

Oxidative stress during stressful heat exposure and recovery in the North Sea eelpout *Zoarces viviparus* L.

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Summary

The interplay between antioxidants, heat shock proteins and hypoxic signaling is supposed to be important for passive survival of critical temperature stress, e.g. during unfavorable conditions in hot summers. We investigated the effect of mild (18°C), critical (22°C) and severe (26°C) experimental heat stress, assumed to induce different degrees of functional hypoxia, as well as the effect of recovery following heat stress on these parameters in liver samples of the common eelpout *Zoarces viviparus*.

Upon heat exposure to critical and higher temperatures we found an increase in oxidative damage markers such as TBARS (thiobarbituric reactive substances) and a more oxidized cellular redox potential, combined with reduced activities of the antioxidant enzyme superoxide dismutase at 26°C. Together, these point to higher oxidative stress levels during hyperthermia. In a recovery-time series, heat-induced hypoxia and subsequent reoxygenation upon

return of the fishes to 12°C led to increased protein oxidation and chemiluminescence rates within the first 12 h of recovery, therein resembling ischemia/reperfusion injury in mammals.

HSP70 levels were found to be only slightly elevated after recovery from sub-lethal heat stress, indicating minor importance of the heat shock response in this species. The DNA binding activity of the hypoxia-inducible transcription factor (HIF-1) was elevated only during mild heat exposure (18°C), but appeared impaired at more severe heat stress. We suppose that the more oxidized redox state during extreme heat may interfere with the hypoxic signaling response.

Key words: hyperthermia, heat shock protein, oxidative stress, redox state, hypoxia inducible factor, temperate fish, common eelpout, *Zoarces viviparus*.

Introduction

Exposure of cold-blooded marine animals to elevated temperatures accelerates mitochondrial respiration and has been shown to increase mitochondrial reactive oxygen species (ROS) formation (Heise et al., 2003; Keller et al., 2004). Excess ROS production by intensively respiring mitochondria is held responsible for cellular damage expected upon exposure of an ectotherm to warming (hyperthermia) and heat stress (Abele et al., 1998, 2002).

However, as more oxygen is consumed in peripheral tissues under severe thermal stress, limitation of oxygen supply to central tissues can occur and produce a state of thermally induced transient hypoxia. Onset of heat-induced hypoxia in body fluids and tissues has been found as animals were warmed beyond their pejus temperatures (T_p), indicating the limits of the species-specific thermal optimum range. These findings have led to the concept of oxygen- and capacity-limited thermal tolerance (for a review, see Pörtner, 2002). In fish,

capacity limits of cardiac performance account for progressive mismatch between oxygen supply and demand in the whole animal during warming (Mark et al., 2002; Farrell, 2002; Lannig et al., 2004), and the decline in cardiac performance could reflect problems with the hearts own oxygen supply (Farrell, 2002). Oxygen extraction by working skeletal muscle during exercise would further increase oxygen demand at high temperatures. When a critical temperature (T_c) is reached, aerobic scope is limited and mitochondria progressively switch to anaerobic energy production, which allows only time limited survival (Zielinski and Pörtner, 1996; Sommer et al., 1997; reviewed in Pörtner, 2002).

Complications can result, when oxygen-deprived tissues are reoxygenated, a situation that resembles ischemia/reperfusion in mammalian tissues (Jones, 1986; McCord, 1988; Erecinska and Silver, 2001; Chi and Karliner, 2004). Elevated ROS formation may, thus, not only result as a consequence of stressful hyperthermia, but also as a consequence of

reoxygenation during recovery (Halliwell and Gutteridge, 1999).

Hypoxic signaling, heat shock response and oxidative stress during warming are interactive cellular processes. Ischemia/reperfusion and ROS production are known to induce heat shock protein (HSP) transcription and synthesis in mammalian cells and to play a 'common denominator role' for both pro- and anti-apoptotic processes under hyperthermia (Flanagan et al., 1998; Katschinski et al., 2000; Skulachev, 2001; King et al., 2002; Kregel, 2002). Therefore, short-term tolerance to oxidative stress provided by an effective antioxidant system (Abele and Puntarulo, 2004), as well as upregulation of HSPs, mediating the refolding of heat-damaged proteins, may support survival during heat stress (Moseley, 1997; Pörtner, 2002).

Moreover, the transcription factor HIF-1 (hypoxia inducible factor), which activates genes involved in angiogenesis, erythropoiesis and glucose metabolism (for reviews, see Wenger, 2000; Semenza, 2004) has been shown to function in heat acclimation of *C. elegans* (Treinin et al., 2003). Loss of the HIF-1 gene abolishes heat resistance in spite of an upregulation of HSP72 in the nematode model and metabolic reorganization mediated by HIF-1 could thus contribute to ameliorate the temperature-induced oxygen limitation upon (sub)-critical warming. Although the causal link between heat and hypoxia resistance may relate to the onset of functional hypoxia during stressful heating, as demonstrated for marine fishes, the mechanistic link has yet to be explored.

The common eelpout is a eurythermal marine fish that offers an appropriate model-system to study the interdependence of oxidative stress, hypoxic signaling and HSP expression under high temperature stress. Acclimation to and stress response to high temperatures and heat-induced hypoxia is well studied in this animal (Zakhartsev et al., 2003; Pörtner et al., 2004). The critical temperature, where anaerobic energy production sets in, was found to range between 22 and 24°C (Van Dijk et al., 1999). Graded short-term exposure to temperatures, mildly and severely elevated over the control temperature should, therefore, make it possible to distinguish between the effect of hyperthermia and the combination of heat and functional hypoxia beyond the critical temperature limit. All parameters were determined in liver tissue, reported to be the most sensitive organ synthesizing HSP70 in response to hyperthermia (King et al., 2002) and also to suffer thermally induced oxygen limitation (Van Dijk et al., 1999).

Materials and methods

Experimental design

Experiment A: graded heat stress and 24 h recovery

Summer-acclimatized eelpout *Zoarces viviparus* L. were caught with bottom traps in shallow waters near the island Helgoland in the German North Sea in June and July 2002. Animals were kept in flow-through aquaria with filtered North Sea water under constant aeration and a natural day:night cycle at 12°C. Before the experimental phase, the fishes were kept

for at least 10 days in the aquaria to recover from handling stress. Eelpouts were fed pieces of fresh white fish once a week.

To mimic acute heat stress, the animals were transferred to 18°C, the high temperature limit in the habitat, which is above T_p (Zakhartsev et al., 2003), as well as to 22°C, which according to Van Dijk et al. (1999) is the critical temperature (T_c), and eventually to 26°C, which represents extreme heat stress where the animals are close to the onset of loss of equilibrium at 27–28°C (Zakhartsev et al., 2003). Exposure time was limited to 2 h. Following heat exposure, 50% of the animals were directly sacrificed, whereas the other half was returned to 12°C and allowed to recover for 24 h.

For tissue sampling, fishes were treated with 0.5 g MS222 per liter of seawater, weighed and killed by cutting through the spine. Tissues were rapidly removed, starting with the most metabolically active (liver) and 100 mg portions were immediately frozen in liquid nitrogen. Samples were stored at –80°C prior to analysis. After sampling, the sex and length of each fish were determined.

