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Altered neuronal activity in the visual processing region of eye-fluke-infected fish

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Research Article

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Abstract

Fish, like most vertebrates, are dependent on vision to varying degrees for a variety of behaviours such as predator avoidance and foraging. Disruption of this key sensory system therefore should have some impact on the ability of fish to execute these tasks. Eye-flukes, such as *Tylodelphys darbyi*, often infect fish where they are known to inflict varying degrees of visual impairment. In New Zealand, *T. darbyi* infects the eyes of *Gobiomorphus cotidianus*, a freshwater fish, where it resides in the vitreous chamber between the lens and retina. Here, we investigate whether the presence of the parasite in the eye has an impact on neuronal information transfer using the *c-Fos* gene as a proxy for neuron activation. We hypothesized that the parasite would reduce visual information entering the eye and therefore result in lower *c-Fos* expression. Interestingly, however, *c-Fos* expression increased with *T. darbyi* intensity when fish were exposed to flashes of light. Our results suggest a mechanism for parasite-induced visual disruption when no obvious pathology is caused by infection. The more *T. darbyi* present the more visual stimuli the fish is presented with, and as such may experience difficulties in distinguishing various features of its external environment.

Introduction

Nearly all fish are dependent on vision, to varying degrees, for performing fundamental tasks such as foraging, avoiding predation and mate selection (Guthrie, 1986). The acquisition, transfer and processing of visual information is part of a complex network of neuronal interactions essential for perception, decision-making and response (Wagner, 2011). When internal or external influences interfere with this process, the ability of a fish to observe and react to environmental cues can be degraded drastically. For example, exposure to certain toxins can result in neuronal stress in the visual processing region of the brain, resulting in fundamental changes to the fish's response to stimuli (Kohler *et al.*, 1995). Vision impairment through events such as increased turbidity or prolonged darkness can severely hinder the foraging ability and predator avoidance behaviours of affected individuals (Reid *et al.*, 1999; Rowe *et al.*, 2003; Knudsen *et al.*, 2006).

Neuronal activity, and thus visual information transfer, can be measured by looking at the expression of genes associated with neuron activity, such as *c-Fos*. The *c-Fos* gene is conserved across all vertebrates and expressed as a result of neuronal activation, thus measurement of the resulting upregulated mRNA or *Fos* proteins is an indirect marker of recent neuronal activity (Dragunow and Faull, 1989; VanElzakker *et al.*, 2008; Wiedmann *et al.*, 2013; Pacheco *et al.*, 2019). In fish model systems, *c-Fos* expression has been used to determine the effect of a variety of stimuli, from toxins/pollutants to aggression displays, on the central nervous system (CNS) of exposed individuals (Salierno *et al.*, 2006; Wai *et al.*, 2006; Topal *et al.*, 2015; Almeida *et al.*, 2019). Rainbow trout *Oncorhynchus mykiss*, for example, show increased *c-Fos* expression in the cerebral and cerebellar regions of the brain when exposed to nickel chloride (Topal *et al.*, 2015). The expression of the *c-Fos* protein expression is altered in the telencephalon and the periaqueductal grey region of the optic tectum of the killifish *Fundulus heteroclitus* when they are exposed to various neurotoxins associated with harmful algal blooms (Salierno *et al.*, 2006).

Measurement of *c-Fos* expression has revealed that parasitic organisms can make alterations to the CNS in non-fish hosts. By measuring *c-Fos* expression, Morales-Montor *et al.* (2004) observed changes in hypothalamic and hippocampal activity in *Taenia solium*-infected mice, which they argued may alter hormonal regulation. The measurement of *c-Fos* expression can also be used to investigate the underlying mechanisms of parasite-mediated behaviour. House *et al.* (2011) used *c-Fos* expression to reveal altered neural activity in the limbic region's sexual arousal pathways of *Toxoplasma gondii*-infected rats exposed to cat urine, the parasite's definitive host. Though it had previously been shown that *T. gondii*-infected rats can be attracted to the odour of cats (Berdoy *et al.*, 2000), this study provided insight into the mechanism of the altered behaviour.

