Macroevolutionary foundations of a recently evolved innate immune defense

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Antagonistic interactions between hosts and parasites may drive the evolution of novel host defenses, or new parasite strategies. Host immunity is therefore one of the fastest evolving traits. But where do the novel immune traits come from? Here, we test for phylogenetic conservation in a rapidly evolving immune trait—peritoneal fibrosis. Peritoneal fibrosis is a costly defense against a specialist tapeworm, Schistocephalus solidus (Cestoda), expressed in some freshwater populations of threespine stickleback fish (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) only to be employed by threespine stickleback against the specialist parasite. We combined literature review on peritoneal fibrosis with a comparative experiment using either parasite-specific, or nonspecific, immune challenge in deliberately selected species across fish tree of life. We show that ray-finned fish are broadly, but not universally, able to induce peritoneal fibrosis when challenged with a generic stimulus (Alumadjuvant). The experimental species were, however, largely indifferent to the tapeworm antigen homogenate. Peritoneal fibrosis, thus, appears to be a common and deeply conserved fish immune response that was co-opted by stickleback to adapt to a new selective challenge.

KEY WORDS: Actinopterygii, comparative experiment, immunity, peritoneal fibrosis, stickleback, vaccination.

The comparative immunology research makes it clear that many of the fastest-evolving and most polymorphic genes in vertebrate species are involved in immunity (Litman and Cooper 2007; Lazaro and Clark 2012; Slodkowicz and Goldman 2020). Most notable is the evolutionary reshuffling of the genes coding Toll-like receptors (Solbakken et al. 2017; Velová et al. 2018), or the diversity of major histocompatibility complex (MHC) genes (Malmstrøm et al. 2016; Radwan 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, 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?

Vertebrates possess, in principle, two functionally distinct strategies to cope with infection according to parasite type combining innate and adaptive immunity (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 helmint 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 (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. 2021). 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 (Gause et al. 2013; De Lisle and Bolnick 2021). Here, we measure the extent of evolutionary conservation of a key immune phenotype, fibrosis.

Recent findings on interpopulation variation in helminth resistance from threespine stickleback fish (Gasterosteus aculeatus) demonstrate that antihelmintic fibrosis response is a fast-evolving immune trait (Weber et al. 2017a). Stickleback, originally a marine species, has only recently invaded freshwater habitats where it experienced greater risk of acquiring parasitic tapeworm Schistocephalus solidus (Cestoda) through feeding on freshwater copepods (Barber and Scharsack 2010; Rahn et al. 2016). When ingested, the tapeworm larva migrates through the intestinal wall to the peritoneal cavity of the fish and grows to its final size, often >30% the host’s mass (Arme and Owen 1967; 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. 2021).

This presumably beneficial form of resistance has costs in greatly suppressing female gonad development and male reproduction (De Lisle and Bolnick 2021; Weber et al. 2021). 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 the sticklebacks’ range (Weber et al. 2017a). In the other populations, stickleback have apparently adopted a nonfibrotic tolerance response to reproduce despite infection (Weber et al. 2017a). These nonfibrotic populations exhibit active upregulation of fibrosis-suppression genes in response to the tapeworm infection (Lohman et al. 2017; Fuess et al. 2020). The ancestral marine populations come very rarely into contact with S. solidus that do not hatch in saline water (Barber and Scharsack 2010) and do not exhibit observable fibrosis in the wild or in captivity (Hund et al. 2020). These various marine and freshwater populations have been diverging only since Pleistocene deglaciation (~12,000 years), indicating that their fibrosis response has evolved surprisingly quickly for such a fundamental immune process.