Each experimental group comprised 12–16 fish. Fish length varied between 18 and 26 cm (21.5 ± 2.6 ; mean \pm s.d.). Average fish mass was 40.2 ± 20.8 g (132 g max; 16 g min.).

Experiment B: recovery time series

For a second experimental series we tested the effects of different recovery times in order to assess a potentially time-dependent maximum of reoxygenation stress. *Zoarces viviparus* were caught near the island Helgoland in September 2003 and transported to the Alfred-Wegener Institute 2 weeks prior to experimentation, where the fish were kept at 10°C in natural sea water with constant aeration and a day:night cycle of 12 h. Fish were fed live shrimp once a week. Animals were exposed only to 18°C for 2 h to induce thermal stress followed by 0 h, 2 h, 8 h or 12 h recovery at the control temperature (10°C). Samples were taken as described above. Each experimental group comprised 6–8 fishes. Fish length varied between 20 and 27 cm (23.6 ± 2.7 cm). Average fish mass was 50.4 ± 22.2 g (113 g max; 17 g min.).

Determination of thiobarbituric reactive substances

Thiobarbituric reactive substances (TBARS) were determined as a marker of lipid peroxidation by the TBA (thiobarbituric acid) assay using malondialdehyde-(bis)-acetate (MDA, Merck, Darmstadt, Germany) as standard (Uchiyama and Mihara, 1978).

Tert-butyl hydroperoxide-initiated chemiluminescence

Tert-butyl hydroperoxide (tBOOH) was measured, according to the method of Gonzalez Flecha et al. (1991), as an indicator of an imbalance between pro- and antioxidant processes resulting from depletion of antioxidant compounds such as glutathione, vitamin E and vitamin C. Tissue samples were homogenized in 30 mmol l⁻¹ KP_i buffer (pH 7.4) containing 120 mmol l⁻¹ KCl, and centrifuged at 600 g for 10 min. The supernatants were diluted in buffer, containing a

final concentration of 3 mmol l⁻¹ tBOOH and assayed in the dark at room temperature for chemiluminescence (counts min⁻¹) in a liquid scintillation counter (Wallac, GMI INC, Ramsey, MN, USA) in the out-of-coincidence mode, using potassium glass vials kept in the dark for at least 48 h to avoid vial phosphorescence activation by fluorescent light. The chemiluminescence data were determined as counts min⁻¹ and expressed in a tissue-specific curve with y =chemiluminescence and x =time. The area under this curve could be calculated for a time period of 3600 s using the MatLab program (Mathworks Inc., Natick, MA, USA). The results are expressed as arbitrary area units mg⁻¹ protein over the studied period.

Determination of protein carbonyl content

Carbonyl groups were measured as an indication of oxidative damage to proteins according to the method of Levine et al. (1990). Samples were homogenized in 50 mmol l⁻¹ Hepes buffer, pH 7.4, containing 125 mmol l⁻¹ KCl, 1.1 mmol l⁻¹ EDTA, 0.6 mmol l⁻¹ MgSO₄ and protease inhibitors (0.5 mg ml⁻¹ leupeptine, 0.7 µg ml⁻¹ pepstatine, 40 µg ml⁻¹ phenylmethylsulfonyl fluoride, 0.5 µg ml⁻¹ aprotinin) and centrifuged at 100 000 *g* for 15 min. Supernatants were incubated at room temperature for 1 h with 10 mmol l⁻¹ 2,4-dinitrophenylhydrazine (DNTP) in 2 mol l⁻¹ HCl. Blanks were run without DNTP. Afterwards, proteins were precipitated with TCA and centrifuged for 10 min at 10 000 *g*. The protein pellet was washed three times with ethanol:ethylacetate (1:1), resuspended in 6 mol l⁻¹ guanidine hydrochloride in 20 mmol l⁻¹ potassium phosphate (pH 2.3) and incubated at 37°C until complete resuspension. The carbonyl content could be measured spectrophotometrically at 360 nm (molar extinction coefficient ϵ =22 000 mol⁻¹ cm⁻¹).

Determination of reduced and oxidized glutathione

The glutathione status represents the most important determinant for the cellular redox environment (Schafer and Buettner, 2001). The content of reduced glutathione (GSH) and oxidized glutathione (GSSG) was determined according to the method of Fariss and Reed (1987). Frozen tissue was ground in liquid nitrogen and the resulting powder homogenized in 1:10 (w:v) pre-cooled PCA (10% containing 2 mmol l⁻¹ bathophenanthroline-disulphonic acid). After centrifugation at 15 000 *g* for 5 min at 4°C, 500 µl of the supernatant were mixed with 10 µl pH indicator [1 mmol l⁻¹ m-cresol purple, sodium salt, containing 0.5 mol l⁻¹ iodoacetic acid (IAA)]. 50 µl 1 mmol l⁻¹ γ -glutamyl glutamate (in 0.3% PCA) was added as internal standard. The pH was adjusted to 8.5 with 5 mol l⁻¹ KOH (containing 0.3 mol l⁻¹ *N*-morpholine-propanesulfonic acid). The mixture was incubated at room temperature for 45 min, to allow IAA to bind GSH. Subsequently, samples were centrifuged for 5 min at 15 000 *g* at 4°C. 300 µl of the supernatant were added to the double amount of 1% 1-fluor-2,4-dinitrobenzene (diluted in 100% ethanol, HPLC grade) and derivatised in dark vials at room temperature for 24 h. After centrifugation at 7500 *g* for 1 min

at 4°C and filtration through 0.2 µm nylon membrane filters, samples were stored in dark HPLC vials at -20°C.

HPLC determination was carried out on a Beckmann Coulter HPLC System using a NH₂-spherisorp column, 5 µm 240×4 mm (Waters, Eschborn, Germany). Solvent A was 80% methanol and solvent B was sodium acetate stock in 80% methanol (20:80). Sodium acetate stock was prepared by dissolving 500 g sodium acetate in 224 ml Milli-Q water and 695 ml of concentrated HPLC-grade acetic acid. The gradient program was as follows: 10 min hold at 90% A followed by a 25 min linear gradient to 25% A at a flow rate of 1 ml min⁻¹ and 2.3–2.8 psi (1 psi=6.9 kPa) back pressure. Peaks were recorded with a photodiode array detector at 365 nm. Concentrations were calculated using five-point calibration curves for GSH and GSSG standards processed in the same manner as described for the samples.

The redox potential was calculated using the Nernst equation: $(\Delta E = E^{\text{opH}} - (RT - 2.303/nF) \log([GSH]^2/[GSSG]))$ mV, as given by Schafer and Buettner (2001). The calculations were done for the different temperatures of maintenance, considering the temperature induced changes of intracellular pH (pH_i) in fish liver (data taken from Larsen et al., 1997; Sartoris et al., 2003).

