Phylogenetically conserved peritoneal fibrosis response to an immunologic adjuvant in ray-finned fishes

Running title: Peritoneal fibrosis in fish

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ABSTRACT

Maintenance of body homeostasis and protection from infection are fundamental to survival. While recognition of self and non-self appeared early in the evolution of metazoans, immunity remains one of the fastest evolving traits to keep up with dynamic challenges from parasites. The immune system thus intertwines ancient innate immune pathways with recently evolved adaptive pattern-recognition units. Here, we focus on peritoneal fibrosis, an effective, yet costly, defense to eliminate infection by a specialist tapeworm parasite, *Schistocephalus solidus* (Cestoda), observed in only some populations of threespine stickleback (*Gasterosteus aculeatus*, Perciformes). We asked whether stickleback fibrosis is a derived species-specific trait or an ancestral immune response that was widely distributed across ray-finned fish (Actinopterygii). First, we reviewed literature published on fibrosis in fish in general and found that peritoneal fibrosis specifically is very rarely reported in ray-finned fish. Then, we experimentally tested for peritoneal fibrosis with parasite-specific and non-specific immune challenges in deliberately selected species across fish tree of life. Peritoneal injection with a common non-specific vaccination adjuvant (Alum) showed that most of the tested species were capable to develop fibrosis. On the other hand, the species were largely indifferent to the tapeworm antigen homogenate. One specific fish clade – Characidae - did not respond to any of the treatments. We therefore show that despite being rarely reported in the literature, peritoneal fibrosis is a common and deeply conserved fish response to a non-specific immune challenge. We outline directions for further research on mechanisms and evolution of peritoneal fibrosis in fish, and also discuss new perspective on peritoneal fibrotic pathology in human patients.

IMPACT STATEMENT

Immunity is a crucial and rapidly evolving system due to the coevolutionary arms race between pathogens and their hosts. Yet, many key features of the vertebrate immune system are ancient. This apparent contradiction raises a key question: are immune functions widely similar among animals, or rapidly evolving to each populations' needs? To address this question, we used experimental immune challenges to evaluate an immune response (peritoneal fibrosis) in phylogenetically diverse set of fish species. Peritoneal fibrosis can be a major form of pathology in humans as well. In some populations of threespine stickleback, peritoneal fibrosis is induced by cestode infection (or injection of cestode proteins or alum adjuvant), and serves to limit cestode growth. First, we performed a comprehensive literature search and show that peritoneal fibrosis has not been widely documented previously in other fish species. We then experimentally tested the ability of 17 species, drawn from across the fish tree of life, to initiate the peritoneal fibrosis response to artificial immune challenges. Our results show that the peritoneal fibrosis response towards a general immune challenge (Alum adjuvant injection) is an ancestral trait in fish, but has been lost entirely in some clades. In contrast, only few species initiated fibrosis when exposed to protein from a tapeworm specialized to infect stickleback. Our comparative experiment thus brings new insights into the evolution of peritoneal fibrosis in fish, showing that it is a common response to a non-specific cue, which can be employed in responding to species-specific parasites.

INTRODUCTION

The comparative immunology research available to date makes it clear that many of the fastest-evolving and most polymorphic genes in vertebrates are involved in immunity (Litman and Cooper 2007). Most notable is the diversity and evolutionary reshuffling of the genes coding Toll-like receptors (TLR) (Solbakken et al. 2017; Velová et al. 2018), or proteins of the major histocompatibility complex (MHC) (Roth et al. 2020). Conversely, the broad outlines of innate immunity are ancient, such as one of the most ancestral immune cytokines, transforming growth factor β (TGF-β), which seems to be conserved across the animal kingdom (Herpin et al. 2004). And yet, even some highly conserved immune genes and processes have been lost or changed past recognition in certain vertebrate clades, such as the loss of MHCII genes in the Atlantic cod (Malmstrøm et al. 2013). This contrast between deep evolutionary conservation, and rapid co-evolutionary dynamics, is puzzling. What features of the immune system are highly conserved, and what are evolutionarily labile? Here, we measure the extent of evolutionary conservation of a key immune phenotype, fibrosis.

Vertebrates possess, in principle, two functionally distinct strategies combining innate and adaptive immunity to cope with infection according to parasite type (Flajnik and Du Pasquier 2004; Allen and Maizels 2011). Type 1 immune response is triggered by fast reproducing pathogens, as microbes, with the aim to quickly eliminate the infection through pro-inflammatory trajectory (Allen and Maizels 2011). On the other hand, type 2 immune response is typically directed to reduce the effect of a multicellular parasite, such as a helminth worm, by containment and encapsulation (Allen and Maizels 2011; Gause et al. 2013). Type 2 immunity largely shares signaling pathways with tissue repair and wound healing (Gause et al. 2013; Thannickal et al. 2014). Perpetual tissue damage and wound healing may, however, result in excessive accumulation of fibrous connective matter called

fibrosis (Wynn and Ramalingam 2012; Thannickal et al. 2014). This fibrosis has been found to effectively suppress growth of certain parasites, or even lead to parasite death (Weber et al., in preparation). However, the benefits of tissue repair and parasite containment can come with a cost from chronic type 2 immune response during persistent or recurrent infections, which may develop into serious health issues or even death (Wynn and Ramalingam 2012; Gause et al. 2013). We used both literature review and experimental immune challenges to document the evolution of fibrosis across the fish tree of life.

Recent findings on inter-population variation in helminth resistance from threespine stickleback fish (*Gasterosteus aculeatus*) suggest that anti-helminthic fibrosis responses may be a fast-evolving immune trait (Weber et al. 2017a). Stickleback are originally a marine species of fish that has only recently invaded freshwater habitats across Northern Hemisphere, especially in post-glacial coastal areas. These freshwater colonists experience greater risk of acquiring a tapeworm (*Schistocephalus solidus*, Cestoda), because they feed on freshwater cyclopoid copepods that are the tapeworm's first host (Barber and Scharsack 2010; Rahn et al. 2016). When ingested, the tapeworm larva migrates through intestinal wall to peritoneal cavity of the fish and grows to its final size, often >30% the host's mass (Ritter et al. 2017). The threespine stickleback is the obligate intermediate host of this specialized parasite. Some populations of stickleback have evolved a capacity to suppress *S. solidus* growth by encapsulating it in fibrotic tissue, sometimes leading to successfully killing and eliminating the parasite (Weber et al. 2017b).

