202 2.7 Assessment of whether the behaviour of hybrid fish spitting fish is persistent

203 In the previous experiments, we observed that PS had obvious behaviour
204 of spitting fish (Fig. 3c and Supplementary Movie 3). This behaviour is very
205 interesting and important, but is it persistent? We set up a feeding experiment
206 using small fish (*C. auratus* (Fig. 5a)) for nine days, and PS still had obvious
207 spiting behaviour after catching the small *C. auratus* fishes (Fig. 5c)). For nine
208 days, we fed not only fish but also blood worms (Fig. 5b, 0.0171 ± 0.0006 ,
209 Chironomidae larvae, a soft-bodied aquatic insect) to simulate a palatable food
210 shortage, but not a complete absence, in the natural environment. Specific
211 experimental methods are described in Supplementary method 4. We counted
212 the daily catch, intake, and spitting of each PS for small fish.

213 2.8 Mechanism explaining why hybrid fish spitted fish

214 Two mechanisms may explain why PS spitted small fish: the small fish
215 tasted bad or they were difficult to chew. To explore this mechanism, we
216 selected approximately 50 g of *C. carp* (Fig. 6a) and cut the back muscle into

217 small pieces (Fig. 6b) without bone, instead of using small fish. We took PS that
218 had the obvious behaviour of spitting small fish in the last experiment as the
219 experimental fishes (n=7). Other than the small fishes that were replaced with
220 small pieces of *C. carp* muscle, the other feeding and statistical schemes were
221 the same as those in Section 2.7. However, the experiment lasted only three
222 days. We counted the average number of daily foraging (ANDF) and the SR of
223 the 7 experimental fishes used in Section 2.7 and this experiment, which was
224 equivalent to the former serving as a control group for the latter, by the following
225 formulas:

$$226 \quad \quad \quad ANDF = N/T$$

227 where N represents the total number of prey captured by PS during the
228 experiment, and T represents the number of days of the experiment.

229 Next, to investigate whether prey size also leads to fish-spitting behaviour
230 in PS, the SR of PS to different sizes of meat and fish was quantified. The
231 specific experimental methods are described in Supplementary method 5.

232 Then, we compared the pharyngeal teeth details of PP, SW and PS and
233 quantified the maximum opening distance between their pharyngeal teeth and
234 their puncture ability based on the following principle: for the same pressure
235 and a smaller force area, the greater the pressure. We quantified the following
236 indicators: the maximum opening width between pharyngeal teeth (MOWPT),
237 the development degree of hook pharyngeal teeth (DDHPT) and the grinding
238 surface area of pharyngeal teeth (GSAPH); these values were calculated using

239 the following formulas:

$$240 \quad MOWPT = TW/HW$$

$$241 \quad DDHPT = TL'/TL$$

$$242 \quad GSAPH = S'/S$$

243 where TW represents the maximum width distance between pharyngeal teeth,
244 HW represents head width; T represents average length of 5 lateral
245 pharyngeal teeth, TL' represents the average length of the hooked portion at
246 the tip of the lateral 5 pharyngeal teeth; and S represents the basal area of all
247 pharyngeal teeth, S' represents the grinding surface area of all pharyngeal
248 teeth. Further information on these parameters is provided in Supplementary
249 figure 2.

250 We quantified the foraging-related traits (Supplementary Table 7, 20
251 measured traits and 17 standardized traits) of all fishes (n=32) in Section 2.7 to
252 explore whether a correlation exists between these traits, and these indicators
253 included the TNC (total number of captures), TNI (total number of ingestions),
254 TNSF (total number of spitting fish) and SR by Spearman's correlation in SPSS
255 21.0.

256 2.8 Statistical analyses

257 The mean \pm standard deviation (SD) was used to represent the
258 unannotated quantitative data, and the other data are annotated in the table or
259 graph notes. One-way analysis of variance (ANOVA) was used to analyse the
260 data of three independent experiments. Spearman's correlation method was

261 used to analyse the correlation. All the data obtained above were measured
262 and calculated using SPSS software version 19. Tukey's test was used to
263 analyse the difference. All graphs were generated by the Origin software
264 version 2019b.

265

266 **3. RESULTS**

267 3.1 Morphology

268 Regarding the external and skeletal morphology, most PS traits were
269 between PP and SW. Direct observation, Tukey's test or PCA all supported the
270 above data (Figs. 1-2, Supplementary Tables 2). It is worth mentioning that the
271 tail length of PS is longer than that of the parents, which is the main reason why
272 PCA2 of PS is different from that of the parents in the PCA of external
273 morphology (Supplementary Tables 2 and 4). Specific morphological
274 descriptions are provided in Supplementary result 1.

275 3.2 Comparison of foraging habits

276 In Fig. 3, for small fish, the FL of PP was extremely significantly ($P < 0.01$)
277 higher than those of SW and PS. The latter two ingested very few small fishes,
278 and no significant difference ($P \geq 0.05$) was found between them. For tough
279 periphytic algae, the FL of SW was extremely significantly ($P < 0.01$) higher
280 than those of PP and PS. PP did not ingest periphytic algae, and some PS
281 individuals may have ingested a small amount of periphytic algae; however, no
282 significant difference was observed between them ($P \geq 0.05$). The FL of

283 periphytic algae (spirogyra) in SW was relatively low, probably because it was
284 not the most suitable periphytic algae for SW; however, the FL of SW on
285 spirogyra was still extremely significantly higher than that of PP or PS (Figs. 3e
286 and 4e).

287 Interestingly, we found a large amount of small fish debris in the PS
288 aquarium tank (Fig. 3c), while little debris was noted in the tanks with SW and
289 PP, suggesting that one of the reasons why fish intake by PS intake was low
290 was spitting of fish.