Determination of enzymatic antioxidants

Superoxide dismutase activity (SOD EC: 1.15.1.1) was determined according to Livingstone et al. (1992), using a xanthine oxidase/cytochrome *c* assay at 20°C as well as at the respective stress temperature of each experimental group. 1 Unit SOD reduces the increase in extinction of superoxide-mediated reduction of oxidized cytochrome *c* by 50% (measured at 550 nm).

Glutathione peroxidase activity (GPX) was determined at 20°C in a coupled optical test according to the method of Günzler and Flohe (1985).

Protein content

The protein content of the samples was determined according to Bradford (1976) using bovine serum albumin as a standard.

Western blot analysis

Nuclear extraction protocols, developed for rainbow trout cells (Soitamo et al., 2001), are not applicable to small tissue samples. Thus, EMSA and western blotting were carried out with whole cell extracts, prepared as described by Vuori et al. (2004).

50 mg of liver tissues were homogenized in 200 µl buffer C [20 mmol l⁻¹ Hepes, pH 7.8, 0.42 mol l⁻¹ NaCl, 1.5 mmol l⁻¹ MgCl₂, 0.2 mmol l⁻¹ EDTA, 0.5 mmol l⁻¹ phenylmethylsulfonyl fluoride (PMSF), 0.5 mmol l⁻¹ 1,4 dithiothreitol (DTT), 25% glycerol, 2 µg ml⁻¹ leupeptine, 2 µg ml⁻¹ antipaine, 2 µg ml⁻¹ pepstatine, 2 µg ml⁻¹ aprotinin, 1 mmol l⁻¹ Na₃VO₄] and centrifuged at 16 100 *g*, 30 min, 4°C.

20 µg protein of whole cell extracts per well were run on 7.5% LiDS-PAGE (lithium dodecyl sulfate-polyacrylamide

gel electrophoresis) at 40 mA and transferred to a Whatman 3MM filter paper + nitrocellulose membrane 'sandwich' for semi-dry blotting (1 h; 0.4 A; 13 V). Membranes were blocked for 1 h at room temperature with 3% non-fat dry milk in PBS 0.3% Tween 20, rinsed three times for 10 min with PBS 0.3% Tween 20 and incubated with the primary antibody overnight at 4°C followed by 1 h at room temperature. Polyclonal antibodies directed against the N terminus of rainbow trout HIF-1 α as described in Soitamo et al. (2001) were used at a dilution of 1:2000 in 1% BSA PBS + 0.02% NaN₃. Afterwards, the membranes were washed and incubated for 3 h at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham Biosciences, Golden, NJ, USA), dilution 1:7500 in 3% non-fat dry milk in PBS 0.3% Tween 20. After washing the membranes, the signals were detected by enhanced chemiluminescence (ECL; Amersham Biosciences). Signal intensities of HIF-1 protein bands were calculated from autoradiographed film, using a Chemi-Imager digital camera and software (Alpha Innotech Co., San Leandro, CA, USA). Calculation of the percentage intensity was based on densitometry of gel images, with the sum of the bands from all experimental groups taken as 100% value.

Electromobility shift assay

Following the fish protocol of Soitamo et al. (2001), the promoter region of the human erythropoietin (EPO) gene was used as the HIF-sensitive DNA probe (5'-GCCCTACGT-GCTGTCTCA-3'). 5'-endlabeling of the sense strand (2 pmol μl^{-1} DNA) was done with 10 U μl^{-1} T4 polynucleotide kinase and [γ -³²P]dATP (10% v:v) over 15 min at 37°C. After removing unincorporated nucleotides by gel filtration (Sephadex G-25), probes were annealed with 2 pmol μl^{-1} antisense strand in 10 mmol l⁻¹ Tris-HCl, pH 8.0, 1 mmol l⁻¹ EDTA, 5 mmol l⁻¹ MgCl₂.

Electromobility shift assay (EMSA) DNA-protein binding reactions were carried out for 30 min on ice in a total volume of 20 μl , containing 10 μg cell extract, 0.1 μg μl^{-1} carrier DNA [poly(dI-dC)], DNA binding buffer (10 mmol l⁻¹ Tris-HCl, pH 7.5, 50 mmol l⁻¹ KCl, 50 mmol l⁻¹ NaCl, 1 mmol l⁻¹ MgCl₂, 1 mmol l⁻¹ EDTA, 5 mmol l⁻¹ DTT, 5% glycerol), 1 μl ³²P-endlabeled DNA probe, 1 μl Bromophenol Blue (1.5%). Samples were run on 4% non-denaturing glycerolpolyacrylamide gel (4% acrylamide, 1% glycerol, 0.33 \times TBE buffer (89 mmol l⁻¹ Tris, 89 mmol l⁻¹ boric acid, 5 mmol l⁻¹ EDTA). Electrophoresis was performed at 150 V and room temperature, for 2 h in 0.33 \times TBE buffer. Dried gels were autoradiographed (Kvietikova et al., 1995). Calculation of the percentage intensity was based on densitometry of gel images, with the sum of all bands taken as 100% value.

For testing the specificity of the human EPO enhancer by supershift experiments, 1 μl of polyclonal antibody (against the N terminus of rainbow trout HIF-1 α , see above) was added to the EMSA reaction mixture with liver extracts from *Z. viviparus*. In the presence of the antibody, the EMSA signal was markedly reduced, indicating that antibody interaction with the HIF-1 α subunit minimized specific binding of the

HIF-1 dimer to the EPO probe (K. Heise, S. Puntarulo, M. Nikinmaa, M. Lucassen, H.-O. Pörtner and D. Abele, unpublished data).

Moreover, the signal was erased by previously incubating the reaction mixture for 15 min with a 2500-fold excess of unlabeled probe prior to addition of the labeled EPO probe. An excess of mutated EPO probe M18 (5'-TTGCCCTAAAA-GCTGTCTCAG-3'; Gorr et al., 2004) was added to minimize non-specific (but not HIF) binding (K. Heise, S. Puntarulo, M. Nikinmaa, M. Lucassen, H.-O. Pörtner and D. Abele, unpublished data). In this control experiment, radioactivity was detected and quantified in dried gels with a phosphor storage image system (FLA-5000; Fuji, Tokyo, Japan) and the AIDA software package (raytest, Straubenhardt, Germany).

Statistics

All values are given as means \pm standard deviation. Differences between experimental groups were analyzed by student's *t*-test using Statview 5.0 (SAS Institute Inc., Cary, NC, USA). A value of $P < 0.05$ was considered to be statistically significant.

Results

Experiment A: graded heat stress and 24 h recovery

Behavior during heat stress and recovery

Observations of behavior included mobility level (frequency of spontaneous swimming) and swimming speed observed during 2 h exposure to heat stress. Eelpouts at 18°C maintained mobility and swimming speed at control level or even higher. At 22°C fishes showed spontaneous mobility but at a reduced speed compared to 12°C. Exposure to 26°C led to an even more obvious slow-down of swimming and to severely reduced spontaneous mobility. However, directly after returning the fish to 12°C for recovery, there was no difference in swimming speed and mobility between unstressed controls and all heat exposed groups. No fish died during heat exposure or recovery.