This presumably beneficial form of resistance has costs, however, in greatly suppressing female gonad development and male reproduction (De Lisle and Bolnick, in preparation; Weber et al., in preparation). These costs may explain why the intensive peritoneal response to *S. solidus* has evolved in only some lake populations, and in some geographic regions of

118 the sticklebacks' range (Weber et al. 2017a). In the other populations, stickleback have
119 apparently adopted a non-fibrotic tolerance response to reproduce despite infection (Weber et
120 al. 2017a). These non-fibrotic populations exhibit active up-regulation of fibrosis-suppression
121 genes in response to cestode infection (Lohman et al. 2017; Fuess et al., in preparation). The
122 ancestral marine populations come very rarely into contact with *S. solidus* which does not
123 hatch in saline water (Barber and Scharsack 2010), and do not exhibit observable fibrosis in
124 the wild or in captivity (D. Bolnick, unpublished data; Hund et al. (2020)). These various
125 marine and freshwater populations have been diverging only since Pleistocene deglaciation
126 (~12,000 years), indicating that their fibrosis response has evolved surprisingly quickly for
127 such a fundamental immune process.

128 The peritoneal fibrosis can reliably be provoked in both fibrotic and non-fibrotic
129 populations of stickleback by a generalized immune challenge (injection with a non-specific
130 Alum adjuvant) (Hund et al. 2020), while only the resistant populations initiate fibrosis in
131 response to tapeworm protein injection. So, the physiological capacity to initiate fibrosis
132 seems conserved in stickleback and its sensitivity to the tapeworm fast-evolving. We
133 therefore wished to determine whether this peritoneal fibrosis is similarly labile, or conserved
134 across a broader range of fish species. In particular, *S. solidus* is a specialist parasite with
135 complex life cycle that invades fish peritoneal cavity (Barber and Scharsack 2010). Peritoneal
136 fibrosis could be a stickleback-specific immune trait, or may be co-opted from a widely
137 conserved response. Is then peritoneal fibrosis an ancestral character state dating back to the
138 origin of teleosts, or beyond?

139 To address this question, we begin with a broad literature review of fibrosis in fishes to
140 systematically summarize documented instances for the first time. Then, we present an
141 experimental test of how widely ray-finned fish (Actinopterygii) exhibit peritoneal fibrosis

142 response. We chose a diverse set of species, deliberately meant to be spread across the
143 phylogenetic tree of Actinopterygii. Drawing on a recent experimental study of fibrosis
144 response in stickleback (Hund et al. 2020), we challenged selected fish species with a
145 generalized immune stimulant (Alum vaccination adjuvant), and with antigens from a
146 specialist helminth parasite (*S. solidus*) that induces peritoneal fibrosis in some populations of
147 threespine stickleback.

148

149 **Literature review: Peritoneal fibrosis in fish is known but not well documented.**

150 Our first aim was to gather an overview of publication record encompassing peritoneal
151 fibrosis in fish. To do so, we performed two searches using Web of Science database on
152 September 30, 2019. The first search was oriented towards parasite-induced fibrosis in fish
153 with terms: (parasit*) AND (fibro*) AND ((teleost) OR (fish)). The other literature search
154 was focused on fibrosis in fish in general while we tried to avoid articles on human subjects:
155 (fibrosis AND (fish OR teleost) NOT (human)).

156 We collected 1459 entries in total, of which 1335 articles were retained after double
157 entries removal. We considered an article to be suitable for our study if it was on ray-finned
158 fish (Actinopterygii) and contained information on any signs related to fibrosis, like organ
159 adhesion, spontaneous proliferation of fibroblasts (fibroma, fibrosarcoma), healing fibroplasia
160 (scarification), or encapsulation (of a parasite or an implant, for example), that could be
161 inferred from the title or article abstract. Encapsulation typically meant that an extra-bodily
162 particle was surrounded with a layer of fibroblasts and extracellular matter (e.g., collagen
163 fibers). We then sorted the suitable articles, by going into their main text, according to three
164 criteria - the explicit presence of fibrosis or capsule ("fibrosis only", "capsule only", "both",

165 “neither”), its topology (“viscera”, “other”, or “multiple”) and assumed cause (“parasite”,
166 “toxicity”, “treatment”, “tumor”, or “unknown”). For topology (location) classification, we
167 took internal organs related to excretory system, digestive tract, or reproduction (kidney, liver,
168 gas bladder, gut, gonads, including also peritoneum) as “viscera” and the remaining organs or
169 tissues, such as gills, skin, muscle, brain, heart, etc. as “other”. When tissues of both types
170 were affected, we labelled article as “multiple”. We then grouped articles with respect to the
171 given cause of the fibrosis-related marks or encapsulation, where “parasite” was for infection
172 with a uni- or multi-cellular parasite, “toxicity” was when the study monitored known
173 environmental pollution (e.g., heavy metals), “treatment” was for deliberate manipulation
174 with fish body or their living conditions (e.g., adding estrogen into water to test the effect on
175 male physiology), “tumor” was when fibrosis happened spontaneously (e.g., fibrosarcoma)
176 and “unknown” pooled studies where the cause could not be identified. We extracted fish
177 species names and sorted them into orders and higher taxonomic categories according to the
178 recent phylogenetic resolution of the Actinopterygii tree of life by Hughes et al. (2018) and
179 Rabosky et al. (2018). We then used this dataset to offer an insight into the published
180 literature on fish fibrosis. Table 1 shows a summary of articles with regard to different fish
181 phylogenetic groups, the presence of fibrosis and/or encapsulation, its location and cause. For
182 brevity, we pooled less represented fish orders (with <6 articles) into “other” category and
183 also grouped articles with species from more orders into “multiple”.

Table 1. Overview of the literature search.

184

185 In the articles we collected, general fibrotic response was documented from a wide
186 array of ray-finned fish (Actinopterygii). We found 375 out of the 1335 articles (i.e. 28%) to
187 be suitable for our study. The most-represented species came from the orders Cypriniformes

188 (61 [including articles with multiple species]; 16% of relevant articles) and Salmoniformes
189 (51; 14%) (Table 1). Authors identified signs of fibrosis during an autopsy, though some
190 cases were observed from fish integument as well (e.g. capsules of skin parasites). Most of the
191 suitable articles reported parasitism (197; 53%) or treatment (81; 22%) as the cause. Overall,
192 fibrosis was present on its own (175; 47%) or in combination with encapsulation (22; 6%).
193 We recorded, however, also large number of cases of encapsulation without additional signs
194 of fibrosis (116; 31%) (Table 1). This demonstrates that fibrotic pathology is not a widely
195 required side-effect of encapsulation.

196 The extent of fibrosis was usually described only qualitatively, along with the identity
197 of the affected organs or tissues. Taking articles related to fibrosis (reporting fibrosis alone or
198 in combination with encapsulation, 198 articles), its incidence was mainly confined to visceral
199 organs (152 cases, 77%). Visceral fibrosis was, however, mainly interstitial fibrosis often
200 represented by tissue scarification after damage. We found only 7 articles that were dealing
201 with peritoneal fibrosis specifically. These seven articles are diverse with regard to species
202 taxonomic position and the cause of the fibrosis response (e.g., tapeworm infection,
203 vaccination, radio-transmitter implantation) (Table 1).

