

RESEARCH ARTICLE

Living in flowing water increases resistance to ultraviolet B radiation

Ensiyeh Ghanizadeh-Kazerouni¹, Craig E. Franklin² and Frank Seebacher^{1,*}

ABSTRACT

Ultraviolet B radiation (UV-B) is an important environmental driver that can affect locomotor performance negatively by inducing production of reactive oxygen species (ROS). Prolonged regular exercise increases antioxidant activities, which may alleviate the negative effects of UV-B-induced ROS. Animals naturally performing exercise, such as humans performing regular exercise or fish living in flowing water, may therefore be more resilient to the negative effects of UV-B. We tested this hypothesis in a fully factorial experiment, where we exposed mosquitofish (*Gambusia holbrooki*) to UV-B and control (no UV-B) conditions in flowing and still water. We show that fish exposed to UV-B and kept in flowing water had increased sustained swimming performance (U_{crit}), increased antioxidant defences (catalase activity and glutathione concentrations) and reduced cellular damage (lipid peroxidation and protein carbonyl concentrations) compared with fish in still water. There was no effect of UV-B or water flow on resting or maximal rates of oxygen consumption. Our results show that environmental water flow can alleviate the negative effects of UV-B-induced ROS by increasing defence mechanisms. The resultant reduction in ROS-induced damage may contribute to maintain locomotor performance. Hence, the benefits of regular exercise are ‘transferred’ to improve resilience to the negative impacts of UV-B. Ecologically, the mechanistic link between responses to different habitat characteristics can determine the success of animals. These dynamics have important ecological connotations when river or stream flow changes as a result of weather patterns, climate or human modifications.

KEY WORDS: Reactive oxygen species, Exercise, Locomotion, Antioxidants, Habitat modification

INTRODUCTION

UV-B is a ubiquitous environmental driver that can damage proteins, DNA and membranes directly (Bancroft et al., 2007), or by inducing excessive production of reactive oxygen species (ROS) (Kulms et al., 2002; Lesser, 2006). Oxidative damage to membranes and proteins occurs when ROS production outpaces the capacity of cellular antioxidant defences (Constantini, 2014; Lesser, 2006). UV-B-induced ROS damage to muscle proteins results in impaired locomotor performance (Ghanizadeh Kazerouni et al., 2015, 2016). Physical activity increases ROS production by contracting muscle acutely (Powers and Jackson, 2008). However, chronic low- to mid-

level physical activity (exercise) increases antioxidant defences, such that oxidative stress is reduced with chronic exercise (Gram et al., 2015; Radak et al., 2013). Defences against oxidative stress in muscle include enzymatic antioxidants like superoxide dismutase (SOD) and catalase (CAT), and non-enzymatic ROS scavengers such as glutathione (GSH; Sen, 1995; Steinbacher and Eckl, 2015). SOD converts the superoxide anion to hydrogen peroxide, which is converted to water and oxygen by glutathione peroxidase and CAT (Lesser, 2006). It is possible that the increased antioxidant capacity resulting from exercise also contributes to the reduction of UV-B-induced ROS damage to muscle and mitochondria.

Exercise is defined as movement that increases energy expenditure above basal levels (Booth et al., 2012), and which leads to a training effect that includes increased metabolic capacities and muscle contractile function (Davison, 1997; Gundersen, 2011; Korzeniewski and Rossiter, 2015). In a natural context, exercise is necessary for fitness-related activities such as behavioural interactions, dispersal and predator–prey interactions (Irschick and Garland, 2001; Martin et al., 2015; Sinclair et al., 2014). UV-B-mediated oxidative damage to muscles and the resulting decreases in locomotor performance therefore can have far-reaching ecological consequences. However, the effect of UV-B may be habitat specific, and habitats that require high levels of physical activity may confer protection against ROS damage via the protective effects of exercise. In aquatic systems, water flow is a physical factor that increases the requirement for increased locomotor activity, which in turn can increase metabolic rate and locomotor performance because of training effects (Dalziel et al., 2015; Davison, 1997). It is possible, therefore, that fish living in flowing water also have increased antioxidant activities, which would increase resilience to UV-B-induced ROS damage compared with fish living in still water environments.