Antioxidant capacity in the liver

Superoxide dismutase (SOD) activity, measured at standardized temperature of 20°C (Fig. 1), was unchanged from control levels after heat exposure to 18°C and 22°C, but significantly reduced after 2 h at 26°C ($P = 0.04$). Partial recovery of the reduced SOD activity was achieved by 24 h maintenance at 12°C ($P = 0.3$ compared to control, $P = 0.06$ compared to 26°C). Also when assayed directly at stress temperature (data not shown), no significant difference in SOD activities between the control group and any of the heat stressed groups was found. However, again, the activity drop between 18°C/22°C and 26°C ($P \leq 0.03$) was observed, indicating that the critical temperature for the enzyme had been reached. This was confirmed by Q_{10} values being 1.3 between 12°C and 22°C and 0.3 between 22°C and 26°C. Glutathione peroxidase (GPX) activities were assayed at 20°C and did not show significant changes between groups compared to control levels at any exposure temperature because of a high inter-

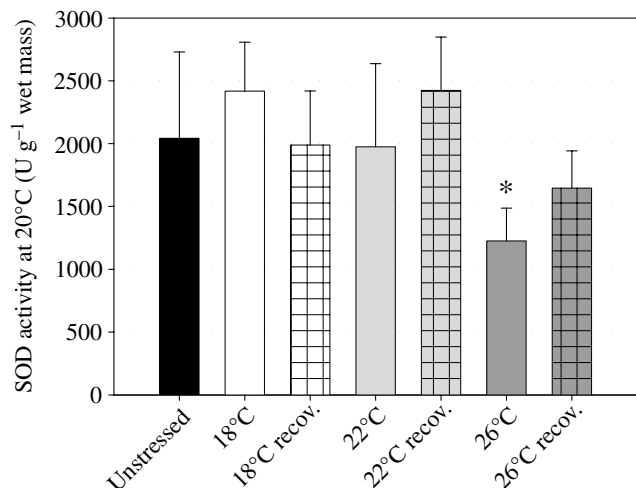


Fig. 1. Superoxide dismutase (SOD) activity in *Z. viviparus* liver samples after 2 h of exposure to 18°, 22° and 26°C and after 24 h recovery (recov.) from the respective temperature. Enzyme activity was measured at 20°C. Values are means \pm s.d., $N=3-5$; *significantly different from the unstressed group, $P<0.05$.

individual variability ($P>0.16$, 4.3 ± 2.2 U g⁻¹ wet mass in the unstressed control fish).

The total glutathione content (controls: 1.2 ± 0.3 $\mu\text{mol g}^{-1}$ wet mass, data not shown) as well as the contents of reduced (GSH, control: 0.9 ± 0.6 $\mu\text{mol g}^{-1}$ wet mass, data not shown) and oxidized (GSSG, Fig. 2A) glutathione did not show significant changes following heat exposure or recovery from heat stress (total glutathione: $P>0.35$, GSH: $P>0.18$). The rise in GSSG upon recovery from 26°C ($P=0.05$, $N=6$; Fig. 2A) was not statistically significant because of a relatively low number of available fish samples. The redox potential ΔE (Fig. 2B) remained close to control values at 18°C ($P=0.28$) and during recovery from 18°C ($P=0.77$). Acute warming to 22°C ($P<0.01$) and 26°C ($P=0.03$) led to a significantly more oxidized cellular redox state. After each high temperature exposure, ΔE values were back to control level within 24 h of recovery ($P>0.33$).

Oxidative damage parameters in the liver

The *ex vivo* susceptibility of lipids to oxidation can be evaluated by exposing them to a source of free radicals such as tBOOH (Dotan et al., 2004). The main reactive species detected by the assay of tBOOH-initiated chemiluminescence, according to Gonzalez Flecha et al. (1991), are the dimol emission of singlet oxygen and the photon emission from excited carbonyl groups formed during lipid peroxidation. The assay evaluates the imbalance between pro- and antioxidant processes resulting from depletion of low-molecular mass antioxidant compounds such as glutathione, ascorbate and α -tocopherol.

Unaltered tBOOH-initiated chemiluminescence rates in all experimental groups (Fig. 3A) indicate that pro-oxidant processes occurring during stress were well balanced by

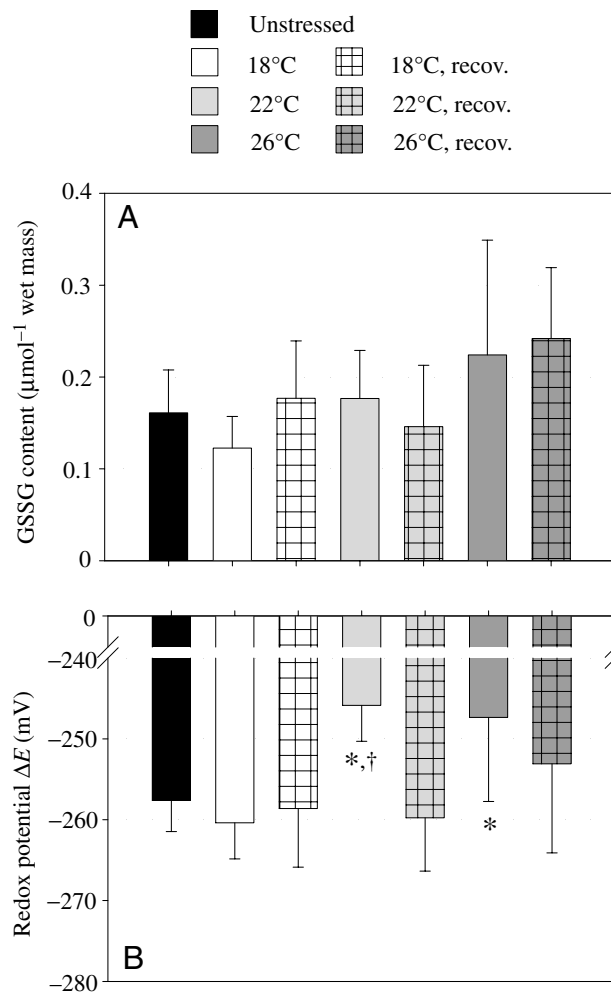


Fig. 2. Glutathione redox chemistry in *Z. viviparus* liver samples after 2 h of exposure to 18°, 22° and 26°C and after 24 h recovery (recov.) from these temperatures. (A) Oxidized glutathione (GSSG) content. (B) Redox potential ΔE at *in situ* temperature and *in situ* intracellular pH (adopted from Sartoris et al., 2003; Larsen et al., 1997), calculated after Schaffer and Buettner (2001). Values are means \pm s.d., $N=4-7$; *significantly different from the unstressed group, †significantly different from the respective recovery group, $P<0.05$.

antioxidants during high temperature exposure and recovery. However, thiobarbituric acid reactive substances (TBARS) as markers of lipid peroxidation were elevated over control levels after all heat treatments and remained elevated throughout recovery (P -values: 0.06 at 18°C, 0.04 at 22°C, <0.01 at 26°C, 0.02 at recovery after 18°C, 0.03 at recovery after 22°C, 0.04 at recovery after 26°C; Fig. 3B). Differences between stressed groups were insignificant. TBARS content was slightly lower after 24 h of recovery than in the respective heat stress group, but still recovery levels were significantly above control values (12°C).