The peritoneal fibrosis can reliably be provoked in both fibrotic and nonfibrotic populations of stickleback by a generalized immune challenge (injection with a nonspecific Alum adjuvant), whereas only the resistant populations initiate fibrosis in response to tapeworm protein injection (Hund et al. 2020). So, the physiological capacity to initiate fibrosis seems conserved in stickleback and its sensitivity to the tapeworm fast-evolving. We therefore wished to determine whether this peritoneal fibrosis is similarly labile, or conserved across a broader range of fish species. One possibility is that peritoneal fibrosis is unique to stickleback, which is the only fish species to host S. solidus, an unusual parasite in that it invades the fish peritoneal cavity (Barber and Scharsack 2010). Or, fibrosis may be a widely conserved trait that stickleback have uniquely co-opted to deal with this specialist parasite species. Is peritoneal fibrosis an ancestral character state dating back to the origin of teleosts, or unique to stickleback?

To address this question, we begin with a broad literature review of fibrosis in fishes to systematically summarize documented instances for the first time. Then, we present an experimental test of how widely ray-finned fish (Actinopterygii) exhibit peritoneal fibrosis response toward an immune challenge based on a recent experimental study of fibrosis response in stickleback (Hund et al. 2020).

Methods

LITERATURE REVIEW

We searched for articles mentioning fibrosis or encapsulation from Actinopterygii using Web of Science database. We then sorted the collected suitable articles according to the presence of fibrosis or encapsulation, its topology, and cause. We also extracted the taxonomic information and present the results in a pivot table. For detailed methods, please see Appendix.

SPECIES SELECTED FOR THE EXPERIMENT

To conduct a phylogenetically broad assay of peritoneal fibrosis response in ray-finned fishes (Actinopterygii), we deliberately chose a diverse set of species spread across the phylogenetic tree of Actinopterygii. We experimentally vaccinated 17 species of fish (Table 1). These species were chosen to achieve broad phylogenetic diversity, but were restricted to commercially available small fish (body length of 2–10 cm, and live weight 0.2–6.0 g). We obtained most of the fish from a fish reseller. A local trout hatchery donated fingerlings of rainbow trout (Oncorhynchus mykiss). We received eggs of the turquoise killifish (Nothobranchius furzeri; population MZCS222) from a stock retained at the Institute of Vertebrate Biology CAS in Brno, Czech Republic. We hatched and maintained the killifish according to breeding protocol (Polačik et al. 2016) until they reached 2 months when fully mature. We also included wild-caught threespine stickleback (Gasterosteus aculeatus) that came from two distinct lake populations (Hosta and a’ Bharpa), both originating from North Uist, Scotland, UK, provided by Andrew MacColl (University of Nottingham, UK), and maintained at our housing facility for 6 months before the experiment.

FISH HOUSING

We standardized housing conditions across most of the species. We planned to have five to seven individuals per species per
Table 1. Species selected for the experimental test of peritoneal fibrosis response.

| Species # | Common name      | Species                      | Order         | Higher taxonomic rank | Control (PBS) | Tapeworm homogenate | Alum |
|-----------|------------------|------------------------------|---------------|-----------------------|---------------|---------------------|------|
|           |                  |                              |               |                       | N | W<sub>total</sub> | N | W<sub>total</sub> | N | W<sub>total</sub> | Origin        |
| 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 | Hyphessobrycon 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  | Tateurmudina ocellicada      | 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 helleri          | 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          |
| x         | Hosta stickleback (10d) |                         |               |                       |               |         |         |         |         | Wild          |

(Continued)
Table 1. (Continued).

| Species # | Common name | Species                        | Order                  | Higher taxonomic rank | Control (PBS) | Tapeworm homogenate | Alum | Origin |
|-----------|-------------|--------------------------------|------------------------|-----------------------|---------------|---------------------|------|--------|
|           |             |                                |                        |                       | \( N \)       | \( W_{total} \)     | \( N \) | \( W_{total} \) | \( N \) | \( W_{total} \) |        |
| 15        | a’ Bharpa stickleback | *Gasterosteus aculeatus* | Perciformes            | Eupercaria            | 6             | 0.27 (0.05)         | 6     | 0.29 (0.06)  | 6     | 0.30 (0.13)  | Wild   |
| x         | 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 featherback | *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 |