204 From this literature survey, we conclude that fibrosis is known in a number of fish
205 species. Yet, the peritoneal fibrosis is very scarcely reported which limits our ability to draw
206 broader conclusions about its evolutionary history or its function. To reach more systematic
207 understanding of peritoneal fibrosis evolution specifically, we initiated a phylogenetically
208 structured experimental study of ray-finned fish fibrotic response to immune challenges.

209

210

211 **METHODS**

212 **Species selected for the experiment**

213 To conduct a phylogenetically broad assay of peritoneal fibrosis response in ray-finned fishes
214 (Actinopterygii), we experimentally vaccinated 17 species of fish (Fig. 1, listed in Table 2).
215 These species were chosen to achieve broad phylogenetic diversity, but were restricted to
216 commercially available small fish (body length of 1-4", or 2-10 cm, and live weight 0.2-6.0
217 g). We obtained most of the fish species from a local ornamental fish reseller. Local trout
218 hatchery donated fingerlings of rainbow trout (*Oncorhynchus mykiss*). We received eggs of
219 the turquoise killifish (*Nothobranchius furzeri*, population MZCS 222) from a stock retained
220 at the Institute of Vertebrate Biology CAS in Brno, Czech Republic. We hatched and
221 maintained the killifish according to breeding protocol (Polačik et al. 2016) until they reached
222 two months when fully mature. We also included wild-caught threespine stickleback
223 (*Gasterosteus aculeatus*) that came from two distinct populations (Loch Hosta and Loch a'
224 Bharpa), both originating from North Uist, Scotland, UK, provided by Andrew MacColl
225 (University of Nottingham, UK) and maintained at the University of Connecticut, CT, USA
226 for six months before the experiment.

Table 2. Species selected for the experimental test.

227

228 **Fish housing**

229 We standardized housing conditions across most of the species. We planned to have 5-7
230 individuals per species per treatment. We prepared 20-gal (~76 L) tanks with reverse-osmosis
231 (RO) water conditioned to conductivity between 700-800 µS/cm with sea salt (Instant
232 Ocean®). We adjusted salinity for marine species to 35 mg/kg. Each tank contained air driven

233 sponge filter and heater with temperature set to 25 °C (water temperature ranged between
234 24.5-26 °C). We also provided a seaweed-like plastic shelter for fish. We fed fish every
235 morning with frozen bloodworms (Chironomidae), mysis shrimp (Mysidae), or dried sushi
236 nori seaweed (*Pyropia* sp.) according to species-specific diet requirements. We held the
237 rainbow trout at water temperature of 12 °C as our standard temperature would be stressful to
238 them. Similarly, stickleback are sensitive to high temperatures and we therefore kept them in
239 their original recirculation system at 19 °C and 1900 µS/cm throughout the treatment. All the
240 other fish were habituated to the common tank setup for at least five days before treatment.

241

242 **Reagents**

243 We used three different treatments to test the peritoneal fibrosis response across different fish
244 species: a saline solution control, purified cestode protein, and Alum which is a generalized
245 stimulant used in immunization vaccines. These treatments are based on a recent experiment
246 evaluating between-population differences in peritoneal fibrosis in threespine stickleback
247 (Hund et al. 2020). All three treatments were delivered via peritoneal injection (the site of *S.*
248 *solidus* infection).

249 The control treatment was an injection of 1X phosphate-buffered saline (PBS) (20 µL
250 per 1 g of species average of live weight), which was also the solution for delivering the other
251 treatments. The second treatment consisted of tapeworm antigen homogenate (abbreviated
252 TH) suspended in PBS. Hund et al. (2020) showed that injection of 9 mg of TH per 1kg live
253 fish mass (0.009 mg/g) induced rapid fibrosis only in tapeworm-resistant stickleback
254 populations. We obtained *S. solidus* tapeworms dissected from wild-caught threespine
255 stickleback (Gosling Lake, Vancouver Island, BC, Canada). We used two tapeworms to

256 prepare the homogenate, sonified them in PBS on ice and then centrifuged the suspension at
257 4000 rt/min at 4 °C for 20 minutes. We assessed overall protein concentration in the upper
258 fraction of the solution using RED 660™ protein assay (G-Biosciences) measured in
259 triplicates and then diluted the sample to 0.45 mg/mL. We aimed at injecting 20 µL of the
260 solution per 1 g of live fish weight and to obtain the desired dose 0.009 mg of tapeworm
261 protein for 1 g of fish weight (or 9 mg/kg, as in Hund et al. (2020)). We then aliquoted the
262 homogenate in 0.6-ml Eppendorf tubes and stored at -20 °C for later injections.

263 The third treatment was a 1% Alum solution (20 µL per 1 g of species average of fish
264 live weight). Alum promotes activation of innate immune response and is commonly used as
265 a vaccine adjuvant (Kool et al. 2012). We dissolved 2% AlumVax Phosphate (OZ
266 Biosciences) in 1:1 with PBS. This concentration of Alum induces peritoneal fibrosis in
267 marine and freshwater stickleback population irrespective of their tapeworm resistance (Hund
268 et al. 2020).

269

270 **Injections**

271 At their arrival to our fish facility (University of Connecticut, CT, USA), we weighed each
272 fish species on a balance to 0.01 g (Gene Mate GP-600) to estimate total volume of solution
273 to be injected per individual (based on species average live mass). We injected 20 µL of the
274 solution per 1 g of average live weight with Ultra-Fine insulin syringes. We always filled
275 syringes aseptically under laminar flow cabinet 1-2 days before the injections, stored them at
276 4 °C and used 1 syringe per individual. We injected fish peritoneally through their left flank
277 after anaesthesia with MS-222 (200 mg/L for up to two minutes). Fish were then allowed to
278 recover from anaesthesia in a highly aerated water from their tank. We returned them to their

279 original tank once they were swimming upright, but typically after more than five minutes
280 after the injection. The different treatment groups were held in separate tanks. All aspects of
281 the experiment were approved in advance by the University of Connecticut Institutional
282 Animal Care and Use Committee (IACUC Protocol A18-008).