The carbonyl content of the liver reflects oxidative modifications of proteins (Fig. 3C) and was similar in all groups with the exception of higher carbonyl levels being found after recovery from 18°C ($P=0.03$ compared to controls).

Hypoxic signaling and molecular defense in the liver

Higher levels of heat shock protein (HSP70) were detected only after recovery from 26°C ($1.5 \pm 0.2\%$ signal intensity, $N=5$) compared to control levels ($1.0 \pm 0.3\%$, $N=4$, $P=0.05$) and to the 26°C heat stress group ($1.0 \pm 0.3\%$, $N=5$, $P=0.02$).

The HIF-1 α protein could be detected in all experimental groups, including unstressed controls and recovering specimens (data not shown), and did not show temperature-dependent concentration changes. Electromobility shift assays (EMSA), demonstrating DNA binding of HIF-1 to the human erythropoietin enhancer, reached only weak signal intensities following heat stress (Fig. 4A, lanes 6–11). DNA binding of HIF-1 significantly above control levels was found only after recovery from exposure to 18°C ($P=0.04$; Fig. 4B). HIF-1 DNA binding in *Z. viviparus* was generally enhanced at more reduced cellular redox potential. Fig. 4C documents a linear relationship (Statview 5.0, linear regression: $P=0.03$; $r^2=0.3$) between ΔE and HIF-DNA binding intensity. The plotted data are from this and from a parallel study, in which fishes from the same batch were exposed to cold temperatures.

Experiment B: recovery from exposure to 18°C

The time course of various parameters during the recovery phase was investigated using a batch of eelpout collected in September 2003. Liver SOD activities of unstressed controls were assayed at 20°C (3470 ± 750 U g⁻¹ wet mass) and at 10°C (2600 ± 480 U g⁻¹ wet mass) and remained unchanged after exposure to 18°C and subsequent recovery ($P>0.08$; data not shown).

Glutathione parameters from the recovery time series are presented in Fig. 5. The GSH content was significantly increased over controls following exposure to 18°C ($P<0.01$, Fig. 5A). Throughout recovery GSH remained significantly above controls ($P=0.04$ after 2 h recovery, $P=0.01$ after 8 h recovery, $P=0.01$ after 12 h recovery) but lower than in the heat stressed group. Owing to high variability within the control group, GSSG content was only insignificantly higher than 10°C controls upon warming and in the recovery groups

Table 1. Statistical comparison of oxidative stress markers in liver samples from unstressed controls and 18°C exposed *Z. viviparus* obtained during the graded heat stress experiment (A) and during the recovery time series (B)

	Unstressed controls		Fish exposed to 18°C	
	A vs B	P	A vs B	P
SOD activity at 20°C	A<B	0.01	A<B	<0.01
GSH content	A=B	0.72	A<B	<0.03
GSSG content	A=B	0.14	A<B	<0.01
Total glutathione	A=B	0.49	A<B	<0.01
2GSSG/GSH	A=B	0.06	A<B	0.03
Redox potential ΔE	A=B	0.40	A=B	0.34
Carbonyl content	A>B	<0.01	A>B	<0.01

Parameters are compared in summer (A) vs autumn (B).

(Fig. 5B). However, as GSH and GSSG had both increased, the total glutathione content was significantly higher in the group exposed to 18°C and in all recovery groups (compared with controls: 18°C, $P<0.01$; 2 h recovery $P<0.04$; 8 h recovery $P<0.01$; 12 h recovery, $P<0.01$; Fig. 5C). Constant glutathione

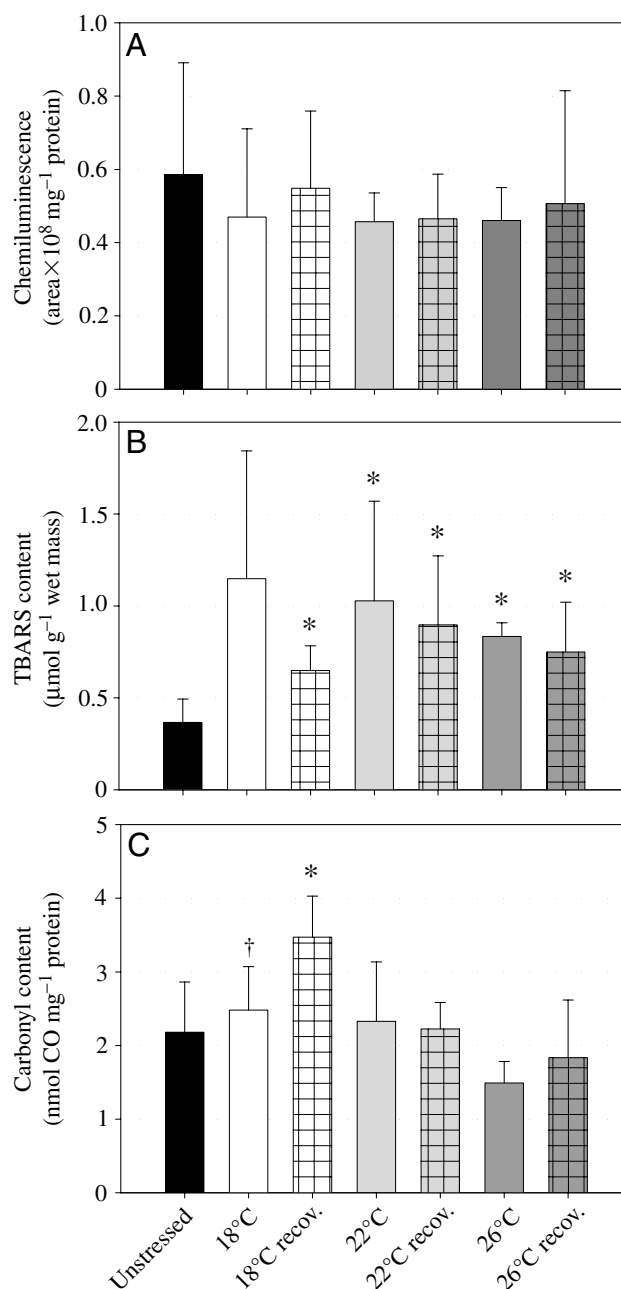


Fig. 3. Oxidative stress markers in *Z. viviparus* liver samples after 2 h of exposure to 18°, 22° and 26°C and after 24 h recovery (recov.) from these temperatures. (A) Tert-butyl hydroperoxide (tBOOH)-initiated chemiluminescence indicating imbalance between pro- and antioxidant processes. (B) Thiobarbituric reactive substances (TBARS) content, indicating lipid peroxidation. (C) Carbonyl content, indicating protein oxidation. Values are means \pm s.d., $N=4-6$; *significantly different from the unstressed group, †significantly different from the respective recovery group, $P<0.05$.

redox ratio (2 GSSG/GSH) and redox potential (ΔE) indicate maintenance of liver redox state at 18°C and throughout recovery (Fig. 5D,E). Liver oxidative stress parameters of the recovery time series are given in Fig. 6. A significant increase in tBOOH-initiated chemiluminescence was found after 12 h recovery from 18°C ($P<0.01$; Fig. 6A). The protein carbonyl content was significantly increased following 8 h of recovery

at control temperature ($P=0.04$) and returned to control levels within 12 h of recovery from warming ($P=0.41$; Fig. 6B). TBARS were not measured in this experiment.