We show the sample size, \( N \), and average with standard deviation (SD) for live weight before dissection in grams, \( W_{total} \), in each experimental treatment. Note that Species # corresponds to species number in Figure 2. 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 treatment for 10 days (“10d”) indicated by “x.” The clown featherback (*Chitala ornata*) were extremely fibrosed (level 3) before the experiment started. The kissing gourami (*Helostoma temminckii*) developed white spot disease during the experiment. The taxonomic position of the family within the higher taxonomic group is not well resolved.
treatment. We prepared 20-gal (~76 L) tanks with reverse osmosis water conditioned to conductivity between 700 and 800 µS/cm with sea salt (Instant Ocean®). We adjusted salinity for marine species to 35 mg/kg. Water temperature ranged between 24.5 and 26°C. We also provided a seaweed-like plastic shelter for fish. We fed fish every morning with frozen bloodworms (Chironomidae), mysis shrimp (Mysidae), or dried sushi nori seaweed (Pyropia sp.) according to species-specific diet requirements. We held the rainbow trout at water temperature of 12°C as our standard temperature would be stressful to them. Similarly, stickleback is sensitive to high temperatures and we therefore kept them in their original recirculation system at 19°C and 1900 µS/cm throughout the treatment. All the other fish were habituated to the common tank setup for at least 5 days before treatment.

REAGENTS

Drawing on a recent experimental study of fibrosis response in stickleback (Hund et al. 2020), we challenged selected fish species with a generalized immune stimulant (Alum vaccination adjuvant), or with antigens from a specialist helminth parasite (S. solidus) that induces peritoneal fibrosis in some populations of threespine stickleback. Saline solution served as a control. All three treatments were delivered via peritoneal injection (the site of S. solidus infection).

The control treatment was an injection of 1× phosphate-buffered saline (PBS) (20 µL per 1 g of species average of live weight), which was also the solution for delivering the other treatments. The second treatment consisted of tapeworm antigen homogenate (abbreviated TH) suspended in PBS. Hund et al. (2020) showed that injection of 9 mg of tapeworm protein per 1 kg live fish mass induced rapid fibrosis in tapeworm-resistant stickleback populations. We obtained S. solidus tapeworms dissected from wild-caught threespine stickleback (Gosling Lake, Vancouver Island, BC, Canada). We sonified two tapeworms in PBS on ice and then centrifuged the suspension at 4000 rpm at 4°C for 20 min. We measured overall protein concentration in the upper fraction using RED 660™ protein assay (G-Biosciences) and then diluted the sample to 0.45 mg/mL. We aimed at injecting 20 µL of the solution per 1 g of live fish weight and to obtain the desired dose of tapeworm protein for fish weight (Hund et al. 2020). We then aliquoted the homogenate in Eppendorf tubes and stored at −20°C for later injections. Using the tissue of S. solidus, we wanted to test whether the stickleback response to this specialized parasite is unique among other fishes. It is possible that S. solidus protein contains distinctive antigens that any fish species would recognize as foreign.

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

INJECTIONS

At their arrival to our fish facility, we weighed each fish species on a balance to 0.01 g (Gene Mate GP-600) to estimate total volume of solution to be injected per individual. We injected 20 µL of the solution per 1 g of average species live weight with Ultra-Fine insulin syringes. We injected fish peritoneally through their left flank after anesthesia with MS-222 (200 mg/L for up to 2 min). Fish were then allowed to recover from anesthesia in a highly aerated tank water. We returned them to their original tank once they were swimming upright, but typically after more than 5 min after the injection. The different treatment groups were held in separate tanks because peritoneal fibrosis is a specific response to the treatment unaffected by common housing conditions (Hund et al. 2020).