291 3.3 Hybrid vs parents in foraging fish or periphytic algae

292 In the PS vs SW experiment of foraging periphytic algae, the FAT of PS was
293 extremely significantly higher ($P < 0.01$) than that of SW (Fig. 4c). The reason
294 why the data presented double peaks may be due to the individual differences
295 in periphytic algae foraging of PS, i.e., either they were interested at the
296 beginning or not interested at all. The AF of PS was significantly lower ($P = 0.02$)
297 than that of SW (Fig. 4d), the FL was extremely significantly lower ($P = 0.002$)
298 than that of SW (Fig. 4e), and the FE was significantly lower ($P = 0.037$) than
299 that of SW (Fig. 4f). In summary, PS showed low interest in foraging for
300 periphytic algae and had low foraging efficiency.

301 In the PS vs PP experiment for foraging fish, the SRFA ($P = 0.219$) and SRTA
302 ($P = 0.167$) of PS were not significantly different from those of PP; the SR of PS
303 was extremely significantly higher ($P < 0.01$) than that of PP (Fig. 4g); the FAT
304 ($P = 0.459$) and the FST ($P = 0.161$) of PS were not significantly different from

305 those of PP; the FAT2 was extremely significantly higher ($P<0.01$) than that of
306 PP (Fig. 4h); and the AF of PS was extremely significantly lower ($P<0.01$) than
307 that of PP (Fig. 4i). In summary, PS showed greater interest in first foraging for
308 fish but had a high SR, which caused PS to be negative in later predation.

309 3.4 Whether the behaviour of hybrid fish spitting fish is persistent

310 As shown in Fig. 5, at the beginning of the experiment, most PS had the
311 behaviours of catching, spitting and ingesting small fish. However, as the
312 experiment proceeded, the number of PS with these behaviours decreased,
313 and only a few fish retained these persistent behaviours by the end of the
314 experiment (Figs. 5d, 5e, 6f and 5j); thus, this pattern was the main reason for
315 the decline in the average number of daily captures, spitting and ingestion (Figs.
316 5g, 5h and 5i). In summary, the behaviours of catching, spitting and ingesting
317 small fish by most PS were not persistent.

318 3.5 Mechanism of fish spitting by hybrid fish

319 No significant difference ($P=0.702$) was found between the ANDF of fish
320 meat and small fish in the individuals exhibiting persistent capture behaviours
321 (Fig. 6i). However, the SR of fish meat was significantly lower ($P<0.01$) than
322 that of small fish (Fig. 6j), suggesting that the spitting behaviour was not caused
323 by bad taste but by chewing difficulty, which may be caused by pharyngeal tooth
324 structure, prey size, and the maximum opening width between pharyngeal teeth.
325 Therefore, the SR of PS to different sizes of meat and fish was quantified. The
326 small fish weighed the same as small-sized meat, and the medium fish weighed

327 the same as medium-sized meat. The results showed that PS did not spit on
328 large, medium and small meat and had a low SR for small fish but a high SR
329 for medium fish (Fig. 6k).

330 Next, the details of the pharyngeal teeth were compared, and we found that
331 the pharyngeal bone of PP was long and narrow, with widely spaced well-
332 developed conical hooked pharyngeal teeth, and the space was larger between
333 the two pharyngeal bones in the closed mouth. These features are useful for
334 piercing and hooking prey. In contrast, the pharyngeal bone of SW was short
335 and thick, with closely spaced grinding pharyngeal teeth, which were curved
336 and flat at the top, forming a grinding surface, and the space was smaller
337 between the two pharyngeal bones in the closed mouth. These features are
338 useful for grinding periphytic algae. The morphology of the pharyngeal bone in
339 PS was balanced between that of the parents, and it had hooked grinding
340 pharyngeal teeth, which were also intermediate between the parents.

341 The quantitative results support the above morphological description. The
342 MOWPT of PP was extremely significantly higher ($P<0.01$) than that of SW and
343 PS, and no significant difference was observed between them ($P\geq 0.05$, Fig.
344 6l). The DDHPT of PP was extremely significantly higher ($P<0.01$) than that of
345 SW and PS, and PS was extremely significantly higher ($P<0.01$) than SW (Fig.
346 6m). The GSAPH of SW was extremely significantly higher ($P<0.01$) than that
347 of PP and PS, and PS was extremely significantly higher ($P<0.01$) than PP (Fig.
348 6n).

349 Specific correlation analysis descriptions can be found in Supplementary
350 result 2. The results of the correlation analysis can be summarized as follows:
351 (1) the larger the PS was, the more fishes it caught. (2) With the increase in
352 capture number, both the ingestion and the spitting increased; the latter
353 increased more, which indicated that more fishes were spitted. (3) The captured
354 fishes can be ingested or spitted, so there was a negative correlation between
355 the ingestion and spitting rate. (4) The spitting rate did not vary with the capture
356 number. (5) The spitting rate of PS was not correlated with the size and shape
357 of its quantified traits.

358 **4. Discussion**

359 4.1 Intermediate morphology of hybrid fish

360 Morphology is often determined by quantitative traits, therefore the
361 morphology of F1 hybrids is general between parents [1, 15]. In this study, PP
362 and SW had disparate feeding habits and foraging traits, and most food habit-
363 related quantitative traits of PS were between parents, but there were also a
364 few superparent traits, such as the longer tail length of PS (Supplementary
365 Table 2). Interestingly, for PP and SW, the sharp horny front jaw is an invisible
366 trait, which is not exhibited by PS (Fig. 1). In addition, our quantitative analysis
367 screened out a large number of food habit-related traits, which provided a
368 reference for subsequent food habit-related morphological studies of other
369 fishes (Supplementary Tables 1-6).

370 4.2 Enhancement of postzygotic ecological Isolation of parents with extreme

371 ecological niches

372 Hybridization generally occurs between closely related sympatric species,
373 and they generally have ecological niche differentiation and adaptive traits [1,
374 27, 28], which leads to hybrids with intermediate traits that cannot well adapt to
375 the ecological niche of the parents [10, 15]. In previous studies, the ingestion
376 of parental resources by hybrids or their growth performance in the parental
377 environment was generally the mean of both parents [10, 12, 15, 18], which
378 meant the ecological isolation between them was not that great. However, in
379 the above studies, there were no hybrid cases of extreme ecological niche
380 parents, such as carnivorous and phytophagous individuals. As described in
381 the introduction, an increase in genetic distance between parents strengthens
382 intrinsic isolation; therefore, will the increase in ecological niche differences also
383 lead to the increase in ecological isolation intensity?