283

284 **Dissections and scoring fibrosis level**

285 We euthanized fish five days post-injection, using an overdose of MS-222 (500 mg/L, > 5
286 min). We dissected trout at day 10 of treatment because of the lower temperature in their
287 tanks, and stickleback on two occasions, at days 5 and 10 of treatment, as lower temperature
288 slows immune response (Rijkers et al. 1980). We also dissected 1-2 individuals from each
289 species prior the injections to examine species-specific anatomy, assess the baseline level of
290 peritoneal fibrosis before injections, and locate their cephalic kidney. We dissected fish
291 immediately after euthanasia under stereo-microscope, photographed and scored their level of
292 fibrosis, using an ordinal categorical score. The peritoneal fibrosis score ranged between 0
293 and 3. Zero represents the absence of noticeable fibrosis, where the internal organs (liver,
294 intestine, gonads) move freely apart from each other and from the peritoneal wall when
295 moved with tweezers. Level 1 was for organs adhered together forming an interconnected
296 conglomerate that moves as a unit. Level 2 was scored when the internal organs also attached
297 to the peritoneal wall, but it was still possible to free them. Level 3 was the extreme form of
298 organ adhesion where the peritoneal lining tore apart and remained attached to the organs
299 after the body cavity had to be forcibly opened. Note that peritoneal fibrosis levels 1, 2, and 3
300 used here correspond with levels 2, 3, and 4, respectively, used by Hund et al. (2020). For
301 illustration of the 0-4 scale (Hund et al. 2020) see video at https://youtu.be/yKvcRVCSpWI.
302 We took a sample of both cephalic kidneys and peritoneal tissue from the left side of fish

303 body cavity (the injection side) during the dissection for future analysis of gene expression,
304 stored in RNAlater for future analysis. We also recorded total and dissected weight of the
305 dead fish (0.001 g, Sartorius Element ELT202), fish sex (where it could be determined), and
306 the presence of any internal parasites.

307

308 **Data analysis**

309 We formally tested the interaction between the effect of treatment (PBS, TH, Alum) and
310 experimental species identity on individual fibrosis score using generalized least squares
311 (GLS) model (function gls, library "nlme" v.3.1-148, Pinheiro et al. 2018). The response
312 variable was fibrosis level (ordered integers 0-3) scored from the individual's left flank. We
313 set treatment, species and their interaction as fixed model effects. We attempted to originally
314 analyze the ordinal response variable using Cumulative link models (CLM, function clm,
315 library "ordinal" v.2019.12-10, Christensen 2019), but CLM with treatment–species
316 interaction failed to converge due to model singularity (e.g., too many groups with zero
317 variance because all individuals had identical fibrosis scores).

318 To assess phylogenetic signal in the experimental data, we created a species tree based
319 on the recent comprehensive phylogeny of ray-finned fishes by Hughes et al. (2018) with
320 estimated divergence times. We then obtained phylogenetic signal (i.e. the tendency of related
321 species to show similar response) and ancestral character state for two species "traits": the
322 maximum level of peritoneal fibrosis in a species and species relative average response in the
323 positive control (difference between species average fibrosis level in the negative control
324 (PBS) and species average fibrosis in the positive control (Alum)). We measured
325 phylogenetic signal with Pagel's λ (function phylosig, with method specification to "lambda",

326 library "phytools" v.0.7-47 (Revell 2012)). We then estimated ancestral character state with
327 maximum likelihood approach using function anc.ML (specifying Brownian motion mode of
328 evolution, library "phytools"). All analyses were performed in R software v. 4.0.1 (R Core
329 Developmental Team 2019).

330

331

332 **RESULTS**

333 The level of fibrosis differed between experimental treatments and across species (GLS,
334 treatment-species interaction, $F_{34,251}$ = 7.93, P < 0.001) (Fig. 1,2). For most species, we
335 observed no detectable or low fibrosis in control-injected (PBS) fish as well as in the
336 tapeworm homogenate (TH) treatment (Fig. 2). However, there were two species, the
337 common carp (*Cyprinus carpio*) and the channel catfish (*Ictalurus punctatus*), with variable
338 individual response both in the PBS control and TH treatment (Fig. 2).

Figure 1. Ancestral state reconstruction of species maximum peritoneal fibrosis.

339

340 In contrast to the negative control and TH, the positive control (Alum injection) induced
341 strong peritoneal fibrosis in most of the species (15 of 17). The response was typically high to
342 extreme (fibrosis level 2 or 3, Fig. 2). The exceptions were two species of tetras, the Mexican
343 tetra (*Astyanax mexicanus*) and the bleeding-heart tetra (*Hyphessobrycon erythrostigma*),
344 which did not respond to any of the treatments (Fig. 2).

345 Phylogenetic signal for species maximum fibrosis and species relative response to the
346 positive control (average Alum vs. PBS difference) both appeared to be strong, significantly
347 different from random evolution (Pagel's $\lambda > 0.987$ and $P < 0.005$, for both traits). Ancestral

348 state at the base of the ray-finned fishes, i.e. at the divergence between the Senegal bichir
349 (*Polypterus senegalus*) and the other species from our experiment, was estimated 1.862 for
350 the species maximum fibrosis (Fig. 1) and 1.325 for species' relative fibrosis response to the
351 Alum treatment (average difference between Alum vs. PBS).

Figure 2. Peritoneal fibrosis in the experimental fish species.

352

353

354 **DISCUSSION**

355 This study represents one of the first comparative experimental assays of the macroevolution
356 of an immune response. We focused on evaluating the prevalence of peritoneal fibrosis
357 response across fishes, because this response has been shown to contribute to parasite growth
358 suppression and elimination in threespine stickleback. We show that published literature
359 contains little data on peritoneal fibrosis in ray-finned fishes. To fill this gap, we
360 experimentally tested the prevalence of peritoneal fibrosis in a wide array of species across
361 the phylogeny of Actinopterygii. Our immune challenge resulted in a variable level of fibrosis
362 between treatments and among species. Response to homogenate from the stickleback-
363 specialized tapeworm was weak at best. The positive control treatment (Alum), on the other
364 hand, provoked strong peritoneal fibrosis in most of the species tested except one specific
365 lineage – two species of tetras (Characidae, Characiformes). The results therefore suggest
366 that, despite being rarely observed or reported, peritoneal fibrosis is a phylogenetically
367 widespread aspect of fish immunity. Ancestral character state reconstruction indicates that the
368 fibrotic response to the vaccination adjuvant (Alum) is phylogenetically conserved at least to
369 the origin of ray-finned fishes (Fig. 1), estimated around 380 million years ago (Hughes et al.

370 2018). However, the fibrotic response is more pronounced in some fish clades, and absent in
371 others (Characidae).

372

373 **Lack of peritoneal fibrosis in publications across ray-finned fish**

374 The literature search indicated that fish initiate fibrosis most frequently in response to tissue
375 injury and/or parasitism. Thus, we can identify two main roles of fibrosis – maintenance of
376 homeostasis in damaged tissue, and formation of physical barrier around an invader
377 (encapsulation). Indeed, Gause et al. (2013) proposed an evolutionary hypothesis for the
378 origin of parasite encapsulation from the ancestral repair response to tissue mechanical
379 damage. The article collection also contained several cases (almost 1/3 of the articles
380 mentioning fibrosis or encapsulation), where parasite encapsulation happened without more
381 wide-spread fibrosis.