DISSECTIONS AND SCORING FIBROSIS LEVEL

We euthanized fish 5 days postinjection, using an overdose of MS-222 (500 mg/L, >5 min). Because trout and stickleback were kept at lower temperatures, which may slow immune responses (Rijkers et al. 1980), we dissected trout 10 days after injection, and stickleback at both 5 and 10 days. We also dissected one to two individuals from each species prior to the injections to examine species-specific anatomy and assess the baseline level of peritoneal fibrosis before injections. We dissected fish immediately after euthanasia under stereomicroscope, photographed, and scored their level of fibrosis, using an ordinal categorical score. The peritoneal fibrosis score ranged between 0 and 3. Zero represents the absence of noticeable fibrosis, where the internal organs (liver, intestine, gonads) move freely apart from each other and from the peritoneal wall when moved with tweezers. Level 1 was for organs adhered together forming an interconnected conglomerate that moves as a unit. Level 2 was scored when the internal organs also attached to the peritoneal wall but was still possible to free them. Level 3 was the extreme form of organ adhesion where the peritoneal lining tore apart and remained attached to the organs after the body cavity had to be forcibly opened. Note that peritoneal fibrosis levels 1, 2, and 3 used here correspond with levels 2, 3, and 4, respectively, used by Hund et al. (2020). For illustration of the 0–4 scale (Hund et al. 2020), see video at https://youtu.be/yKvcRVCSpWI.

We excluded two species before data analysis. The clown featherback (Chitala ornata) were already extremely fibrosed (level 3) when they arrived to our facility due to unknown reason and the kissing gourami (Helostoma temminckii) developed...
white spot disease during the experiment after they were injected. This made the total number of species tested 17 (Table 1).

**DATA ANALYSIS**

We formally tested the interaction between the effect of treatment (PBS, TH, Alum) and experimental species identity on individual fibrosis score using generalized least squares (GLS) model (function *gls*, library “nlme” version 3.1-148 [Pinheiro et al. 2018]). The response variable was fibrosis level (ordered integers 0–3) scored from the individual’s left flank. We set treatment, species, and their interaction as fixed model effects. We attempted to originally analyze the ordinal response variable using Cumulative link models (function *clm*, library “ordinal” version 2019.12-10 [Christensen 2019]), but models with treatment–species interaction failed to converge due to model singularity.

To assess phylogenetic signal in the experimental data, we created a species tree based on the recent comprehensive phylogeny of ray-finned fishes by Hughes et al. (2018) with estimated divergence times. We measured phylogenetic signal (i.e., the tendency of related species to show similar response) in species maximum level of peritoneal fibrosis with Pagel’s $\lambda$ (function *phylosig*, library “phytools” version 0.7-47 [Revell 2012]). To alleviate the effect of non-Gaussian scale on the estimate, we nonparametrically validated the phylogenetic signal analysis in 10,000 permutations by randomly reassigning the trait values among species and recalculating the corresponding Pagel’s $\lambda$. We reconstructed ancestral state for the response to tapeworm homogenate and the response to Alum as discrete binary traits (presence or absence based on difference between PBS and either TH or Alum treatments). We used maximum likelihood approach with function *ace* (library “phytools”) for the joint estimation (i.e., based on the whole tree topology) of the likelihood at each node of the tree assuming equal rates for gain and loss of peritoneal fibrosis. All analyses were performed in R software version 4.0.1 (R Core Developmental Team 2020).

**Results**

**LITERATURE REVIEW: PERITONEAL FIBROSIS IN FISH IS KNOWN BUT NOT WELL DOCUMENTED**

Our first aim was to gather an overview of publication record encompassing peritoneal fibrosis in fish. In the articles we collected (for detailed methods, see Appendix), general fibrotic response was documented from a wide array of ray-finned fish (Actinopterygii). We found 375 out of the 1335 articles (28%) to be suitable for our study (i.e., articles mentioning fibrosis or encapsulation from Actinopterygii). The most-represented species came from the orders Cypriniformes (61 [including articles with multiple species]; 16% of relevant articles) and Salmoniformes (51; 14%) (Table 2). Authors typically identified signs of fibrosis during an autopsy, although some cases were observed from fish integument as well (e.g., capsules of skin parasites). Most of the suitable articles reported parasitism (197; 53%) or treatment (81; 22%) as the cause. Overall, fibrosis was present either on its own (175; 47%) or in combination with encapsulation of a foreign object (22; 6%).