384 Our results support hypothesis 1, namely, the intermediate foraging
385 morphology of PS did not result in intermediate foraging performance for
386 parental resources, and PS could hardly forage for parental resources. A similar
387 example has been found in natural hybridization of sunfishes, as hybrid
388 individuals exhibited kinematics intermediate between those of the two parental
389 species. However, performance assays indicated that hybrids display
390 performance most similar to that of the worse-performing species for a given
391 parental resource [19]. The difference is that in our research, the poor foraging
392 performance of PS for parental resources was caused not only by the decline

393 in PS foraging ability but, more importantly, by the decrease in foraging activity.

394 The food habit of a species depends not only on heredity and environment,
395 but also experience [29-34]. PS showed less interest in foraging for periphytic
396 algae from the beginning of the experiment, which may be innate. Interestingly,
397 however, PS showed interest in foraging small fishes at the beginning of the
398 experiment, while after the first successful capture, PS had difficulty ingesting
399 the fish, which led to the subsequent decrease in foraging activity. This result
400 may be experiential.

401 4.3 Mechanism of hybrid fish spitting fish

402 The behaviour of PS spitting fish is one of the highlights of this study. Two
403 mechanisms may explain why PS spitted small fish: the small fish tasted bad
404 or were difficult to chew. When fed with bone-free meat, there was almost no
405 spitting behaviour of PS, which invalidated the first hypothesis. Chewing
406 difficulties may be caused by two factors, namely, the prey size is too large or
407 there is a defect in their own traits. No spitting was found when PS was fed
408 different sizes of meat, but when PS was fed medium fish with the same weight
409 as medium meat, they still had a higher spitting rate. However, when PS was
410 fed small fish with the same weight as that of small meat, their spitting rate
411 decreased significantly. In summary, for easy-to-chew meat, regardless of its
412 size, PS will not spit it out; however, for difficult-to-chew fish, PS can only ingest
413 smaller individuals that are easy to chew, indicating that the mechanism of fish
414 spitting in PS may be related to defects in its chewing function.

415 Therefore, we quantified the foraging-related traits of 32 fishes in Section
416 2.7 to explore whether a correlation exists between these traits and the spitting
417 rate. Unfortunately, we did not find any correlation between any trait and the
418 spitting rate, indicating that other non-self factors may also affect the spitting
419 rate, such as the size of food. Regrettably, in Section 2.7, we did not realize that
420 we should subdivide the size of the fish food, as subsequent supplementary
421 experiments proved that it could indeed affect the spitting rate. Interestingly, in
422 PS, the spitting rate did not vary with the total capture number, indicating that
423 regardless of how strong or weak the PS were in predation, they had a similar
424 and weaker ability to ingest small fish, which also reflected the defects of their
425 chewing function. In summary, the above analysis suggested that the
426 mechanism of fish spitting of PS was not related to its own differential traits,
427 and further comparison with parental traits is needed.

428 Therefore, the structure of PS and parental pharyngeal teeth was further
429 quantified. The function of the pharyngeal teeth of carnivorous Cyprinidae
430 fishes is to puncture food [35], similar to canine teeth, which is reflected in the
431 PP. In contrast, the function of the pharyngeal teeth of herbivorous Cyprinidae
432 fishes is to grind food [35], similar to cheek teeth, which is reflected in SW.
433 Regarding pharyngeal tooth puncture ability, that of PS was between that of the
434 parents but not as good as that of PP, indicating that PS may not reach the
435 threshold of puncture fish. In addition, regarding the maximum width of
436 pharyngeal teeth, that of PS was close to that of SW but significantly smaller

437 than that of PP, suggesting that PS can only chew smaller prey than PP. In
438 conclusion, the difficulty of ingesting small fish by PS may be due to the
439 intermediate pharyngeal tooth traits, which do not effectively enable puncture
440 of fish.

441 **5. Conclusion**

442 Will extreme ecological niche differences between parents lead to extreme
443 ecological isolation? This study preliminarily answered this question by
444 comparing the foraging level and foraging behaviour of carnivorous PP,
445 herbivorous SW and their hybrid (PS) associated with parent resources. The
446 external morphology and skeletal morphology of PS were between those of the
447 parents, but the intermediate foraging morphology of PS was not associated
448 with intermediate foraging performance for parental resources, and PS could
449 hardly forage for parental resources. The poor foraging performance of PS for
450 parental resources was caused not only by the decline in PS foraging ability but,
451 more importantly, by the decrease in foraging activity. Interestingly, PS has
452 obvious fish-spitting behaviour, which is a typical example of the contradiction
453 between intermediate traits and parental resources.

454 **Ethics approval and consent to participate**

455 The authors claim that none of the material in the paper has been published
456 or is under consideration for publication elsewhere. The submission is original,
457 and all authors are aware of the submission and agree to its publication in
458 *Frontiers in Zoology*. We declare that there is no conflict of interests regarding
459 the publication of this paper.

460 **Consent for publication**

461 Written informed consent for publication was obtained from all participants.

462 **Availability of data and materials**

463 The datasets used or analysed during the current study are available from
464 the corresponding author on reasonable request.

465 **Competing interests**

466 All authors declare that they have no competing interests.

467 **Funding**

468 This work was funded by the Chongqing graduate scientific research
469 innovation Project (CYB20092) and open project of Key Laboratory of
470 Freshwater Fish Reproduction and Development (Ministry of Education).

