Parasite-induced oxidative stress in liver tissue of fathead minnows exposed to trematode cercariae

A. D. STUMBO*, C. P. GOATER *and* A. HONTELA

Department of Biological Sciences, Water Institute for Sustainable Environments (WISE), University of Lethbridge, 4401 University Drive West, Lethbridge, AB, Canada T1K 3M4

(*Received 18 March 2012; revised 20 April and 15 May 2012; accepted 16 May 2012; first published online 16 August 2012*)

SUMMARY

Although results from field surveys have linked parasites to oxidative stress in their fish hosts, direct evidence involving experimentally infected hosts is lacking. We evaluated the effects of experimental infections with larval trematodes on induction of oxidative stress in fathead minnows, *Pimephales promelas*. Juvenile fish were exposed in the laboratory to the larvae (cercariae) of 2 species of trematode: *Ornithodiplostomum* sp. that develops in the liver, and *O. ptychocheilus* that develops in the brain. For *Ornithodiplostomum* sp., lipid peroxidation concentration in liver tissue increased 5 days after exposure and remained higher than controls until the end of the experiment at 28 days. For *O. ptychocheilus*, liver lipid peroxidation concentration was higher than controls at 5 days, but not thereafter. Sustained elevation in lipid peroxidation concentration for the liver trematode may be explained by direct tissue damage caused by developing larvae in the liver, or by an immune response. These experimental results support those from field studies, indicating that the lipid peroxidation assay may be an effective biomonitor for parasite-induced oxidative stress in fish, and that the nature of the oxidative stress response is species and/or tissue specific.

Key words: lipid peroxidation, metacercariae, *Pimephales promelas*.

INTRODUCTION

Aquatic organisms are exposed to a wide range of natural and anthropogenic stressors that have the potential to disrupt physiological homeostasis. One approach to diagnose and evaluate challenges to homeostasis, both in the field and the laboratory, is to quantify metabolic by-products indicative of physiological or cellular stress. Among such by-products are chemically-reactive molecules associated with oxidative stress. Internal or external stressors such as UV radiation, temperature extremes, contamination, tissue damage, and pathogens enhance the formation of reactive oxygen species (ROS) within specific tissue (Kelly *et al*. 1998; Toyokuni, 2002). Elevated ROS cause damage to the integrity of cell lipids, DNA, and proteins, ultimately leading to pathologies associated with tissue lesions, inflammation, cancer, and other dysfunctions (Kelly *et al*. 1998; Spector, 2000; Velkova-Jordanoska and Kostoski, 2005). By measuring oxidative stress biomarkers such as lipid peroxidation (LPO, a consequence of ROS-induced cell damage) or anti-oxidants (molecules that combat ROS), the mechanisms mediating the damaging effects of stressors can be determined (Kelly *et al*. 1998).

Studies with fish confirm that oxidative stress is a frequent consequence of exposure to various stressors, including anthropogenic toxicants (see reviews in Di Giulio *et al*. 1989; Kelly *et al*. 1998; Oost *et al*. 2003). Miller *et al*. (2007) reported increased oxidative stress via reduced glutathione levels in juvenile rainbow trout subjected to acute selenium exposure and ROS-induced damage has been suggested as a contributing cause of selenium toxicity and pathologies such as larval deformities (Spallholz *et al*. 2004; Muscatello *et al*. 2006). Recent studies have shown that parasites can also cause oxidative stress in fish tissues. Kurtz *et al*. (2006) measured a marker of oxidative protein damage (protein-bound acrolein) in liver of three-spined sticklebacks (*Gasterosteus aculeatus*) experimentally exposed to larval stages of nematodes and trematodes, and demonstrated a negative correlation between oxidative stress and fish body-condition, and a positive correlation with immune activation. However, most of the evidence for parasite-induced oxidative stress is indirect, arising from field studies with fish collected from sites that differ in exposure to a range of parasites. Farmed carp (*Cyprinus carpio*) infected with a cestode had elevated antioxidant levels compared with uninfected carp (Dautremepuits *et al*. 2003), and catfish (*Rhamdia quelen*) infected with a trematode had increased muscle LPO (Belló *et al*. 2000). Both exposure to mercury contamination and nematode infection were associated with increased oxidative stress in yellow perch (Marcogliese *et al*. 2005). Taken together, these results suggest that a variety of parasites can cause oxidative stress in