The aim of this study was to test whether mosquitofish (*Gambusia holbrooki*) living in flowing water are more resilient to the effects of UV-B compared with animals living in still water. We hypothesised that fish from flowing water have higher swimming performance and metabolic scope than fish in still water, which is paralleled by greater antioxidant capacities and lower ROS-induced damage in response to UV-B exposure. To test our hypothesis, we exposed fish to different levels of UV-B and water flow in a fully factorial design.

MATERIALS AND METHODS

Study animals

All experiments were carried out with the approval of The University of Sydney Animal Ethics Committee (approval number L04/1-2013/3/5907). Adult male mosquitofish (*Gambusia holbrooki* Girard 1859; mean±s.e.m. standard length 20.4±0.22 mm) were collected from the field in Australia (33°46′33″S, 151°14′52″E) and kept in plastic tanks (645×423×276 mm) at a density of 1–2 fish per litre for 1–2 weeks before starting

¹School of Life and Environmental Sciences A08, University of Sydney, Sydney, NSW 2006, Australia. ²School of Biological Sciences, University of Queensland, St Lucia, QLD 4072, Australia.

*Author for correspondence (frank.seebacher@sydney.edu.au)

 F.S., 0000-0002-2281-9311

List of symbols and abbreviations

CAT	catalase
GSH	glutathione
MDA	malondialdehyde
M_{O_2}	metabolic rate
PC	protein carbonyl
ROS	reactive oxygen species
SOD	superoxide dismutase
U_{crit}	critical sustained swimming speed
UV-B	ultraviolet B radiation

experiments. Fish were kept at 20°C during all procedures, which was similar to the temperature at their collection site. The photoperiod was 12 h light:12 h dark for all treatments, and fish were fed twice per day with fish flakes (Wardley Tropical Fish Flakes, The Hartz Mountain Corporation, Secaucus, NJ, USA).

Experimental design and treatments

Fish were allocated randomly to one of four treatments: UV-B and no UV-B control, each in still and flowing water. Fish were exposed to treatments for 2–3 weeks. Within treatments, fish were dispersed across three circular tanks (180 mm height and 350 mm diameter; $N=13$ fish per tank), with a plastic cylinder (180 mm height×100 mm diameter) fixed at the centre of each tank to create an annulus that facilitated water flow (see below).

Fish in the UV-B treatment were exposed to visible light plus UV-B, while the control groups received visible light only. UV-B was provided by 120 cm UV-B fluorescent tubes (UVB-313 EL, Q-lab, Cleveland, OH, USA; 280–390 nm) for 3 h each day from 11:00 h to 14:00 h, which coincides with the time of greatest natural UV-B irradiation in the field. The natural dose of solar UV-B radiation in summer is 7.4 W m^{-2} at midday at the collection site (Ghanizadeh Kazerouni et al., 2016), with a total ambient daily dose of 5100 J m^{-2} (Beckmann et al., 2014). Our UV-B lamps were set up to generate a peak irradiance of 3.3 W m^{-2} at the water surface, which is equal to a total daily dose of 1188 J m^{-2} . We chose this lower than maximum ambient dose of UV-B to better reflect the average conditions (Ghanizadeh Kazerouni et al., 2016).

Water flow was generated by a submersible pump (5 W, SP-900; Resun, China) mounted close to the wall of each tank. The pumps created a water flow of 6–8 cm s^{-1} [equivalent to 3–4 body lengths per second (BL s^{-1}), measured with a flow meter; DigiFlow 6710 M, Savant Electronics, Taichung, Taiwan]. We chose this flow rate because it is naturally encountered by mosquitofish (Pyke, 2005) and because it increases sustained swimming performance in this species (Sinclair et al., 2014). Still water tanks were set up identically except that there was no water flow.