471 **Acknowledgements**

472 Thanks to all the authors for contributing to this article.

473 **Authors' contributions**

474 H.R. Gu and Z.J. Wang conceived the ideas and designed the methodology;
475 H.Y. Wang and Y.F. Wang made the experimental design and work in the
476 subsequent supplementary experiment. Y. He took the microCT images; S.H.

477 Deng, X.H. He, Y. Wu, K.Y. Xing and X. Gao contributed to the breeding and
478 feeding of the experimental fishes used in this experiment; H.R. Gu completed
479 all the experiments and data processing and analysis in this study; X.F. He
480 provided constructive guidance to H.R. Gu in morphology; H.R. Gu and Z.J.
481 Wang led the writing of the manuscript. All authors contributed critically to the
482 drafts and provided final approval for publication.
483

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569

570 **Figure legends**

571 **Figure 1.** External characters comparison. (a) The full view of PP. (b-d) The
572 head characters of PP. The full view of SW. (f-h) The head characters of SW. (i)
573 The full view of PS) (j-i) The head characters of SP. (m) The PCA of external
574 characters. The white scale is 1 mm; the black scale is 10 mm. We have used
575 some figures in previous articles (Gu et al. 2019), including (a), (c), (d), (e), (g),
576 (h), (i), (k) and (l).

577 **Figure 2.** Osteal characters comparison. (a-c) The MicroCT image of head
578 characters of PP. (d) The pharyngeal bone of PP. (e-g) The MicroCT image of
579 head characters of SW. (h) The pharyngeal bone of SW. (i-k) The MicroCT
580 image of side head of PS. (l) The pharyngeal bone of SP. (m) The PCA of osteal
581 characters. The scale of MicroCT images is 6 mm, and the scale of pharyngeal
582 bones is 1mm.

583 **Figure 3.** Comparison of foraging habit. (a) Small fish (*S. taeniatus*). (b) Tough
584 periphytic algae (Spirogyra). (c) Small fish debris. (d) The FL (foraging level) of
585 small fishes among PP, SW and PS. (e) The FL (foraging level) of tough
586 periphytic algae among PP, SW and PS. The scale of all figures is 1 mm. The
587 different ** above the boxes differ significantly at $P < 0.01$ based on Tukey test,
588 the height give the mean, the thick lines give the medians, and whiskers
589 indicate mean \pm SE.

590 **Figure 4.** Hybrid vs parents in foraging little fish or periphytic algae. (a) Small
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593 PS vs SW in the foraging level. (f) PS vs SW in the FE (foraging efficiency). (g)
594 PS vs PP in the SRFA (success rate of the first attack), SRTA (success rate of
595 the total attacks) and SR (spitting rate). (h) PS vs PP in the FAT, FST (first
596 success time) and FAT2 (first attack time after the first successful capture). (i)
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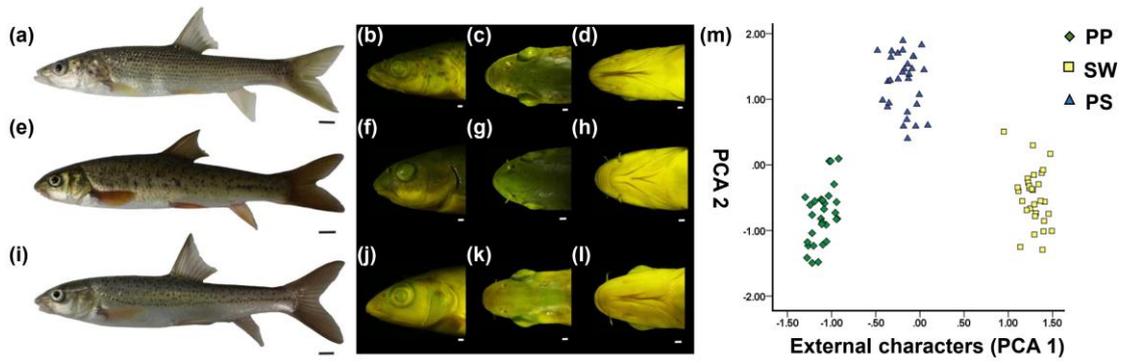
600 **Figure 5.** The changes of the related indicators of foraging fish in hybrid fish
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602 (c) Small fish debris. (d) The trends of captures of every SP with time. (e) The
603 trends of ingestion of every SP with time. (f) The trends of spitting of every SP
604 with time. (g) The mean trend of captures of SP with time. (h) The mean trend
605 of ingestion of SP with time. (i) The mean trend of spitting of SP with time. (j)
606 The trends in the number of SP involved in capture, ingestion and spitting. In
607 (d), (e) and (f), each line represents an individual. The scale of all figures is 1
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610 **Figure 6.** Mechanism of hybrid fish spitting small fish. (a) Small fish (*C. auratus*).
611 (b) A small piece muscle in the back of *C. auratus*. (c-e) The MicroCT image of
612 pharyngeal bones of PP, SW and PS. (f-h) The detail image of grinding surface
613 of pharyngeal bones of PP, SW and PS. (i) The average number of daily

614 foraging (ANDF) for small fish or meat by these SP with a persistent spitting-
615 fish behavior. (j) Compare the spitting rate (SR) of SP between foraging small
616 fish and meat. (k) Compare the SR of SP between foraging S fish (small fish,
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618 0.10 ± 0.01 g), M meat (medium meat, 0.24 ± 0.01 g), B meat (big meat,
619 0.50 ± 0.05 g). (l) The relative maximum opening width between pharyngeal
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621 (DDHPT). (n) The relative grinding surface area of pharyngeal teeth (GSAPH).
622 The scale in (a) is 1 mm, in (b), the meat is 1 mm and the fish is 10 mm, in (c-
623 e) is 0.5 mm, in (f-h) is 2 mm. The different ** above the boxes differ significantly
624 at $P < 0.01$ based on Tukey test, the height give the mean, the thick lines give
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626

