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Original Contribution

Redox regulation in Atlantic cod (*Gadus morhua*) embryos developing under normal and heat-stressed conditions

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ABSTRACT

With regard to predicted oceanic warming, we studied the effects of heat stress on the redox system during embryonic development of Atlantic cod (*Gadus morhua*), with emphasis on the glutathione balance, activities of key antioxidant enzymes, and their mRNA levels. The embryos were incubated at optimal temperature for development (6 °C) or slightly above the threshold temperature (10 °C). The regulation of all the redox-related parameters measured at optimum development was highly dynamic and complex, indicating the importance of both maternal and zygotic contributions to maintaining redox equilibrium. Development at 10 °C caused a significantly higher mortality at the blastula and early gastrula stages, indicating severe stress. Measures of the glutathione redox couple showed a significantly more reduced state in embryos at 10 °C compared to 6 °C at the post-gastrula stages. Mean normalized expression of *nrf2*, *trxred*, *g6pd*, *gclc*, *nox1*, *CuZnsod*, and *mt* in embryos kept at 10 °C revealed stage-specific significantly reduced mRNA levels. Activities of antioxidant enzymes changed both during ontogenesis and in response to temperature, but did not correlate with mRNA levels. As the embryos need a tightly regulated redox environment to coordinate between growth and differentiation, these findings suggest that the altered redox balance might participate in inducing phenotypic changes caused by elevated temperature.

A challenge during embryonic development is the activation and coordination of catabolic and anabolic pathways, while simultaneously managing reactive metabolic by-products, such as reactive oxygen species (ROS) and reactive nitrogen species. ROS are generated mainly through the mitochondrial electron-transport chain, in the endoplasmic reticulum, and through the activity of several enzymes. These oxidants are balanced by reductants, also called antioxidants. Overproduction of ROS and/or lack of antioxidant capacity can shift the delicate cellular and extracellular redox equilibria, leading to irreversible oxidative modifications at the molecular, cellular, and organ levels and consequently the development of pathologies [1]. Changes in temperature have a more profound effect during early life and embryos tend to be more stenothermal than juveniles and adults [2,3]. The embryonic stages are the "thermal bottlenecks" for the Atlantic cod distribution, with optimum development occurring at temperatures around 6 °C, whereas embryos developing at or above threshold temperature (10 °C) are severely stressed [4,5]. As the routine metabolic rate increases with increasing temperature [3], we hypothesized that an increase in seawater temperatures, such as those predicted for the North Atlantic Ocean (UK Climate Projections Briefing Report—UKCIP09 [6]), will affect the redox system in the sensitive cod embryos.

The redox system can be viewed by assuming a redox balance [7–9]. The redox potential of cells is under strict control through regulation of the concentrations and ratios of several redox couples, the most important being the glutathione couple (reduced glutathione (GSH)/oxidized glutathione (GSSG)). GSH/GSSG is present in all cells in millimolar concentrations and usually at ratios higher than 100:1 [9]. Evidence suggests that the concentration and ratio of GSH and GSSG control inter- and intramolecular oxidation states of protein thiols and thereby the amount of S–S bonding, glutathionylation, and phosphorylation of proteins. Changes in the three-dimensional conformation along with the addition of side chains to proteins change their activity, and the regulation of the redox potential (E) can therefore modulate signaling pathways and hence change cell fate. This is in accordance with the finding that differentiating cells have a higher E than proliferating cells [7,10] and treating cells to inhibit growth and induce differentiation increases E, whereas treating them to promote growth reduces E [8]. Furthermore, different cell compartments have different E. The mitochondria are more reduced than the cytosol. The cytosol of cells is in turn more reduced than the extracellular plasma [8]. Other redox couples,

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such as NADPH/$NADP^+$, thioredoxins, and other proteins with cysteine or disulfide cysteine, are also assumed to participate in redox regulation. Their concentrations are at nonequilibrium and the redox potentials are different from that of the GSH/GSSG couple [8].

Several of the components of the redox system are highly regulated by gene expression. The nuclear factor erythroid 2-related factor 2 (encoded by the gene *nrf2*) is a transcription factor that is induced by electrophilic compounds (oxidative stress) and binds to antioxidant-response elements (AREs) to activate transcription of genes for antioxidant enzymes and glutathione-metabolizing enzymes [11]. Among these genes are *gpx2* [12], *gpx3* [1], *gclc* [13], and *trx* [14] (Table 1). Knockout and knockdown experiments on mice have revealed the importance of redox-related genes during embryo development, e.g., the Nrf2 mutant is viable, but exhibits a reduced tolerance for oxidative stress, whereas functional inactivation of other genes connected to the redox balance are embryonic lethal (Table 1).

The purpose of this study was to describe the development of the redox system in Atlantic cod during embryogenesis and assess the effect of incubating eggs at 6 °C (control) compared to 10 °C (heat stressed). Cod eggs were sampled at specific developmental stages from fertilization until hatching. In this study we measured total GSH (tGSH) and oxidized glutathione (GSSG), assuming that this gives an estimate of the redox potential in the embryo. The activities of several key antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) were analyzed. For SOD we measured mitochondrial (MnSOD) and cytosolic (CuZnSOD) SOD, which reduce superoxide anions ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2). CAT and GPx reduce H_2O_2 to water, while oxidizing NADPH and GSH, respectively. GSH can then be reduced by glutathione reductase using NADPH. In addition we measured the mRNA levels of genes related to the redox system: *nrf2*, *gpx1*, *gpx3*, *gpx4b*, *glrx2*, *glrx3*, *trx*, *trxred*, *g6pd*, *gclc*, *gr*, *nox1*, *Mnsod*, *CuZnsod*, *cat*, and *mt* (Table 1).