In a preliminary experiment, we filmed the behaviour of fish (not elsewhere used in the experiment) to ascertain that individuals in the flow tanks experienced a greater level of (swimming) exercise compared with fish in the still water treatment. Briefly, we filmed (C910 camera, Logitech, Japan, filming at 30 frames s^{-1}) fish in six tanks each for the flowing and still water treatment. We filmed fish every 2 h during a day, and determined tailbeat frequencies as an indicator of swimming activity of five randomly chosen fish per tank (Tracker Video Analysis and Modeling Tool, Open Source Physics, www.opensourcephysics.org). Fish had a mean±s.e.m. of 7.87 ± 0.15 tailbeats s^{-1} in the flowing water tank and 0.75 ± 0.10 tailbeats s^{-1} in the still water treatment.

Swimming performance

Critical sustained swimming speed (U_{crit}) was measured in 12 fish from each treatment according to published protocols (Hammill et al., 2004; Seebacher et al., 2015). U_{crit} was measured in a Blazka-style flume consisting of a cylindrical clear Perspex flume (150 mm length and 38 mm diameter). The flume was fitted tightly over the intake end of a submersible pump (12 V DC, iL500, Rule, Hertfordshire, UK) and a bundle of hollow straws at the inlet end of the flume helped maintain laminar flow. The flume and pump were submerged in a plastic tank (645×423×276 mm). A variable DC power source (NP9615, Manson Engineering Industrial, Hong Kong, China) was used to control flow speed in the flume by changing the voltage input into the pump. A flow meter (DigiFlow 6710M, Savant Electronics, Taichung, Taiwan) connected to the outlet of each pump was used to measure water flow in each flume in real-time. Fish swam at an initial flow rate of 0.06 m s^{-1} for 20 min. The flow speed was then increased by 0.02 m s^{-1} every 5 min until the fish were exhausted and could not hold their position in the water column any longer. We stopped the flow for 5–10 s when fish stopped swimming and fell back onto the grid, before increasing the speed to the previous setting again. The trial was terminated after fish stopped swimming for the second time. U_{crit} is reported as BL s^{-1} .

Oxygen consumption

We determined resting and maximum rates of oxygen consumption in 8–10 fish from each treatment. Individual fish were placed inside respirometers consisting of cylindrical clear plastic tubes (15 mm diameter and 100 mm length, 27 ml volume). A peristaltic pump (i150, iPumps, Tewkesbury, UK) circulated water through the respirometers while fish were resting. To determine resting metabolic rate, oxygen concentration inside the respirometers was measured with a sensor spot (PSt3, PreSens, Regensburg, Germany) attached to the inside of the tube. Fibre optic cables connected to an oxygen meter (Witrox, PreSens) were used to monitor the sensor spots. The respirometers were placed in temperature-controlled water baths, and fish were left undisturbed inside for 2 h before measurement of resting oxygen consumption rate (a period sufficient to achieve resting levels; Seebacher et al., 2016). After 2 h, the pump was switched off remotely to stop the water flow and thereby seal the respirometers without disturbing the fish. The dissolved oxygen concentration inside the respirometers was recorded for 15–20 min, and the slope of oxygen depletion was used to calculate resting metabolic rate.

Maximum rates of oxygen consumption were measured in each fish following measurement of resting oxygen consumption and according to a published protocol (Seebacher et al., 2013). Fish were placed in a cylindrical glass respirometer (120 ml volume), which was placed on a magnetic stirring plate. A sensor spot (PSt3, PreSens) was attached to the inside of the glass respirometer and oxygen concentration was monitored by fibre optic cables connected to an oxygen meter (FIBOX 3, PreSens). A magnetic stir bar at the bottom of the respirometer (separated from the fish using a plastic mesh) created water flow. A plastic column was suspended from the lid at the centre of the respirometer to help reduce turbulence. The flow speed was controlled by changing the speed of the stir bar. Speed was increased until the fish could not hold their position in the water column; it was then reduced until the fish could keep their position in the water column and swim steadily. This swimming speed was defined as near-maximum swimming speed. Fish were swum at maximum swimming speed for 5–7 min, and the maximum oxygen consumption was calculated from the

slope of the decrease in oxygen concentration. To check for other possible sources of oxygen consumption during all measures of oxygen consumption, the oxygen concentration of an empty chamber was measured, and when necessary oxygen consumption by the empty chamber was subtracted from the fish data. All respirometers were dried after use and regularly cleaned so that confounding effects were minimal. Metabolic scope was calculated as the difference between maximum and resting metabolic rate.