The extent of fibrosis was usually described only qualitatively, along with the identity of the affected organs or tissues. Taking articles related to fibrosis (reporting fibrosis alone or in combination with encapsulation, 198 articles), its incidence was mainly confined to visceral organs (152 cases, 77%). Fibrosis located around or in the internal organs was, however, mainly interstitial fibrosis often represented by tissue scarification after damage. We found only seven articles that were dealing with peritoneal fibrosis specifically. These seven articles are diverse with regard to species taxonomic position and the cause of the fibrosis response (e.g., tapeworm infection, vaccination, radio-transmitter implantation) (Table 2). From this literature survey, we conclude that fibrosis is known in a number of fish species. Yet, the peritoneal fibrosis is very scarcely reported, which limits our ability to draw broader conclusions about its evolutionary history or its function.

**COMPARATIVE IMMUNOLOGICAL EXPERIMENT: COMMON AND STRONG IMMUNE RESPONSE TO ALUM**

We conducted a phylogenetically structured experimental study of ray-finned fish peritoneal fibrosis in response to immune challenge to reach more systematic understanding of its evolution. The level of fibrosis differed between experimental treatments and across species (GLS, treatment-species interaction, $F_{34,251} = 7.93$, $P < 0.001$) (Fig. 1). For most species, we observed no detectable or low fibrosis in control-injected (PBS) fish as well as in the tapeworm homogenate (TH) treatment (Fig. 1). However, there were two species, the common carp (*Cyprinus carpio*) and the channel catfish (*Ictalurus punctatus*), with variable individual response both in the PBS control and TH treatment (Fig. 1). Also note that in some species (such as *Danio rerio* or *Chromis viridis*), the default fibrosis level was 1 (organs attached together).

In contrast to the negative control and TH, the positive control (Alum) induced strong peritoneal fibrosis in most of the species (15 of 17). The response was typically high to extreme (fibrosis level 2 or 3; Fig. 1). The exceptions were two species of tetras, the Mexican tetra (*Astyanax mexicanus*) and the bleeding-heart tetra (*Hyphessobrycon erythrostigma*), which did not respond to any of the treatments (Fig. 1).

Phylogenetic signal for species maximum fibrosis appeared to be strong, significantly different from random evolution
### Table 2. Overview of the literature search for presence of peritoneal fibrosis in fish.

| Higher taxonomic rank | Order                  | N | Fibrosis only | Capsule only | Both | None | Peritoneal fibrosis | Position | Cause                  |
|-----------------------|------------------------|---|---------------|--------------|------|------|--------------------|----------|------------------------|
| 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                     | 10       | 1                      | 2        |
| Eupercaria            | Centrarchiformes       | 12| 4             | 5            | 3    |      | 1                  | 10       | 1                      | 1        | 12                     |
| Eupercaria            | Moronidae             | 7 | 6             | 1            |      |      | 4                  | 2        | 4                      | 3        | 3                      | 2        |
| Eupercaria            | Perciformes           | 19| 4             | 11           | 1    | 3    | 10                 | 9        | 16                     | 2        | 1                      | 2        |
| Eupercaria            | Siluriformes           | 9 | 5             | 4            |      |      | 4                  | 5        | 1                      | 2        |
| Eupercaria            | Sciaenidae            | 8 | 5             | 3            |      |      | 2                  | 5        | 5                      | 1        |
| Eupercaria            | Scorpaeniformes       | 6 | 3             | 2            | 1    |      | 3                  | 3        | 3                      | 1        | 1                      | 1        |
| Zeiogadaria           | Gadiformes            | 10| 3             | 7            |      |      | 5                  | 4        | 1                      | 9        |
| 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                     |
| 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        | 6                      |
| Pelagia               | Scombriformes         | 9 | 1             | 4            | 2    | 2    | 1                  | 2        | 7                      | 2        |
| Protacanthopterygii   | Salmoniformes         | 50| 29            | 10           | 3    | 8    | 31                 | 19       | 20                     | 2        | 13                     | 7        | 8                      |
| other                  |                        | 53| 26            | 14           | 3    | 10   | 37                 | 13       | 26                     | 6        | 4                      | 11       | 9                      | 1        |
| multiple              |                        | 24| 9             | 6            | 1    | 8    | 13                 | 9        | 2                      | 11       | 4                      | 1        | 7                      | 1        |
| **Total**             |                        | 375| 175          | 116          | 23   | 61   | 7                  | 220      | 144                    | 11       | 197                    | 29       | 81                     | 53       | 15                     |