Materials and methods

Hatchery and sampling conditions

The experiments were conducted in accordance with the Animal Welfare Act of 20 December 1974, No. 73, Chapter VI, Sections 20–22, amended 19 June 2009. The Norwegian legislation conforms in all respects with the basic requirements of the European Convention and fertilized eggs are exempted from the definitions in the regulation. Atlantic cod milt and unfertilized eggs were a gift from Fjord Forsk Sogn, Norway, and samples were fresh-frozen directly in liquid nitrogen before measuring glutathione, antioxidant enzyme activity, and isolation of RNA. Newly fertilized Atlantic cod eggs were collected from the wild-caught brood stock at the Austevoll Research Station, Norway. The eggs were spawned naturally at 7 °C and collected in a filter tank connected to the tank outlet. Eggs were rinsed in fresh filtered and oxygenated seawater and transported for 2 h to the laboratory. In the laboratory the fertilized egg batch was divided between three cone-shaped 16-liter incubation tanks situated in a cold room at 6 °C (5.9 ± 0.5 °C, three tanks). When the eggs reached the early blastula stage, half of the egg batch was transferred from 6 °C to another cold room kept at 10 °C (9.7 ± 0.5 °C, three tanks) for continuous heat stress. To reduce mortality at early blastula, the 10 °C incubation tanks were filled with filtered 8 °C seawater and gradually adjusted to the desired experimental temperature over the next 12 h. Oxygen was supplied to eggs via gently bubbling air from the base of each tank. Dead eggs were removed and the amount was measured, and water was exchanged (70%) on a daily basis. For both the 6 and the 10 °C groups, eggs were collected at fixed embryonic developmental stages and day degrees (DD; days postfertilization multiplied by incubation temperature; see Fig. 1 for the embryonic sampling strategy), for analysis of RNA (≈ 50 eggs) and antioxidant compounds or antioxidant enzyme activities (≈ 200

Table 1
Gene abbreviation, name, and product function for the target genes analyzed.

Abbr.	Name	Function	Mutant/knockout (mice)
nrf2	Nuclear factor erythroid 2-related factor 2	Transcription factor that translocates to the nucleus upon oxidative stress; binds to ARE in several antioxidant genes to increase cellular defense	$Nrf2^{-/-}$ vital, but has reduced tolerance to oxidative stress [29]
gpx1[a]	Glutathione peroxidase 1	Detoxifies H_2O_2; localized in the cytosol and in the mitochondria	$GPx1^{-/-}$ mice develop normally [30]
gpx3[a]	Glutathione peroxidase 3	Detoxifies H_2O_2; localized in the extracellular fluid, kidneys, and embryonic tissues; contains an ARE in the promoter region	$GPx3^{-/-}$ mice develop normally [31]
gpx4b[a]	Glutathione peroxidase 4b	Detoxifies phospholipid hydroperoxide; mainly localized in the membranes, different isoforms	$GPx4^{-/-}$ embryonic lethal [32]
glrx2	Glutaredoxin 2	Mitochondrial; receives e^- from both GSH, thioredoxins, and other proteins with disulfides	No *glrx2* or *glrx3* knockout, $glrx1^{-/-}$ has normal embryo development [33]
glrx3	Glutaredoxin 3	Cytosolic; function same as Glrx2; both key participants in redox signaling mediated by protein thiols and mixed disulfides	$glrx1^{-/-}$ has normal embryo development [33]
trx	Thioredoxin	Small antioxidant molecule; oxidized to reduce other proteins using the thiol group of cysteine; contains an ARE in the promoter region	$trx1^{-/-}$ and $trx2^{-/-}$ embryonic lethal [34]
trxred	Thioredoxin reductase	Catalyzes oxidized form of thioredoxins to produce the reduced form of thioredoxins	$trxred^{-/-}$ vital until gastrula stage [35]
g6pd	Glucose-6-phosphate dehydrogenase	The rate-limiting enzyme of the pentose phosphate pathway; provides NADPH (required as a reductant)	$g6pd^{-/-}$ lethal [36]
gclc	γ-Glutamylcysteine synthetase	Combines Glu and Cys as the first step to produce glutathione; the rate-limiting enzyme in GSH synthesis; contains an ARE in the promoter region	$gclc^{-/-}$ embryonic lethal [37]
gr	Glutathione reductase	Reduces GSSG to its reduced form GSH; NADPH dependent	Reduced-GR mice are sensitive to oxidative damage [44]
nox1	NADPH oxidase 1	Membrane-bound pro-oxidant enzyme; catalyzes superoxide production	Normal embryo development [39]
Mnsod[a]	Mn superoxide dismutase	Mitochondrial; transforms toxic superoxide into H_2O_2	$Mnsod^{-/-}$ embryonic lethal or has heart pathologies [40]
CuZnsod[a]	CuZn superoxide dismutase	Cytosolic; transforms toxic superoxide into H_2O_2	$CuZnsod^{-/-}$ normal development or has increased lethality [41]
cat[a]	Catalase	Detoxifies H_2O_2; mainly localized in the peroxisomes	Normal embryo development [42]
mt	Metallothionein	Cysteine-rich protein; binds heavy metals and oxygen radicals	Normal embryo development [43]

[a] Enzyme activities of gene product were measured, for GPx we measured total GPx activity, for results see Fig. 3.