* Corresponding author: Tel: +403 329 2319. E-mail: anthony.stumbo@uleth.ca

Parasitology (2012), **139**, 1666–1671. © Cambridge University Press 2012
doi:10.1017/S0031182012001023

https://doi.org/10.1017/S0031182012001023 Published online by Cambridge University Press

a range of hosts. However, experimental evidence linking oxidative stress to parasitic infections remains limited.

Ornithodiplostomum sp. is an undescribed species of trematode that encysts within the body cavity of fathead minnows (*Pimephales promelas*; Matisz and Goater, 2010) while its congener *O. ptychocheilus* (Faust), encysts within the optic lobes (Matisz *et al.* 2010). In lakes and ponds in Alberta, these two species typically co-occur in the same individual fish (Sandland *et al.* 2001; Goater, *unpublished observations*). Both species utilize pond snails (*Physa* spp.) as first intermediate host, fathead minnows as second intermediate host, and piscivorous birds as definitive hosts. The development of larval *Ornithodiplostomum* spp. within host tissues is complex (Matisz and Goater, 2010; Matisz *et al.* 2010). For both species, there is an obligate period of development that occurs within the liver or brain (for *Ornithodiplostomum* sp. and *O. ptychocheilus*, respectively), followed by a consolidation and encystment phase that occurs within adjacent tissue. The former phase is associated with rapid rates of growth and differentiation, while the latter is associated with developmental 'resting' and encystment.

Developing *Ornithodiplostomum* metacercariae cause a range of detrimental effects in individual minnows, including reduced growth (James *et al.* 2008), reduced optomotor performance and activity (Shirakashi and Goater, 2005), and reduced survival (Sandland *et al.* 2001). Trematode-induced oxidative stress and associated pathology may provide a unifying mechanism to explain these diverse negative consequences of infection. The purpose of this experiment was to test the hypothesis that larval trematodes cause oxidative stress in minnows, by measuring LPO in liver tissue of fathead minnows exposed to *Ornithodiplostomum* sp. or *O. ptychocheilus* cercariae. First, we evaluated whether temporary development of *Ornithodiplostomum* sp. within liver tissue causes long-term oxidative stress, and whether these effects are dose dependent. To assess if parasite-induced oxidative stress requires direct tissue contact, or is part of a generalized response to infection, we also evaluated oxidative stress in the liver tissue of minnows exposed to *O. ptychocheilus*.

MATERIALS AND METHODS

Experimental infections

Naïve minnows were exposed to known numbers of *Ornithodiplostomum* spp. cercariae following methods described by Sandland and Goater (2000). To initiate experimental infections, young of the year (uninfected) minnows were collected from Goldspring Pond, Alberta (49° 5′ 41·3514″, −111° 59′ 28·6146″), a population known to be infected with metacercariae of *O. ptychocheilus* and *Ornithodiplostomum* sp. In July 2009, 8 one-day-old chickens were force-fed minnow brain or viscera containing large numbers of metacercariae. Three days later, parasite eggs were present in the feces of the chickens. Eggs were collected and processed following the method of Sandland and Goater (2000). The F_1 generation of laboratory reared *Physa* sp. snails were exposed to the miracidia, and reared under standard conditions. For exposure to cercariae, the dilution methods described by Sandland and Goater (2000) were used to estimate the volume of water containing known numbers of 2-h-old cercariae. Exposure of individual minnows occurred within 60 mm Petri dishes for a 2-h period. Previous results in our laboratory indicate that approximately 80% of cercariae used in exposures are recovered subsequently as metacercariae.

