Eye flukes (*Diplostomum spp.*) damage retinal tissue and may cause a regenerative response in wild threespine stickleback fish

Ruth A. Frey[1], Lindsey M. Barrett[1], Lauren Parkin[1], Brittany Blakeley[1], Muriel Ålund[2], Gregory Byford[2], Abigail Euhus[1], Christine Tsarnas[1], Janette W. Boughman[2], and Deborah L. Stenkamp[1]*

[1]Department of Biological Sciences, University of Idaho, Moscow, ID 83844

[2]Department of Integrative Biology, Michigan State University, Lansing, MI 48824

*Author for Correspondence:

Deborah L. Stenkamp, Ph.D.

Professor, Biological Sciences

875 Perimeter Drive MS 3051

University of Idaho

Moscow, ID 83844-3051

USA

dstenkam@uidaho.edu

Abstract

Fish rely upon vision as a dominant sensory system for foraging, predator avoidance, and mate selection. Damage to the visual system, in particular to the neural retina of the eye, has been demonstrated to result in a regenerative response in captive fish that serve as model organisms (e.g. zebrafish), and this response restores some visual function. The purpose of the present study is to determine whether damage to the visual system that occurs in wild populations of fish also results in a regenerative response, offering a potentially ecologically relevant model of retinal regeneration. Adult threespine stickleback were collected from several water bodies of Iceland, and cryosectioned eye tissues were processed for hematoxylin and eosin staining or for indirect immunofluorescence using cell-specific markers. In many of the samples, eye flukes (metacercariae of *Diplostomum spp.*) were present, frequently between the neural retina and retinal pigmented epithelium (RPE). Damage to the retina and to the RPE was evident in eyes containing flukes, and RPE fragments were observed within fluke bodies, suggesting they had consumed this eye tissue. Expression of a cell proliferation marker was also observed in both retina and RPE, consistent with a proliferative response to the damage. Interestingly, some regions of infected retina displayed "laminar fusions," in which neuronal cell bodies were misplaced within the major synaptic layer of the retina. These laminar fusions are also frequently found in regenerated zebrafish retina following non-parasitic (experimental) forms of retinal damage. The stickleback retina may therefore respond to fluke-mediated damage by engaging in retinal regeneration.

Keywords: Stickleback, Diplostomum, Eye, Retina, RPE, Proliferation, Regeneration, Damage, Zebrafish

1. Introduction

Vision is considered the dominant sensory system for many teleost fish (Bowmaker, 2008). The structure and function of fish eyes are similar to those of other vertebrates (Stenkamp, 2015), but also display tremendous diversity as adapted to a wide range of visual environments and functions in foraging, predator avoidance, shoaling, and mate selection (Hofmann and Carleton, 2009; Musilova et al., 2019). A key functional component of the eye is the neurosensory retina, containing light-sensing rod and cone photoreceptor cells, inner retinal neurons for processing, contrast enhancement, and relaying information to the brain, Müller glia for structural and metabolic support, and microglia as the major immune cell type of the retina (Dowling, 1970). At the apical surface, the neural retina abuts the retinal pigmented epithelium (RPE). The RPE provides metabolic support to the retina from the nearby choroidal vasculature and participates in the recycling of visual pigment chromophore as well as photoreceptor outer segment membrane (Baba et al., 2022).

Damage to the fish retina under experimental circumstances, and as shown for model organisms such as goldfish and zebrafish, can result in a regenerative response that repairs the damage (Stenkamp, 2007). Experimental forms of damage include chemical neurotoxins (Fimbel et al., 2007; McGinn et al., 2019; McGinn et al., 2018; Mitchell, 2022; Nagashima et al., 2013; Sherpa et al., 2008; Sherpa et al., 2014; Stenkamp et al., 2001), bright light (Kassen et al., 2007; Qin et al., 2009; Thummel et al., 2011), stab wounds (Fausett and Goldman, 2006; Ramachandran et al., 2011), and selective damage to specific retinal cell types using genetic and pharmacological tools (D'Orazi et al., 2016; Montgomery et al., 2011; White et al., 2017). Each type of damage results in death of retinal neurons (Fimbel et al., 2007; Vihtelic and Hyde, 2000), the accumulation of microglia and other immune cells (Mitchell et al., 2018; Nagashima and Hitchcock, 2021; White et al., 2017), and can also result in re-entry of Müller glia into the cell cycle to undergo one asymmetric cell division (Nagashima et al., 2013). One of the daughter cells remains as a Müller glial cell, while the other generates a transit-amplifying proliferative population that migrates and generates postmitotic retinal neurons to replace those that were lost to the original damage (Powell et al., 2016). The regenerated fish retina is functional (McGinn et al., 2018; Mensinger and Powers, 1999) and new neurons are integrated with synaptic connections to undamaged retinal neurons (D'Orazi et al., 2016; Hitchcock, 1997; McGinn et al., 2018) and to targets within the brain as demonstrated by behavioral studies (Lindsey and Powers, 2007; Sherpa et al., 2008; Sherpa et al., 2014). This function and accuracy of connections is striking, particularly because the histology of the regenerated retina shows abnormalities such as neurons misplaced within synaptic layers (Sherpa et al., 2008; Sherpa et al., 2014), the overproduction of neurons (Powell et al., 2016; Sherpa et al., 2008), and errors in topographic patterning (Cameron and Carney, 2000; Stenkamp and Cameron, 2002; Stenkamp et al., 2001; Stenkamp et al., 2021). In cases of more modest damage, particularly to only rod photoreceptors, the regenerative response is distinct and involves primarily an upregulation of proliferation within a population of rod precursor cells (Montgomery et al., 2011; Morris et al., 2008). Rod precursor cells in undamaged retina of mature fish normally generate new rods at a

slow, regular pace (Stenkamp, 2011). Interestingly, experimental damage to zebrafish RPE can also result in a regenerative response, as was recently demonstrated using a genetic/pharmacological means of RPE ablation (Hanovice et al., 2019).