Interestingly, some parameters showed significant differences between both experimental series in the control and also in the 18°C exposed fish group (Table 1). The carbonyl content was significantly higher in control and 18°C-treated fish of experiment A (graded heat stress, summer experiment) compared to B (recovery time series in fall). This went along with significantly lower SOD activities in experiment A. Further, the increase in GSH and total glutathione content upon exposure to 18°C (Fig. 5A,C) in experiment B, not observed in experiment A (see above), caused a significant difference in the glutathione concentration in the liver between the groups exposed to 18°C in both experiments (Table 1). However, the redox potential remained the same and the control glutathione parameters in the controls did not vary between both experiments.

Discussion

The present study investigates the physiological response of the common eelpout *Zoarces viviparus* to short-term experimental hyperthermia and heat stress, which has been suggested to cause functional hypoxia in central tissues (Pörtner, 2002). Specifically, we addressed the question of whether oxidative stress occurs during hyperthermia and/or during recovery at control temperature and whether antioxidants (AOX), heat shock proteins (HSP) and hypoxic signaling is induced. The interplay between these defense systems is supposed to be important for passive survival of critical temperature stress, e.g. during unfavorable conditions on hot summer days.

Based on the previous evaluation of the thermal biology of *Z. viviparus*, as summarized in Zakhartsev et al. (2003), we chose 18°, 22° and 26°C as stress temperatures, covering the range from subcritical values (18°C) beyond pejus temperatures, where the animals started to lose their aerobic scope, to the critical heat stress limit, where heat-induced

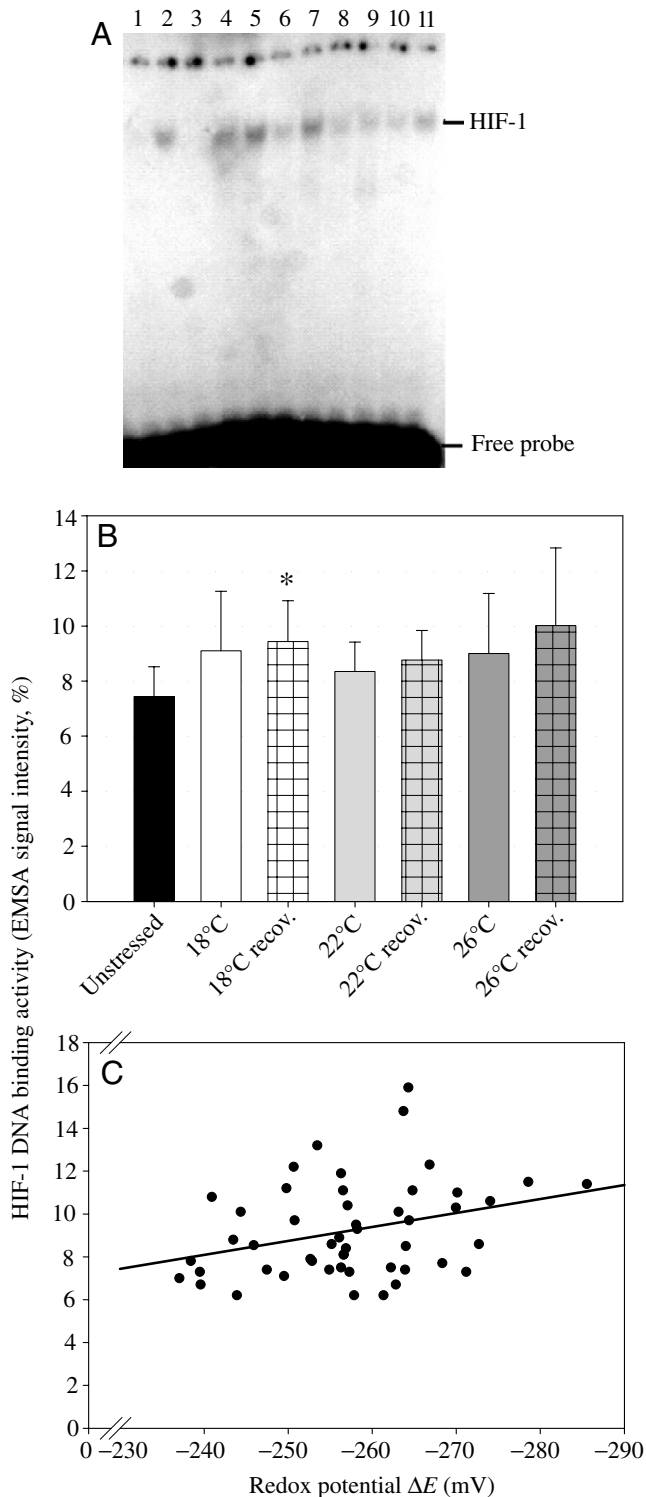


Fig. 4. DNA binding activity of HIF-1 in *Z. viviparus* whole cell extracts from the liver after 2 h of exposure to 18°, 22° and 26°C and after 24 h recovery (recov.) from these temperatures. (A) Signal intensity of HIF-1 DNA binding to the human erythropoietin enhancer. Lanes 1: unstressed; 2: 1°C; 3: 1°C recovery; 4: 5°C; 5: 5°C recovery; 6: 18°C; 7: 18°C recovery; 8: 22°C; 9: 22°C recovery; 10: 26°C; 11: 26°C recovery; data taken from the heat stress experiment (this study) and from cold stress (K. Heise, S. Puntarulo, M. Nikinmaa, M. Lucassen, H.-O. Pörtner and D. Abele, unpublished data). (B) HIF-1 DNA binding. Values are means \pm s.d., $N=4-5$; *significantly different from the unstressed group, $P<0.05$. (C) Linear regression demonstrating increased HIF-1 DNA binding at a more reduced redox environment ($y=0.065x-7.593$; $r^2=0.3$, $N=50$, $P<0.05$, Statview 5.0). Data taken from the heat stress experiment (this study) and from cold stress (K. Heise, S. Puntarulo, M. Nikinmaa, M. Lucassen, H.-O. Pörtner and D. Abele, unpublished data).

hypoxia becomes more severe and anaerobic metabolism sets in (22°C). Finally, animals were exposed to 26°C, which is slightly below the temperature at which Zakhartsev and co-workers found an onset of muscular spasms and loss of equilibrium.

