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RESEARCH ARTICLE

Rates of warming impact oxidative stress in zebrafish (*Danio rerio*)

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ABSTRACT

Potentially negative effects of thermal variation on physiological functions may be modulated by compensatory responses, but their efficacy depends on the time scale of phenotypic adjustment relative to the rate of temperature change. Increasing temperatures in particular can affect mitochondrial bioenergetics and rates of reactive oxygen species (ROS) production. Our aim was to test whether different rates of temperature increase affect mitochondrial bioenergetics and modulate oxidative stress. We exposed zebrafish (*Danio rerio*) to warming from 20°C to 28°C over 3, 6, 24 or 48 h, and compared these with a control group that was kept at constant 20°C. Fish exposed to the fastest (3 h) and slowest (48 h) rates of warming had significantly higher rates of H_2O_2 production relative to the control treatment, and the proportion of O_2 converted to H_2O_2 (H_2O_2/O_2 ratio) was significantly greater in these groups. However, ROS production was not paralleled by differences in mitochondrial substrate oxidation rates, leak respiration rates or coupling (respiratory control ratios). Increased rates of ROS production did not lead to damage of proteins or membranes, which may be explained by a moderate increase in catalase activity at the fastest, but not the slowest, rate of warming. The increase in ROS production at the slowest rate of warming indicates that even seemingly benign environments may be stressful. Understanding how animals respond to different rates of temperature change is important, because the rate determines the time period for phenotypic adjustments and it also alters the environmental thermal signal that triggers compensatory pathways.

KEY WORDS: Mitochondria, Temperature, Reactive oxygen species, Catalase, Oxidative damage, Respiratory control ratio

INTRODUCTION

Temperature variation exerts a strong selection pressure on all organisms, because physiological rates change thermodynamically. Thermal fluctuations are ubiquitous in natural environments, and are expected to increase in frequency and amplitude with climate change. Temperature fluctuations may have an even greater effect on ectothermic physiology than mean temperature changes (Kingsolver and Buckley, 2015; Vasseur et al., 2014). Animals have evolved a range of physiological responses to compensate for thermal variation, for example, from heat shock protein production in response to acute heat stress (Liu et al., 2018), to plasticity of mitochondrial bioenergetics induced over weeks to months (Chung and Schulte, 2015, 2020). Changes in mean temperature are important in altering physiology, but considering the pronounced thermal fluctuation in natural environments, it is equally as important to consider the effects of temperature change over different time scales (Kingsolver and Buckley, 2017). The efficacy of compensatory responses to thermal variation depends on the relationship between the rate of temperature change and the time course of the phenotypic response. It is therefore critical to characterise how different rates of thermal change modulate the capacity for physiological responses.

Understanding the effect of thermal change on mitochondrial bioenergetics is important because changes to energy supply can have direct effects on animal performance and fitness (Chung and Schulte, 2020; Iftikar et al., 2014). The physiological consequences of warming are of particular interest because increasing temperatures increase cellular energy demands, and can also cause cellular damage as temperatures approach critical thermal maxima (Huey et al., 2012; Salin et al., 2016; Schulte, 2014). Like all biochemical rates, mitochondrial respiration rates tend to increase with increasing temperature, but increases in mitochondrial flux can also increase the production of reactive oxygen species (ROS) and lead to oxidative stress (Abele et al., 2002; Banh et al., 2016). Mitochondrial respiration is a significant producer of ROS (Zhang and Wong, 2021) and it is likely that increased mitochondrial O_2 flux during heating underlies increases in ROS generation (Banh et al., 2016; Roussel and Voituron, 2020). Low levels of ROS are important for cell signaling, but if their production exceeds scavenging capacities by antioxidants, ROS can damage lipids, proteins and DNA, and potentially trigger cell death (Costantini, 2019). Increased oxidative damage can have important consequences for animal performance by reducing fitness-related traits such as swimming performance and escape speed from predators (Ghanizadeh-Kazerouni et al., 2016; Janssens and Stoks, 2014).

Cells primarily defend from excessive ROS via a suite of enzymatic and non-enzymatic antioxidants (Halliwell, 2011). The primary ROS generated within the mitochondria is superoxide, which is rapidly converted to H_2O_2 by superoxide dismutase (SOD). H_2O_2 diffuses across membranes and readily disperses to other cellular compartments. H_2O_2-consuming antioxidants such as catalase, which is found in peroxisomes as well as inside mitochondria (Dickinson and Chang, 2011; Rindler et al., 2016), are therefore critical to preventing oxidative damage (Mailloux and Harper, 2011). Importantly, the expression and activity of enzymatic antioxidants can be upregulated in response to oxidative stress caused by environmental drivers such as food availability, UV exposure and seasonal temperature change (Dar et al., 2019; Ghanizadeh-Kazerouni et al., 2016; Pavlović et al., 2010). However, upregulation and biosynthesis of antioxidants, as well as the reactivation of H_2O_2 scavengers, are not instantaneous and there is some time lag (Dar et al., 2019; Halliwell, 2011). The efficacy of antioxidant defenses may therefore vary with rates of warming, which may alter the impact of different rates of ROS production.