The experiment was set up as an 'infection' × 'time-period' factorial involving 108 naïve minnows separated at random into 9 groups. Treatments consisted of 36 fish exposed to dechlorinated water (control), 20 *Ornithodiplostomum* sp. cercariae (low-dose), or 100 *Ornithodiplostomum* sp. cercariae (high-dose) on 12 September 2009. Each group of 36 fish was separated at random into 3 groups of 12, corresponding to dates of dissection at 5, 10, or 28 days post-infection (p.i.). At each of these intervals, 2 minnows were prepared for histological analysis to assess metacercariae development and intensity. Livers from the remaining fish were removed under a dissecting microscope and placed on ice in homogenization microcentrifuge tubes. Samples were stored at −80 °C until analysis. It was not possible to mechanically isolate host tissue from parasite tissue due to the small size of developing metacercariae. Thus, our estimates of lipid peroxidation in host tissue assume that the contribution from parasite tissue is negligible.

We incorporated an additional group of 36 minnows exposed to 100 *O. ptychocheilus* cercariae into the overall design. A limited supply of fish and cercariae restricted our ability to complete a full 'time × dose × species' design. The 36 fish were necropsied in the same manner as described above at corresponding post-infection time-intervals (i.e. 5, 10 and 28 days).

Lipid peroxidation assay

Lipid peroxidation in liver tissue was evaluated with the BIOXYTECH® LPO-586 Assay (OXIS International, Inc., Portland, USA; catalogue no. 21012), following the methods described by Miller *et al.* (2007). In this assay, malondialdehyde (MDA), the end product of the LPO process, is used as an indicator for the concentration of LPO within tissue (Esterbauer *et al.* 1991). LPO concentrations were quantified by the reaction of MDA with n-methyl-2-phenylindole at 45 °C and 586 nm, and LPO is expressed as μmol MDA/mg of protein.

https://doi.org/10.1017/S0031182012001023 Published online by Cambridge University Press

Histopathology

Standard histological sectioning of *Ornithodiplostomum* sp.-infected minnows was used to provide a qualitative assessment of ontogenic changes in metacercariae growth and site selection, and to verify infection levels at 5, 10 and 28 days p.i. Histological sections of 2 randomly selected minnows were prepared from each of the 6 treatments. To allow for complete fixative penetration, minnow heads were removed immediately rostral to the operculum, and tails were removed just caudal to the visceral cavity (Matisz and Goater, 2010). Bodies were fixed in 10% neutral-buffered formalin for 7 days, and decalcified in 0·1M EDTA titrant for 14 days. These samples were dehydrated in ethanol prior to paraffin embedding. Each fish was serially sectioned along its sagittal plane (10 μm thickness). Sections were deparaffinized and stained with Mayer's haematoxylin and eosin Y. All sections were examined by light microscopy using a Zeiss axiocam digital camera mounted onto a Zeiss axioskop 40 microscope.

Statistical analysis

The data were log transformed prior to analyses to meet the assumptions of normality. A two-way ANCOVA was performed using Predictive Analytics SoftWare v.18 with 'time-period' of liver dissections (5 days, 10 days, or 28 days p.i.) and treatment (control, *Ornithodiplostomum* sp.-low, or *Ornithodiplostomum* sp.-high) evaluated as fixed effects, and LPO (μmol MDA/mg of protein) as the dependent variable. Minnow weight at necropsy was evaluated as a covariate. Least significant difference (LSD) pairwise comparisons were used to test differences between pairs of means within significant fixed effects. A second two-way ANCOVA was used to evaluate differences in LPO concentration between *O. ptychocheilus*-exposed and uninfected fish.

RESULTS

Results from the first two-way ANCOVA showed that the concentration of LPO in liver tissue was not affected by the interaction between time and infection with *Ornithodiplostomum* sp. cercariae ($F_{4,69}=1{\cdot}37$, $P=0{\cdot}255$). LPO concentrations were also not affected by time-period ($F_{2,69}=1{\cdot}35$, $P=0{\cdot}246$) and the covariate was not significant ($F_{1,69}=0{\cdot}097$, $P=0{\cdot}757$). However, LPO concentration in liver tissue was significantly affected by infection with *Ornithodiplostomum* sp. ($F_{2,69}=5{\cdot}66$, $P=0{\cdot}005$; Fig. 1). The LSD pairwise comparisons indicated that the 39% difference in LPO concentration between controls and lightly infected hosts was significant ($P=0{\cdot}006$), as was the 35% difference between controls and heavily infected fish ($P=0{\cdot}013$). The difference in LPO concentration between lightly and heavily infected fish