We show fish phylogenetic group, the presence of fibrosis and/or encapsulation, its location, and cause. For brevity, we pooled less represented fish orders (with less than six articles) into “other” category and also grouped articles with species from more orders into “multiple.” Highlighted in gray are records of peritoneal fibrosis (column) and articles from Characiformes (row). Characiformes include the two tetra species that did not respond with peritoneal fibrosis to any of the treatments in our immune challenge experiment.

1 *Incertae sedis*—the taxonomic position of the family within the higher taxonomic group is not well resolved.
**Figure 1.** Peritoneal fibrosis in the experimental fish species. Individual points in the species plots show recorded level of peritoneal fibrosis scored from their left flank (the side of the injection). The fibrosis level was scored on an ordinal scale 0–3 and the jitter was used to show all data points. Note that the three-spine stickleback (*G. aculeatus*) that stayed in the experiment for 10 days are shown in gray as they did not enter data analysis. For completeness, we also present data for two unused species in gray—kissing gourami (*Helostoma temminckii*) and clown featherback (*Chitala ornata*). Full version of the abbreviated species names can be found in Table 1. The *G. aculeatus* B is for a’ Bharpa population and *G. aculeatus* H for Hosta population.

![Data plot](image)

**Table 1.** Summary of fibrosis score by species and treatment.

| Species          | Control | TH   | Alum |
|------------------|---------|------|------|
| *P. senegalus*   |         |      |      |
| *Cyp. carpio*    |         |      |      |
| *Da. rerio*      |         |      |      |
| *I. punctatus*   |         |      |      |
| *A. mexicanus*   |         |      |      |
| *Hy. erythrostigma* |     |      |      |
| *On. mykiss*     |         |      |      |
| *Sp. nematoptera* |     |      |      |
| *T. ocellicauda*  |         |      |      |
| *Chr. viridis*   |         |      |      |
| *Sa. fasciatus*  |         |      |      |
| *X. helleri*     |         |      |      |
| *N. furzeri*     |         |      |      |
| *Or. niloticus*  |         |      |      |
| *G. aculeatus* B |         |      |      |
| *G. aculeatus* H |         |      |      |
| *L. macrochirus* |         |      |      |
| *Di. nigroviridis* |     |      |      |
| *He. temminckii* |         |      |      |
| *Chi. ornata*    |         |      |      |

**Discussion**

This study represents one of the first phylogenetically broad multispecies comparative experimental assays of the macroevolution of an immune response. We focused on evaluating the prevalence of peritoneal fibrosis in response to TH treatment was 0.013 (observed only in carp), in response to Alum the likelihood was 0.987 (Fig. 2).
of peritoneal fibrosis response across fishes. It has been shown to contribute to parasite growth suppression and elimination, and being highly evolutionary dynamic in postglacial lake populations of threespine stickleback (Weber et al. 2017a,b). We show that published literature contains little data on peritoneal fibrosis in ray-finned fishes. To fill this gap, we experimentally tested the prevalence of peritoneal fibrosis in a wide array of species deliberately chosen to represent a broad sample across the phylogeny of Actinopterygii. Our immune challenge resulted in a variable level of fibrosis between treatments and among species. Response to homogenate from the stickleback-specialized tapeworm was weak at best. This finding confirms that the use of fibrosis pathways in response to *S. solidus* is a recently evolved trait. The positive control treatment (Alum), on the other hand, provoked strong peritoneal fibrosis in most of the species tested except one specific lineage—two species of tetras (from family Characidae, Characiformes). The results therefore suggest that, despite being rarely observed or reported, the capacity for peritoneal fibrosis is a phylogenetically widespread aspect of fish immunity. The peritoneal fibrotic response is probably phylogenetically conserved at least to the origin of ray-finned fishes (Fig. 2), estimated around 380 million years ago (Hughes et al. 2018).