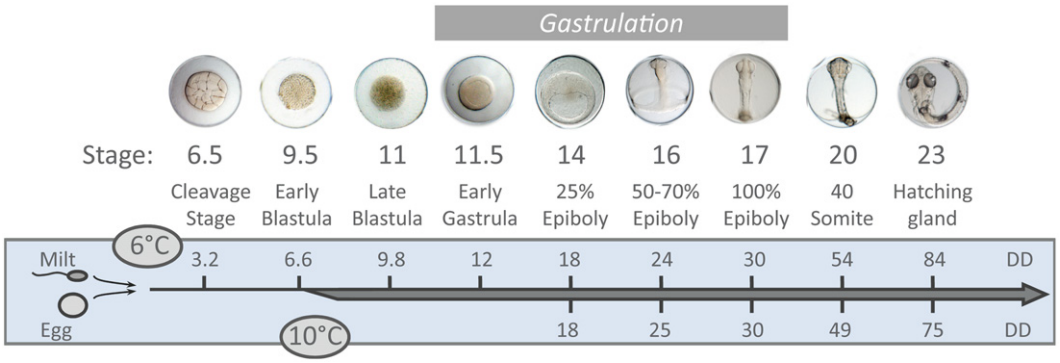

Fig. 1. Sampled stages of Atlantic cod (*Gadus morhua*) embryos staged after Hall et al. [45]. Development is given in day degrees postfertilization (DD) at 6 and 10 °C incubation temperature. Samples were collected to measure the low-molecular-weight redox compound glutathione (tGSH, GSSG), antioxidant enzyme activity (GPx, tSOD, MnSOD, CuZnSOD, catalase), and RNA extraction followed by quantitative real-time PCR analysis of *nrf2*, *gpx1*, *gpx3*, *gpx4b*, *glrx2*, *glrx3*, *trx*, *trxred*, *g6pd*, *gclc*, *gr*, *nox1*, *Mnsod*, *CuZnsod*, *cat,* and *mt*.

eggs per analysis). Staging of the samples was achieved using light microscopy (Olympus SZX12 connected to a Nikon digital sight DsFi1 camera) and described according to Hall et al. [45] (Fig. 1). For the sampled eggs, the surrounding seawater was removed using a glass pipette and samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

Enzyme and GSH/GSSG analysis

To assess the redox status of the developing cod embryos we measured the tGSH and GSSG and the activities of GPx, tSOD, MnSOD, CuZnSOD, and CAT (see Table 1, footnote).

To analyze tSOD and tGSH, frozen samples (105 ± 15 mg; $n \approx 100$ eggs) were placed in Eppendorf tubes (2 ml) with 800 µl ice-cold homogenization buffer (50 mM potassium phosphate, 0.95 mM EGTA, pH 7.2) and a metal ball and then homogenized (30 shakes per second for 1 min) in a ball mill (Retsch MM301 ball mill; Haan, Germany). The homogenate was then centrifuged (5 min, 1500 g, 4 °C). For tSOD, 100 µl of the supernatant was transferred to a new tube and 100 µl of ice-cold buffer (50 mM potassium phosphate, pH 7.2, 400 mM mannitol, 130 mM sucrose, 0.95 mM EGTA) was added. To measure tGSH, the remaining homogenate was centrifuged again (15 min, 10,000 g, 4 °C) and 100 µl of clear supernatant was transferred to a new tube with 300 µl of 5% (w/v) metaphosphoric acid. The tube was then shaken by hand and centrifuged again (10 min, 10,000 g, 4 °C) to remove protein, and the clear supernatant was transferred to a new tube for storage.

GSSG was measured by weighing frozen sample (50 ± 11 mg) into an Eppendorf tube and then immediately adding 50 µl ice-cold scavenger (*N*-ethylmaleimide pyridine derivative solution, Cat. No. GT40c; Oxford Biomedical Research, Oxford, UK) and 50 µl ice-cold saline (9 g L^{-1} NaCl in ddH_2O). Samples were homogenized as for tSOD and tGSH, and then 400 µl of 5% (w/v) metaphosphoric acid was added to each tube, and the tubes were shaken by hand and then centrifuged (10 min, 20,000 g, 4 °C). The clear top layer was transferred to a new tube for storage.

To analyze CAT and GPx enzymes samples were weighed (225 ± 91 mg) into tubes and a twofold dilution of ice-cold homogenization buffer (50 mM potassium phosphate, 0.5 mM EDTA, pH 7.2) was added. Samples were homogenized using a metal homogenization ball (30 shakes per second for 2 min) and centrifuged (15 min, 10,000 g, 4 °C), and the clear supernatant was extracted. For CAT analyses the supernatant was used without further modification. For GPx analysis, 66 µl of the supernatant was transferred to a new 1.5-ml Eppendorf tube with 33 µl of ice-cold buffer (50 mM potassium phosphate, pH 7.6, 10 mM EDTA, 3 mM dithiothreitol). For all enzymes the extracts were frozen on dry ice immediately upon preparation and stored at −80 °C for a maximum of 1 month before analysis.