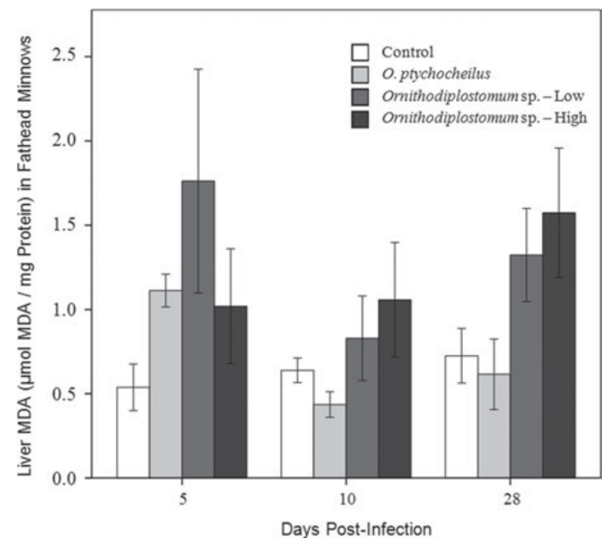

Fig. 1. Lipid peroxidation (malondialdehyde, MDA, mean ± S.E.) at 5, 10 or 28 days post-infection in liver tissue of fathead minnows exposed to cercariae-free water (Control), *Ornithodiplostomum ptychocheilus* cercariae, 20 *Ornithodiplostomum* sp. cercariae (Low-dose), or 100 *Ornithodiplostomum* sp. cercariae (High-dose).

was not significant ($P=0{\cdot}779$). This suggests that oxidative stress caused by *Ornithodiplostomum* sp. infection is independent of dose and time (Fig. 1).

The two-way ANCOVA of results from the *O. ptychocheilus* treatment showed that the concentration of LPO in liver tissue was not significant relative to time-period ($F_{2,44}=1{\cdot}109$, $P=0{\cdot}339$), treatment ($F_{1,44}=0{\cdot}253$, $P=0{\cdot}617$), or covariate ($F_{1,44}=0{\cdot}121$, $P=0{\cdot}730$). The interaction between time-period and treatment was significant ($F_{2,44}=3{\cdot}900$, $P=0{\cdot}028$, Fig. 1) due to the peak in LPO concentration at 5 days, followed by a decline to levels similar to controls at later time-periods.

Metacercariae counts in low and high dose treatments were significantly different ($P<0{\cdot}001$; $15\pm1{\cdot}6$ and $68{\cdot}6\pm6{\cdot}4$, respectively). Large numbers of small, unencysted *Ornithodiplostomum* sp. metacercariae were observed within the parenchyma of the liver at 5 days p.i. (Fig. 2A). At this time, a distinctive gap was evident between the metacercariae body and adjacent liver tissue. By 10 days p.i., unencysted metacercariae were still present within liver tissue. Qualitatively, these metacercariae were larger than at 5 days p.i. and the gap between the parasite and the adjacent liver tissue was still present (Fig. 2B). By 28 days pi, the metacercariae had increased further in size (Fig. 2C). Metacercariae were now absent from the parenchyma of the liver (although some were observed along its outer edge), located instead throughout the body cavity. All 28-day old metacercariae were enveloped by a distinctive cyst wall. These qualitative observations correspond with our previous assessment of *Ornithodiplostomum* sp. metacercariae growth and migration in minnows (Matisz and Goater, 2010).

https://doi.org/10.1017/S0031182012001023 Published online by Cambridge University Press