Increasing temperature can also increase the rate of proton leak (leak respiration) across the inner mitochondrial membrane, which

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dissipates the proton gradient without ATP synthesis (Jastroch et al., 2010). Increased leak respiration reduces the efficiency of mitochondrial ATP production, but it can also reduce ROS production by lowering the proton motive force (Mailloux and Harper, 2011). Leak respiration includes passive proton conductance and active proton transport mediated by membrane-bound uncoupling proteins, which can be upregulated in response to ROS production (Jastroch et al., 2010). However, it is unclear how rapidly uncoupling can be induced and whether alterations to leak respiration can play a role in mitigating heating-induced oxidative stress.

The importance of mitochondrial ROS production in constraining animal performance in warming environments therefore depends on the dynamics between the rate of temperature change and ROS generation, and the time course of compensatory responses. Our aim was to determine the effect of the rate of warming on mitochondrial bioenergetics and rates of H_2O_2 production, antioxidant (catalase) activity, and oxidative damage to proteins and lipids. We tested the following alternative hypotheses: (a) warming increases mitochondrial respiration and H_2O_2 production rates, and either (b) fish that experience more rapid rates of temperature increase will have less time to upregulate catalase antioxidant activities and therefore incur greater oxidative damage, or (c) the fastest rates of warming will lead to disproportionately higher proton leak rates, resulting in no difference between rates of H_2O_2 production or oxidative damage between slow and fast rates of temperature increase.

MATERIALS AND METHODS

Animal husbandry

All experiments were conducted with the approval of the University of Sydney Animal Ethics Committee (approval number 2017/1200). Adult zebrafish [*Danio rerio* (Hamilton 1822)] were purchased from a commercial supplier (Livefish, Bundaberg, QLD, Australia). All fish were kept in large opaque plastic tanks (645×423×276 mm) at 23–24°C for at least 5 days before the temperature was gradually lowered to 20°C over 24 h. Fish were maintained at 20°C for at least 3 weeks before being moved to smaller experimental tanks (370×250×190 mm) in groups of 6–9 fish. Groups were left undisturbed for 1 week before warming treatments were initiated. There were a total of 35 experimental tanks (i.e. 6–8 tanks per treatment group). Fish were fed to satiation once daily with commercial flake food (TetraMin Tropical Flakes, Tetra, Blacksburg, VA, USA) and kept on a 14 h:10 h light:dark cycle.

Experimental design

We conducted four warming treatments, each increasing from 20°C to 28°C over a time period of 3, 6, 24 and 48 h (which equates to rates of 2.67, 1.33, 0.33 and 0.17°C h^{-1}, respectively). Additionally, a control group of fish was kept at constant 20°C (Fig. 1). The temperature range of 20–28°C is well within the natural range experienced by the species, and is not stressful per se (López-Olmeda and Sánchez-Vázquez, 2011). We chose two short and two longer rates to compare rapid diurnal temperature spikes with more gradual changes. The fastest rate of increase (8°C over 3 h) was considerably slower than typical ramp rates used in critical thermal maxima experiments (Morgan et al., 2018) because we did not want to subject fish to critical heat stress. Immediately after completion of each temperature treatment, we euthanised fish and measured mitochondrial bioenergetics and simultaneous H_2O_2 production rates (n=12–15 pairs of fish per treatment; see below). A different subset fish was maintained at 28°C for 3 days after the completion of each temperature treatment, and we then measured oxidative damage to proteins (protein carbonyls, n=13–15 fish per treatment) and lipids (lipid peroxidation, n=12–15) and catalase antioxidant activity (n=13–15) to determine whether there was a prolonged effect of different rates of temperature change on oxidative balance.

Warming treatments

The rate of temperature increase was controlled by implementing a 'bang-bang' feedback controller on a custom-built hardware and software platform. A Raspberry Pi 3 (Model B+, Raspberry Pi Ltd, Cambridge, UK) was connected to a rack power distribution unit (Digital Loggers Inc., Santa Clara, CA, USA), which controlled the power supply to independent aquarium heaters (Eheim Jager GmbH, Germany) that were placed in the treatment tanks. The Raspberry Pi was also connected to waterproof digital temperature sensors (DS18B20, DFRobot, Shanghai, China) that were suspended into individual treatment tanks. Software was written in the Python programming language (Python Software Foundation, https://www.python.org/), which caused the aquarium heaters to switch on or off depending on the temperature sensor readings and the programmed rate of temperature increase; the script is available at https://github.com/jordanwjolly/rpi-digital-water-bath.git. The temperature sensor readings were independently validated using a thermometer (K-type thermocouple thermometer, Hanna Instruments, Woonsocket, RI, USA). We conducted pilot experiments using heaters with different power output (25–150 W) to optimise the accuracy of the rate of temperature change (Fig. 1).