**LACK OF PERITONEAL FIBROSIS IN PUBLICATIONS**

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

Our study was motivated by interpopulation variation in threespine sticklebacks’ ability to encapsulate parasitic tapeworm *S. solidus* (Weber et al. 2017b). The interpopulation variation...
probably stems from an evolutionary trade-off between the benefit of resistance (early encapsulation of the worm) and the risk of organ adhesion, excessive fibroblast proliferation, and ultimately partial sterility in the stickleback (De Lisle and Bolnick 2021; Weber et al. 2021). Previous records on peritoneal fibrosis in threespine stickleback are mostly lacking (but see Hoffman 1975, p. 175), although the phenomenon is found in numerous populations across the species’ circumpolar range. This is in line with the literature search, where we found only very few studies reporting peritoneal fibrosis in fish. In these few articles, peritoneal fibrosis was associated with serious intrusion of body integrity, for example, radio-transmitter implantation (Mangan 1998), vaccination with bacteria (Colquhoun et al. 1998), or, similarly to the stickleback, tapeworm infection (Abdelsalam et al. 2016). Apparently, the stress has to be intensive and/or chronic to trigger peritoneal fibrosis. The question thus remained whether peritoneal fibrosis is that rare and highly specific response across fish species. We used a phylogenetically informed immune challenge experiment with selected representatives across the fish tree of life to answer this question.

PERITONEAL FIBROSIS IN RESPONSE TO VACCINATION ADJUVANT WAS PREVALENT IN MOST FISH SPECIES

We successfully triggered peritoneal fibrosis in most of the fish species tested with the positive control (Alum). Alum is a commonly used vaccination adjuvant that causes influx of multiple types of immune cells into the injected region and alerts individual’s immune system (Kool et al. 2012). Yet, the particular mechanism of how Alum promotes vaccination is still unknown. In the case of peritoneal fibrosis, the Alum crystals may in fact act as an irritating agent that stimulates the type 2 immune response leading to containment of the body nonself (Gause et al. 2013). Although Alum itself is not a natural challenge, it is an immune stimulant that induces a broadly relevant inflammatory response that is widely used in defense against diverse parasites and pathogens (Thannickal et al. 2014). The widespread response to the Alum injection demonstrates that the capacity for peritoneal fibrosis is distributed across ray-finned fish phylogeny. Absence of fish peritoneal fibrosis in the published literature may thus stem from high specificity (peritoneal cavity invasion), low severity/chronicity of the common stressors, or the phenomenon may simply have been overlooked as it was until recently in stickleback.

THE EXPERIMENTAL EXCEPTIONS

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

General indifference to the TH treatment
Schistocephalus solidus is a parasite specialist of the threespine stickleback. The cue to trigger peritoneal fibrosis in threespine stickleback in response to the tapeworm is probably specific, as shown by some populations that fail to respond to certain genotypes of S. solidus (Weber et al. 2017a). The parasite community in North Uist stickleback is relatively rich and includes S. solidus (Rahn et al. 2016). The Scottish stickleback were also observed to show peritoneal fibrosis in the wild, with a’ Bharpa population having strong response but Hosta population none (A. MacColl, pers. comm.). The tapeworm protein effective dose used here (9 mg/g of fish live weight) triggered strong fibrosis in a naturally fibrotic lake population from Vancouver Island (Hund et al. 2020), but it is possible that the Scottish stickleback do not recognize tapeworms from western Canada. The specific cue that triggers peritoneal fibrosis in response to the tapeworm infection is unknown, although presumably protein based, and the work on its identification is currently ongoing. It may still be as well possible that the expression of peritoneal fibrosis might be suppressed by stickleback in some nonfibrotic populations (Lohman et al. 2017; Fuess et al. 2020).