With the possible exception of stab wounds, the experimental approaches to retinal and RPE injury do not replicate the types of damage that would occur to the retina in wild fish. Is the regenerative response active and adaptive in cases of ecologically relevant retinal damage? A common parasitic disease that damages eye tissues of many species of fish involves infection by species of the trematode genus *Diplostomum. Diplostomum spp.* ("eye flukes") have a three-host life cycle, with the adult parasite form using obligate hosts of fish-eating birds such as grebes, loons, and gulls, and with lymnaeid snails and fish as intermediate hosts (Lester and Huizinga, 1977) (Fig. 1). The first larval forms (cercariae) emerge from the snails and penetrate the gills of fish (Hoglund, 1995; Whyte et al., 1991). Cercariae then shed their tails to become the second larval forms (metacercaria) and migrate to the eye via the circulatory system (Cavaleiro et al., 2012; Karvonen et al., 2003; Padros et al., 2018). The metacercariae populate the capsule of the ocular lens (Cavaleiro et al., 2012; Karvonen et al., 2004; Scharsack and Kalbe, 2014; Ubels et al., 2018), and/or the vitreous cavity of the eye (Blasco-Costa et al., 2014), and/or the space between the RPE and retina (subretinal space) (Lester and Huizinga, 1977; Padros et al., 2018; Ubels et al., 2018). The metacercariae grow in size (Lester and Huizinga, 1977), most likely by feeding on eye tissues (Lester and Huizinga, 1977; Padros et al., 2018). Parasites of the lens and vitreous cavity can obscure the light pathway through the eye or cause further damage to the lens resulting in opaque cataracts (Karvonen et al., 2004; Shariff et al., 1980). Parasites within the subretinal space can cause retinal detachment and subsequent retinal damage (Lester and Huizinga, 1977; Padros et al., 2018), as well as direct damage to the RPE (Padros et al., 2018). Parasitic load in these locations has been shown to impair visual function of infected fish (Owen et al., 1993; Ubels et al., 2018), leading to changes in feeding behavior (Vivas Munoz et al., 2021) and predator avoidance (Gopko et al., 2017), likely making infected fish easier targets for the parasites' bird hosts.

In the present study we evaluate retinal structure and markers of retinal cell types and proliferation in threespine stickleback sampled from freshwater and coastal marine environments, and that have active *Diplostomum spp.* infections. We find that the retinas infected with fluke metacercariae display regions of tissue damage and retinal detachment, as observed in previous studies (Lester and Huizinga, 1977; Padros et al., 2018; Ubels et al., 2018), and further that the parasites likely consume RPE (Padros et al., 2018). Interestingly, this damage is accompanied by localized proliferative responses that resemble the response to damage documented in model organisms. Finally, portions of the infected retinas show histological features similar to those of regenerated retinas.

2. Materials and Methods

2.1 Animals.

Threespine stickleback fish (*Gasterosteus aculeatus*) were caught from inland spring-fed and glacially-fed lakes, and coastal marine environments in Iceland, using minnow traps, in the summers of 2016, 2017, and 2018 (Table 1) (Alund et al., 2022). Water temperature and salinity were measured at each location. Healthy adult fish were transported to Michigan State University and maintained for 2-4 weeks in mixed-sex tanks, separated by population (Table 1), at the temperature and salinity measured onsite in Iceland (4-14°C; 0-1 ppt salinity for freshwater fish, 31 ppt salinity for marine fish), with continuous water flow. They received once daily ad libitum feedings with frozen bloodworms. Ten light-adapted stickleback from each population were humanely sacrificed for tissue collection, at which time sex was recorded. Whole eyes were removed with curved forceps, the corneas were punctured, and eyes immersed in 4% paraformaldehyde in phosphate-buffered (pH 7.4) saline (PBS), overnight at 4°C. Eyes were washed three times in PBS, then shipped overnight to the University of Idaho in PBS. Eyes were then photographed and lenses removed. Presence of fluke metacercariae within the eye was noted at this time if flukes were clearly visible within the eyecup or were released from the eye at the time of lens removal. Eyecups were washed for 10 min in PBS containing 5% sucrose, and then in a graded series of 5-20% sucrose, and cryoprotected overnight at 4°C in 20% sucrose. Eyecups were then incubated at room temperature for 30 min in a 2:1 solution of 20% sucrose in phosphate buffer:OCT (optimal cutting temperature embedding medium; Sakura Finetek). Eyecups were embedded and frozen in this solution, using supercooled (with liquid N_2) methylbutane, and stored at -20°C until sectioning. Cryosectioning (7 µm) was performed using a Leica CM4050 cryostat and sections were collected onto SuperFrost slides (Thermofisher). Slides were dried in a vacuum desiccator for at least two hours and then stored at -20°C until further processing.