Mitochondrial bioenergetics and H_2O_2 measurements

Immediately after the warming treatments, fish were euthanised by anaesthesia with MS-222 (0.3 g l^{-1}, pH 7.0 with $NaHCO_3$) followed by cervical dislocation, and tail muscle was dissected and skinned. Tissue was homogenised in a Teflon-glass Potter–Elvehjem homogeniser in nine volumes of isolation buffer consisting of 140 mmol l^{-1} KCl, 20 mmol l^{-1} HEPES, 5 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} EGTA, 1 mmol l^{-1} ATP and 0.5 g l^{-1} bovine serum albumin (BSA), pH 7.0 (DosSantos et al., 2013); we did not add proteases to the buffer. The homogenate was centrifuged at 1400 ***g*** for 5 min to settle cellular debris, and the supernatant containing mitochondria was saved. The supernatant was centrifuged at 4°C and 9000 ***g*** for 9 min and the mitochondrial pellet was resuspended in ice-cold respiration buffer containing 110 mmol l^{-1} sucrose, 60 mmol l^{-1} KCl, 0.5 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} taurine, 10 mmol l^{-1} KH_2PO_4 and 0.5 g l^{-1} BSA at pH 7.1 at a ratio of 2000 µl g^{-1} tissue.

Mitochondrial respiration was measured with an Oroboros Oxygraph 2-k high-resolution respirometry system (Oroboros Instruments, Innsbruck, Austria). Oxygen sensors were calibrated daily with air-saturated respiration buffer and an anoxic solution containing saturated baker's yeast in deionised H_2O. We pooled two fish from the same treatment for each individual measurement to increase the signal-to-noise ratio of ROS emission measurements, which was determined by pilot studies. Each pooled sample was measured at both 20 and 28°C test temperatures, and the order of test temperatures was randomised. Two identical 2 ml respiration chambers were measured in parallel. We first added substrates that fuel complex I (5 mmol l^{-1} pyruvate and 2 mmol l^{-1} malate) and complex II (10 mmol l^{-1} succinate) to measure state II respiration rates. We then added 0.5 mmol l^{-1} ADP to assess maximal phosphorylating state III respiration rates. F_O/F_1 ATPase was

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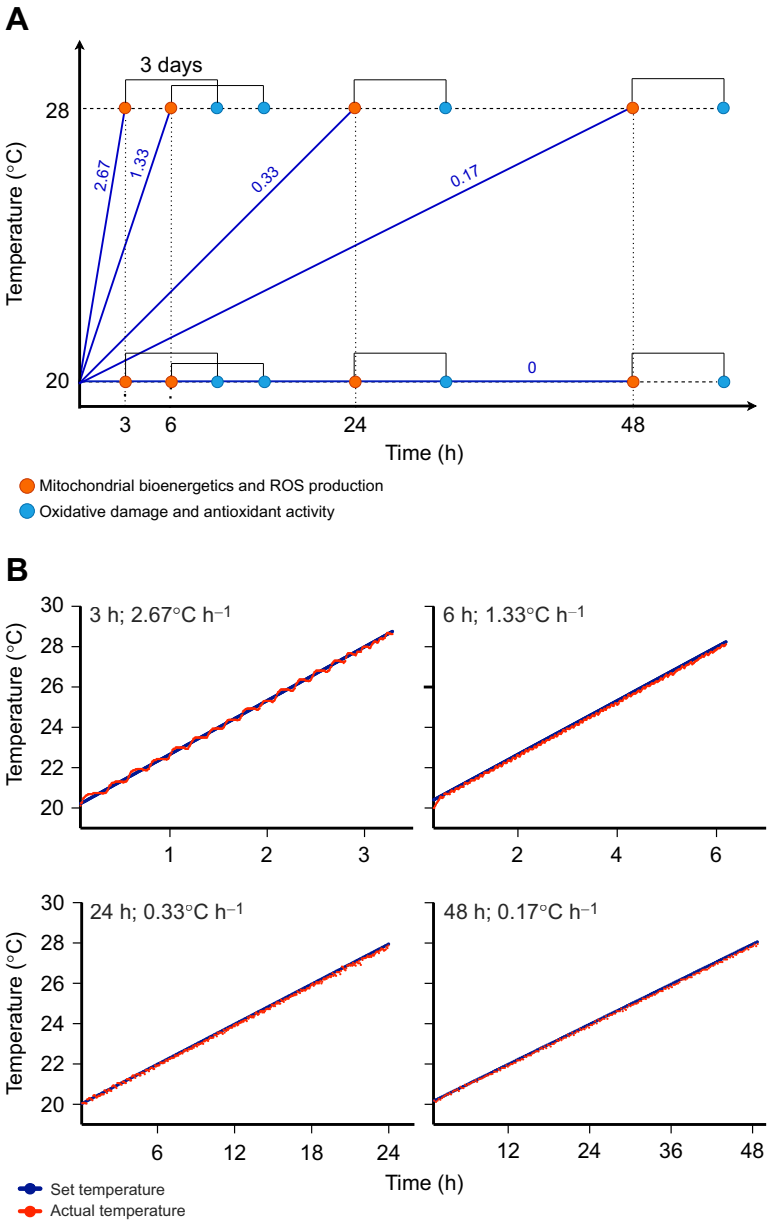