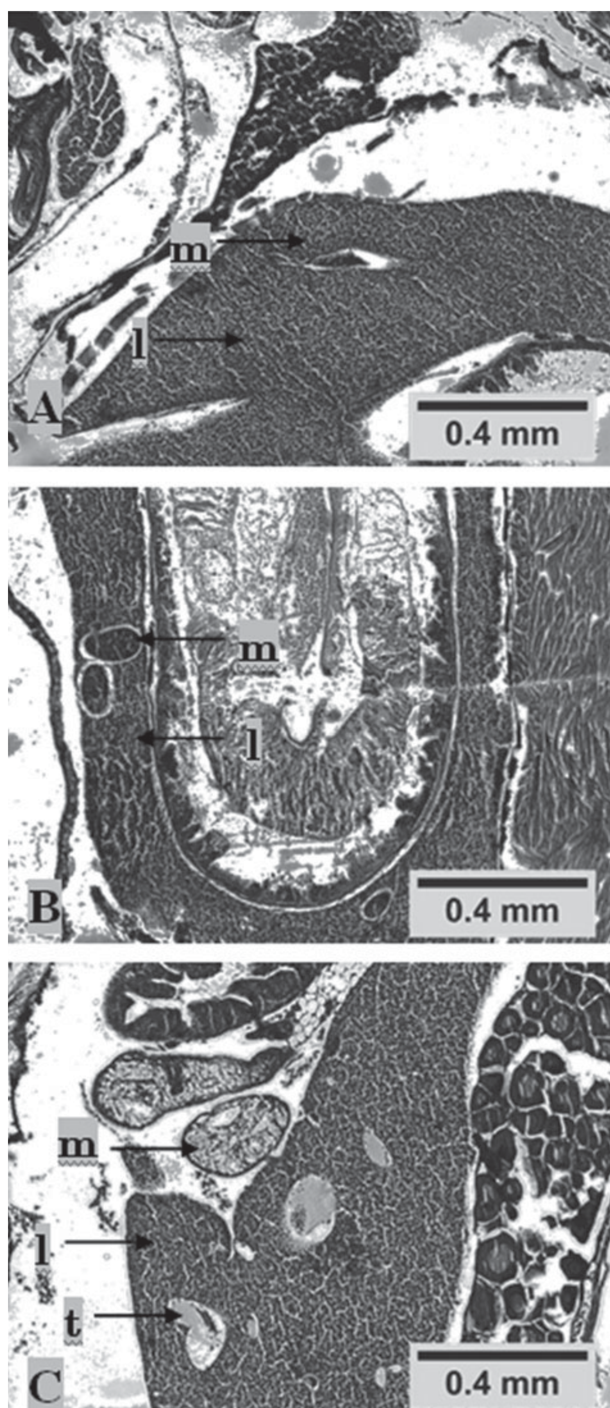

Fig. 2. Histological cross-sections of fathead minnows infected with *Ornithodiplostomum* sp. at 5 days (A), 10 days (B), and 28 days (C) post-infection. Liver tissue (l), *Ornithodiplostomum* sp. metacercariae (m), and metacercariae migration tracks (t) are indicated by arrows.

DISCUSSION

The results from this experimental study provide the first direct evidence for metacercariae-induced alteration in tissue lipid peroxidation in fish. Since LPO is a well-characterized indicator of oxidative stress, the results suggest that *Ornithodiplostomum* spp. infection causes elevated concentrations of ROS in fathead minnow liver tissue. These results support findings from several correlative studies involving parasite-induced oxidative stress in aquatic organisms (e.g. Belló *et al*. 2000; Dautremepuits *et al*. 2003). The results also support the use of the LPO assay for investigating interactions between parasites and environmental stressors under natural conditions.

This is the first study to evaluate oxidative stress throughout the various stages of larval parasite development. For minnows exposed to *Ornithodiplostomum* sp. cercariae, LPO concentrations diverged from controls at 5 days post-exposure, corresponding to the period when all metacercariae were observed within the parenchyma of the liver. At this time, the metacercariae are unencysted and an extensive network of microvilli envelope the entire worm, presumably to support feeding (Matisz and Goater, 2010). Thus, maximum differences in LPO concentrations between infected and uninfected minnows coincided with the period of maximum metacercariae development in the liver (Matisz and Goater, 2010). The difference persisted at least to 28 days post-exposure, by which time all metacercariae had reached the encysted stage outside the liver. An implication of these results is that elevated liver LPO likely occurs in fathead minnows that are exposed to *Ornithodiplostomum* spp. cercariae in natural lakes in late summer/early fall each year (Sandland *et al*. 2001).