Diplostomid trematodes often reside in the eyes of fish as metacercariae where they can inflict variable levels of visual impairment, at times due to direct damage to the host's tissues (Paperna, 1991; Williams and Whitaker, 1997; Grobbelaar *et al.*, 2015; Blasco-Costa and Locke, 2017; Locke *et al.*, 2018). For example, *Diplostomum* spp. often reside in the lens of

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their host resulting in the formation of cataracts, with their opacity increasing with intensity of infection, therefore further reducing vision (Chappell, 1995; Wall, 1998; Karvonen *et al.*, 2004; Fig. 1B). Considering the important role vision plays in the environmental perception of many fish species, it is not surprising that parasite-impaired vision has been shown to hinder the host in a variety of ways (Guthrie, 1986; Owen *et al.*, 1993; Karvonen *et al.*, 2004; Seppälä *et al.*, 2004, 2005, 2008; Voutilainen *et al.*, 2008; Ubels *et al.*, 2018). It has been demonstrated that rainbow trout *O. mykiss* infected by *Diplostomum* spp. are more susceptible to predation and form less cohesive shoals compared to uninfected fish (Seppälä *et al.*, 2004, 2008). A further example of this is the reduced foraging success of Arctic charr *Salvelinus alpinus* infected with *Diplostomum spathaceum*, as evidenced by reduced prey consumption and delayed reaction time to prey in individuals with greater cataract opacity (Voutilainen *et al.*, 2008). Closely related diplostomids in the genus *Tylodelphys* reside in the humours of the eye rather than the lens and as such do not cause obvious pathology in the form of cataract formation (Stumbo and Poulin, 2016; Muñoz *et al.*, 2017, 2019). However, it has been shown recently that *Tylodelphys clavata* impairs the reaction distance of infected fish to prey and even reduces their ability to compete with uninfected conspecifics (Muñoz *et al.*, 2017, 2019). *Tylodelphys* spp. when present in the humours move about freely (Blasco-Costa *et al.*, 2017), and as such if there is physical pathology associated with infection it is likely to be more subtle than *Diplostomum* spp. causing cataracts in the lens.

In New Zealand, the only described diplostomid, *Tylodelphys darbyi*, infects the eyes of its second intermediate fish host, the common bully *Gobiomorphus cotidianus* (Blasco-Costa *et al.*, 2017; Presswell and Blasco-Costa, 2020). *Tylodelphys darbyi* is relatively large (>1 mm) in comparison with other species of the genus and freely move about the vitreous chamber of the eye between the lens and the retina (Blasco-Costa *et al.*, 2017). There does not appear to be any physical pathology associated with *T. darbyi* infection, but the parasite does appear to obscure vision by occupying the open region of the vitreous chamber (Fig. 1; Stumbo and Poulin, 2016). During the day, the parasite obstructs ~75% of the observed retinal surface while at night *T. darbyi* settles to the lower portion of the eye, reducing obstruction to ~30% (Stumbo and Poulin, 2016). It was suggested by Stumbo and Poulin (2016) that this diel behaviour could cause fish to be predated at higher rates due to increased visual obstruction. However, it has recently been demonstrated that while fish spend more time actively moving about in the open with higher infection levels there does not appear to be an impact of the parasite on anti-predator behaviour (Ruehle and Poulin, 2019; Ruehle and Poulin, 2020). Furthermore, Stumbo and Poulin (2016) postulated that the metacercariae were layering within the chamber, i.e. lying superimposed instead of adjacent to each other, as the amount of retinal surface area obstruction was independent of parasite intensity (Fig. 1D). Thus, the amount of light reaching the retina may be determined by the number of *T. darbyi* metacercariae within the eye of an infected fish due to layering and not overall retinal obstruction. If this is the case, then it is possible that impairment of visual information transfer would increase at higher infection levels. Therefore, we hypothesize that the number of *T. darbyi* metacercariae not only affects the amount of light reaching the retina, due to layering of individuals, but also influences neuronal activity induced by this light stimulus, measured as *c-Fos* expression, in the visual processing region in the brain of infected bullies. Here, we test the prediction that with decreased light and visual information reaching the retina, regions of the brain involved in visual processing would be less active and thus show lower *c-Fos* levels.

Materials and methods

Infected bullies were collected with minnow traps from Lake Hayes (44°58′08.0″S, 168°48′44.6″E) in Central Otago, New Zealand, in February of 2016, and transported to the University of Otago in aerated coolers. The fish were housed together in 30-L aquaria for 2 weeks, at 11 ± 2°C and with a 12/12 L/D light cycle, and fed commercial pellets (Ridley AquaFeeds Pty Ltd., Narangba, Qld., Australia) *ad libitum*. The 2-week holding period should have ensured that most metacercariae had reached infectivity to their avian definitive host. Tanks were provided with PVC pipe shelters (4 cm diameter × 10 cm length) to reduce stress and aggression, and a 75% water change was performed on alternate days. The fish ranged between 45 and 94 mm in length (mean ± S.D. = 63 ± 13 mm), and were selected at random for the following tests.