627 **Figures**

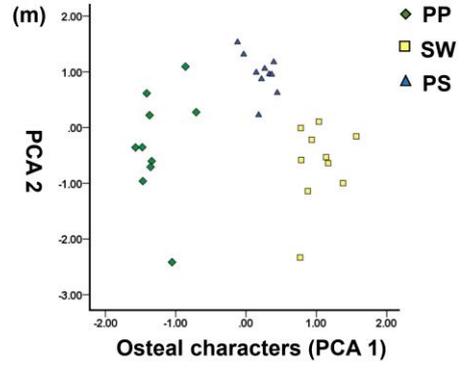
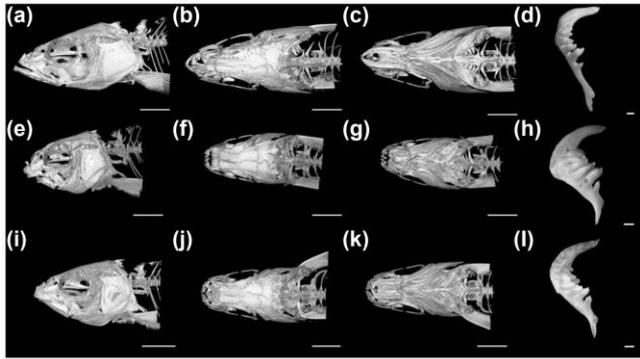
628 **Fig. 1**



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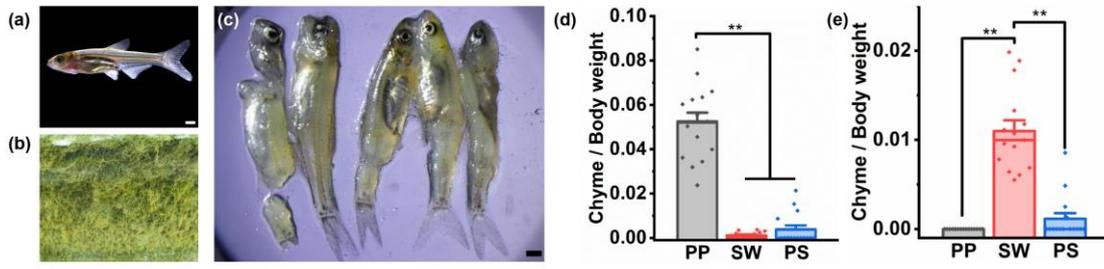
631 **Fig. 2**



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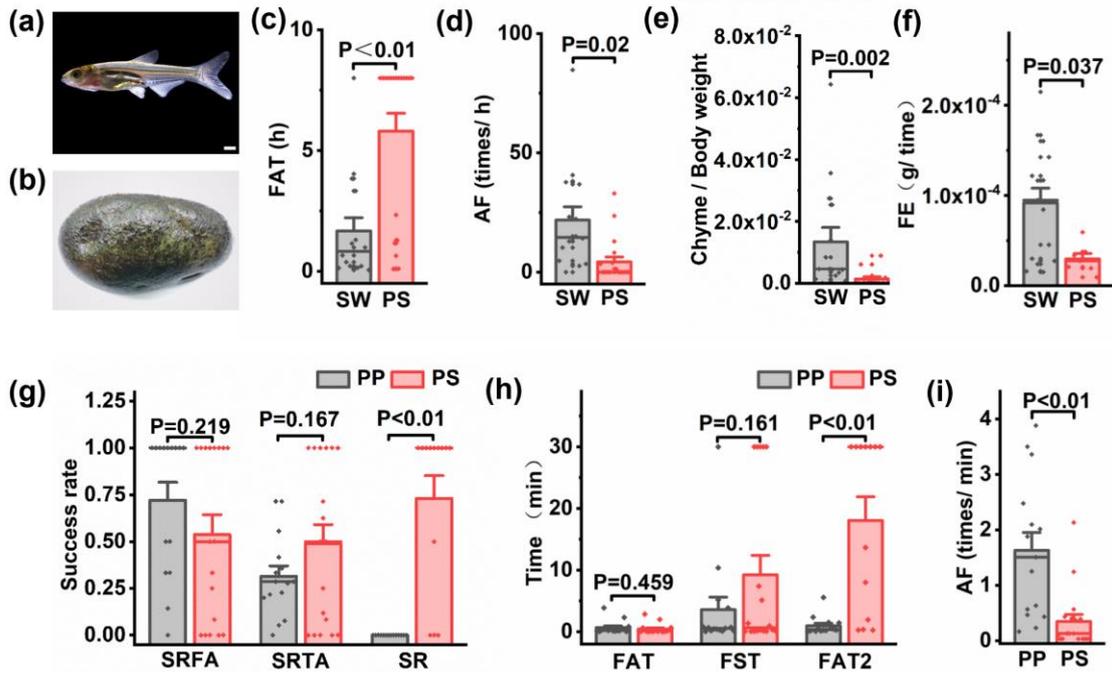
634 **Fig. 3**



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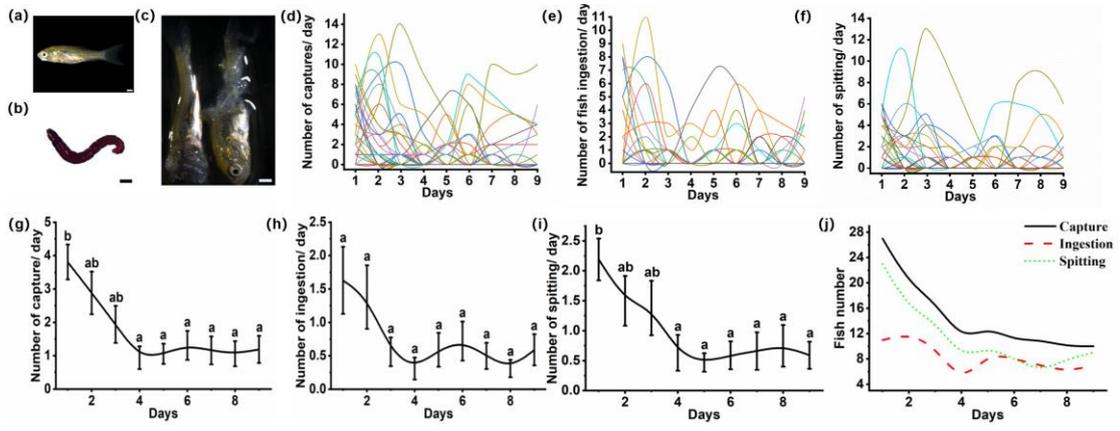
637 **Fig. 4**