Stickleback were collected with permission from landowners in Iceland, and with collecting permits from Vantajökulsþjóðgarður National Park and Fjállabak Nature Reserve; fish were imported to the U.S. under permits from the Icelandic Food and Veterinary Authority and the U.S. Fish and Wildlife Service. All procedures involving live animals were approved by the Michigan State University Animal Care and Use Committee.

2.2 Hematoxylin and Eosin (H&E) Staining.

Slides were rehydrated in PBS and then incubated in hematoxylin (Sigma-Aldrich) for 1-5 min and rinsed with tap water. They were then incubated in eosin (Sigma-Aldrich) for 1-2 min followed by a rinse in distilled water. Slides were dehydrated in an increasing EtOH series, and then with 100% xylene, and mounted in Permount (Fisher Scientific). Eyecups that had been noted to contain metacercariae, as well as those not noted to contain metacercarieae (at the time of dissection) were used. Some of the latter eyes, when sectioned, showed fluke infections were indeed present, while others did not (Table 1).

2.3 Indirect Immunofluorescence Staining.

Antibody staining was performed as previously described (Dhakal et al., 2015; Stevens et al., 2011). In brief, slides were incubated with blocking solution (20% goat serum in PBS with 0.1% Triton X-100) for 30 min, and then incubated with primary antibody overnight at 4°C in 1% goat serum/0.1% Triton X-100/PBS. Primary antibody was detected using a Cy3-conjugated secondary antibody (1:200), and 0.5-1.0 ug/mL 4'6-diaminidino-2-phenylindole (DAPI) was included in this step to label nuclei. Slides were mounted with Vectashield mounting medium (Vector labs). Eyecups that had been noted to contain metacercariae, as well as those not noted to contain metacercariae were used. Control sections were processed in the same manner, but without primary antibody in the solution applied for the overnight step.

Primary antibodies included mouse monoclonal zpr1, which targets Arrestin3a (Renninger et al., 2011) in red- and green-sensitive double cone photoreceptors (Larison and Bremiller, 1990) (1:100, Zebrafish International Resource Center; ZIRC); mouse monoclonal anti-Glutamine synthetase (GS), which labels Müller glia (Peterson et al., 2001) (1:500, BD Biosciences); mouse monoclonal anti-Proliferating cell nuclear antigen (PCNA), which labels cells in S-phase (1:200; Sigma-Aldrich); mouse monoclonal anti-HuC/D, which labels retinal ganglion cells and amacrine cells (Dhakal et al., 2015) (1:200-1:40; Molecular Probes/Life Technologies/Thermofisher); and mouse monoclonal anti-Synaptic vesicle 2 (SV2), which labels presynaptic terminals (1:2000, Developmental Studies Hybridoma Bank).

In the case of anti-PCNA staining, an antigen retrieval step was incorporated prior to the blocking step as follows. Slides were incubated in PBS with 0.1% Triton X-100 for 15 min at room temperature, then for 10 min in 10 mM sodium citrate pH 6.0 containing 0.05% Tween 20 at 95-100°C. Slides were allowed to cool in the citrate buffer for 10 min, and then rinsed in PBS with 0.1% Triton X-100.

2.4 Microscopy and Photography.

Whole eyes and lenses were imaged on either a Nikon SMZ-1500 with a QImaging MicroPublisher 3.3 RTV camera, or on a Leica M165 FC stereomicroscope with a DFC3000 G camera. Fixed individual metacercariae, and stained retinal cryosections were imaged on a Leica DM2500 compound microscope using a Leica DFC7000T camera, under differential interference contrast (DIC; Nomarski), brightfield, or epifluorescence optics, as appropriate. Figures were assembled using PhotoShop CS6.

3. Results

3.1 Presence of eye fluke metacercariae in eyes of threespine stickleback.

Whole, fixed stickleback eyes were dissected to remove lenses and continue tissue processing. During this procedure, opaque profiles were frequently evident within the eyecup (Fig. 2A), and/or were released from the eye during dissection, possibly having been present within the vitreal cavity (Fig. 2B) (Table 1). These profiles were oval in shape and ranged in length from 200 to 800 μm. Upon further evaluation, these profiles showed superficial features consistent with identity as the metacercariae of digenean trematodes, most likely of the *Diplostomum* genus, which often infect eyes of threespine stickleback and other freshwater teleosts in northern climates (Cavaleiro et al., 2012). These anatomical features include prominent oral and ventral suckers and an excretory pore (Fig. 2C) (Cavaleiro et al., 2012). Fish collected from freshwater lake populations were more likely to be infected than marine populations [Table 1; X^2 (1, N = 216) = 9.9399, p = 0.001617]. Within freshwater populations, those collected from spring-fed lakes were no more or less likely to be infected than those collected from glacial lakes [Table 1; X^2 (1, N = 148) = 0.0461, p = 0.830023], and those collected from lowland lakes were no more or less likely to be infected than those collected from highland lakes [Table 1; X^2 (1, N = 148) = 0.0461, p = 0.830023]. Males and females were equally likely to be infected [X^2 (1, N = 148) = 1.1135, p = 0.291312].