Light exposures

Fish ($n = 22$) were placed individually into 1 L beakers with 500 mL of water. The exterior of each beaker was wrapped in opaque tape to prevent fish from observing external information. Black plastic sheeting was placed on all sides of the shelving unit on which the beakers were maintained, limiting outside light, which itself was kept a low enough level to minimize visual stimulation while still allowing the observer to perform the experiment. After a 2 h acclimation period, a flashing light (135 lumens, 10 Hz) positioned 20 cm above the containers was activated from outside the black plastic sheeting for 15 min. Control fish ($n = 8$) were treated in the same way except for no light stimulus was provided.

Tissue preparation and parasite recovery

Following the experiments, fish were euthanized with MS-222 (tricaine mesylate, 1 g L^{-1}) 45-min after trials, allowing time for the upregulation of the *c-Fos* protein, and then dissected for parasite load and removal of the brain. *Tylodelphys darbyi* metacercariae were recovered from the humours in the eyes during dissection under a microscope. The cranium was separated from the body and the dorsal portion was dissected away, exposing the brain, which was then prepared following methods modified from Bosch *et al.* (2001) and Topal *et al.* (2015) for later observation of *c-Fos* activity. Once removed from the cranium, brains were fully submerged in 10% neutral buffered formalin containing 2.5% acrolein for 12 h. After this period, the brains were fully removed and placed into a fresh solution of 2.5% acrolein for 2 h. Brains were then transferred to paraformaldehyde for 2 h, followed by a series of four 15 min washes in potassium phosphate-buffered saline (KPBS). Following this, brains were placed into a 30% sucrose solution until embedding.

Once brains had sunk to the bottom of the solution, indicating full sucrose saturation, they were placed individually into histology moulds containing Cryomatrix™ freezing medium, and frozen using liquid nitrogen-cooled isopropyl alcohol and stored at −80°C. Sectioning was performed using a cryomicrotome (Thermo Scientific, Isceon 89). The sections were cut to 8 μm thickness along the dorsal plane, then mounted onto slides (4/slide) and stored at −80°C.

Immunohistochemistry

Tissue sections of the optic tectum mounted on slides were randomly selected for each fish. Initially, slides were placed in distilled water and incubated at 30°C for 60 min to remove Cryomatrix™, and then rinsed in KPBS for 2 min with mild