382 Our study was motivated by inter-population variation in threespine sticklebacks'
383 ability to encapsulate parasitic tapeworm *S. solidus* (Weber et al. 2017b). The inter-population
384 variation probably stems from an evolutionary trade-off between the benefit of resistance
385 (early encapsulation of the worm) and the risk of organ adhesion, excessive fibroblast
386 proliferation, and ultimately partial sterility in the stickleback (Weber et al., in preparation;
387 De Lisle and Bolnick, in preparation). Previous records on peritoneal fibrosis in threespine
388 stickleback mostly lacking (but see Hoffman 1975, p.175), although the phenomenon is found
389 in numerous populations across the species' circumpolar range. This is in line with the
390 literature search, where we found only very few studies reporting peritoneal fibrosis in fish. In
391 these few articles, peritoneal fibrosis was associated with serious intrusion of body integrity,
392 e.g., radio-transmitter implantation (Mangan 1998), vaccination with bacteria (Colquhoun et

393 al. 1998), or, similarly to the stickleback, tapeworm infection (Abdelsalam et al. 2016).
394 Apparently, the stress has to be intensive and/or chronic to trigger peritoneal fibrosis. The
395 question thus remained whether peritoneal fibrosis is that rare and highly specific response
396 across fish species. We used a phylogenetically informed immune challenge experiment with
397 selected representatives across the fish tree of life to answer this question.

398

399 **Peritoneal fibrosis in response to vaccination adjuvant was prevalent in most fish species**

400 We successfully triggered peritoneal fibrosis in most of the fish species tested with the
401 positive control (Alum). Alum is a commonly used vaccination adjuvant that causes influx of
402 multiple types of immune cells into the injected region and alerts individual's immune system
403 (Kool et al. 2012). Yet, the particular mechanism of how Alum promotes vaccination is still
404 unknown. In the case of peritoneal fibrosis, the Alum crystals may in fact act as an irritating
405 agent that stimulates the type 2 immune response leading to containment of the body non-self
406 (Gause et al. 2013). The universal response to the Alum injection demonstrates that the
407 capacity for peritoneal fibrosis is widely distributed across ray-finned fish phylogeny.
408 Absence of fish peritoneal fibrosis in the published literature may thus stem from high
409 specificity (peritoneal cavity invasion), low severity/chronicity of the common stressors, or
410 the phenomenon may simply have been overlooked as was until recently in stickleback. In
411 addition, even though *S. solidus* is a species-specific parasite of the threespine stickleback, the
412 peritoneal fibrosis response itself is not necessarily triggered by a specific antigen (Hund et al.
413 2020). It rather seems that its expression might be regulated (Lohman et al. 2017; Fuess et al.,
414 in preparation).

415

416 **The experimental exceptions**

417 *Individual variation.* The homogenate prepared from *S. solidus* tapeworm caused
418 peritoneal fibrosis only in two tested species and one population of the threespine stickleback.
419 In common carp (*Cyprinus carpio*) and channel catfish (*Ictalurus punctatus*), the level of
420 peritoneal fibrosis varied among individuals both in the TH and negative control (PBS)
421 treatments. We recorded similar pattern also in threespine stickleback from Loch Hosta
422 population after 10 days post-injection. The response was comparable between TH and the
423 negative control (PBS). Based on the individual variation and the pre-treatment dissections, it
424 seems like different individuals might be more or less sensitive to the injections in the carp,
425 catfish and Loch Hosta stickleback. Individual variation in these three groups contrasts with
426 the largely uniform response exhibited in each treatment by the remaining species.

427 *General indifference to the TH treatment*. *Schistocephalus solidus* is a parasite specialist
428 of the threespine stickleback. The cue to trigger peritoneal fibrosis in threespine stickleback in
429 response to the tapeworm is probably specific, as shown by some populations that fail to
430 respond to certain genotypes of *S. solidus* (Weber et al. 2017a). Parasite community in North
431 Uist stickleback is relatively rich and includes *S. solidus* (Rahn et al. 2016). The Scottish
432 stickleback were also observed to show peritoneal fibrosis in the wild, with a' Bharpa
433 population having strong response and Hosta population absent (A. MacColl, pers. comm.).
434 The tapeworm protein effective dose used here (9 mg/g of fish live weight) triggered strong
435 fibrosis in a naturally fibrotic lake population from Vancouver Island (Hund et al. 2020), but
436 it is possible that the Scottish stickleback do not recognize tapeworms from western Canada.
437 The specific cue that triggers peritoneal fibrosis in response to the tapeworm infection is
438 unknown, though presumably protein-based, and the work on its identification currently
439 ongoing.

440 *Absence of peritoneal fibrosis response.* Our experimental data show that peritoneal
441 fibrosis is widespread across fish phylogeny, except for two related species – the Mexican
442 tetra (*Astyanax mexicanus*) and the bleeding-heart tetra (*Hyphessobrycon erythrostigma*),
443 which did not respond to any of the treatments. The adaptation of some populations of
444 Mexican tetra to the freshwater cave systems offers an attractive interpretation for the absent
445 peritoneal response. The underground water habitats are traditionally viewed as poor in
446 nutrients and local communities, including parasites (Culver and Pipan 2009). Thus, taking
447 into account the risks associated with the peritoneal fibrosis described in the threespine
448 stickleback, maintenance of such response could be too costly for the Mexican tetra.
449 However, the cave environment of wild populations of Mexican tetras does not have to be as
450 poor as assumed (Simon et al. 2017). More importantly, we provide an evidence that the lack
451 of the peritoneal fibrosis response might be more general among Characiformes, recording
452 null response in the South-American bleeding-heart tetra as well. The non-specific immunity
453 of these two tetra species might therefore differ from the other ray-finned fish, and is a
454 tempting target for more research. The family Characidae containing the two tetras consists of
455 over a thousand species widely distributed in fresh waters from Texas, USA to Argentina. It
456 would be interesting to uncover phylogenetic extent of the absence of peritoneal fibrosis in
457 more detail, including maybe also African lineages of Characiformes. Interestingly, our
458 literature search indicates that Characiformes are able to encapsulate parasites and also show
459 signs of (interstitial) tissue fibrosis (Table 1).