The elevation in LPO detected in the present study may involve direct damage to liver tissue caused by developing *Ornithodiplostomum* sp. We did not assess tissue damage in this study, but Matisz and Goater (2010) and Matisz *et al*. (2010) showed extensive damage during the developmental phase of *Ornithodiplostomum* spp., followed by rapid tissue repair or regeneration. There is no direct evidence that these metacercariae feed on liver tissue, although the transient structure of the tegument during the development phase strongly suggests a feeding function (Conn *et al*. 2008; Matisz and Goater, 2010). Further, our observations that the gap between the tissue and developing metacercariae expands during the development phase suggests that tissue adjacent to developing worms is damaged, although perhaps temporarily. When cellular damage occurs, platelets involved in tissue repair release ROS to recruit additional platelets, a process known as redox signalling (Palmer and Paulson, 1997). Thus, both species of *Ornithodiplostomum* may induce tissue damage and oxidative stress resulting in lipid peroxidation.

An alternative explanation for the observed increase in oxidative stress in infected fish is host immunity. Cercariae and metacercariae of strigeiod trematodes elicit a variety of complex immune responses in fish (Stables and Chappell, 1986; Whyte *et al*. 1990), although the nature of the fathead immune response to *Ornithodiplostomum* spp. is not known. Further, sticklebacks exposed to parasites showed increased immune activation via respiratory

https://doi.org/10.1017/S0031182012001023 Published online by Cambridge University Press

burst (Scharsack *et al*. 2007). Respiratory bursts occur when leukocytes come in contact with foreign organisms such as bacteria, fungi, and parasites, resulting in the release of ROS (Muñoz *et al*. 1998; Wang *et al*. 2010) reducing the viability and affecting the development of certain parasites (Wilson *et al*. 1994; Allen and Fetterer, 2002). A positive correlation between the levels of immune activation and levels of oxidative stress has been demonstrated in sticklebacks exposed to various endoparasites (Kurtz *et al*. 2006). Further experimental studies are required to confirm the mechanistic link between parasite infection, the immune response, and oxidative stress.

Exposure to the brain-encysting trematode, *O. ptychocheilus*, also caused an increase in LPO in minnows. This is an important finding because it indicates that oxidative stress can be induced in non-target host tissue. Marcogliese *et al*. (2005) described a similar off-target effect in the livers of perch infected with a muscle-encysting metacercariae. These authors suggested a general inflammation response to encysting worms as a possible mechanism. If this is the case for *O. ptychocheilus*, then development of pre-encysted worms within the optic lobes may lead to a general inflammation response that is observed within liver tissue. An important follow-up question is to evaluate the singular versus combined effects of both species of *Ornithodiplostomum* spp. on oxidative stress in minnows. More generally, we need to understand whether parasite-induced oxidative stress increases as the number of different types of parasites in host tissue increases.

In addition to providing support for the LPO assay as an indicator of stress due to parasitism, our experimental results also support field-based approaches that seek to uncover the importance of cumulative or combined stressors on natural fish populations. Marcogliese *et al*. (2005) showed that a combination of parasites and pollutants such as mercury lead to higher levels of ROS than either stressor alone. Jacobson *et al*. (2003) showed synergistic effects leading to lowered immune function in chinook salmon when exposed to polychlorinated biphenyls (PCBs) and trematodes. Stress due to parasites is often neglected in studies of cumulative effects, but as our results indicate, specialist parasites that are a regular feature of most freshwater fish populations can reduce physiological performance (for review see Marcogliese and Pietrock, 2011), even at low rates of exposure. Thus, oxidative stress due to exposure to multiple environmental stressors, including parasites and pollutants, may be an underlying mechanism of decreased performance in natural fish populations.

ACKNOWLEDGEMENTS

We thank Heather Bird, Chelsea Matisz, and Lana Miller for laboratory assistance. This study was performed in accordance with regulations of the University of Lethbridge Animal Care Committee (permit # 1015).

FINANCIAL SUPPORT

Funding support from the Natural Sciences and Engineering Research Council of Canada to C.P.G. (grant number 40130) and A.H. (grant number 40220) is gratefully acknowledged.