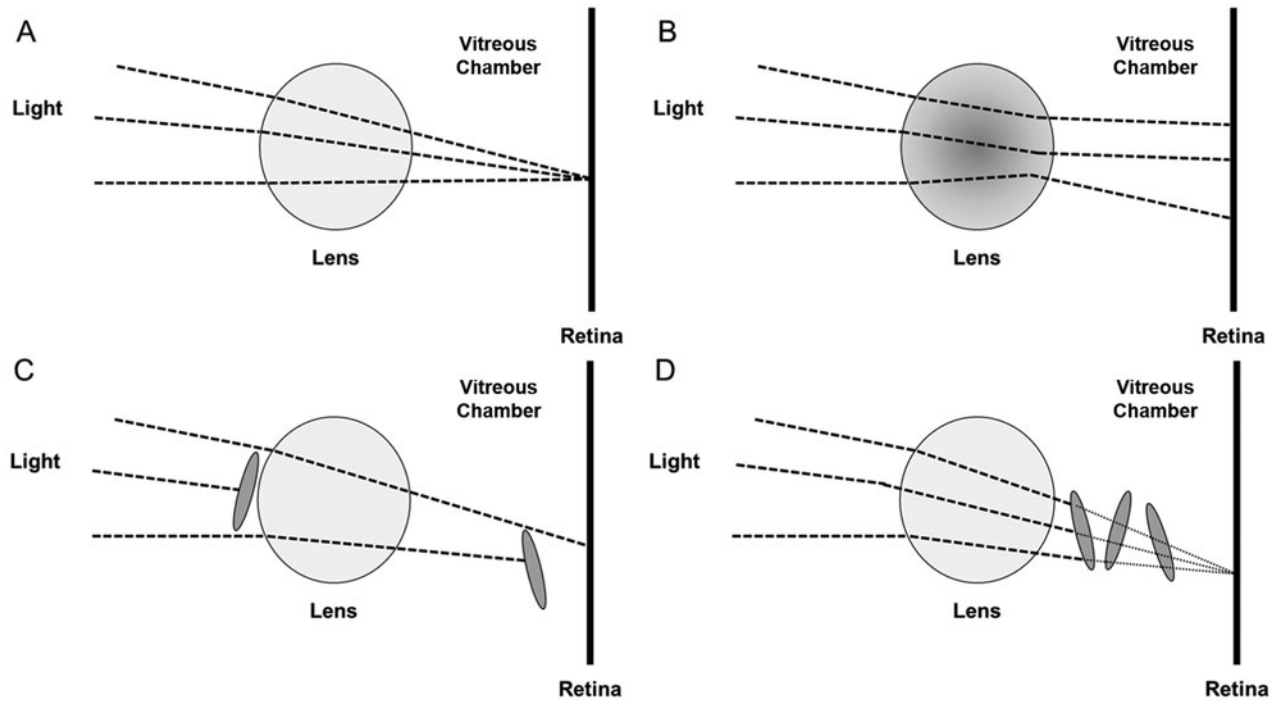

Fig. 1. (A) Light refraction through spherical lens in healthy fish eye. (B) Hypothetical light refraction in fish eye with cataracts in the lens. (C) Hypothetical light disruption by metacercariae moving freely within humours of a fish eye. (D) Hypothetical light disruption by semi-transparent metacercariae layered between the lens and the retina of a fish eye.

agitation. A hydrophobic circle was drawn around the tissue sample with a PAP pen before the remaining steps were performed on the slides. A 3-fold, 2 min rinse with KPBS was performed between the application of each solution unless otherwise noted. In order to reduce non-specific background staining, endogenous peroxidases were blocked by incubation with 0.3% H_2O_2 in 10% methanol in KPBS for 30 min. Incubation with Tween™ (polyoxyethylene (20) sorbitan monolaurate) was then carried out for 5 min to increase the permeability of the cells. A 20 min incubation in 2% normal horse serum in KPBS (Sigma-Aldrich, MO, USA) was used to further reduce non-specific binding. The primary antibody (polyclonal goat *anti-c-Fos*, 1:200 in 2% normal horse serum, Santa Cruz Biotechnology, TX, USA) was then immediately applied to the slides, which were left to incubate at room temperature for 60 min, and then at 8°C for 48 h.

Following incubation with the primary antibody, the secondary antibody (biotinylated horse anti-goat, 1:500 in KPBS, Vector Laboratories, CA, USA) was applied to the tissue and left to incubate at room temperature for 60 min. An avidin–biotin complex (Vectastain ABC standard kit, Vector Laboratories, CA, USA) was then applied for 60 min. Finally, nickel-DAB chromogen was applied for 20 min to allow for visualization of *c-Fos* expression and then rinsed with distilled water. Slides were left to dry for 12 h in the dark, after which mounting solution (Shandon IMMU-MOUNT, REF 990402) was applied with a cover slip and sealed with a clear lacquer.

Data analysis

Images of the optic tectum sections were acquired using a compound microscope (Olympus BX51) at 10× magnification, with a mounted camera (Olympus U-TV0.5XC-3). Analysis focused on the stratum marginale (SM), stratum opticum (SO) and stratum fibrosum et griseum superficiale (SFGS) (Fig. 2), as these regions are predominantly associated with vision and directly linked to the retinal ganglion cells (RGCs), which are the neurons responsible for transmitting visual information from the eye to the brain (Northmore, 2011). The periaqueductal grey region was disregarded as it was prone to tearing during histology and is largely a motor area. ImageJ (1.48v, U.S. National Institutes of Health, Bethesda, Maryland, USA) was used to determine the area of the analysed tissue (Fig. 2). *c-Fos* is expressed in cell nuclei, and these nuclei are revealed as black dots in stained tissue (Fig. 3). The number of nuclei with increased expression of *c-Fos* was counted manually. A generalized linear model with the quasi-Poisson distribution was performed with R 3.2.3 (R Core Team, 2016) with the number of metacercariae (mean of both eyes) per fish and treatment (control or light exposed) used as predictors, and the number of *c-Fos* + nuclei per mm^2 of tissue treated as the response. Fish length was treated as a proxy for age and used as a covariate.