Fig. 1. Schematic representation of the experimental design. (A) Fish were exposed to one of five different linear temperature treatments: increase from 20°C to 28°C over 3, 6, 24 and 48 h, or a control held at constant 20°C (solid blue lines). Rates of temperature increase (°C h^{-1}) are shown in blue next to each treatment. Mitochondrial bioenergetics and ROS production rates were measured immediately after the completion of the temperature increase (orange circles), and oxidative damage to proteins and lipids, and catalase antioxidant activity were measured after 3 days at 28°C (light blue circles). (B) Representative examples of temperature treatments regulated by the RPi temperature control system. Different rates of temperature increase were programmed (blue circles and lines) using an RPi temperature control system, and the actual recorded temperatures (red circles and lines) in the tank are shown for comparison. Rates of temperature increase are shown in the top left corner of each panel.

inhibited by adding 0.5 mmol l^{-1} oligomycin to assess state IV respiration. Finally, we completely uncoupled the mitochondria by titrating 0.5 µmol l^{-1} of carbonyl cyanide 4-(trifluoromethyoxy) phenylhydrazone (FCCP) until respiration no longer increased. The respiratory control ratio (RCR) was calculated as the ratio of state III to state IV respiration rates. Protein content of the mitochondrial solution was measured using a Bradford assay (Sigma-Aldrich, Castle Hill, Australia).

We measured mitochondrial H_2O_2 production simultaneously with bioenergetic measurements. H_2O_2 production is proportional to changes in the fluorometric signal generated by Amplex Red (Cayman Chemical, Ann Arbor, MI, USA). Following the addition of isolated mitochondria and respiration buffer (see above), we added 10 µmol l^{-1} Amplex Red and 2 U horseradish peroxidase. We also added 5 U of superoxide dismutase to convert residual superoxide to H_2O_2. After allowing the baseline fluorometry signal to stabilise, we calibrated the Amplex Red signal by titrating 0.1 µmol l^{-1} of H_2O_2 to generate a standard curve from which H_2O_2 emission from experimental samples was determined. In preliminary assays, we optimised the amount of isolated mitochondria added to the respiration chambers. We then proceeded with measurements of mitochondrial respiration (as above), while measuring H_2O_2 emission rates at the same time. After the addition of FCCP, we added two 0.1 µmol l^{-1} volumes of H_2O_2 to ensure that the fluorescence signal remained in the linear range of the standard curve. We calculated the H_2O_2/O_2 ratio as the ratio of H_2O_2 emission rate (pmol H_2O_2 s^{-1} mg^{-1} protein) to the respiration rate (nmol O_2 s^{-1} mg^{-1} protein).

Oxidative damage

We measured protein carbonyl content and lipid peroxidation 3 days after the warming treatment as indicators of protein and membrane damage, respectively. Skeletal muscle samples were collected as above and frozen in liquid nitrogen and stored at −80°C. Muscle tissue was homogenised (in a TissueLyser LT, Qiagen, USA) in 1:9 (w/v) phosphate buffer containing 100 mmol l^{-1} KCl and 1 mmol l^{-1} EDTA (pH 7.4), followed by centrifugation for 10 min at 10,000 ***g***. We measured protein carbonyls using a commercial kit

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(MAK094, Sigma-Aldrich) following the manufacturer's instructions. Protein carbonyl groups are formed from the oxidation of proteins by ROS and are therefore an indicator of oxidative damage to proteins. Samples (n=13–16 fish per treatment group) were measured in duplicate on a 96-well plate and absorbance was measured at 375 nm in a microplate reader (CLARIOstar, BMG Labtech, Ortenburg, Germany). Total protein concentration was measured with a Bradford assay (Sigma-Aldrich), and protein carbonyl content was normalised to total protein content.

Lipid peroxidation was estimated by measuring malondialdehyde (MDA), which is an index of thiobarbituric acid-reactive substances that are formed as a by-product of lipid degradation by ROS. We measured MDA concentration in muscle tissue using a commercial kit (MAK085, Sigma-Aldrich) following the manufacturer's instructions. Samples (n=12–15 fish per treatment group) were measured in duplicate on a 96-well plate and absorbance was measured at 532 nm using a microplate reader (CLARIOstar, BMG Labtech). A standard curve of known MDA concentrations was used to extrapolate the sample MDA concentration, which was expressed as nmol MDA mg^{-1} tissue.