The SOD, GPx, and CAT activities were analyzed with commercial kits (Items 706002 (SOD), 703102 (GPx), 707002 (CAT); Cayman Chemical Co., Ann Arbor, MI, USA). To measure the MnSOD activity, 2 mM potassium cyanide was added to tSOD extracts to inhibit CuZnSOD activity. The MnSOD measures were subtracted from the tSOD measures to attain the CuZnSOD measures. The GSH and GSSG were analyzed with a commercial kit (Product GT40; Oxford Biomedical Research). Total protein concentrations of supernatants (except GSSG) were measured with a Coomassie Brilliant Blue reagent kit (Bio-Rad Laboratories, Hercules, CA, USA). All samples were analyzed spectrometrically for absorbance in ELISA plates with a microplate reader (iEMS Reader Ms; Labsystems, Finland) measuring CuZnSOD/MnSOD at 450 nm, GPx at 340 nm, CAT at 531 nm, GSH/GSSG at 405 nm, and protein at 531 nm.

Reverse transcription–quantitative real-time PCR (qPCR)

The procedure for reverse transcription followed by qPCR and gene quantification during embryo development was performed as previously described [5]. Selected redox-related genes and gene product functions are given in Table 1. Gene accession numbers, forward and reverse primer sequences, amplicon sizes, and qPCR efficiencies are given in Table 2. A total RNA input concentration of 500 ± 5 ng was used for each reaction. We report the cycle threshold (C_T) values (i.e., relative to total RNA input of 500 ± 5 ng per reaction) from the cleavage stage (3.2 DD postfertilization) until the hatching gland stage (84 DD). The genes *ef1α*, *ubi*, and *tub2* were used for normalization of target genes as described previously [5]. Mean normalized expression (MNE) is given from 25% epiboly because of the increased zygotic gene activation of the reference genes at the midblastula transition. GeNorm was used to calculate the MNE [15] of the 16 target genes: *nrf2*, *gpx1*, *gpx3*, *gpx4b*, *glrx2*, *glrx3*, *trx*, *trxred*, *g6pd*, *gclc*, *gr*, *nox1*, *Mnsod*, *CuZnsod*, *cat*, and *mt* (Tables 1 and 2).

Statistical treatment

Statistica software (version 10, 2008; Statsoft, Inc., Tulsa, OK, USA,) was used for statistical analysis. For enzyme activities and mRNA levels of the selected redox genes the differences between developmental stages or temperature effects were analyzed by repeated-measures analysis of variance (rm ANOVA), followed by the Tukey HSD post hoc test. Differences between temperature

Table 2
Gene abbreviation, GenBank accession number, forward and reverse primer sequences, primer amplicon size. and qRT-PCR efficiency for target genes analyzed.

Abbr.	Accession No.	Forward primer	Reverse primer	Amplicon size	PCR efficiency
nrf2	GmE100127i38552	TCGCAGTAGGAGCTGGATGA	CTCCGGTCTGTCCTTGGAAA	87	1.94 ± 0.10
gpx1	EX725875	CCAAATATGGACGGCATAGGA	CAAACGCTACAGCCGGAACT	101	2.00 ± 0.05
gpx3	EX724801	CGTTCTCGGGTTTCCCTGTA	GCTCAAACAGCGGGAACGT	125	1.99 ± 0.05
gpx4	EX721840	CCCTGTGGAAGTGGCTGAAG	CATCCAAGGGTCCGTATCTCTT	129	1.90 ± 0.03
glrx2	EX741839	GTAGGATGGCCAAAAATGTGTTTA	GGCCCCAGTCATCTGAGCTA	116	1.85 ± 0.07
glrx3	EX727686	GATCTGAATGAGCGCCTGAAG	GAAGCTGCTGAACTGGATGCT	144	1.97 ± 0.03
trx	EX729450	ACCGCAACGTGGTCTTCCT	ATTCGCCCCAGCAAAGTTATC	134	1.92 ± 0.05
trxred	EG641174	AGACTCCAAACGGTCAGGAGGTA	GAGTTCACCGTGGCCAACA	126	2.02 ± 0.05
g6pd	EX743332	CCGGAAAACACGCACTTTGT	GAAGAAGGCCGACAGAGACTCA	123	1.88 ± 0.04
gclc	ES480178	CGAGAACGAGTCCGATCACTT	GTCAGTCAGCTGCACCTCCAT	133	2.01 ± 0.08
gr	EX728920	TCACGCTCACCACCAAGGA	GTGTGGAGGCCAGTCGTGTT	121	1.91 ± 0.01
nox1	EX727949	GCCTATATGATTGGCCTGATGAC	GCTGTGCTGAGTGGGTCGTA	107	1.99 ± 0.13
Mnsod	GE905819	ATGTGGCCTCCTCCATTGAA	GCATCACGCCACCTATGTCA	129	1.86 ± 0.03
CuZnsod	CO541611	CATGGCTTCCACGTCCATG	CGTTTCCCAGGTCTCCAACAT	133	1.92 ± 0.04
cat	DQ270487	GCCAAGTTGTTTGAGCACGTT	CTGGGATCACGCACCGTATC	101	2.02 ± 0.03
mt	CO542775	CCTTGCGACTGCACCAAGA	CAGTTTAGGCAGGTGCATGATG	62	1.96 ± 0.06

Annotations are as of June 2011, based on best hits against the NCBI nr protein database (BlastX).