Hematoxylin and eosin (H&E) stained cryosections, and DAPI stained sections of stickleback eyes also revealed the presence of fluke metacercariae residing within the RPE (Fig. 2D), and frequently within the subretinal space between the neural retina and the RPE (Fig. 3; Supplemental Fig. 1) (Table 1). The H&E staining further revealed structures typical of *Diplostomum* metacercariae, such as oral and ventral suckers and pharyngeal cavities. The suckers and oral cavities were sometimes associated with fragments of RPE (Fig. 1D), suggesting that the metacercariae adhered to or traveled along or within the RPE, and may have consumed the RPE as well, as suggested by (Padros et al., 2018).

3.2 Histology of retina and RPE in fluke-infected eyes of threespine stickleback.

We processed eyes that were observed to contain metacercariae upon dissection for cryosectioning and H&E staining, as well as eyes that did not appear to contain metacercariae. Of the former, all of those sectioned also showed metacercariae within the subretinal space. Of the eyes without matacercarie visible within the eyecup, four of 62 showed metacercariae within the subretinal space (Table 1), suggesting that eye flukes can invade this space even when other metacercariae were not visible within the eyecup. The parasites may have been hidden from investigator view within RPE or present in the retinal periphery, which was not inspected during dissection.

Retinas not observed to contain flukes (58 of the non-infected eyes were examined as cryosections using H&E or a nuclear stain) displayed normal histological appearances, with hematoxylin-stained nuclear layers separated by eosin-positive synaptic (plexiform) layers (Fig.

3A). The RPE associated with the retina also appeared normal and healthy, such that the outer segments of photoreceptors were embedded within microvilli of the apposing RPE (Fig. 3A).

The retinas of fluke-infected fish displayed a wide range of histological conditions. For example, some regions of these retinas and RPE appeared to have a normal, healthy appearance, similar to those of uninfected stickleback eyes (Fig. 3B). In contrast, other regions were disrupted by the presence of metacercariae (Fig. 3C-E; Supplemental Fig. 1). Observed disruptions included apparent direct invasion/ripping of retinal tissue (Fig. 3C), disorganization of retinal tissue such that retinal layers were distorted (Fig. 3C), the presence of large vacuoles within the retina, predominantly in the outer retina (Fig. 3D), suggesting cellular stress and damage and possible loss of cells and an inflammatory response. The most common location of flukes was within the subretinal space (Fig. 3C, E; Supplemental Fig. 1), causing localized retinal detachments. Occasionally metacercariae were observed within the choroidal vasculature (Fig. 3E). Finally, some regions were so disrupted that the RPE was virtually absent, possibly consumed by the parasites (Fig. 3E) (Padros et al., 2018). The retinal damage observed may therefore be secondary to this loss of RPE and detachment of retina from RPE, as the RPE has essential functions for supporting a healthy neural retina (Chidlow et al., 2022). We also note that infected eyes tended to be fragile and possibly susceptible to dissection and histology artefacts.

Another interesting set of histological conditions was observed in some of the retinas of fluke-infected eyes. In this set of conditions, the neural retina remained apposed to the RPE, and showed little or no evidence of cellular stress. However, the laminar organization of the retina appeared disrupted, in primarily two distinct ways. The first was the presence of multiple nuclei spanning the inner plexiform layer (Fig. 3F, G), which we refer to as laminar fusions. The second was an overabundance and disorganization of nuclei within the ganglion cell layer (Fig. 3H). Interestingly, these histological conditions and features (laminar fusions; overproduction of retinal cells) have been well described in retinas of goldfish and zebrafish that have regenerated following experimental forms of retinal damage (Hitchcock et al., 1992; Maier and Wolburg, 1979; Powell et al., 2016; Raymond et al., 1988; Sherpa et al., 2008; Sherpa et al., 2014). These findings raise the possibility that retinas of threespine stickleback may respond to fluke-mediated damage through the deployment of a regenerative response similar to that described for teleost model organisms (Lahne et al., 2020b). We did not observe laminar fusions or overabundance of retinal cells in uninfected eyes (Fig. 3A).

3.3 Distribution of markers of Müller glia and cone photoreceptors in retinas of fluke-infected stickleback.

We stained retinal cryosections of fluke-infected eyes using indirect immunofluorescence methods and anti-Glutamine synthetase (GS) antibodies, which label the processes of Müller glia in zebrafish (Peterson et al., 2001). In eyes that appeared to be uninfected, GS-positive processes spanned the thickness of the nuclear layers of the retina and

beyond, to form the outer limiting membrane (OLM) at the level of photoreceptor inner segments, and the inner limiting membrane (ILM), vitreal to the ganglion cell layer (Fig. 4A).

As with the results of H&E staining, we observed subtle distinctions in staining appearance for GS immunoreactivity. In some retinal regions, GS-positive processes appeared similar to those observed in uninfected eyes (Fig. 4B). In other regions of fluke-infected eyes, particularly in cases where metacercariae were nearby in the subretinal space, staining was more variable, and dim or absent from regions apical to the outer nuclear layer, suggesting that the OLM had sustained damage, or the GS antigen had been degraded or shifted from the most apical process of the Müller glia (Fig. 4C). The ILM in some cases was not solidly stained (Fig. 4C).