Catalase antioxidant activity

Catalase is an antioxidant enzyme that converts H_2O_2 to water and oxygen. Catalase activity was measured in a UV-Vis spectrophotometer (Ultrospec 2100, GE Healthcare, Australia) connected to a temperature-controlled water bath (BL-30, Thermoline, Australia) by tracking the decrease in H_2O_2 over time at 240 nm. We measured activity of each sample (n=13–15 per experimental group) at 20 and 28°C test temperatures; we used tissue homogenate from fish used for assays of oxidative damage. Catalase activity was measured by adding the supernatant to an assay reaction mixture containing 50 mmol l^{-1} phosphate buffer (pH 7.8) and 50 mmol l^{-1} H_2O_2 (Barata et al., 2005). Each assay was conducted in duplicate. We monitored the absorbance at 240 nm for 3 min, and catalase activity was calculated as 1 µmol of substrate converted into product per minute (1 enzyme unit) per mg wet tissue mass.

Statistical analysis

We analysed all data with permutational analyses of variance using the lmPerm package in R (https://CRAN.R-project.org/package=lmPerm). Permutational analyses use the data per se for inferences without making assumptions about the underlying population distributions, so that there are no test statistics associated with probability estimates (Drummond and Vowler, 2012). Permutational analyses are preferable to frequentist statistics, particularly when data stem from mixed or unknown distributions or sample sizes are low relative to the total number of samples in the population (Ludbrook and Dudley, 1998). We used the rate of temperature increase (5 levels: 8°C over 3, 6, 24 or 48 h, and control) and test temperature (20 and 28°C) as independent factors to analyse mitochondrial respiration rates, and H_2O_2 production during maximal substrate oxidation (state III) and leak respiration (state II and IV). Individual fish ID was included as a random factor to account for repeated measures of each sample at the two test temperatures. We also determined the effects of the rate of temperature increase and the test temperature on RCR, and the H_2O_2/O_2 ratio at each state of mitochondrial respiration. We conducted a one-way permutational ANOVA to test whether there was an effect of the rate of temperature increase on protein carbonyl and MDA content.

Finally, we conducted a two-way permutational ANOVA with the rate of temperature increase and test temperature as predictors of catalase activity. As above, we included fish ID as a random factor to account for the repeated measures at the two test temperatures. We identified one individual (2 data points, one for each test temperature) as an outlier in the dataset (Quinn and Keough, 2002). We deemed these data points to be negatively influential using Cook's distance values >4/n–k, where k is the number of predictors, and omitted this individual from the analysis. *Post hoc* comparisons were conducted for significant effects, and we used a one factor permutational ANOVA to compare each treatment group with the control group.

RESULTS

Mitochondrial bioenergetics

The rate of temperature increase did not significantly alter mitochondrial respiration rates during substrate oxidation (state III) or leak respiration (state II and state IV), or change RCRs from different treatment groups (Fig. 2, Table 1). There was a significant increase in mitochondrial respiration rates with increasing test temperature, while RCRs decreased (main effect of test temperature; Fig. 2, Table 1).

ROS production

There was a significant effect of the rate of temperature increase on ROS (H_2O_2) production rates during all states of mitochondrial respiration (Fig. 3A–C, Table 2). In particular, *post hoc* tests showed that the fastest (3 h) and slowest (48 h) rates of temperature increase led to higher H_2O_2 production rates during state II and III respiration

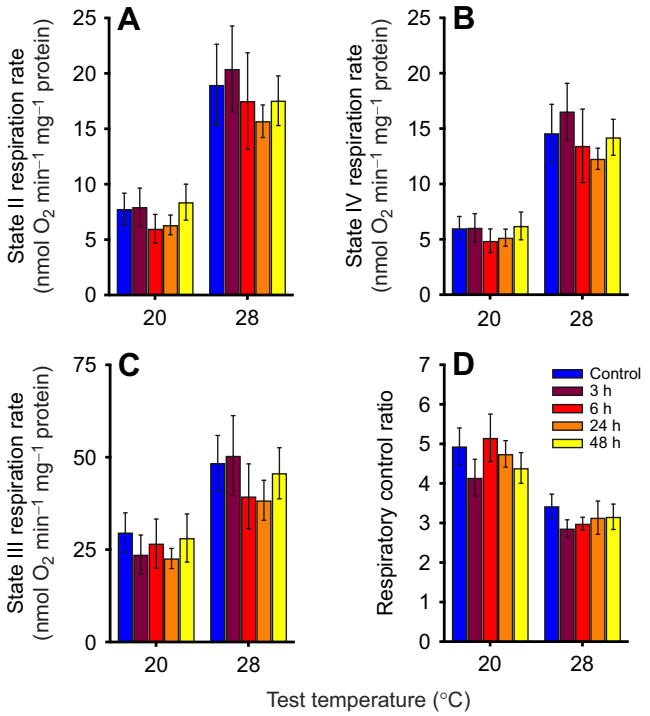

Fig. 2. The effect of the rate of temperature increase on mitochondrial bioenergetics. There was no effect of the rate of temperature increase (different coloured bars) on leak (A) state II or (B) state IV respiration, (C) maximal rates of substrate oxidation (state III) or (D) mitochondrial coupling (respiratory control ratios, RCRs). However, there was a significant increase in all respiration rates with increasing test temperature, while RCRs decreased. Means±s.e.m. are shown and n=12–15 fish per treatment group.