460

461 **Outlook**

462 One particular avenue for future research would be to understand proximate mechanisms of
463 peritoneal fibrosis in general. Examining why tetras do not express peritoneal fibrosis even

464 after Alum peritoneal injection, while other tested species did, could help to better understand
465 the regulation of the response. The lack of cue recognition does not seem probable as Alum is
466 a non-specific (general) immune challenge. Is then the absence caused by down- or up-
467 regulation of specific immune pathways, or their complete absence? This is a particularly
468 pressing question with potential clinical application. Long-term peritoneal dialysis in human
469 patients with kidney failure may occasionally lead to peritoneal fibrosis and pathological
470 encapsulating peritoneal sclerosis (EPS) (Braun et al. 2012). EPS is a rare complication of the
471 peritoneal dialysis characteristic with thickened peritoneal membrane, bowel obstruction; and
472 is fatal in majority of the diagnosed cases (Kawanishi and Moriishi 2005). Ray-finned fish
473 share the main immune domains with human from jawed vertebrate radiation (Litman et al.
474 2005) and the peritoneal fibrosis appears superficially similar to EPS (fibroblast activation,
475 fibrin production). Perhaps further research on tetras could offer a new perspective in the
476 pursuit of the lethal human EPS elimination. Research into fish species that activate fibrosis
477 in response to specific immune challenges (e.g., *S. solidus*) may help identify molecular
478 patterns that trigger fibrosis.

479

480 **Conclusion**

481 As proposed by Gause et al. (2013), fibrosis is probably an ancient trait evolved from wound
482 healing; the formerly repairing mechanism now suits also coping with endoparasites. We
483 showed that, despite being rarely reported in the published literature, the potential to develop
484 peritoneal fibrosis is widespread across fish phylogeny and it can be triggered through a
485 general treatment (Alum peritoneal injection) in almost all tested fish. The comparative
486 immunology experiment, such as the one we performed, is particularly powerful and broad
487 approach to infer historical origin, evolutionary rate of the immune traits, and to identify

interesting atypical lineages (Weber and Agrawal 2012). By investigating those exceptions, we may consequently focus on documenting genetic mechanisms and adaptive value of different character states.

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AUTHOR CONTRIBUTIONS

DB and MV designed the study, MV conducted experimental work, performed data collection and analysis, MV and DB wrote the manuscript.

CONFLICT OF INTEREST

510 The authors have declared no conflict of interest.

511

512 **DATA ACCESSIBILITY**

513 Data will be made publicly available after acceptance along with the statistical code under
514 DOI: 10.6084/m9.figshare.12619367.

515

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622 **TABLES**

623 Table 1. Overview of the literature search for presence of peritoneal fibrosis in fish. Highlighted in gray are records of peritoneal fibrosis

624 (column) and articles from Characiformes (row). Characiformes include the two tetra species that did not respond with peritoneal fibrosis

625 to any of the treatments in the immune challenge experiment.

Higher taxonomic rank	Order	N	Fibrosis or capsule				peritoneal fibrosis	Position			Cause				
			fibrosis only	capsule only	both	neither		viscera	other	multiple	parasite	toxicity	treatment	tumor	unknown
Anabantaria	Anabantiformes	9	6	1	2			4	4	1	4		5		
Carangaria	Pleuronectiformes	18	11	4		3	1	10	8		10	3	1	2	2
Elopomorpha	Anguilliformes	13	6	6	1		1	10	3		10	1	2		
Eupercaria	Centrarchiformes	12	4	5	3			10	1	1	12				
Eupercaria	Moronidae*	7	6	1				4	2	1	3	1	1		2
Eupercaria	Perciformes	19	4	11	1	3		10	9		16		2	1	
Eupercaria	Spariformes	9		5		4		4	5		5			4	
Eupercaria	Sciaenidae*	8	5	3				2	5	1	5		2		1
Eupercaria	Scorpaeniformes	6	3	2		1		3	3		3	1	1	1	
Zeiogadaria	Gadiformes	10	3	7				5	4	1	9		1		
Otophysa	Characiformes	12	2	8		2		7	5		8	1	1	2	
Otophysa	Cypriniformes	56	23	19	5	9		32	24		26	5	17	8	
Otophysa	Siluriformes	26	21	3		2		21	5		11	1	12	2	
Ovalentaria	Cichliformes	11	7	1	1	2		8	3		2	3	4	2	
Ovalentaria	Cyprinodontiformes	12	8	2	1	1		6	6		5	1	6		
Ovalentaria	Mugiliformes	11	1	4		6	1	1	9	1	4		1	6	
Pelagiaria	Scombriformes	9	1	4	2	2	1	2	7		7			2	
Protacanthopterygii	Salmoniformes	50	29	10	3	8	1	31	19		20	2	13	7	8

	other	53	26	14	3	10	1	37	13	3	26	6	11	9	1
	multiple	24	9	6	1	8	1	13	9	2	11	4	1	7	1
TOTAL		375	175	116	23	61	7	220	144	11	197	29	81	53	15

626 **incertae sedis* – the taxonomic position of the family within the higher taxonomic group is not well resolved

627

628 Table 2. Species selected for the experimental test of peritoneal fibrosis response. We show the sample size, N, and average with standard
629 deviation (SD) for live weight before dissection in grams, W_{total}, in each experimental treatment. Note that Species # corresponds to species
630 number in Fig. 1. We also present data for two species that we did not use in the analysis as well as for the stickleback that were kept in the
631 treatment for 10 days ("10d"). The clown feather-back (*Chitala ornata*) were extremely fibrosed (level 3) before the experiment started.
632 The kissing gourami (*Helostoma temminckii*) developed white spot disease during the experiment.