We next processed retinal cryosections for indirect immunofluorescence with the zpr1 antibody, which labels cone Arrestin3a, found in double cones of teleost fish (Larison and Bremiller, 1990; Renninger et al., 2011). Retinas of eyes that appeared to be uninfected were characterized by a pattern of staining largely similar to the zpr1 staining pattern within zebrafish and goldfish retinas (Larison and Bremiller, 1990; Stenkamp et al., 1997), with labeling of outer segments and inner segments of double cones, and of their synaptic terminals of within the inner plexiform layer (Fig. 4D).

More than one type of staining pattern was observed in fluke-infected eyes. Some retinal regions showed staining of outer and inner segments as well as synaptic terminals, but this staining was uneven and cone morphology distinct from that seen in uninfected retinas (Fig. 4E). Other regions, generally those near metacercariae, showed a truncated staining pattern, with little or no evidence of either the presence of outer segments or the localization of the antigen to outer segments (Fig. 4F).

3.4 A marker of cell proliferation in the retinas of fluke-infected stickleback.

Because the H&E staining showed evidence of retinal and RPE damage, as well as histological features typical of regenerated retina, we wished to further test the hypothesis that stickleback retinas deployed a regenerative response to this damage. In teleost model organism systems, damage to photoreceptors only – particularly chronic, ongoing damage – results in rod precursors of the outer nuclear layer increasing their rate of proliferation and generating new rod photoreceptors to replace those lost to damage (Montgomery et al., 2011; Morris et al., 2005; Song et al., 2020). More catastrophic (acute) damage, especially that which involves damage to the inner retina, instead results in Müller glia re-entering the cell cycle to produce progenitors that proliferate and generate new neurons to replace those lost to damage (Lahne et al., 2020b; Montgomery et al., 2011). Therefore, we processed retinas for indirect immunofluorescence using an antibody targeting Proliferating cell nuclear antigen (PCNA), present in cells that are in S-phase. The apparently uninfected eyes showed limited and sporadic labeling within the outer nuclear layer (see inset to Fig. 5C), consistent with the

303 presence of rod precursors, which have a slow rate of proliferation in adult fish retinas and
304 generate new rods as the fish eye grows (Stenkamp, 2011).

305 In contrast, fluke-infected eyes showed regions in which PCNA-positive nuclei were
306 scattered throughout the outer nuclear layer and were present in greater abundance (Fig. 5A-
307 C), consistent with a rod precursor-based proliferative response to damage that may generate
308 new rod photoreceptors at an accelerated pace. In comparison with eyes that were not
309 observed to contain flukes (Fig. 5C inset), this proliferation activity appeared to be regionally
310 distributed, and likely in response to damage rather than constitutive. In addition, we observed
311 regions that included PCNA-positive nuclei within the inner retinal layer, and occasionally in
312 clusters (Fig. 5D-F). These latter observations are consistent with a Müller-glia-based
313 proliferative response that may generate several types of new neurons. Again, these responses
314 were regionally variable, as some regions of infected retinas showed little to no proliferation
315 (Fig. 54F inset).

316 The RPE of zebrafish, when damaged, can also regenerate through a proliferative
317 response (Hanovice et al., 2019), and so we examined sections for PCNA labeling within RPE.
318 Healthy-appearing RPE did not contain PCNA-positive nuclei (Fig. 6A-D). In contrast, PCNA-
319 positive nuclei were present in damaged RPE (Fig. 6F-H). These damaged regions of RPE were
320 highly disorganized, however, making definitive identification of RPE nuclei challenging.

321 ***3.5 Neuronal markers in regions of fluke-infected stickleback retinas with laminar fusions.***

322 The presence of PCNA in the outer and inner retinal layers of fluke-infected eyes was
323 consistent with a regenerative response to metacercariae-inflicted retinal damage, and the H&E
324 staining revealed apparent laminar fusions, which are also seen in regenerated zebrafish retinas
325 (Sherpa et al., 2014). To further characterize the laminar fusions, which are often seen in the
326 inner retina, contain neurons, and disrupt continuity of synaptic layers, we processed retinas
327 for indirect immunofluorescence using antibodies that target HuC/D (Elavl3), found in retinal
328 ganglion cells and amacrine cells, and Synaptic vesicle 2 (SV2), found in presynaptic terminals.
329 In uninfected eyes, the HuC/D antibody stained cells within the ganglion cell layer and inner
330 boundary of the inner nuclear layer, suggesting these were HuC/D-positive ganglion and
331 amacrine cells (Fig. 7A). In some regions of infected retinas, HuC/D-positive cells were also
332 localized to laminar fusions (Fig. 7B), similar to the situation in regenerated zebrafish retinas
333 (Sherpa et al., 2014).

334 In uninfected eyes, SV2-positive material was found in both plexiform layers of the
335 neural retina (Fig. 7C). In some regions of infected retinas, anti-SV2 stained the plexiform layers
336 but was largely excluded from the laminar fusions (Fig. 7D).

337

4. Discussion

The main findings of this study include: 1) Fluke metacercariae infect the eyes of threespine stickleback sampled from several types of environments in Iceland; 2) The metacercariae can also invade the subretinal space, likely causing retinal detachment (Padros et al., 2018); 3) Metacercariae may inflict direct damage upon the RPE and retina, possibly consuming RPE (Padros et al., 2018); 4) Infected retinas can respond to this damage by proliferation within the outer and inner nuclear layers; 5) The patterns of cell proliferation and the presence of laminar fusions within fluke-infected eyes suggest that their retinas may respond to damage by engaging a regenerative response.