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Table 1. Results from the statistical analysis of mitochondrial bioenergetics

Source	State II	State III	State IV	RCR
$TestT_1$	**<0.001**	**<0.001**	**<0.001**	**<0.001**
$Rate_4$	0.956	0.919	1	0.375
TestT:Rate	1	0.6144	0.727	0.799

Probabilities of the effects of the rate of temperature increase (Rate) and test temperature (TestT) on state II, state III and state IV respiration rates, and the respiratory control ratio (RCR) are shown. Numerator degrees of freedom are presented as subscripts with the factors in the source column. Significant (P<0.05) effects are in bold.

(Fig. 3A–C) compared with the control group. H_2O_2 production also increased significantly with increasing test temperature at all states of mitochondrial respiration (Fig. 3, Table 2).

Similarly, there was a significant effect of the rate of temperature increase on the estimated proportion of O_2 that was converted to H_2O_2 (H_2O_2/O_2) during state II and III respiration, but not state IV respiration (Fig. 3D–F, Table 2). In particular, the H_2O_2/O_2 ratio was significantly higher in the fastest (3 h) and slower (24 and 48 h) rates of temperature increase compared with controls (Fig. 3D,E). There was a significant effect of test temperature on H_2O_2/O_2 for all states of respiration except during state III (Fig. 3, Table 2).

Oxidative damage and catalase activity

There were no significant differences in lipid peroxidation (P=0.514; Fig. 4A) or protein carbonyl content (P=0.437, Fig. 4B) in fish from different warming treatments. There was an effect at one-tailed significance of the rate of temperature increase on catalase activity (P=0.0596). Similarly to the results for ROS emission rates, catalase activity was highest at the fastest rate of temperature increase (3 h) (Fig. 4C). There was no significant main effect or interaction of test temperature on catalase activity (P=0.451).

DISCUSSION

We have shown that the rate of warming influences mitochondrial ROS emission, which indicates that the transient characteristics of temperature change have important physiological consequences for animals living in variable environments. Unexpectedly, we found that both the absolute amount of H_2O_2 produced and the proportion of O_2 converted to H_2O_2 were higher at the fastest and slowest rates of warming. The rate of temperature change therefore has non-linear effects on oxidative stress (Schulte et al., 2011), where extreme ends of the temporal range, i.e. the faster and slower changes, promote mitochondrial ROS emission. However, increased ROS emission did not lead to sustained oxidative damage, which may be explained at least in part by upregulated catalase antioxidant activity in the fastest, but not the slowest rates of warming. Hence, not only the amplitude but also the rate of thermal change can have potentially costly physiological impacts, indicating that thermal transients are an important aspect of habitat quality.

The finding that both the absolute rate of ROS emission, and the H_2O_2 produced per O_2 consumed were significantly higher in the fastest and slowest rates regardless of acute test temperature cannot be explained by variation in leak respiration. We detected no differences between treatments in leak respiration, and therefore we reject the hypothesis that increased leak respiration rates and decreased mitochondrial coupling are correlated with decreased rates of ROS emission (Mueller et al., 2011). Increased leak respiration was therefore not a protective mechanism preventing ROS emission, but may reflect a loss in membrane integrity. Our results thus beg the question of what are the mechanisms that caused higher rates of ROS emission only in the fastest and slowest rates of warming. Most mitochondrial ROS are produced by complex III, and electron transfer is facilitated by contact between cytochrome *c*1 and cytochrome *c* (Pérez-Mejías et al., 2020). It is conceivable that changes in temperature alter the physical contact between subunits and thereby modify electron leak and ROS emission; however, this suggestion is speculative.

Higher rates of ROS emission in fish exposed to the fast rates of warming are not unexpected from the literature, which links rapid heating and heat stress to oxidative stress (Abele et al., 2002; Liu et al., 2018; Roussel and Voituron, 2020). However, our results differ because we used more gradual ramp rates within a normal