Species #	Common name	Species	Order	Higher taxonomic rank	Control (PBS)		Tapeworm homogenate		Alum		Origin
					N	W_{total}	N	W_{total}	N	W_{total}	
1	Senegal bichir	*Polypterus senegalus*	Polypteriformes	Cladistia	6	3.98(0.75)	6	4.09(0.71)	6	4.09(0.72)	captive-bred
2	common carp	*Cyprinus carpio*	Cypriniformes	Otophysa	7	1.87(0.34)	7	1.72(0.46)	7	1.68(0.33)	captive-bred
3	zebrafish	*Danio rerio*	Cypriniformes	Otophysa	6	0.29(0.04)	7	0.29(0.08)	7	0.28(0.06)	captive-bred
4	channel catfish	*Ictalurus punctatus*	Siluriformes	Otophysa	7	2.24(0.53)	7	2.28(0.62)	7	2.15(0.39)	captive-bred
5	Mexican tetra	*Astyanax mexicanus*	Characiformes	Otophysa	6	0.82(0.17)	6	0.90(0.16)	6	0.91(0.08)	captive-bred
6	bleeding-heart tetra	*Hyphesobrycon erythrostigma*	Characiformes	Otophysa	5	0.97(0.24)	6	0.98(0.36)	6	1.09(0.21)	wild
7	rainbow trout	*Oncorhynchus mykiss*	Salmoniformes	Protacanthopterygii	5	4.53(0.88)	5	4.24(1.03)	5	4.21(1.00)	captive-bred
8	pajama cardinalfish	*Sphaeramia nematoptera*	Kurtiformes	Gobiaria	5	2.22(0.09)	5	2.21(0.45)	5	2.30(0.68)	captive-bred
9	peacock gudgeon	*Tateurndina ocellicauda*	Gobiiformes	Gobiaria	5	0.54(0.09)	4	0.68(0.11)	4	0.57(0.03)	captive-bred
10	green chromis	*Chromis viridis*	Pomacentridae*	Ovalentaria	4	1.37(0.11)	5	1.42(0.39)	5	1.35(0.35)	wild
11	jewelled blenny	*Salarias fasciatus*	Blenniiformes	Ovalentaria	4	1.57(0.54)	5	1.32(0.50)	3	1.68(0.97)	wild
12	turquoise killifish	*Nothobranchius furzeri*	Cyprinodontiformes	Ovalentaria	6	0.79(0.39)	6	0.77(0.38)	5	0.76(0.27)	captive-bred
13	green swordtail	*Xiphophorus hellerii*	Cyprinodontiformes	Ovalentaria	6	1.43(0.28)	6	1.29(0.16)	6	1.47(0.34)	captive-bred
14	Nile tilapia	*Oreochromis niloticus*	Cichliformes	Ovalentaria	7	2.18(0.69)	7	2.07(0.81)	7	1.95(0.70)	captive-bred
15	Hosta stickleback	*Gasterosteus aculeatus*	Perciformes	Eupercaria	6	0.31(0.24)	6	0.28(0.14)	6	0.30(0.16)	wild
	Hosta stickleback (10d)			Eupercaria	6	0.36(0.14)	6	0.38(0.19)	6	0.38(0.17)	wild

15	a' Bharpa stickleback	*Gasterosteus aculeatus*	Perciformes	Eupercaria	6	0.27(0.05)	6	0.29(0.06)	6	0.30(0.13)	wild
	a' Bharpa stickleback (10d)			Eupercaria	5	0.37(0.07)	6	0.33(0.07)	5	0.30(0.03)	wild
16	blue-gill sunfish	*Lepomis macrochirus*	Centrarchiformes	Eupercaria	6	0.85(0.14)	6	0.79(0.18)	6	0.77(0.15)	captive-bred
17	spotted green pufferfish	*Dichotomyctere nigroviridis*	Tetraodontiformes	Eupercaria	3	1.49(0.27)	4	1.40(0.38)	4	1.29(0.36)	captive-bred
x	clown feather-back	*Chitala ornata*	Osteoglossiformes	Osteoglossomorpha	1	4.70	0	-	0	-	captive-bred
x	kissing gourami	*Helostoma temminckii*	Anabantiformes	Anabantaria	5	4.56(0.78)	0	-	1	5.66	captive-bred

633 **incertae sedis* – the taxonomic position of the family within the higher taxonomic group is not well resolved