REFERENCES

Allen, P. C. and Fetterer, R. H. (2002). Recent advances in biology and immunobiology of *Eimeria* species and in diagnosis and control of infection with these coccidian parasites of poultry. *Clinical Microbiology Reviews* **15**, 58–65.

Belló, A. R., Belló-Klein, A., Belló, A. A., Llesuy, S. F., Robaldo, R. B. and Bianchini, A. (2000). Lipid peroxidation induced by *Clinostomum detruncatum* in muscle of the freshwater fish *Rhamdia quelen*. *Diseases of Aquatic Organisms* **42**, 233–236.

Conn, D. B., Goater, C. P. and Bray, D. (2008). Developmental and functional ultrastructure of *Ornithodiplostomum ptychocheilus* diplostomula (Trematoda: Strigeoidea) during invasion of the brain of the fish intermediate host, *Pimephales promelas*. *Journal of Parasitology* **94**, 635–642.

Dautremepuits, C., Betoulle, S. and Vernet, G. (2003). Stimulation of antioxidant enzymes levels in carp (*Cyrinus carpio* L.) infected by *Ptychobothrium* sp. (Cestoda). *Fish and Shellfish Immunology* **15**, 467–471.

Di Giulio, R. T., Washburn, P. C., Wenning, R. J., Winston, G. W. and Jewell, C. S. (1989). Biochemical responses in aquatic animals: a review of determinants of oxidative stress. *Environmental Toxicology and Chemistry* **8**, 1103–1123.

Esterbauer, H., Schaur, R. J. and Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology and Medicine* **11**, 81–128.

James, C. T., Noyes, K. J., Stumbo, A. D., Wisenden, B. D. and Goater, C. P. (2008). Cost of exposure to trematode cercariae and learned recognition and avoidance of parasite risk by fathead minnows. *Journal of Fish Biology* **73**, 2238–2248.

Jacobson, K. C., Arkoosh, M. R., Kagley, A. N., Clemons, E. R., Collier, T. K. and Casillas, E. (2003). Cumulative effects of natural and anthropogenic stress on immune function and disease resistance in juvenile chinook salmon. *Journal of Aquatic Animal Health* **15**, 1–12.

Kelly, S., Havrilla, C., Brady, T., Abramo, K. H. and Levin, E. (1998). Oxidative stress in toxicology: established mammalian and emerging piscine model systems. *Environmental Health Perspectives* **106**, 375–84.

Kurtz, J., Wegner, K. M., Kalbe, M., Reusch, T. B. H., Schaschl, J., Hasseiquist, D. and Milinski, M. (2006). MHC genes and oxidative stress in sticklebacks: an immuno-ecological approach. *Proceedings of the Royal Society of London, B* **273**, 1407–1414.

Marcogliese, D., Brambilla, L. G., Gagne, F. and Gendron, A. D. (2005). Joint effects of parasitism and pollution on oxidative stress biomarkers in yellow perch *Perca flavescens*. *Diseases of Aquatic Organisms* **63**, 77–84.

Marcogliese, D. J. and Pietrock, M. (2011). Combined effects of parasites and contaminants on animal health: parasites do matter. *Trends in Parasitology* **27**, 123–130.

Matisz, C. E. and Goater, C. P. (2010). Migration, site selection and development of *Ornithodiplostomum* sp. metacercariae (Digenea: Strigeoidea) in fathead minnows (*Pimephales promelas*). *International Journal for Parasitology* **40**, 1489–1496.

Matisz, C. E., Goater, C. P. and Bray, D. (2010). Migration and site selection of *Ornithodiplostomum ptychocheilus* (Trematoda: Digenea) metacercariae in the brain of fathead minnows (*Pimephales promelas*). *Parasitology* **137**, 719–731.

Miller, L. L., Wang, F., Palace, V. P. and Hontela, A. (2007). Effects of acute and subchronic exposures to waterborne selenite on the physiological stress response and oxidative stress indicators in juvenile rainbow trout. *Aquatic Toxicology* **83**, 263–271.