A moderate proportion of the threespine stickleback sampled from distinct Icelandic populations showed fluke metacercariae present within the eyecup and/or the subretinal space. While in some cases only one or a few parasites were observed, in other cases high numbers of flukes were seen, particularly within the subretinal space. The metacercaria form is non-reproductive, and so the variability in parasite load is likely a function of initial numbers of invading cercaria and/or number of infection events (Pennycuick, 1971; Vivas Munoz et al., 2021). In alignment with these observations, the degree of retinal and RPE damage of fluke-infected stickleback was highly variable, at times even within each individual eye. Types of damage included retinal detachment where the parasites occupied the subretinal space, thinning of the RPE, thinning of the photoreceptor layer, loss of photoreceptor outer segments, and direct disruption of retinal structure/tearing of the retina. Swelling of retinal cells and the formation of eosin-positive spaces within nuclear layers also suggests some type of toxic or osmotic insult, and/or loss of metabolic support to the retina via the RPE.

Use of the RPE as a nutrient source by metacercariae was previously suggested within infected Arctic charr, due to the presence of pigmented material within the flukes (Lester and Huizinga, 1977; Padros et al., 2018). We observed similar evidence of RPE material present within the parasites. Previous investigators had noted damage to RPE and damage to photoreceptors, but it was unclear whether the photoreceptor damage was related to the retinal detachment and loss of metabolic support by the RPE, or due to actual ingestion of photoreceptor components by the parasites (Padros et al., 2018). Unfortunately, in the course of this study we discovered that the (anti-mouse) secondary antibodies used, label metacercariae, and this artifact prevents us from making any conclusions regarding consumption of neural retina tissues by the flukes. However, RPE cells (as well as photoreceptors) have abundant membrane lipid content, and may represent a nutrient- and calorie-rich foraging strategy for metacercariae of *Diplostomum spp.* In addition, the disruption of the interaction between RPE and retina is known to cause metabolic stress in retinal cells, leading to degeneration and death of photoreceptors (Chidlow et al., 2022; Hanovice et al., 2019). We attempted to further examine the retinas for signs of reactive gliosis (Thomas et al., 2016) and inflammation/invasion by immune cells (Mitchell et al., 2018), but the markers available (Glial fibrillary acidic protein, GFAP within Müller glia; 4C4 and anti-L-plastin to label

immune cells) that are effective on zebrafish tissues were not effective for staining the stickleback tissues.

A well-documented type of retinal response to damage in fish is the process of retinal regeneration, which takes place in fish model organisms upon experimentally-induced retinal damage (Lahne et al., 2020b). The present study provides some evidence that a similar process, at least at the level of cellular proliferation, may take place in a natural setting in response to damage by a parasitic infection. We observed regions of retina in which many nuclei of the outer nuclear layer and some nuclei of the inner nuclear layer were PCNA-positive, indicating entry into the cell cycle and replication of DNA. The pattern of PCNA staining resembled that seen following experimentally induced damage to photoreceptors in zebrafish and is consistent with the observed regions of tissue damage in the stickleback retinas. For examples of PCNA staining in zebrafish retina following experimental damage, see Figure 1A of (Lahne et al., 2020a), Figure 1D-F of (Nelson et al., 2013), and Figure 6A-P of (Montgomery et al., 2011). Because our samples came from wild populations, and due to the limited existence of markers and other tools available for stickleback retina, we were unable to verify whether these PCNA-positive nuclei in the inner nuclear layer represented or were derived from Müller glia, as they are in zebrafish (Lahne et al., 2020b). A further limitation is that the fate of these proliferating cells cannot be known. A useful next step would be to undertake experimental infection of threespine stickleback in a laboratory setting, as in (Gopko et al., 2017; Scharsack and Kalbe, 2014), and use a nucleotide incorporation strategy (McGinn et al., 2019; McGinn et al., 2018; Sherpa et al., 2008; Sherpa et al., 2014; Vihtelic and Hyde, 2000) to label cells that were in S phase at the time of administration to determine if these cells generated new neurons. This, accompanied by the quantification of proliferating cells in infected vs. uninfected retinas would further test the hypothesis that proliferation is a component of a regenerative response to parasitic damage. We noted that some proliferative responses were exclusively localized to the outer nuclear layer, while others included the inner nuclear layer. The former may either represent the response of only the rod precursor cells to the loss or damage of rods, similar to that seen in zebrafish models in which predominantly rods are affected (Montgomery et al., 2011; Morris et al., 2005) or in which chronic, slow photoreceptor degeneration occurs (Song et al., 2020).

As an alternative, but indirect means to note the presence of regenerated neurons, we examined fluke-infected stickleback retinas for evidence of laminar fusions, in which cell bodies are misplaced within synaptic layers. In regenerated zebrafish and goldfish retinas, laminar fusions are most common in the inner plexiform layer, between the inner nuclear layer and ganglion cell layer [see Figure 3 of (Hitchcock et al., 1992)], and consist of cells that can be labeled with neuronal markers [see Figure 4 of (Sherpa et al., 2014)]. Hematoxylin and eosin-stained retinal sections of infected stickleback indeed displayed laminar fusions, as well as regions of disorganized retinal ganglion cell layer, which are also found in regenerated zebrafish retina (Sherpa et al., 2014). Staining of stickleback retina sections with markers of neurons (HuC/D) and presynaptic terminals (SV2) extended these findings, indicating that laminar

fusions contained neurons and disrupted the synaptic layers. We acknowledge that the laminar fusions may also – or instead – be the consequence of parasites burrowing through the thickness of the retina, leaving disrupted tissue in their wake.