Ethical statement

We used naturally infected fish for our experiments, because the complete life-cycle of *T. darbyi* is not known. For this reason, we aimed to minimize our sample sizes while keeping them large enough to achieve statistical requirements. *Gobiomorphus cotidianus* is the most abundant and widespread endemic, freshwater fish in New Zealand (McDowall, 1990) and >100 can be recovered in a single trap night, so the numbers we used in this experiment ($n < 40$) are not likely to impact the population. In addition, the Lake Hayes population has a *T. darbyi* prevalence of near 100% (Stumbo and Poulin, 2016), allowing us to collect smaller numbers of fish and still guarantee infection. Fish were kept in holding tanks at stocking densities of <2 L per fish and provided with enough shelter (i.e. 10 cm PVC pipes) that each individual had a territory to itself. Common bullies take longer to acclimate (e.g. pale colouration) to holding conditions when not in a group and without adequate shelter (author's per. observation), so these conditions were intended to reduce stress. Fish were held in captivity for no more than 4 months and at the

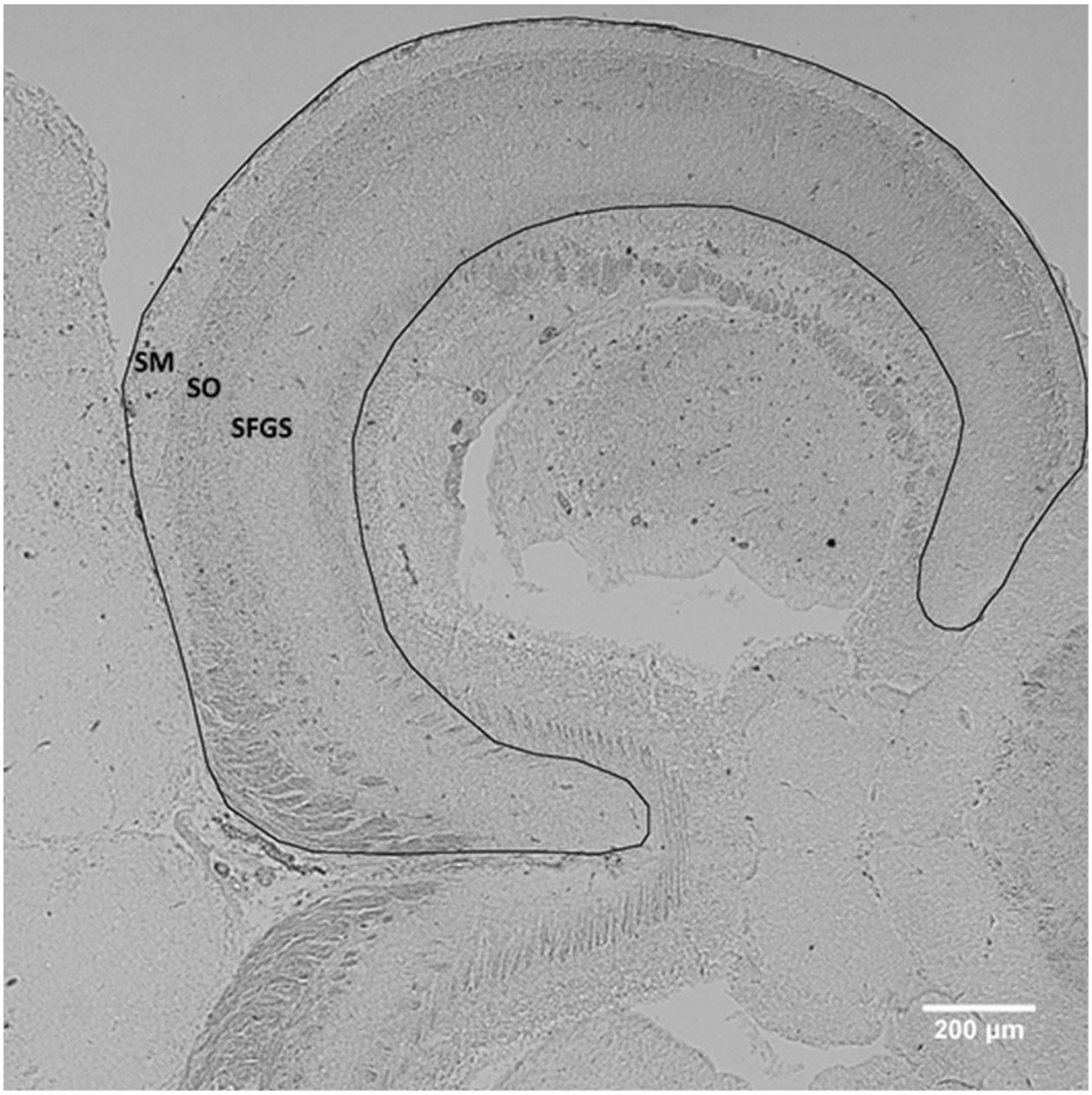

Fig. 2. Histological section of the brain of a common bully *Gobiomorphus cotidianus*. The outline indicates the visual processing region investigated for neural activation *via c-Fos* protein staining, comprising the SM, SO and SFGS as defined by Northmore (2011).

end of the experiment all individuals were humanely killed *via* overdose of MS-222 (1 g L^{-1}). These methods were approved by the University of Otago Animal Ethics Committee (no. 52/15).

Results