ROS damage: protein carbonyl and lipid peroxidation

To measure ROS-induced damage, fish were killed by immersion in a buffered MS222 solution (0.4 g l^{-1} ; Sigma-Aldrich, Castle Hill, NSW, Australia) and tail muscle was dissected and stored at -80°C for biochemical assays. Lipid peroxidation was measured in tail muscle of seven fish from each treatment group using a commercial kit (MAK085, Sigma-Aldrich) and following the manufacturer's instructions. The lipid peroxidation assay is based on measuring malondialdehyde (MDA), the end product of the lipid peroxidation chain, and the amount of lipid peroxidation in each sample was expressed as nmol MDA per mg tissue.

Protein carbonyl concentration was determined in tail muscle of seven fish from each treatment group using a commercial kit (MAK094, Sigma-Aldrich) and following the manufacturer's instructions. The protein carbonyl assay is based on measuring a stable carbonyl group formed as a result of ROS-oxidised proteins. The amount of protein carbonyl in each sample was reported as nmol protein carbonyl per mg total protein. To determine the concentration of total protein, a bicinchoninic acid protein assay kit (BCA1 and B9643, Sigma-Aldrich) was used following the manufacturer's instructions.

Antioxidants: GSH, SOD and CAT

The concentration of total GSH was determined in tail muscle of nine fish from each treatment using a commercial kit (CS0260, Sigma-Aldrich) and following the manufacturer's instructions. Reduced glutathione (GSH) is the main form of GSH present inside cells. GSH stabilises ROS by donating an electron, which leads to the formation of glutathione disulphide (GSSG). The level of GSH in each sample was reported as nmol GSH per mg tissue.

CAT enzyme activity was determined in tail muscle of nine fish from each treatment group and according to published protocols (Barata et al., 2005; Yakovleva et al., 2004). Tissues were homogenized in 1:9 (w/v) 100 mmol l^{-1} phosphate buffer (pH 7.4) containing 100 mmol l^{-1} KCl and 1 mmol l^{-1} EDTA, and homogenates were centrifuged at 4°C for 10 min at $10,000 \text{ g}$. The supernatant was used to measure CAT activity in an assay medium containing 50 mmol l^{-1} phosphate buffer (pH 7.8) and $30\% \text{ H}_2\text{O}_2$. The reaction was monitored for 3 min at 240 nm and the CAT activity was reported as μmol of substrate converted into product per minute (=1 unit) per mg tissue.

The activity of total SOD (Cu/Zn, Mn and FeSOD) was determined in tail muscle of nine fish from each treatment group using a commercial kit (706002, Cayman Chemicals, MI, USA) and following the manufacturer's instructions; 1 U of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical, and the SOD activity in each sample is reported as units per mg tissue.

Statistical analysis

Data were analysed by permutational analysis of variance in the package *lmp* in R. We conducted a two-factor permutational analysis of variance with UV-B treatment (UV-B and no-UV-B)

and flow treatment (flowing water and still water) as fixed factors to test the effects of UV-B and flow on swimming performance, metabolic rate, GSH concentration, CAT activity, SOD activity, lipid peroxidation and protein carbonyl concentration. We used standard length as a covariate in the analysis of U_{crit} , and mass as a covariate in the analysis of oxygen consumption. A significance level of 0.05 was used for all analyses, and the data are presented as means \pm s.e.m.

RESULTS

Swimming performance and metabolic rate

Flowing water significantly increased U_{crit} of fish in both UV-B and no-UV-B control treatments (main effect of flowing water; Table 1, Fig. 1A). There was a significant main effect of UV-B on sustained swimming performance, where fish exposed to UV-B had significantly lower U_{crit} than fish not treated with UV-B (Table 1, Fig. 1A).

There was no effect of UV-B or water flow on resting metabolic rate, maximum metabolic rate or metabolic scope (Table 1, Fig. 1B–D).