Absence of peritoneal fibrosis response
Our experimental data show that peritoneal fibrosis is widespread across the fish phylogeny, except for two related species—the Mexican tetra (Astrynax mexicanus) and the bleeding-heart tetra (Hyphessobrycon erythrostigma), which did not respond to any of the treatments. The adaptation of some populations of Mexican tetra to the freshwater cave systems and lower parasitic burden could partially explain the observed pattern (Peuß et al. 2020). Considering the risks associated with the peritoneal fibrosis described in the threespine stickleback, maintenance of such response could be too costly for the Mexican tetra. Interestingly, the cave Mexican tetras exhibit marked reduction of visceral adipose tissue immunopathology compared to the surface populations (Peuß et al. 2020). However, we provide evidence that the absence of the peritoneal fibrosis might be more prevalent among Characiformes: neither of the tetra species in our study did not...
respond to Alum. The nonspecific immunity of these tetra species might therefore differ from the other ray-finned fish, and is a tempting target for more research, although we cannot yet generalize to many populations of each species, or across the entire clade. The family Characidae containing the two tetras consists of over a thousand species widely distributed in fresh waters from Texas, USA to Argentina. It would be interesting to uncover the mechanism for and phylogenetic extent of the absence of peritoneal fibrosis in more detail. Interestingly, our literature search indicates that Characiformes are able to encapsulate parasites and also show signs of (interstitial) tissue fibrosis (Table 2).

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

AUTHOR CONTRIBUTIONS
DIB and MV designed the study. MV conducted experimental work and performed data collection and analysis. MV and DIB wrote the manuscript.

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DATA ARCHIVING
Data supporting this article are available along with the statistical code at DRYAD (https://doi.org/10.5061/dryad.d2547d83c) and FigShare (https://doi.org/10.6084/m9.figshare.12619367).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

APPENDIX
Literature Review on Peritoneal Fibrosis in Fish:
Methods
To gather an overview of publication record encompassing peritoneal fibrosis in fish, we performed two searches using Web of Science database on September 30, 2019. The first search was oriented toward parasite-induced fibrosis in fish with terms: (parasit* AND (fibro*) AND ((teleost) OR (fish))). The other literature search was focused on fibrosis in fish in general while we tried to avoid articles on human subjects: (fibrosis AND (fish OR teleost) NOT (human)).

We collected 1459 entries in total, of which 1337 articles were retained after double entries removal. We considered an article to be suitable for our study if it was on ray-finned fish (Actinopterygii) and contained information on any signs related to fibrosis, such as organ adhesion, spontaneous proliferation of fibroblasts (fibroma, fibrosarcoma), healing fibroplasia (scarification), or encapsulation (of a parasite or an implant, e.g.), that could be inferred from the title or article abstract. Encapsulation typically meant that an extra-bodily particle was surrounded with a layer of fibroblasts and extracellular matter (e.g., collagen fibers). We then sorted the suitable articles, by going into their main text, according to three criteria—the explicit presence of fibrosis or capsule (“fibrosis only,” “capsule only,” “both,” “none”), its topology (“viscera,” “other,” or “multiple”), and assumed cause (“parasite,” “toxicity,” “treatment,” “tumor,” or “unknown”). For topology (location) classification, we took internal organs related to excretory system, digestive tract, or reproduction (kidney, liver, gas bladder, gut, gonads, including also peritoneum) as “viscera” and the remaining organs or tissues, such as gills, skin, muscle, brain, heart, and so on, as “other.” When tissues of both types were affected, we labeled the article as “multiple.” We then grouped articles with respect to the given cause of the fibrosis-related marks or encapsulation, where “parasite” was used for infection with a uni- or multicellular parasite, “toxicity” was used when the study monitored known environmental pollution (e.g., heavy metals), “treatment” was used for deliberate manipulation with fish body or their living conditions (e.g., adding estrogen into water to test the effect on male physiology), “tumor” was used when fibrosis happened spontaneously (e.g., fibrosarcoma), and “unknown” pooled studies where the cause could not be identified. We extracted fish species names and sorted them into orders and higher taxonomic categories according to the recent phylogenetic resolution of the Actinopterygii tree of life by Hughes et al. (2018) and Rabosky et al. (2018). We then used this dataset to offer an insight into the published literature on fish fibrosis.
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