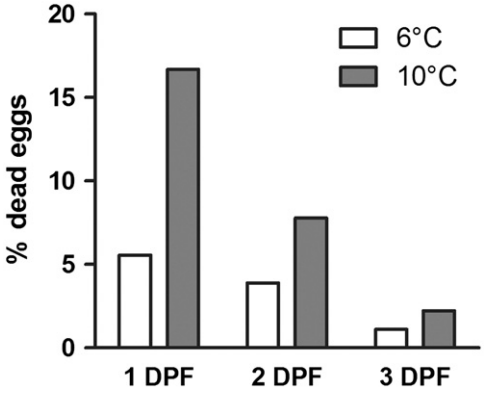

Fig. 2. Difference in Atlantic cod (*G. morhua*) embryo mortality expressed as percentage of dead eggs when incubated at 6 °C (control) and 10 °C (heat stress). Samples were collected at days postfertilization (DPF).

treatments at individual stages were also tested with *t* tests, as differences may be masked by high variation in single sampling points in rm ANOVA. Differences were considered significant at $p < 0.05$.

Results

Mortality

The transfer of early blastula stage embryos from 6 to 8 °C seawater followed by an increase in temperature to 10 °C over a 12-h period resulted in a threefold increase in dead eggs compared to the 6 °C control group (Fig. 2). At early gastrula and 25% epiboly the total amount of dead eggs in the 10 °C long-term heat exposure was twofold higher than in the 6 °C group. Thereafter the mortality was low in both temperature groups.

Glutathione amount and redox enzyme activities

The tGSH (Fig. 3A) was stable from the cleavage and early blastula stage until 100% epiboly. From 100% epiboly until the hatching gland stage, there was a large increase in tGSH in both groups ($p < 0.001$). Only at 100% epiboly was there a difference between the treatments, with tGSH being higher in the 10 °C group than in the 6 °C group ($p < 0.01$). The concentration of oxidized glutathione (Fig. 3B) at 6 °C was below the detection limit of the method until 100% epiboly, but then increased sharply until the 40-somite stage ($p < 0.001$), with no further change in GSSG until the hatching gland stage. The concentration of GSSG was stable during ontogeny at 10 °C and significantly lower at 10 °C compared to 6 °C at the 40-somite and hatching gland stages ($p < 0.01$).

The activity of CuZnSOD (Fig. 3C) in cod eggs was higher than the activity of MnSOD (Fig. 3D), throughout the embryonic period, and was responsible for the majority of the total SOD (Fig. 3E) activity. At 6 °C, there was a gradual increase in both CuZnSOD and tSOD from the cleavage stage until 100% epiboly ($p < 0.002$) and a decrease from 100% epiboly until the hatching gland stage ($p < 0.001$). At 10 °C, only tSOD had a significant increase ($p < 0.01$) during gastrulation, and the peak activity appeared one stage earlier, e.g., at 50–70% compared to 100% epiboly found in the 6 °C group. Both tSOD and CuZnSOD showed a sharp decrease in activity between 50–70 and 100% epiboly. tSOD activity was higher in the 10 °C versus the 6 °C group at 25% epiboly ($p < 0.05$), whereas at 100% epiboly both total and CuZnSOD activities were higher in the 6 °C versus the 10 °C group ($p < 0.01$). MnSOD activity did not show significant changes during ontogeny. However, the activity at 10 °C was higher than that at 6 °C in the late blastula and 25% epiboly stages ($p < 0.02$).

Glutathione peroxidase activity (Fig. 3F) was gradually reduced from the cleavage stage until early gastrula ($p < 0.05$), was stable until 50–70% epiboly, and then increased until 100% epiboly ($p < 0.001$) in the 6 °C group. Further, until the hatching gland stage, there was another significant reduction of GPx activity ($p < 0.01$). At 10 °C, the activity of GPx was stable from late blastula until the 40-somite stage and then it increased abruptly ($p < 0.01$). GPx activity was higher at 100% epiboly ($p < 0.001$) and lower at the hatching gland stage ($p < 0.05$) in the 6 °C versus the 10 °C group.

Catalase activity (Fig. 3G) did not change significantly during ontogeny at either temperature, but the activity was higher at 10 °C, compared to 6 °C at 100% epiboly ($p < 0.04$).

Ontogenetic levels of redox-associated mRNA

To clearly elaborate the dynamics of the embryonic mRNA levels, which encode redox-related functions, we report the C_T of poly(A)-mRNA levels relative to standardized 500 ± 5 ng RNA per reaction from cleavage stage (3.2 DD, Fig. 1) until hatching gland stage (84 DD) at optimum developmental temperature (6 °C control; Fig. 4). All quantified mRNA levels were highly dynamic