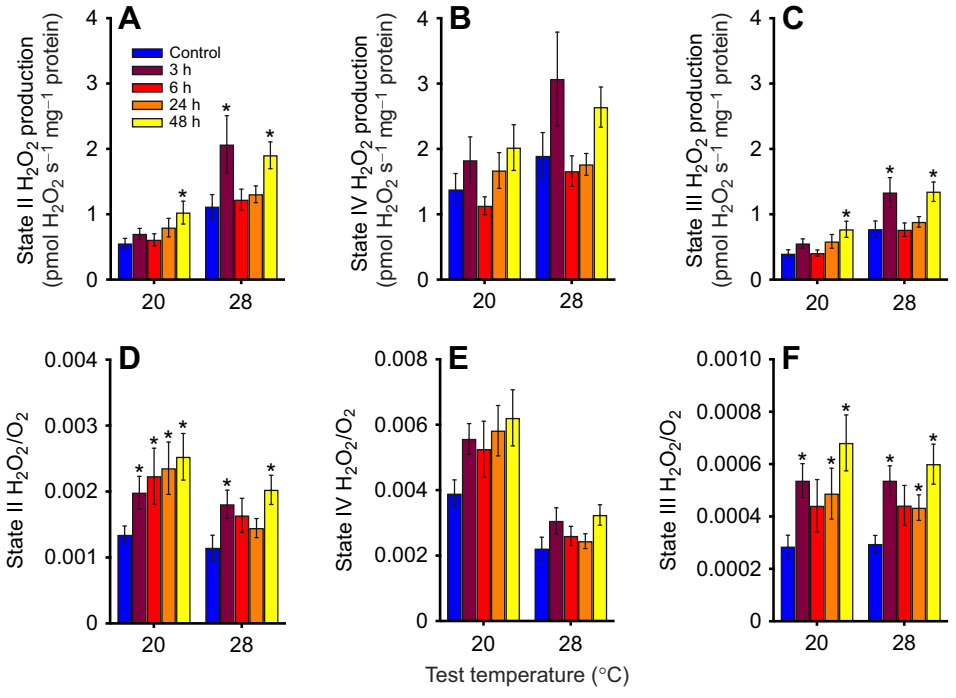

Fig. 3. The effect of the rate of temperature increase on rates of H_2O_2 production. There were significant effects of the rate of temperature increase (different coloured bars) on H_2O_2 production during leak (A) state II and (B) state IV rates, and (C) during maximal substrate state III rates. Increasing test temperature significantly increased H_2O_2 production during all respiration rates. There was also a significant effect of the rate of temperature increase on the amount of H_2O_2 produced per oxygen consumed (H_2O_2/O_2 ratio) during (D) state II and (F) state III rates but not (E) state IV rates. Asterisks indicate significant differences from the control for each test temperature. Means±s.em.. are shown and n=12–15 per treatment.

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Table 2. Results from the statistical analysis of mitochondrial ROS production

	State II		State III		State IV	
Source	H_2O_2	H_2O_2/O_2	H_2O_2	H_2O_2/O_2	H_2O_2	H_2O_2/O_2
$TestT_1$	**<0.001**	**0.002**	**<0.001**	1	**0.026**	**<0.001**
$Rate_4$	**0.029**	**0.028**	**0.001**	**<0.001**	**0.025**	0.118
TestT:Rate	0.162	0.421	0.222	0.987	0.585	0.862

Probabilities of the effects of the rate of temperature increase (Rate) and test temperature (TestT) on H_2O_2 production rates and the H_2O_2/O_2 ratio during state II, state III and state IV respiration are shown. Numerator degrees of freedom are shown as subscripts with factors in the source column. Significant (P<0.05) effects are in bold.

tolerance range for zebrafish (López-Olmeda and Sánchez-Vázquez, 2011), rather than thermal extremes. We thereby emulated short-term, environmentally common fluctuations. It is therefore particularly interesting that we found such strong differences in ROS emission between warming rates.

A potential mechanism mediating the relationship between rapid heating and increased ROS emission is increased mitochondrial oxidative activity resulting from both the direct thermodynamic effect on reaction rates and the increased cellular energy demand (Abele et al., 2002; Roussel and Voituron, 2020). However, we did not find differences in mitochondrial oxidative capacities between treatments. It is possible, nonetheless, that endogenous antioxidants within the mitochondria can instead account for differences in ROS between treatments. Catalase is predominantly found within peroxisomes, but it also occurs within mitochondria (Dickinson and Chang, 2011; Munro et al., 2016; Rindler et al., 2016). Changes in catalase activity could therefore explain differences in ROS emission in isolated mitochondria. Furthermore, glutathione peroxidase and thioredoxin-dependent pathways also play an important role in scavenging H_2O_2 within the mitochondria (Munro et al., 2016). Hence, higher rates of ROS emission in isolated mitochondria at the fastest rate of warming may have been due to insufficient time to upregulate of H_2O_2-consuming antioxidants within the mitochondrial matrix. Upregulation of catalase and glutathione peroxidase in response to chronic heat stress exposure can be rapid, but the time course varies depending on the specific experimental conditions (Liu et al., 2018; Yang et al., 2010). In fish red muscle, the thermal sensitivity of H_2O_2 emission is also greater than that of antioxidant processes, which can at least partly explain the non-linear dynamics (Banh et al., 2016).