Response to graded heat stress and recovery

Induction of antioxidant enzymes is an important line of defense against oxidative stress in biological systems (Storey, 1996; Parihar et al., 1997) but can be compromised under temperature stress because of thermal impairment of protein function (reviewed by Abele and Puntarulo, 2004). In the present study, unchanged SOD and GPX activities were recorded after exposure to 22°C, whereas particularly SOD was reduced under extreme hyperthermia (26°C; Fig. 1). Similarly, impairment, especially of SOD, above T_c has been reported for different marine invertebrates (Abele et al., 1998,

2001, 2002). This loss of enzymatic antioxidant activity beyond critical temperatures might relate to heat-induced protein denaturation or disturbances of protein synthesis (Pörtner, 2002; Kregel, 2002). Interestingly, although GPX activity was not significantly elevated, the cellular redox potential, which is mainly determined by the ratio of oxidized to reduced glutathione (Schafer and Buettner, 2001), was more oxidized under critical hyperthermia at 22°C and 26°C (Fig. 2B). Presumably this relates to spontaneous, non-enzymatic GSH oxidation by emerging ROS under hyperthermia. It may also be due to loss of function of the enzyme glutathione reductase, which re-converts oxidized to reduced glutathione.

The glutathione-based antioxidant effect is thought to be protective in the hydrophilic protein fraction and, indeed, protein oxidation was not elevated at critically high temperatures ($\leq 22^\circ\text{C}$). In the hydrophobic lipid fraction, the

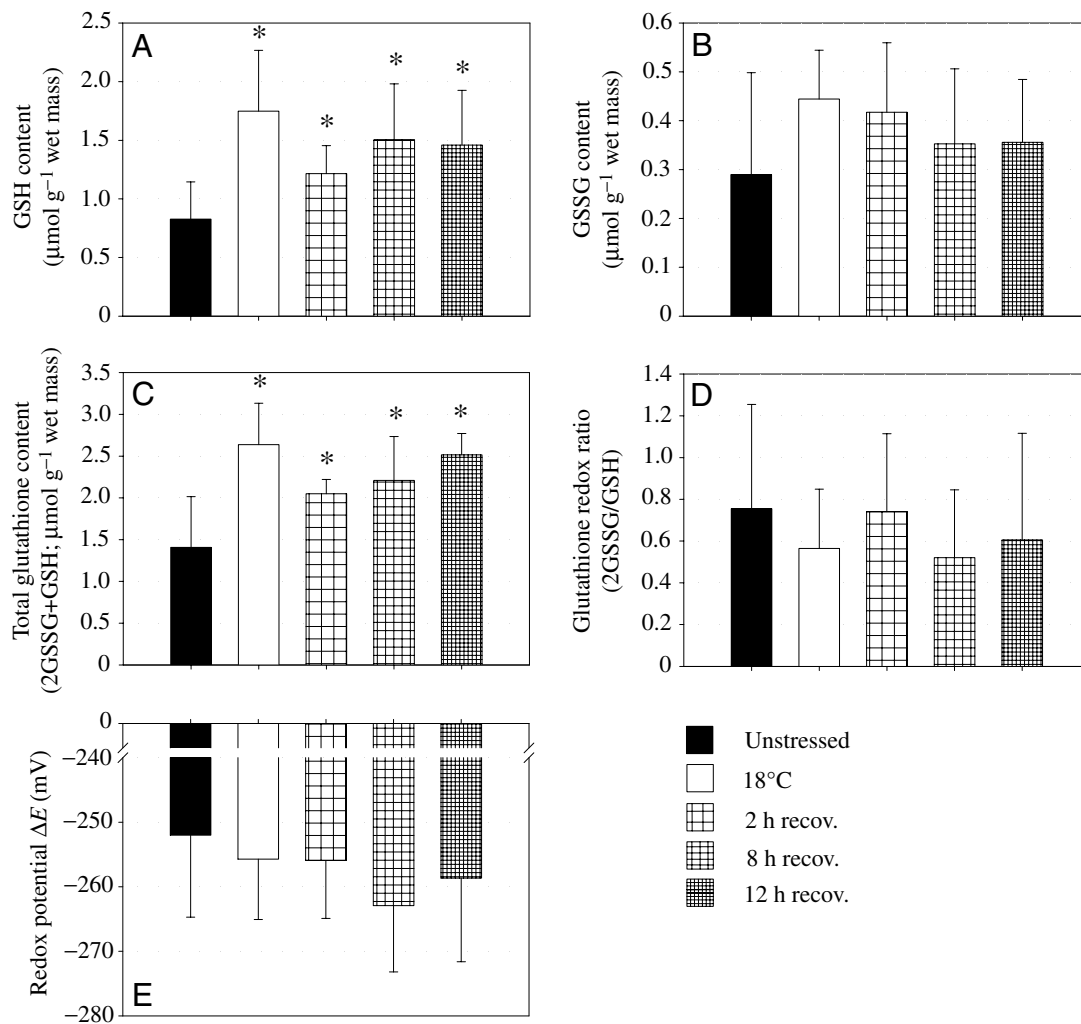


Fig. 5. Glutathione redox chemistry in *Z. viviparus* liver samples after 2 h of exposure to 18°C and after 2, 8 or 12 h of recovery (recov.). (A) Reduced glutathione (GSH) content. (B) Oxidized glutathione (GSSG) content. (C) Total glutathione content (2GSSG+GSH). (D) Glutathione redox ratio (2GSSG/GSH). (E) Redox potential (ΔE) at *in situ* temperature and *in situ* intracellular pH (adopted from Sartoris et al., 2003; Larsen et al., 1997), calculated after Schaffer and Buettner (2001). Values are means \pm s.d., $N=4-5$; *significantly different from the unstressed group, $P<0.05$.

antioxidant glutathione is much less soluble and therefore less effective, and TBARS levels rose significantly over controls at 22°C and higher. In agreement with these findings, glutathione depletion in heat stressed marine sponges resulted in a loss of antioxidant protection (Bachinski et al., 1997) and the same effect has been observed in mammalian systems (Freeman et al., 1990). The elevated TBARS levels occurred although ROS formation and antioxidant defense were kept in balance as deduced from unchanged chemiluminescence intensity in the homogenate assay. Higher TBARS levels in heat stressed eelpout may, moreover, be supported by an impairment of TBARS degradation and elimination in heated fish. However, higher lipid peroxidation by elevated mitochondrial ROS formation seems more probable as our recent *in vitro* studies with isolated mitochondria from marine invertebrates have clearly documented elevated ROS formation rates at rising temperatures (Abele et al., 2002; Heise et al., 2003; Keller et al., 2004). Similar effects could be expected in fish, but, as yet, there is no direct evidence.