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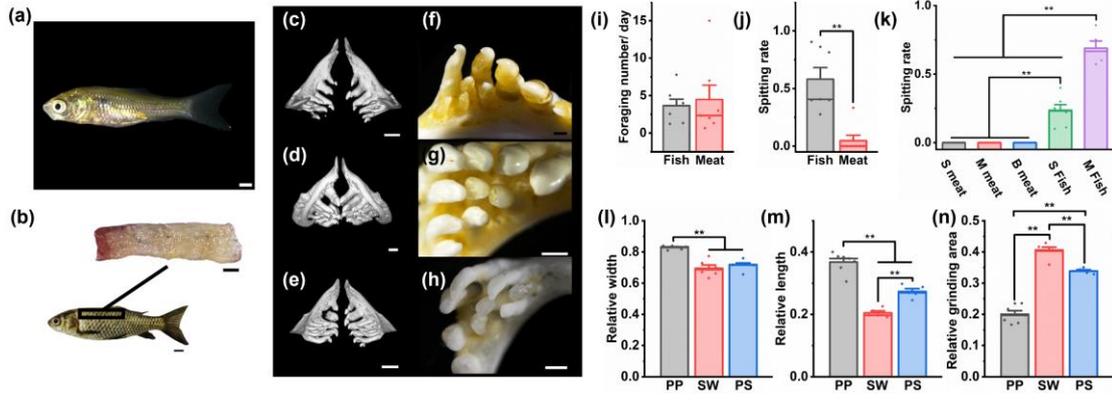
640 **Fig. 5**



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643 **Fig. 6**



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Figures

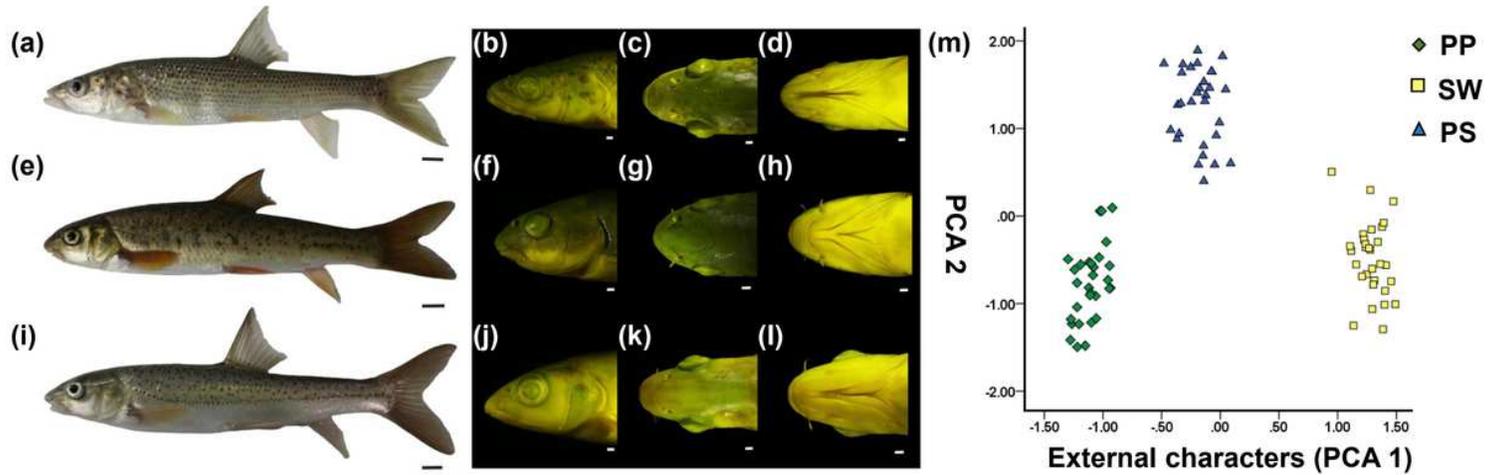


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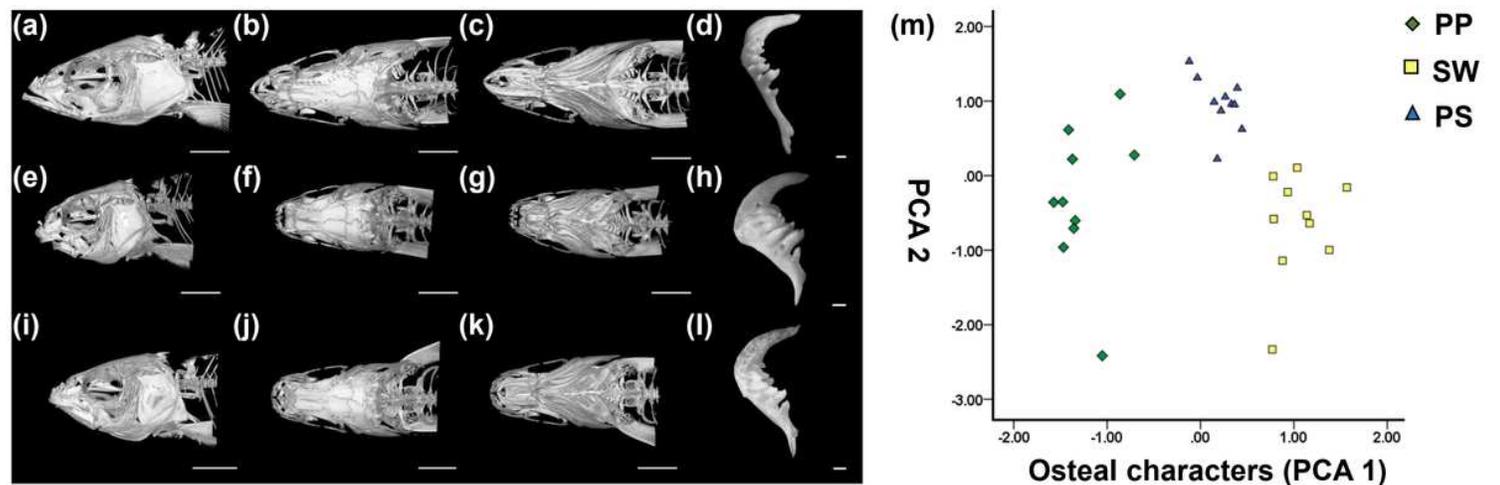