ROS damage: protein carbonyl and lipid peroxidation

Fish exposed to UV-B had significantly higher lipid peroxidation and protein carbonyl concentration than control fish (main effect of UV-B; Table 1, Fig. 2). Protein carbonyl concentration was significantly lower in UV-B-exposed fish living in flowing water (significant flow \times UV-B interaction; Table 1, Fig. 2B). Fish kept in flowing water also had significantly decreased lipid peroxidation in both UV-B and control groups (main effect of flowing water; Table 1, Fig. 2A).

Antioxidants: GSH, SOD and CAT

Fish from the flowing water treatments had significantly higher CAT activity and GSH concentration in both UV-B and control groups compared with fish from the still water treatments (main effect of flowing water; Table 1, Fig. 3A,B). However, fish from the UV-B treatment groups showed significantly reduced CAT activity and GSH concentration (main effect of UV-B; Table 1, Fig. 3A,B). There was no effect of UV-B or water flow on SOD activity (Table 1, Fig. 3C).

DISCUSSION

We show that fish living in flowing water had increased antioxidant defences, reduced oxidative damage and increased swimming

Table 1. Results from two-way permutational analyses of variance

	d.f.	<i>P</i>		
		UV-B	Flow	UV-B \times flow
U_{crit}	1,43	<0.001*	<0.001*	0.15
Resting \dot{M}_{O_2}	1,33	0.91	0.34	0.49
Maximum \dot{M}_{O_2}	1,33	0.25	0.98	0.73
MS	1,33	0.22	0.98	0.56
MDA	1,24	<0.001*	0.002*	0.18
PC	1,24	<0.001	0.71	0.027*
CAT	1,32	<0.001*	<0.001*	0.19
GSH	1,32	0.038*	0.030*	0.27
SOD	1,32	0.25	0.88	0.88

Analyses of variance were used to test the effects of water flow and UV-B radiation (UV-B) on critical sustained swimming performance (U_{crit}), resting metabolic rate (\dot{M}_{O_2}), maximum metabolic rate, metabolic scope (MS), lipid peroxidation (MDA), protein carbonyl (PC), catalase (CAT) activity, glutathione (GSH) concentration and superoxide dismutase (SOD) activity. An asterisk indicates a significant result.

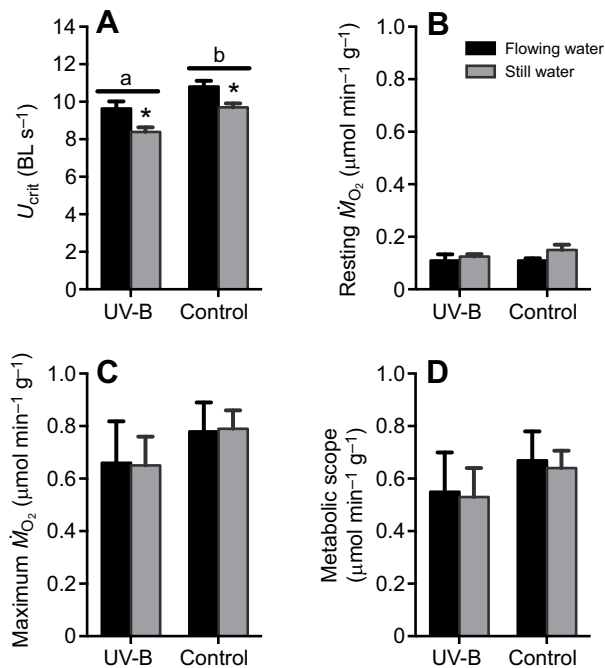


Fig. 1. Effect of ultraviolet B radiation (UV-B) and water flow on swimming performance and metabolic rate. (A) Flowing water significantly increased critical sustained swimming speed (U_{crit}) compared with still water treatment in both UV-B and control groups, but fish exposed to UV-B showed significantly lower U_{crit} than control fish. There was no effect of UV-B or water flow on resting metabolic rate (\dot{M}_{O_2} ; B), maximum metabolic rate (C) and metabolic scope (D). An asterisk indicates significant differences between flowing and still water treatments. Horizontal lines and letters above bars indicate significant differences between UV-B and control groups. Swimming performance was measured in 12 fish per treatment and metabolic rate in 8–10 fish per treatment. Means+s.e.m. are shown.