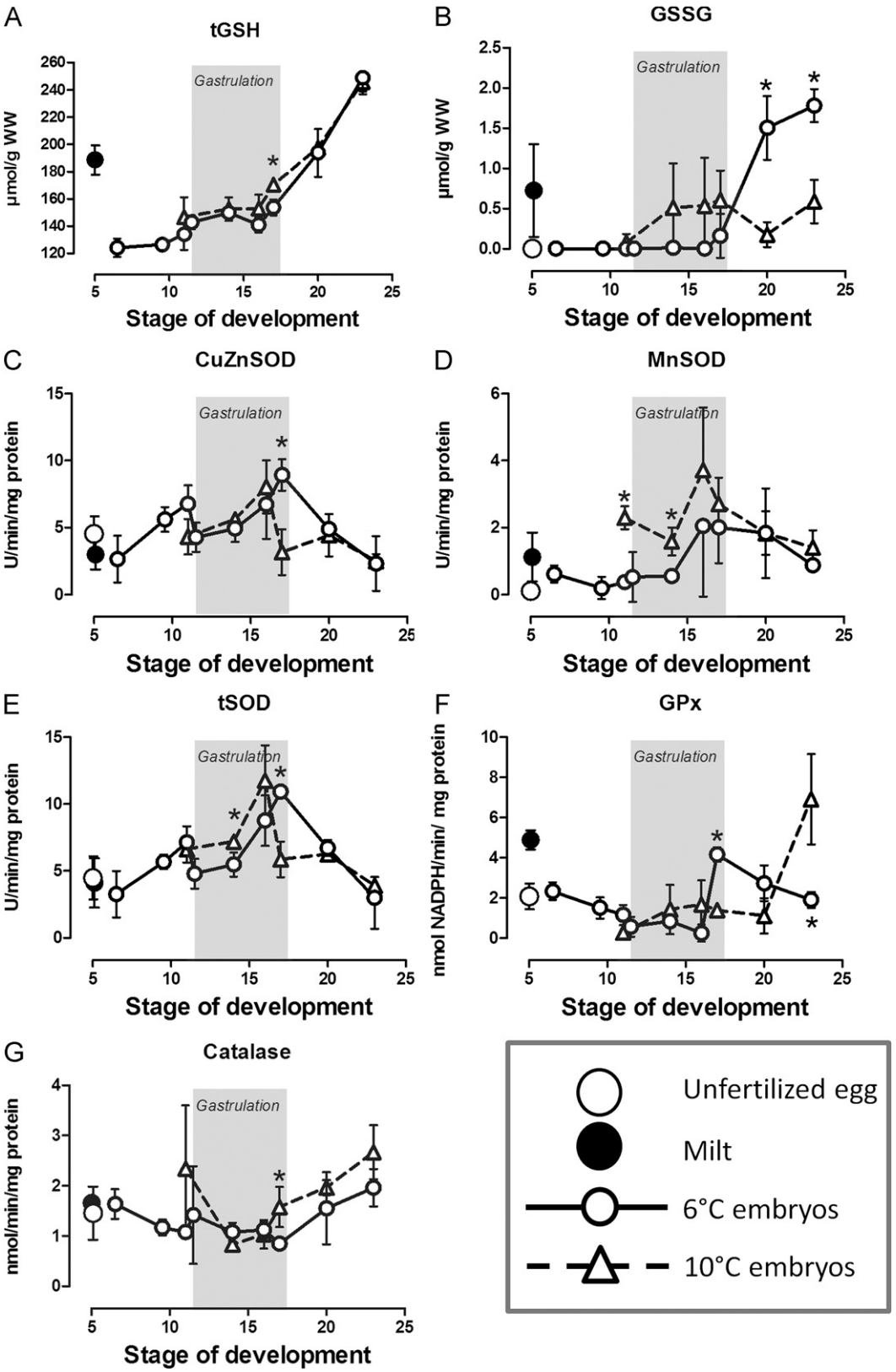

Fig. 3. Developmental increase in oxidized glutathione was observed in the control group but not in the heat-stressed group. Measures of (A) total glutathione (tGSH, µmol/g wet wt), (B) oxidized form of glutathione (GSSG, µmol/g wet wt), and the antioxidant enzyme activity of (C) glutathione peroxidase (GPx, nmol NADPH/min/mg protein), (D) total superoxide dismutase (tSOD, U/min/mg protein), (E) MnSOD (U/min/mg protein), (F) CuZnSOD (U/min/mg protein), and (G) catalase (nmol/min/mg protein) in Atlantic cod (*G. morhua*) milt and unfertilized eggs and during embryonic development at 6 °C (control) and 10 °C (heat stress) are shown. The amount of tGSH in unfertilized eggs was 49.6 ± 7.0 µmol/g wet wt. Development is given in stages (see Fig. 1) and the data points represent means ± SD of three independent groups.