Contrary to our hypotheses, increased mitochondrial ROS emission was not associated with sustained oxidative damage to tissue proteins or lipids at either the fastest or slowest rates of warming. A concomitant increase in catalase antioxidant activity in the fastest rate of warming may partially explain this lack of damage. However, the induction of other antioxidants, particularly peroxidases and peroxiredoxins (Treberg, 2021) may have also played a role. Furthermore, the time course of changes in oxidative damage and apoptosis of damaged cells can be rapid (Liu et al., 2018). We chose to measure oxidative damage 3 days after completion of warming treatments to assess whether any changes in ROS emission led to prolonged oxidative costs, but this delay also meant that we could have missed transient changes in damage, antioxidant activities and any repair processes as well. Oxidative damage and induced defences increase rapidly (<24 h) following heat stress in fish, but can also decrease to near control levels within 24 h in a non-stressful environment (Lushchak and Bagnyukova, 2006a,b; Zhao et al., 2022). In light of our results, it would be interesting to determine the temporal dynamics of a range of oxidative damage and antioxidant markers at greater resolution.

Surprisingly, fish exposed to the slowest rate of warming also exhibited significantly higher rates of ROS emission. Water temperature in shallow freshwater environments typically oscillates in daily cycles owing to solar radiation so that fish in these environments commonly experience, and should be tolerant to, thermal fluctuations within this range. The rate of temperature change may have been too gradual so that it did not reach a signal-to-noise detection threshold necessary to trigger thermosensory signalling cascades (Ahern, 2013; Little et al., 2020). Our finding and speculated explanation are interesting because gradually increasing temperatures are typically thought to be less 'stressful' owing to the greater time for physiological compensatory adjustments (Mascaró et al., 2019). However, if signal-to-noise ratios prevent compensatory responses, environments perceived to be benign may actually be quite stressful physiologically.

Although we did not detect a direct cost of oxidative damage, the increased H_2O_2/O_2 ratios in the fastest and slowest rates of warming indicate that a greater proportion of oxygen consumed was converted to ROS rather than ATP, thereby reducing the efficiency of cellular energy transformation. There can be a considerable energetic cost incurred by the biosynthesis of antioxidants, and the reductive energy required to reactivate and maintain high concentrations of ROS-scavenging molecules (Mailloux and Harper, 2011). Diurnal thermal fluctuations are a common feature of many freshwater aquatic habitats, and environments such as ephemeral pools can fluctuate by as much

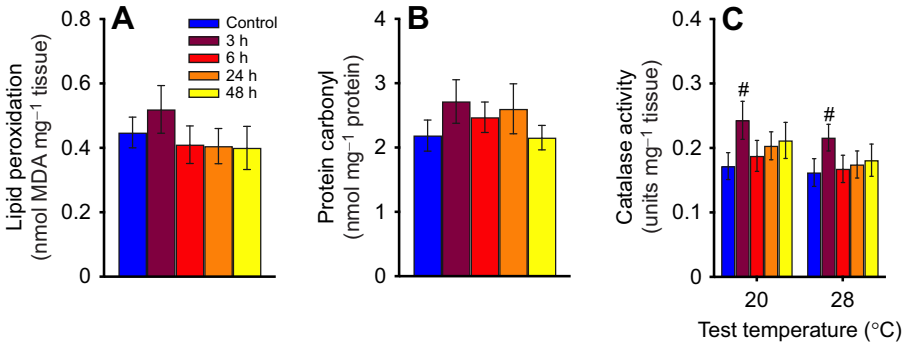

Fig. 4. The effect of the rate of temperature increase on oxidative damage and catalase activity. There were no significant effects of the rate of temperature increase (different coloured bars) on (A) lipid peroxidation estimated as MDA content or (B) protein carbonyl content. (C) The rate of temperature increase had an effect on catalase activity at a one-tailed significance (indicated by #), but there were no effects of test temperature. Means±s.e.m. are shown and n=12–15 per treatment group.

as 20°C over a 24 h period (Niehaus et al., 2006). Anthropogenic change is predicted to increase thermal variability and stochasticity with more extreme local fluctuations (Vasseur et al., 2014). Understanding how animals can respond to different rates of temperature change is important, because the rate determines the time period for phenotypic adjustments and it also alters the environmental thermal signal that triggers compensatory pathways. Resolving the temporal relationship between environmental fluctuations and phenotypic responses will provide a predictive framework for forecasting the resilience of populations to future change.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: I.L., F.S.; Methodology: I.L., G.Y.L., J.J.; Software: J.J.; Validation: F.S.; Formal analysis: I.L., F.S.; Investigation: I.L., G.Y.L.; Resources: F.S.; Data curation: I.L.; Writing - original draft: I.L., F.S.; Writing - review & editing: I.L., G.Y.L., J.J., F.S.; Supervision: F.S.; Project administration: F.S.; Funding acquisition: F.S.

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Data availability

Data are available from the Dryad digital repository (Seebacher et al., 2022): doi:10.5061/dryad.fqz612jv0.

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