performance when exposed to UV-B. Hence, there is a ‘transfer’ of benefit across responses to different environmental stimuli. This finding is interesting because it shows that the mechanistic link between different habitat characteristics can determine ecological success of animals. Locomotor performance is closely related to fitness (Husak, 2006; Le Galliard et al., 2004), so the increase in sustained locomotion is likely to have important ecological consequences. Relative locomotor performance of predator and prey, for example, determines predation success (Grigaltchik et al., 2012), and therefore UV-B-induced decreases in locomotor performance increase the likelihood of being captured by predators and, vice versa, any increases in prey performance should increase survival.

UV-B reduced U_{crit} in fish in both flowing and still water but, as we hypothesised, fish exposed to UV-B had significantly higher U_{crit} in flowing water than in still water. This result indicates that the increased antioxidant capacities resulted in reduced ROS damage and increased U_{crit} . Interestingly, fish exposed to UV-B in flowing water had similar swimming performance to control fish in still water. Water flow thereby negated the negative effects of UV-B. However, despite increased defences and reduced damage, UV-B still reduced exercise-enhanced locomotor performance.

Contrary to our hypothesis, metabolic scope was not affected by UV-B or exercise. It would have been expected that sustained exercise increases maximal oxygen consumption rate (Joyner and Coyle, 2007). However, it may be that the intensity of exercise in our treatments was not high enough to enhance maximal metabolic rate. Our results showing that U_{crit} increased in the absence of an increase

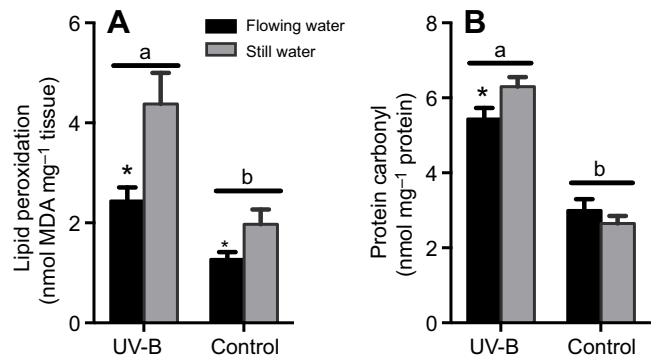


Fig. 2. Effect of UV-B and water flow on ROS-induced damage. Fish exposed to UV-B showed significantly higher lipid peroxidation (A) and protein carbonyl concentrations (B) than fish not treated with UV-B. However, there was a main effect of flowing water on lipid peroxidation, where fish in flowing water had significantly lower lipid peroxidation than fish in still water in both UV-B and control groups (A). Protein carbonyl concentration was significantly lower in flowing water for fish exposed to UV-B (UV-B×flow interaction; B). An asterisk indicates significant differences between flowing and still water treatments. Horizontal lines and letters above bars indicate significant differences between UV-B and control groups. Lipid peroxidation and protein carbonyl concentrations were measured in 7 fish per treatment. Means+s.e.m. are shown.

in maximal metabolic rate and metabolic scope indicate that sustained performance is relatively independent from mitochondrial ATP supply. Exercise can also increase locomotor performance by improving muscle efficiency – that is, the amount of oxygen used for a given amount of force produced (Joyner and Coyle, 2007; Lichtwark and Wilson, 2005; Salin et al., 2015). Additionally, exercise can alter contractile properties and fatigue resistance by modulating calcium cycling dynamics and fibre-type composition (Anttila et al., 2008; Gundersen, 2011; Place et al., 2015). Calcium cycling dynamics, in particular, are important in determining force production and fatigue resistance, and hence swimming performance (Gundersen, 2011). Fast release of calcium from the sarcoplasmic reticulum increases force production but decreases fatigue resistance (Seebacher et al., 2012). Increased rates of re-sequestration of calcium into the sarcoplasmic reticulum by the sarco(endo)plasmic reticulum ATPase (SERCA) improves both sprint and sustained locomotion (Seebacher and Walter, 2012). ROS can interfere with calcium release dynamics from the sarcoplasmic reticulum and the calcium sensitivity of troponin (Cheng et al., 2016). Hence, UV-B-induced ROS may reduce swimming performance by interfering with calcium cycling dynamics, although we have shown previously that UV-B did not affect SERCA activity in mosquitofish (Ghanizadeh Kazerouni et al., 2015).