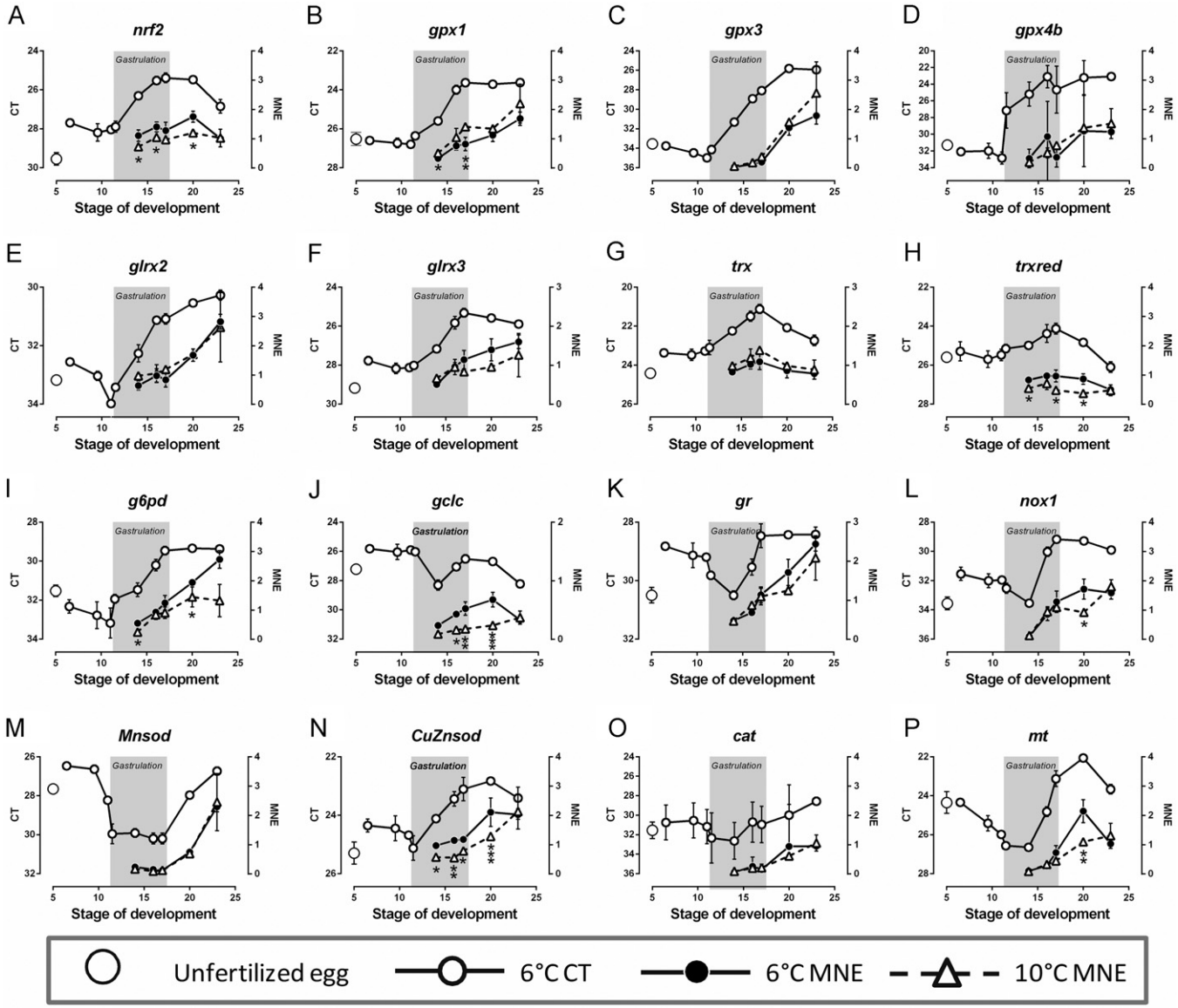

Fig. 4. mRNA levels presented as raw cycle threshold (CT, left *y* axis) and mean normalized expression (MNE, right *y* axis) determined by qPCR of (A) *nrf2*, (B) *gpx1*, (C) *gpx3*, (D) *gpx4b*, (E) *glrx2*, (F) *glrx3*, (G) *trx*, (H) *trxred*, (I) *g6pd*, (J) *gclc*, (K) *gr*, (L) *nox1*, (M) *Mnsod*, (N) *CuZnsod*, (O) *cat*, and (P) *mt* in Atlantic cod (*G. morhua*) unfertilized eggs and during normal embryonic development at 6 °C control temperature and during continuous thermal stress at 10 °C. The C_T values of each gene are relative to a total RNA input of 50 (± 0.5) ng/μl. MNE of target genes is normalized against *ef1α*, *ubi*, and *tub2* as reference genes and is given from stage 14 (25% epiboly) to stage 23 (hatching gland stage). Data points represent means $\pm$ SD of three independent groups of approximately 50 eggs.

in their mRNA profiles from fertilization until hatching gland stage (6 °C: $p < 0.001$).

The mRNA level for *nrf2* (Fig. 4A) was stable from the cleavage stage until early blastula, then increased until 50–70% epiboly ($p < 0.001$), was stable until the 40-somite stage, and then decreased to the hatching gland stage ($p < 0.001$). MNE of *nrf2* increased slightly between 25% epiboly and the 40-somite stage at both temperatures ($p < 0.05$) and then decreased to the hatching gland stage in the group held at 6 °C ($p < 0.01$), but was stable at 10 °C. MNE was significantly lower at 10°C than at 6°C ($p < 0.01$, rm ANOVA), with significant differences at 25% epiboly, 50–70% epiboly, and the 40-somite stage ($p < 0.05$, *t* tests).

According to the mRNA level, *gpx1*, *gpx3*, and *gpx4b* (Figs. 4B–D) all showed stable levels during the cleavage and blastula stages and a gradual increase in mRNA level during the gastrula stages ($p < 0.01$) and had stable levels after the gastrula stages. The increase in mRNA level started at late blastula for *gpx4b* and at early gastrula for *gpx1*. The increase lasted until 50–70% epiboly for both enzymes. For *gpx3*, the increase lasted from early gastrula until the 40-somite stage. The MNE of *gpx1* at 6 °C showed that mRNA level relative to the reference genes increased gradually between 25% epiboly and the hatching gland stage ($p < 0.001$). At 10 °C *gpx1* showed two periods of increase, one from 25% epiboly to 100% epiboly ($p < 0.05$) and one from the 40-somite stage until the hatching gland stage ($p < 0.05$). Generally there were higher mRNA levels at 10 °C compared to 6 °C ($p < 0.05$, rm ANOVA), but using *t* tests there were stage-specific significant differences only at 25 and 100% epiboly ($p < 0.05$). The MNE data for *gpx3* were stable during the gastrula stages and gradually increased from 100% epiboly to the hatching gland stage ($p < 0.001$, both temperatures combined). There was no effect of temperature on MNE of *gpx3*. MNE of *gpx4b* did not change during ontogeny at 6 °C, but increased significantly between 25% epiboly and the 40-somite stage ($p < 0.01$) at 10 °C. There was, however, no general effect of temperature or difference between temperature groups at single developmental stages in MNE of *gpx4b*.