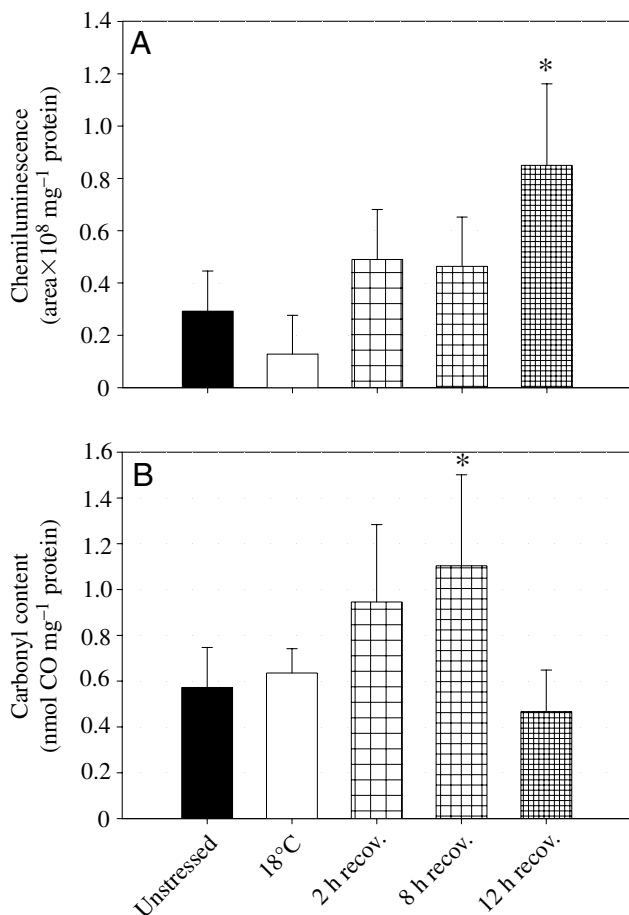


Fig. 6. Oxidative stress markers in *Z. viviparus* liver samples after 2 h of exposure to 18°C as well as after 2, 8 or 12 h of recovery (recov.). (A) Tert-butyl hydroperoxide (tBOOH)-initiated chemiluminescence, indicating imbalance between pro- and antioxidant processes. (B) Carbonyl content, indicating protein oxidation. Values are means \pm s.d., $N=3-5$; *significantly different from the unstressed group, $P<0.05$.

A heat shock response, notably of HSP70, has been demonstrated in a number of studies using fish cells (Arai et al., 1994; Airaksinen et al., 1998, 2003) and also in whole animal experiments with marine (Dietz and Somero, 1992) and freshwater fish (Molina et al., 2000), exposed to a variety of stresses including heat (for a review, see Iwama et al., 1998). We found only insignificantly elevated HSP70 levels after recovery from 26°C, which supports the view that no major inactivation of native proteins had occurred at this temperature. This conclusion is in line with the protein oxidation remaining close to control levels in all groups. Thus, in the studied eelpout, HSP70 was either not induced although T_c was reached, or by 24 h into the recovery phase it was too late to detect the heat shock response.

Recent results by Treinin et al. (2003) demonstrated that the transcription factor HIF-1 is essential for heat acclimation in *Caenorhabditis elegans* and probably also in rat and mouse. Stabilization or activation of HIF-1 occurring within 1 h of heat exposure in mice liver and kidney appeared to be mediated by increased HSP90 levels (Katschinski et al., 2002). We hypothesized that heat stress could activate hypoxic signaling also in the North Sea eelpout *Z. viviparus*. However, only sub-critical warming in the pejus temperature range (18°C) caused a significant induction of HIF-1 activity (Fig. 4A,B), whereas higher temperatures produced only weak EMSA signals. We, therefore, conclude that metabolic reorganization to improve oxygen supply can only be functional in the pejus range of this fish species (15–22°C; Zakhartsev et al., 2003) in which aerobic scope diminishes. The observed transition of *Z. viviparus* liver tissue to anaerobic metabolism on longer warming beyond the T_c by Van Dijk et al. (1999) is a consequence of extreme hypoxia and probably induced *via* reversible enzyme phosphorylation leading to accumulation of glycolytic substrates, but not controlled by HIF-1-induced upregulation of glycolytic enzymes (Semenza et al., 1994). Moreover, other transcription factors such as Sp1 and Sp3 could be responsible for the temperature-mediated induction of glycolytic enzymes as shown for β -enolase and pyruvate kinase M in mammalian muscle cells subjected to hypoxia (Discher et al., 1998).

In addition, as HIF-1 DNA binding was generally higher at more reduced redox state (Fig. 4C), the more oxidized conditions at 22°C and 26°C (Fig. 2B) might 'switch-off' the hypoxic signal, as proposed by Abele (2002), and thereby prevent the more complex HIF-1-induced physiological response. In any event, redox sensitivity and the presence of the protein in normoxic samples suggest that HIF-1 could have currently unknown normoxic functions in fish (Nikinmaa and Rees, 2005).

Recovery time series

We did not observe any increase of oxidative stress markers (chemiluminescence and TBARS, Fig. 3A,B) at 24 h of recovery from heat stress in any group, except for the higher carbonyl content after recovery from 18°C (Fig. 3C). Thus, on first sight, heat-induced hypoxia does not mimic the ischemia/reperfusion pattern of oxidative stress in mammals. To see

whether oxidative damage repair might have been faster and already finished within 24 h of recovery from high temperature stress, we performed a recovery-time series with eelpout caught in the autumn.

An unaltered redox potential (ΔE) in all groups (Fig. 5E) confirmed our previous finding that neither sub-critical temperature stress (18°C) itself, nor the subsequent recovery phase disturbed the cellular redox milieu. Increased chemiluminescence rates and carbonyl contents during recovery intervals of up to 12 h (Fig. 6A,B) confirmed our prediction that repair of oxidative damage after heat stress could occur before 24 h. The distinct time patterns of both parameters can be explained by the different underlying processes. Chemiluminescence depends on the progressive exploitation of small molecule antioxidants under ongoing oxidative stress, obviously still active after 12 h. Protein oxidation depends on oxidative stress and induction of repair mechanisms, i.e. proteasomal degradation and new synthesis (Dröge, 2002), which set in after about 10 h of recovery, so that the 12 h values were already back to control levels in the recovery time series with autumn animals. Taking into account that exactly the same protein carbonyl parameters were still elevated after 24 h in summer animals exposed to 18°C illustrates the complex interaction of various repair systems and the unknown importance of seasonal preconditioning. Thus, it is possible that seasonal changes in the protein turnover between the experimental fishes in the first batch caught in summer 2002 (kept at 12°C) and the second batch caught in autumn 2003 (kept at 10°C), account for the variations.

Conclusions

Our data present a first indication that heat-induced hypoxia and reoxygenation upon recovery in the North Sea eelpout may bring about similar complications as ischemia/reperfusion events in mammals. We have not tested repeated thermal stress, which the fishes may experience when trapped in shallow areas or under otherwise unfavorable conditions, and during which the stress effect may emerge more clearly. In any event, hypoxic signaling and subsequent metabolic reorganization to counterbalance thermal oxygen limitation seems to be effective only in the pejus temperature range, while it appears impaired at critical and higher temperatures (22°C and 26°C), presumably because of the more oxidized cellular redox state.

The fishes, most probably, do not die from oxidative damage, although SOD was impaired and lipid peroxidation was significantly increased upon critical heating. However, oxidative stress effects, leading to a more oxidized cellular redox state under critical heat exposure (−245 mV vs −260 mV in controls), can exacerbate the hypoxic deficit by impairment of a more active HIF-1 signal.

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