Muscatello, J. R., Bennett, P. M., Himbeault, K. T., Belknap, A. M., Janz, D. M. (2006). Larval deformities associated with selenium accumulation in northern pike (*Esox lucius*) exposed to metal mining effluent. *Environmental Science and Technology* **40**, 6506–6512.

Muñoz, P., Calduch-Giner, J. A., Sitja-Bobadilla, A., Alvarez-Pellitero, P. and Perez- Sanchez, J. (1998). Modulation of the respiratory

https://doi.org/10.1017/S0031182012001023 Published online by Cambridge University Press

burst activity of Mediterranean sea bass (*Dicentrarchus labrax* L.) phagocytes by growth hormone and parasitic status. *Fish and Shellfish Immunology* **8**, 25–36.

Oost, R., v. d., Beyer, J. and Vermeulen, N. P. E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology* **13**, 57–149.

Palmer, H. and Paulson, K. E. (1997). Reactive oxygen species and antioxidants in signal transduction and gene expression. *Nutrition Reviews* **55**, 353–361.

Sandland, G. J. and Goater, C. P. (2000). Development and intensity-dependence of *Ornithodiplostomum ptychocheilus* metacercariae in fathead minnows (*Pimephales promelas*). *Journal of Parasitology* **86**, 1056–1060.

Sandland, G. J. and Goater, C. P. (2001). Parasite-induced variation in host morphology: brain-encysting trematodes in fathead minnows. *Journal of Parasitology* **87**, 267–272.

Sandland, G. J., Goater, C. P. and Danylchuk, A. (2001). Population dynamics of *Ornithodiplostomum ptychocheilus* metacercariae in fathead minnows (*Pimephales promelas*) from 4 northern-Alberta lakes. *Journal of Parasitology* **87**, 744–748.

Scharsack, J. P., Kalbe, M., Harrod, C. and Rauch, G. (2007). Habitat-specific adaptation of immune responses of stickleback (*Gasterosteus aculeatus*) lake and river ecotypes. *Proceedings of the Royal Society of London* **274**, 1523–1532.

Shirakashi, S. and Goater, C. P. (2005). Chronology of parasite-induced alteration of fish behaviour: effects of parasite maturation and host experience. *Parasitology* **130**, 177–183.

Spallholz, J. E., Palace, V. P. and Reid, T. W. (2004). Methioninase and selenomethionine but not Se-methylselenocysteine generate methylselenol and superoxide in an *in vitro* chemiluminescent assay: implications for the nutritional carcinostatic activity of selenoamino acids. *Biochemical Pharmacology* **67**, 547–554.

Spector, A. (2000). Review: Oxidative stress and disease. *Journal of Ocular Pharmacology and Therapeutics* **16**, 193–201.

Stables, J. N. and Chappell, L. H. (1986). The epidemiology of diplostomiasis in farmed rainbow trout from north-east Scotland. *Parasitology* **92**, 699–710.

Toyokuni, S. (2002). Reactive oxygen species-induced molecular damage and its application in pathology. *Pathology International* **49**, 91–102.

Velkova-Jordanoska, L. and Kostoski, G. (2005). Histopathological analysis of liver in fish (*Barbus meridionalis petenyi* Heckel) in reservoir Trebenista. *Croatian Natural History Museum* **14**, 147–53.

Wang, D., Malo, D. and Hekimi, S. (2010). Elevated mitochondrial reactive oxygen species generation affects the immune response via hypoxia-inducible factor-1α in long-lived *Mclk1*$^{+/-}$ mouse mutants. *The Journal of Immunology* **184**, 582–590.

Whyte, S. K., Chappell, L. H. and Secombes, C. J. (1990). Protection of rainbow trout, *Oncorhynchus mykiss* (Richardson), against *Diplostomum spathaceum* (Digenea): the role of specific antibody and activated macrophages. *Journal of Fish Diseases* **13**, 281–291.

Wilson, M. E., Andersen, K. A. and Britigan, B. E. (1994). Responses of *Leishmania chagasi* promastigotes to oxidant stress. *Infection and Immunity* **62**, 5133–5141.

https://doi.org/10.1017/S0031182012001023 Published online by Cambridge University Press