While the current study does not directly demonstrate retinal regeneration in response to damage by fluke metacercariae, the results are consistent with this interpretation and provide a natural context for retinal regeneration in wild fish populations. Therefore, the threespine stickleback may have the means to restore some retinal structure in regions that have undergone fluke-related damage, potentially allowing some visual function amid the fluke infection, and possibly representing an ecologically relevant example of retinal repair in wild fish.

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Table 1. Presence of fluke metacercariae in threespine stickleback sampled from Icelandic populations.

Water source	Population[1]	Nickname	Collection year	# Eyes with flukes evident during dissection / # eyes collected	# Additional eyes with flukes revealed by sectioning / # sectioned[2]	Total # fluke-infected eyes sectioned and/or examined
Marine	Þangskálavatn	Thanga	2016	0/10	0/6	0
	Þangskálavatn	Thanga	2017	0/10	0/3	0
	Eyrar	Eyrar	2017	3/10	0/3	3
	Lónslón	Lon	2018	0/10	0/1	0
	Hófðvatn	Hofdi M	2018	0/10	0/1	0
	Midfjardará	Midfar	2018	1/10	0/4	1
	Hraunsfjórður	B Tide	2018	0/8	0/2	0
Lowland spring-fed	Hófðvatn	Hofdi LS	2016	6/9	1/2	7
	Berserkjahraunvatn	Berserk	2018	0/10	0/1	0
	Hópsvatn	Hops	2018	6/9	0/2	6
	Hraunsfjóðarvatn	Hraunf	2018	0/10	0/2	0
	Stemmulón	Stemu	2018	2/10	0/1	2
	Lítill Lónspollur	Litla Lon	2018	0/10	0/3	0
Highland spring-fed	Frostastaðavatn	Frosta	2016	0/10	0/3	0
	Galtaból	Galta	2018	1/10	1/3	2
Lowland glacial	Lágarfljót	Fljot	2018	1/10	0/1	1
	Grimsá	Grimsa	2018	2/10	1/2	3
Highland glacial	Blautaver	Blauta	2016	0/10	0/7	0
	Hrauneyjalón	Hrauny	2016	1/10	0/7	0
	Hvitárvatn	Hvita	2018	4/10	0/1	4
	Kýlingavatn	Kyling	2018	1/10	0/2	1
	Þristikla	Pristi	2018	6/10	1/4	7

[1] Populations sampled include several also sampled in (Alund et al., 2022).

[2] Denominators in this column include only eyecups not observed to contain flukes during dissection.

Figure Legends

Figure 1. The life cycle of *Diplostomum spp.* ("eye flukes"). **A.** Sexual reproduction of adult parasites within the intestine of an avian definitive host. **B.** Fertilized egg. **C.** Miracidium, seeking the first intermediate host, where it will give rise to sporocysts. **D.** Asexual reproduction of sporocysts in a lymnaeid snail. **E.** Cercaria, released from snail, seeking the second intermediate host, where it will shed its tail and develop as the second larval stage. **F.** Metacercaria residing within the fish eye. **G.** The life cycle is completed when an avian definitive host eats an infected fish.

Figure 2. Eye fluke metacercariae present in eyes of threespine stickleback fish. **A.** View of paraformaldehyde-fixed stickleback eye (from Hvita) following removal of lens, showing flukes (arrows) visible within the eyecup. **B.** View of lens (L) removed from a fixed stickleback eye (from Hvita), showing fluke that was recently released from the eyecup along with the lens (arrow). **C.** Differential interference contrast (DIC/Nomarski) image of two flukes that were released from the eye (stickleback from Hofdi LS) during dissection. Ventral views show oral (O) and ventral (V) suckers and excretory pore (EP). **D.** Hematoxylin and eosin-stained section (7 µm thickness) of stickleback eye (from Hofdi LS), showing a fluke present within and disrupting the retinal pigmented epithelium (RPE), with the ventral sucker apparently clinging to a fragment of RPE (asterisk in center of image), and possibly some RPE within the oral cavity (middle of white circle at anterior of metacercaria). Fluke shown in 2D is the same as that shown in Fig. 3C. Scale bars: A, 1.0 mm; B, 1.0 mm; C, 200 µm; D, 50 µm.