The mean number of *T. darbyi* metacercariae per fish among bullies exposed to the flashing light stimulus ranged from 0 to 17.5, with an average ± S.D. of 2.7 ± 4.1 metacercariae. One individual possessed an average of 55 metacercariae per eye, and therefore was treated as an outlier and removed from further analysis. Metacercariae numbers in control fish ranged from 0 to 14.5, with an average ± S.D. among individuals of 5.75 ± 5.5.

The model revealed a significant difference in the number cells exhibiting *c-Fos* expression between treatment groups ($t = 2.059$, $P = 0.047$; Fig. 4), with control fish possessing fewer *c-Fos*-expressing cells. The number of metacercariae was not a significant predictor of *c-Fos* expression among control fish ($t = 0.402$, $P = 0.690$). The number of metacercariae among fish exposed to the light stimulus, however, was a highly significant predictor ($t = 6.075$, $P < 0.001$), with a positive relationship observed between metacercariae number and increased *c-Fos* expression. There was no significant interaction between metacercariae number and treatment ($t = 1.168$, $P = 0.251$). Fish length also had no significant effect on *c-Fos* expression ($t = 0.153$, $P = 0.879$).

Discussion

Tylodelphys darbyi is large (>1 mm) and tends to inhabit the lower portion of the vitreous chamber, extending into the central region of the vitreous chamber in a parallel orientation to the lens (Stumbo and Poulin, 2016; Blasco-Costa *et al.*, 2017). This coupled with the lack of a significant effect of metacercariae number on the amount of retinal obstruction (Stumbo and Poulin, 2016) indicates sagittal layering of metacercariae between the lens and the retina. We hypothesized that with increasing *T.*