Figure 2

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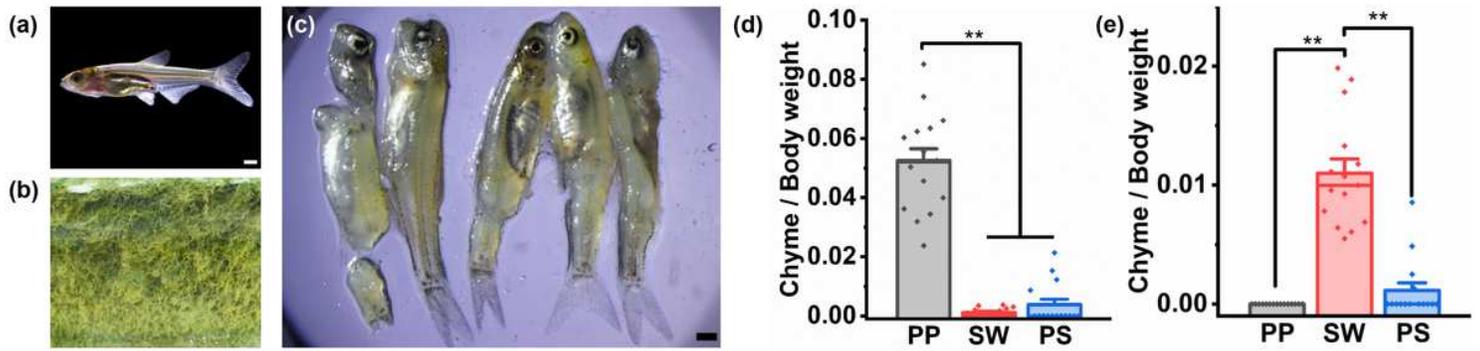


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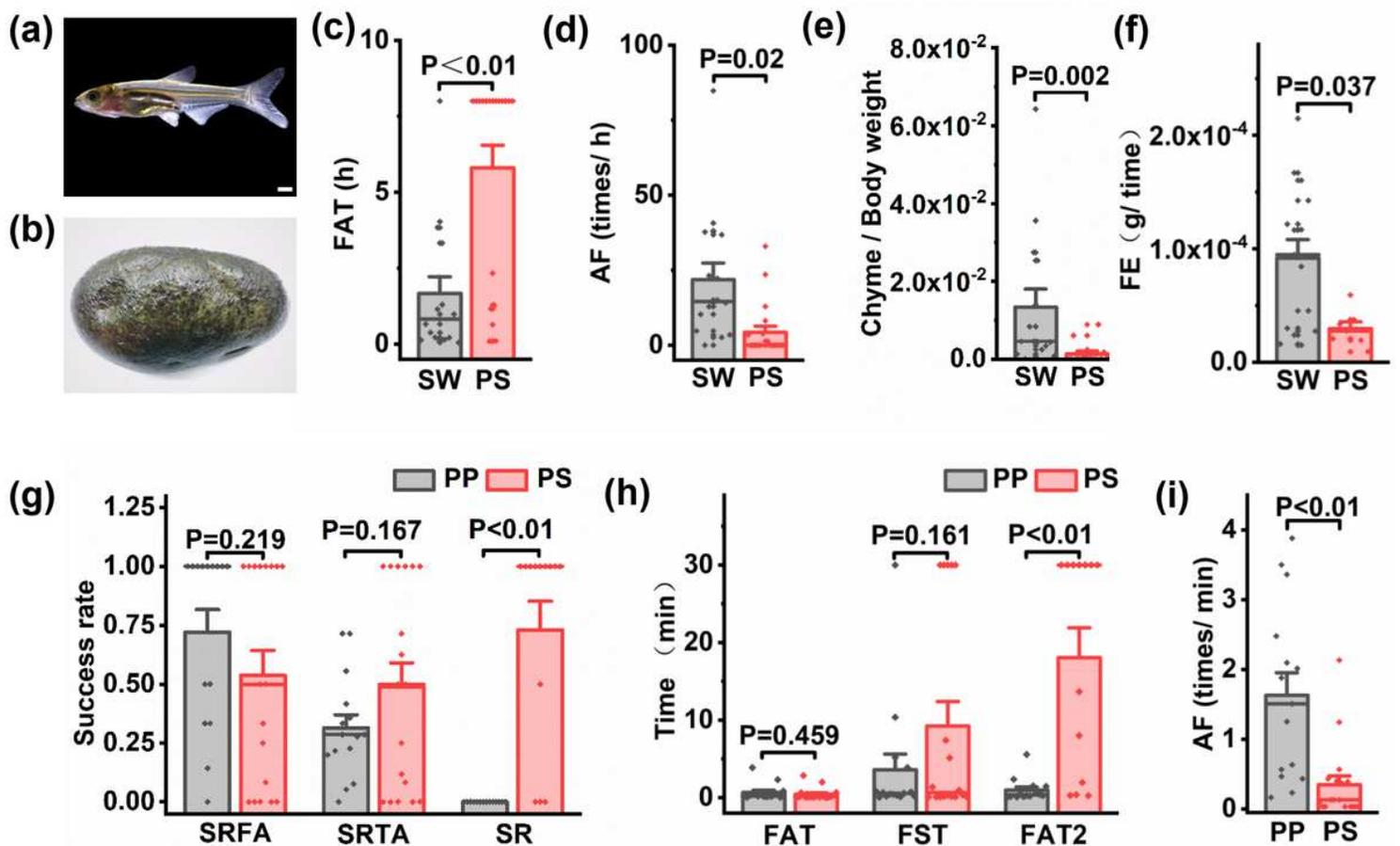