Figure 3. Histology (hematoxylin and eosin staining) of sectioned (7 µm thickness) stickleback retina and retinal pigmented epithelium (RPE) **A.** Retina and RPE of uninfected eye (from Hrauny), showing normal retinal lamination and RPE histology. **B.** Region of fluke-infected eye (from Hofdi LS) showing normal retinal lamination and RPE histology. Flukes were located >100 µm from region shown. **C.** Region of fluke-infected eye (from Hofdi LS) showing presence of a metacercaria (same as in Fig. 2D, but rotated orientation) surrounded by disrupted and damaged retina and RPE. **D.** Region of fluke-infected eye (from Galta) showing major damage to retinal organization. Numerous flukes were located <100 µm from retina, within subretinal space (but not in imaged area). **E.** Low-magnification view of fluke-infected eye (from Galta) showing region of major damage to retina and RPE, and the presence of numerous metacercariae; one fluke appears to occupy the choroid (CH). **F.** Region of fluke-infected stickleback eye (from Hofdi LS) showing a "laminar fusion" (LF), a histological feature typical of regenerated retina, in which nuclei are present in the inner plexiform layer (IPL). **G.** An additional example of a laminar fusion in a stickleback retina (from Hofdi LS). H. Region of fluke-infected stickleback eye (from Hofdi LS), in which disorganized, supernumerary nuclei appear within the ganglion cell layer (GCL). For panels F, G, and H, flukes were located >100 µm from regions shown. ONL, outer nuclear layer; INL, inner nuclear layer. Scale bars: A (applies to all others except E), 20 µm; E, 50 µm.

Figure 4. Indirect immunofluorescence of Müller glia and cone photoreceptor staining within sectioned (7 µm thickness) stickleback retina. All sections were counterstained with DAPI to show positions of retinal nuclei. **A.** Anti-glutamine synthetase (GS) staining of a retina of an uninfected eye (from Berserk) showing normal distribution of the GS antigen throughout Müller glia, extending from the outer limiting membrane (OLM) to the inner limiting membrane (ILM). **B.** A region of a fluke-infected eye (from Hofdi LS) also showing normal distribution of the GS antigen. **C.** Anti-GS staining of a region of a fluke-infected eye (from Pristi), with fluke metacercaria positioned in the interface between retina and RPE (arrow), showing possibly reduced GS antigen in the OLM. **D.** Zpr1 staining (targets arrestin3a) of a retina of an uninfected eye (from Thanga) showing normal distribution of the antigen within double cones in outer segments (OS), inner segments (IS), and synaptic terminals within the outer plexiform layer (OPL). **E.** A region of a fluke-infected eye (from Galta) showing somewhat disorganized distribution of the antigen. **F.** Zpr1 staining of a region of a fluke-infected eye (from Grimsa), with fluke metacercaria positioned in the interface between retina and RPE (arrow), showing shortened cones and fewer nuclei within the outer nuclear layer (ONL). INL, inner nuclear layer, GCL, ganglion cell layer. Scale bars: A (applies to B-E), 20 µm.

Figure 5. Cell proliferation in retinas of fluke-infected eyes of threespine stickleback. Sections (7 µm thickness) were counterstained with DAPI to show positions of retinal nuclei. **A-C.** Anti-PCNA (**A**) and DAPI (**B**) staining of a region of a fluke-infected eye (from Grimsa) showing PCNA+ nuclei (**C**; merged image) within the outer nuclear layer (ONL), possibly representing a response by rod precursors to photoreceptor damage. Inset to C is a merged image of a region an uninfected eye (from Berserk) showing no damage and very few PCNA-positive nuclei (arrows). **D-F.** Anti-PCNA (**D**) and DAPI (**E**) staining of a region of a fluke-infected eye (from Galta) showing PCNA+ nuclei (**F**; merged image) within the ONL, along with clusters of nuclei and individual nuclei within the inner nuclear layer (INL), possibly representing a more robust regenerative response. Inset to F is a merged image of a region from the same eye showing no damage and no PCNA-positive nuclei. AF, autofluorescence of photoreceptor inner segments. Scale bars: A (applies to B-F), 20 µm; Insets to C and F, 20 µm.

Figure 6. Cell proliferation in damaged RPE of fluke-infected eyes of threespine stickleback. Sections (7 um thickness) were counterstained with DAPI to show positions of nuclei associated with the RPE. **A-D.** Region of healthy RPE (from Grimsa): brightfield (BF) image (**A**), PCNA (**B**), and DAPI (**C**) staining. No PCNA-positive nuclei are present in this region of RPE (**D**; merged image; three DAPI-positive nuclei are indicated with arrows). **E-H.** Region of damaged RPE (from Grimsa): brightfield image (**E**), PCNA (**F**), and DAPI (**G**) staining. Several PCNA-positive nuclei are associated with the damaged RPE (**H**; merged image), two are indicated with arrows. Scale bar: A (applies to all), 20 µm.

Figure 7. Neuronal and synaptic markers in retinal regions of laminar fusions (LF) of fluke-infected eyes of threespine stickleback. Sections (7 µm thickness) were counterstained with DAPI to show positions of retinal nuclei. **A.** Anti-HuC/D and DAPI staining of uninfected retina

(from Hrauny) showing anti-HuC/D staining within neurons of the inner nuclear layer (INL) and ganglion cell layer (GCL). **B.** A region of a fluke-infected eye (from Pristi) showing HuC/D+ neurons within a laminar fusion (LF) joining the INL to the GCL. **C.** Anti-SV2 and DAPI staining of retina of uninfected eye (from Hofdi), with labeling of the outer plexiform layer (OPL) and inner plexiform layer (INL). **D.** A region of a fluke-infected eye (from Pristi) showing tissue in which the SV2+ OPL and IPL are interrupted by the presence of cell nuclei. Scale bar: A (applies to all), 20 μm.

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