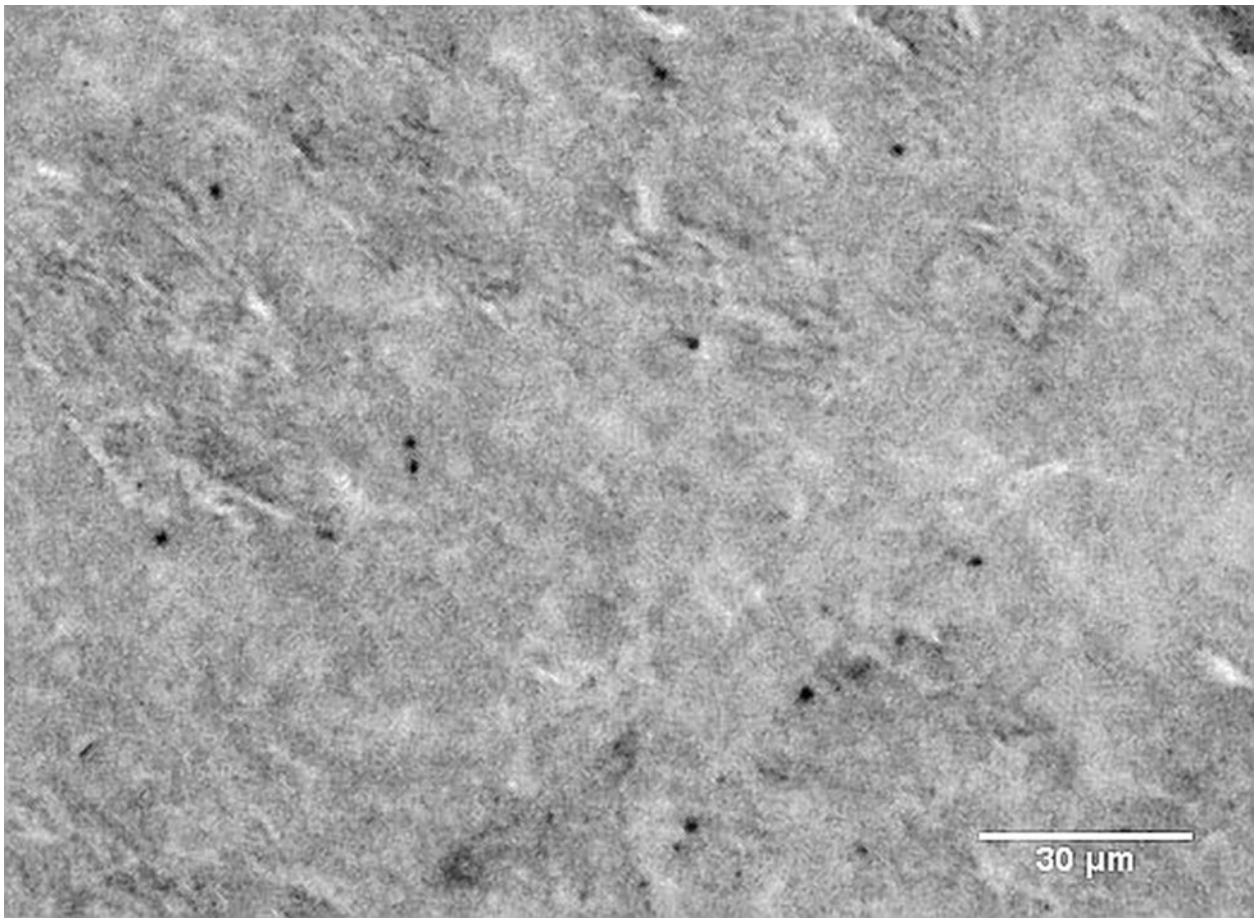

Fig. 3. Histological section of common bully *G. cotidianus* brain after immunohistochemistry staining. Each black dot represents the expression of the *c-Fos* protein in a cell nucleus, indicating recent neuron activity.

darbyi intensity, the amount of visual stimuli reaching the retina would decrease due to this layering of metacercariae regardless of the amount of overall retinal obstruction. It was expected, as a result, that with decreased light and visual information, regions of the brain involved in visual processing would be less active (i.e. lower *c-Fos* expression). Interestingly, we found the opposite pattern, i.e. an increase in *c-Fos* expression, and therefore neuronal activity, with higher parasite intensities, possibly suggesting that fish are receiving an overabundance of visual information. Although this may seem counterintuitive, it suggests a mechanism for parasite-induced visual disruption when no obvious pathology is induced by the parasite. Simply, we propose that the more parasites present in the eyes, the more visual stimuli the fish is presented with, and as such the fish may experience difficulties in distinguishing various features of its external environment. Ruehle and Poulin (2020) showed that at higher *T. darbyi* infections the amount of time a bully spent moving about in the open increased, potentially due to a greater need to explore their environment compared to those with fewer parasites. Our findings here provide a possible mechanism for this by showing increased neuronal activity with higher infection levels.

A by-product of infection, such as the metabolic waste of metacercariae, may be responsible for *c-Fos* protein expression pattern in a similar manner to when fish are exposed to toxins like pentylenetetrazole or nickel chloride (Baraban *et al.*, 2005; Topal *et al.*, 2015), with higher levels of infection resulting in an accumulation of the by-product and increased protein expression. This seems unlikely, however, considering that *c-Fos* expression was significantly lower in control fish not exposed to the light stimulus and was not linked to metacercariae number in these fish. Another possibility is that with the light stimulus, the metacercariae are causing a stress response in the fish. Stress from handling (Salierno *et al.*, 2006) or a startle response (Bosch *et al.*, 2001) has been shown to result in a greater number of cells with *c-Fos* expression, though this was in the periaqueductal grey region and the telencephalon which are primarily motor regions of the brain (Northmore, 2011). Also, the same studies did not find elevated *c-Fos* expression in the visual region of the optic tectum (SFGS, SO), the area analysed here (Northmore, 2011). This suggests that the observed upregulation of *c-Fos* in exposed fish is more likely a result of an increased amount of information being transferred from the RGCs to the optic tectum, with the metacercariae themselves acting as a source of visual information.