In humans, chronic exercise caused similar increases in antioxidant capacities and reduced ROS damage, and one of the recognised benefits of regular exercise is that it reduces oxidative stress (Gram et al., 2015; Radak et al., 2013). Our results indicate that the transfer of the increased antioxidant capacity to improve resilience to the negative effects of UV-B radiation is an additional benefit of regular exercise beyond its direct health-promoting effects (Booth et al., 2012; Hawley and Holloszy, 2009; Joseph et al., 2016). At an ecological level, our findings indicate that fish from streams that experience greater levels of physical activity are more resilient to UV-B. Alterations to flow regimes of rivers and streams therefore may have pronounced effects on the resilience of animals to UV-B and their fitness. Local weather patterns and regional and

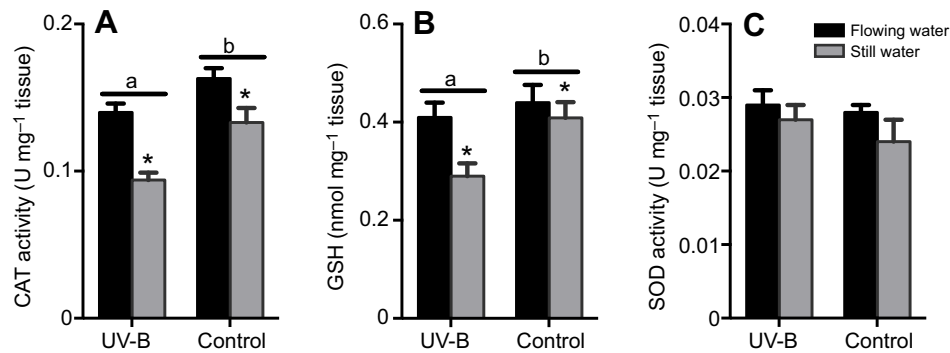


Fig. 3. Effect of UV-B and water flow on antioxidant capacities. Flowing water significantly increased catalase (CAT, A) and glutathione (GSH, B) activities compared with still water treatment in both UV-B and control groups. There was a main effect of UV-B on CAT activity and GSH concentration, and both were lower in fish exposed to UV-B (A,B). There was no effect of UV-B or flow on superoxide dismutase (SOD) activity (C). An asterisk indicates significant differences between flowing and still water treatments. Horizontal lines and letters above bars indicate significant differences between UV-B and control groups. All responses were measured in 9 fish per treatment. Mean+s.e.m. are shown.

global climate patterns, such as El Niño and climate warming, can interact to alter rainfall and thereby flow rates of water bodies (Helmuth et al., 2014; Mol et al., 2000). Human modifications such as the construction of dams will compound climate effects and also cause interactions between flow and other drivers such as temperature (Macnaughton et al., 2016). We show that decreased flow, as well as changes in temperature (Ghanizadeh Kazerouni et al., 2016) can make fish more vulnerable to the negative effects of UV-B.

In conclusion, exposure to water flow increased resistance to the deleterious effects of UV-B while simultaneously enhancing locomotor capacity, which may promote fitness gains relative to sedentary individuals in still water. It is therefore possible that a decrease of water flow because of environmental changes results in reduced regular physical activity and antioxidant capacity, which exacerbates the negative effects of UV-B on performance and fitness.

Competing interests

The authors declare no competing or financial interests.

Author contributions

F.S. and C.E.F. conceived the ideas, F.S. designed the experiments and wrote the manuscript, E.G.-K. conducted the experiments, and E.G.-K. and C.E.F. revised the manuscript.

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Data availability

Data are available from the Dryad Digital Repository (Ghanizadeh-Kazerouni et al., 2017): <http://dx.doi.org/10.5061/dryad.4g200>

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