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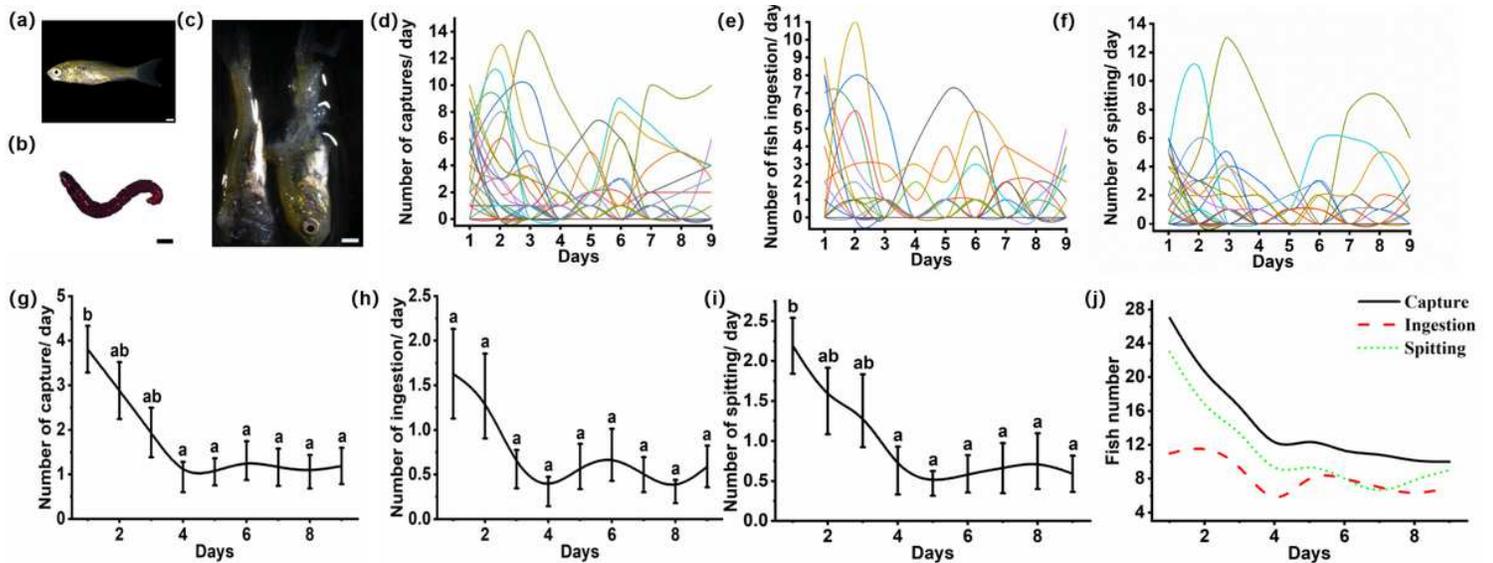


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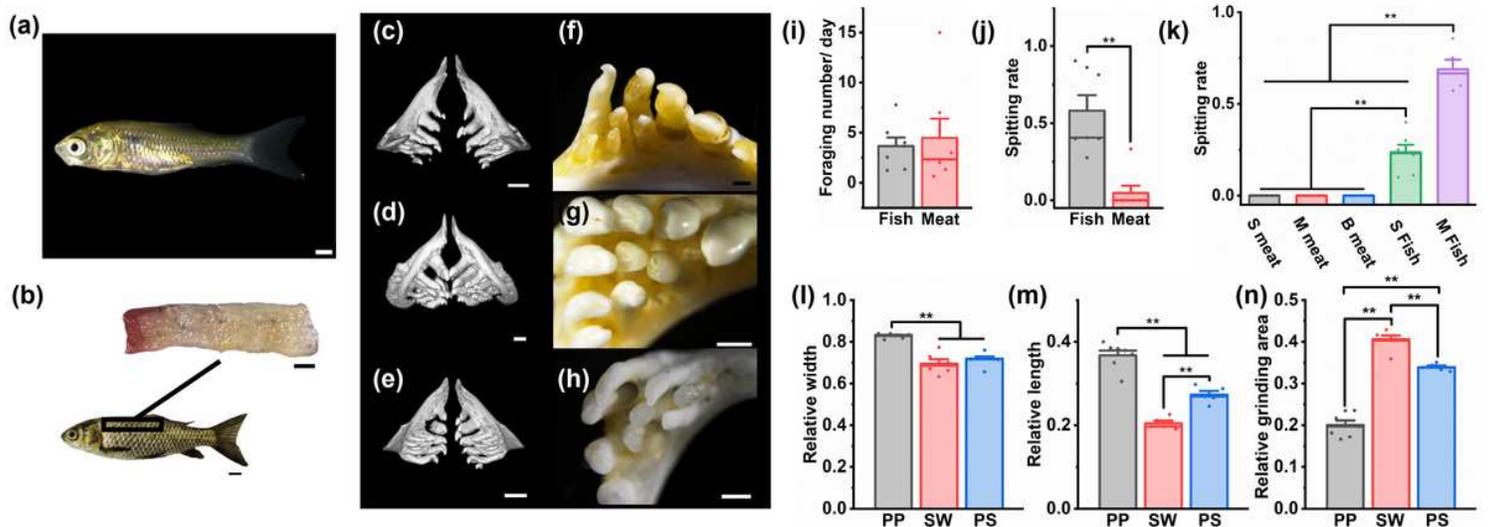


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