Light passing across the boundary between two media with different refractive indices is refracted to varying degrees, altering the light's course. Discounting the unlikely possibility that a metacercaria has the exact same refractive index as the vitreous humour surrounding it, the angle of the light passing through a parasite must be altered. This scattering of light would also likely be cumulative, intensifying with greater numbers of metacercariae. Additionally, the movement of the metacercariae must result in a change in the number of parasite bodies the light must pass through to reach the photoreceptors at any retinal location over time. These two mechanisms (light refraction and shifting light intensity) may produce a more complex stimulus than simply the flashing light alone, resulting in a greater amount of visual information proportional to the number of parasites per eye.

Visual acuity is dependent on a complex system of information processing starting within the eye. Briefly, visual information is

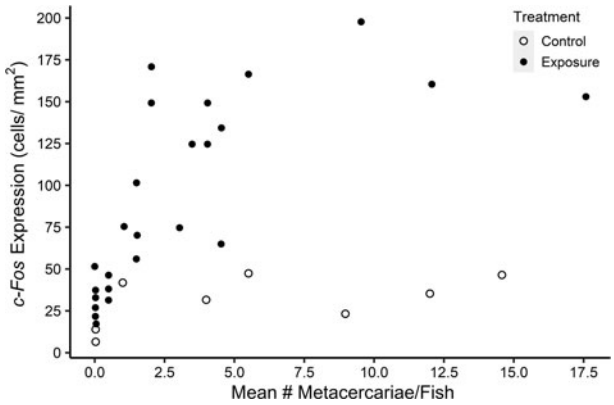

Fig. 4. Neuronal activity as indicated by the number of cell nuclei showing *c-Fos* expression as a function of mean metacercariae number per eye in *Tylodelphys darbyi*-infected *G. cotidianus*. Fish were either subjected to a visual stimulus (exposed) or left undisturbed (control).

first received by the photoreceptors, relayed to the bipolar cells and subsequently to the RGC. However, two additional cell types, the horizontal cells and amacrine cells, can integrate and modulate information at the photoreceptor–bipolar cell and bipolar cell–RGC interfaces, respectively (Levine, 2011). Thus, by the time a visual signal is received by the RGCs, significant processing has already taken place. These photoreceptors are also tightly packed within the retina, allowing for greater spatial resolution (Hirsch and Curcio, 1989). The receptive field of archer fish *Toxotes jaculatrix*, for example, is as low as a tenth of a degree in certain regions of the retina, allowing for high-sampling frequency (Ben-Simon *et al.*, 2012). Even slight changes in the angle of received information would result in reduced spatial resolution across the photoreceptor layer. It is likely, then, that *T. darbyi* metacercariae, although increasing the amount of visual information being processed, are decreasing the quality of the external information *via* information saturation.

The visual cortex is tied to other processing regions of the brain, and the observed increase in neuronal activation may have consequences for *T. darbyi*-infected bullies beyond impaired visual processing. A simplified example is that neuron activity in the SFGS is relayed to the torus longitudinalis (TL), which then signals the SM, resulting in an open loop between the optic tectum and the TL (Northmore, 2011). The TL, as well as being part of visual processing, is part of eye movement control and is linked to the premotor cortex (Northmore *et al.*, 1983; Wullimann, 1994). Prolonged neural stimulation in the visual cortex can also lead to neural fatigue, weakening neural response and visual acuity (Maffei *et al.*, 1973; Carandini, 2000). Neuronal stress resulting from visual information saturation may then weaken the visual processing ability of the fish, in turn impairing or altering its motor control.

Here, we have shown that fish harbouring *T. darbyi* demonstrate increased *c-Fos* expression at higher intensities of infection. We propose that high numbers of metacercariae in the eyes of fish result in increased scattering of incoming light before it reaches the retina, overwhelming the fish's visual processing centres. Although our results do not allow us to conclude that the amount of information reaching the RGC changes due to *T. darbyi* intensity, it is possible the quality of any external information will be reduced. This lends support to the suggestion that infection can impair the fish's ability to detect approaching predators presented in Stumbo and Poulin (2016) even though no impact has been observed thus far (Ruehle and Poulin, 2019). Furthermore, our findings provide a possible mechanism for why higher intensities of *T. darbyi* correlate with fish spending more time moving in the open (Ruehle and Poulin, 2020).

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Conflict of interest. The authors declare no conflict of interest.

Ethical standards. These methods used were approved by the University of Otago Animal Ethics Committee (no. 52/15).

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