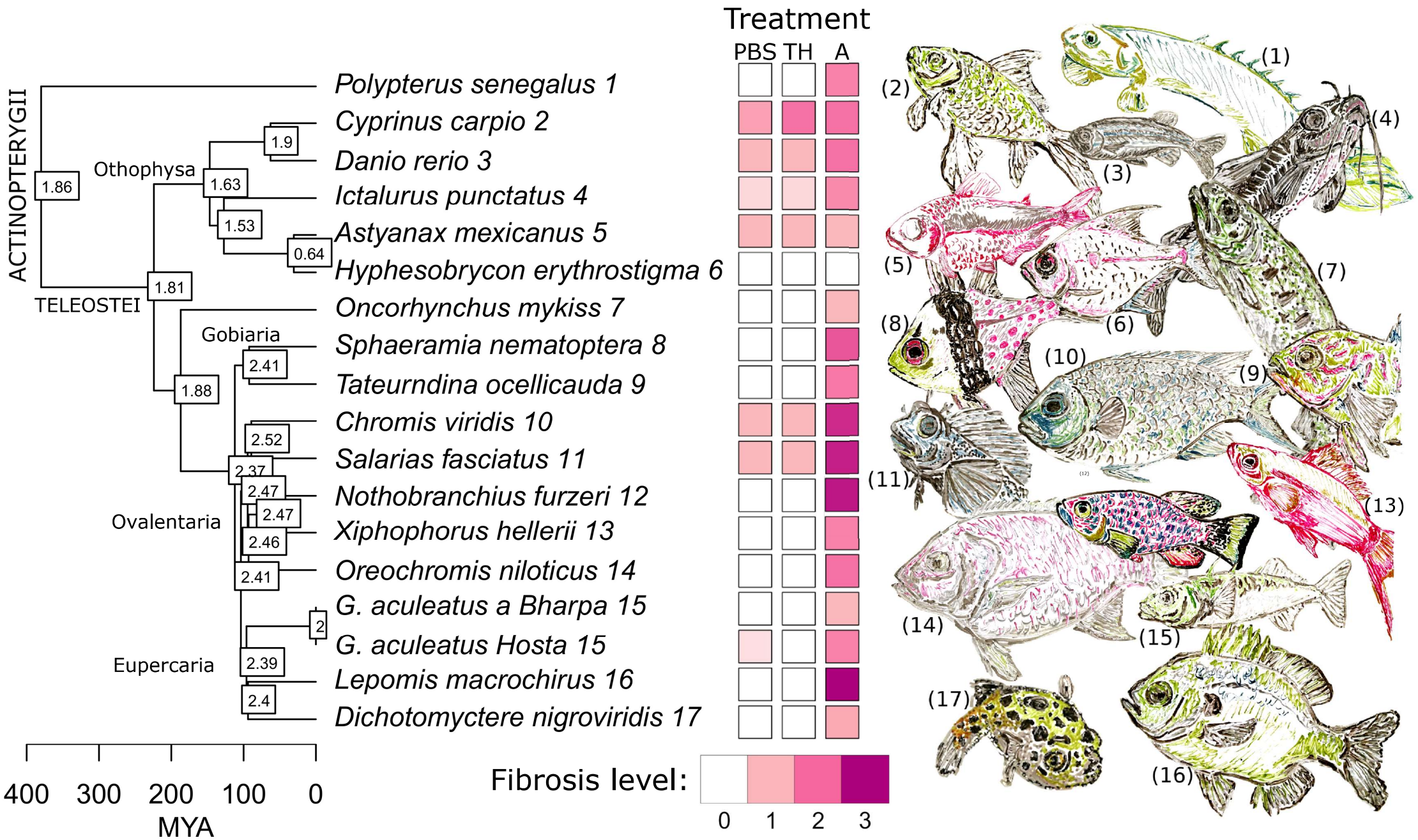
634 **FIGURE CAPTIONS**

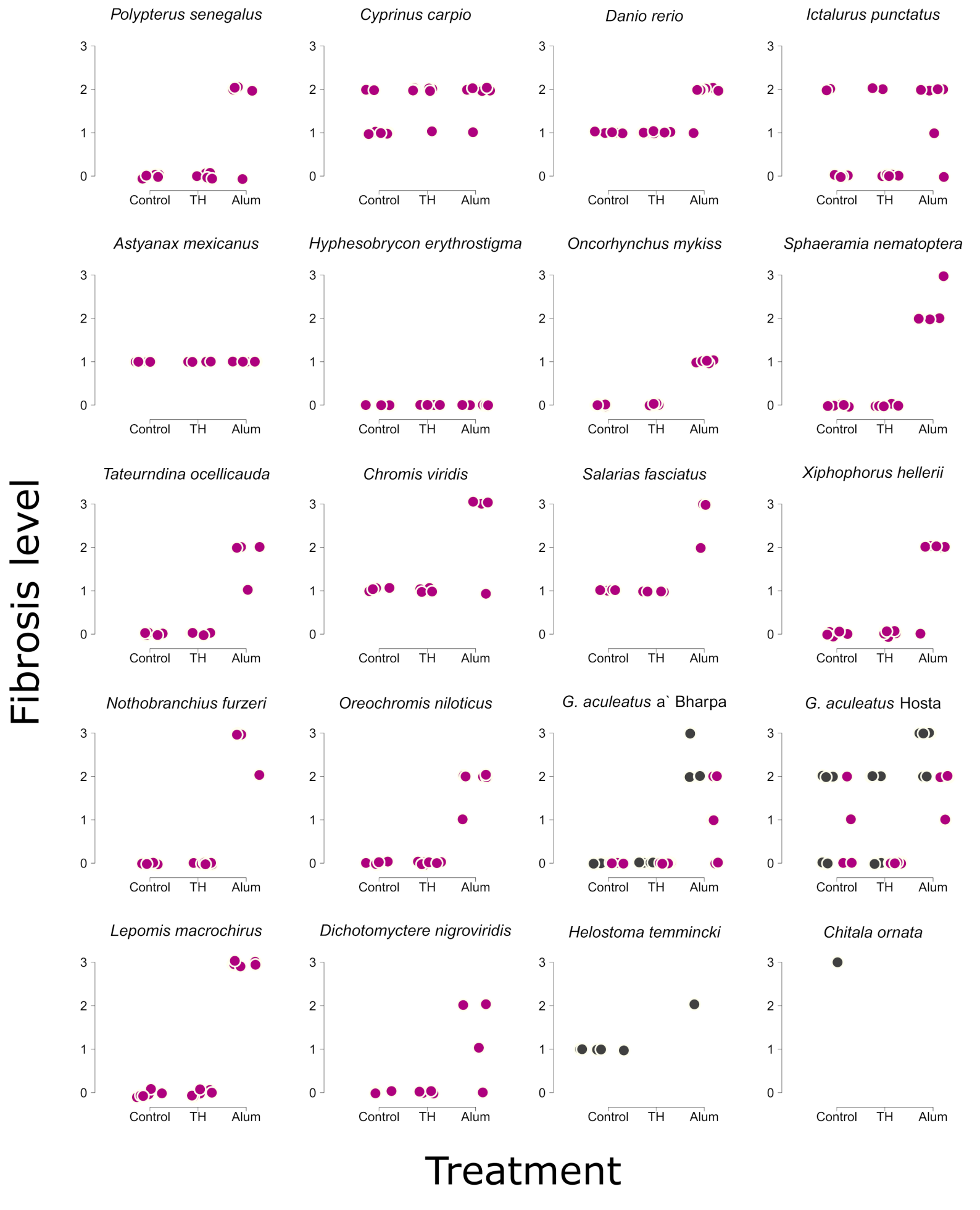
635 Figure 1. Ancestral state reconstruction of species maximum peritoneal fibrosis. The values at
636 the branching nodes give estimate of the ancestral level of maximum peritoneal fibrosis level.
637 Tree structure and branch lengths are based on recent reconstruction of phylogeny of ray-
638 finned fishes (Actinopterygii) (Hughes et al. 2018). The squares of different intensity of
639 purple color show average level of peritoneal fibrosis per treatment in each tested species.
640 Treatment abbreviations are PBS: phosphate-buffered saline solution (control), TH: tapeworm
641 antigen homogenate, A: Alum vaccine adjuvant. Fish species drawings by M. F. Maciejewski
642 (not to scale).

643

644 Figure 2. Peritoneal fibrosis in the experimental fish species. Individual points in the species
645 plots show recorded level of peritoneal fibrosis scored from their left flank (the side of the
646 injection). Note that the three-spine stickleback (both populations of *G. aculeatus*) that stayed
647 in the experiment for 10 days are shown in grey as they did not enter data analysis. For
648 completeness, we also present data for two unused species in grey - kissing gourami (*H.*
649 *temminckii*) and clown feather-back (*Ch. ornata*).

650


Treatment
PBS TH A
ACTINOPTERYGII
TELEOSTEI
Othophysa
Gobiaria
Ovalentaria
Eupercaria
1.86
1.9
1.63
1.53
0.64
1.81
1.88
2.41
2.52
2.37
2.47
2.47
2.46
2.41
2
2.39
2.4
Polypterus senegalus 1
Cyprinus carpio 2
Danio rerio 3
Ictalurus punctatus 4
Astyanax mexicanus 5
Hyphesobrycon erythrostigma 6
Oncorhynchus mykiss 7
Sphaeramia nematoptera 8
Tateurndina ocellicauda 9
Chromis viridis 10
Salarias fasciatus 11
Nothobranchius furzeri 12
Xiphophorus hellerii 13
Oreochromis niloticus 14
G. aculeatus a Bharpa 15
G. aculeatus Hosta 15
Lepomis macrochirus 16
Dichotomyctere nigroviridis 17
400 300 200 100 0
MYA
Fibrosis level:
0 1 2 3
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
(10)
(11)
(12)
(13)
(14)
(15)
(16)
(17)


Polypterus senegalus
Cyprinus carpio
Danio rerio
Ictalurus punctatus
Astyanax mexicanus
Hyphesobrycon erythrostigma
Oncorhynchus mykiss
Sphaeramia nematoptera
Tateurndina ocellicauda
Chromis viridis
Salarias fasciatus
Xiphophorus hellerii
Nothobranchius furzeri
Oreochromis niloticus
G. aculeatus a` Bharpa
G. aculeatus Hosta
Lepomis macrochirus
Dichotomyctere nigroviridis
Helostoma temmincki
Chitala ornata
0
1
2
3
Control
TH
Alum
Fibrosis level
Treatment