Glutaredoxins (*glrx2 and glrx3*) (Figs. 4E, 4F) had mRNA level profiles similar to those of *gpx1* and *gpx4b*, with stable to slightly decreased levels during the cleavage and blastula stages ($p < 0.001$ and $p > 0.05$, respectively), an increase in mRNA levels during the gastrula stages until 50–70% epiboly ($p < 0.001$), and then a stabilization. The MNE of *glrx2* was not affected by temperature during the gastrula stages and showed an increase from 100% epiboly until the hatching gland stage ($p < 0.001$). *glrx3* had different MNE profiles at the two temperatures. At 6 °C, the mRNA level increased gradually from 25% epiboly until the hatching gland stage ($p < 0.001$), whereas at 10 °C there was no change in *glrx3* during development. Temperature generally affected the MNE of *glrx3* ($p < 0.05$, rm ANOVA); however, there were no significant differences at single developmental stages.

Thioredoxin (*trx*; Fig. 4G) mRNA level resembled those of the *gpx's* to some extent, with a stable level in the cleavage and blastula stages and an increase during the gastrula stages until 50–70% epiboly ($p < 0.001$). However, *trx* displayed a gradual decrease in mRNA level from 100% epiboly until the hatching gland stage ($p < 0.001$). The MNE of *trx* also increased from 25 to 100% epiboly ($p < 0.01$) and decreased from 100% epiboly until the hatching gland stage ($p < 0.001$). There was no general effect of temperature and no difference between temperature groups at single developmental stages on the MNE of *trx*.

The mRNA level of thioredoxin reductase (*trxred*; Fig. 4H) showed a stable level at the cleavage and blastula stages, an increase during the gastrula stages ($p < 0.01$), and a decrease from 100% epiboly until the hatching gland stage ($p < 0.001$ and $p < 0.05$ for 6 and 10 °C, respectively). The MNE of *trxred* at 6 °C was stable during gastrulation and decreased from 100% epiboly until the hatching gland stage ($p < 0.05$). At 10 °C there was no change in MNE during ontogeny. MNE was generally lower at 10°C compared to 6 °C ($p < 0.001$, rm ANOVA), with significant differences at 25 and 100% epiboly and at the 40-somite stage ($p < 0.05$, *t* tests).

The mRNA level for glucose-6-phosphate dehydrogenase (*g6pd*; Fig. 4I) was stable at the cleavage stage until late blastula, then increased from late blastula until 100% epiboly ($p < 0.001$), and then stabilized until the hatching gland stage. *g6dh* displayed a significant increase in level between late blastula and early gastrula at 6 °C ($p = 0.05$). MNE of *g6ph* at 6 °C increased significantly from 25 to 100% epiboly ($p = 0.02$) and then further from 100% epiboly until the hatching gland stage ($p < 0.001$). MNE at 10 °C did not change during development. The overall MNE was lower at 10 °C compared to 6 °C ($p < 0.001$, rm ANOVA) and was significantly lower at 25% epiboly and the hatching gland stage ($p < 0.05$, *t* tests).

The mRNA for *gclc* (Fig. 4J) showed a high and stable level from the cleavage stage until early gastrula. The level then decreased sharply until 25% epiboly ($p < 0.001$) and then increased again during the remaining gastrula stages ($p < 0.001$), whereas a decrease from the 40-somite stage until the hatching gland stage was also observed ($p < 0.001$). The MNE of *gclc* at 6 °C gradually increased from 25% epiboly until the 40-somite stage ($p < 0.01$) and then decreased until the hatching gland stage ($p < 0.01$). At 10 °C the MNE was stable except for a significantly higher level at the hatching gland stage than in the gastrula period ($p < 0.05$). The temperature effect was highly significant ($p < 0.001$, rm ANOVA), with the 6 °C group having higher overall level at all stages except the hatching gland stage ($p < 0.01$, *t* tests).

The mRNA level for *gr* (Fig. 4K) was stable during the cleavage and blastula stages, then showed a sharp decrease from late blastula until 25% epiboly ($p < 0.001$), followed by a sharp increase from 25 to 100% epiboly ($p < 0.001$), and then a stabilization until the hatching gland stage. MNE increased gradually from 25% epiboly until the hatching gland stage at both temperatures ($p < 0.001$) with no significant differences in expression at the different temperatures.

The mRNA level of *nox1* (Fig. 4J) was stable during the cleavage and blastula stages, but decreased from late blastula until 25% epiboly ($p < 0.05$) and then increased again during the remaining gastrula stage ($p < 0.001$). From 100% epiboly until the hatching gland stage, the level was stable. The MNE of *nox1* at 6 °C increased gradually from 25% epiboly until the 40-somite stage ($p < 0.001$) and stabilized from the 40-somite stage to the hatching gland stage. At 10 °C, there was an increase in MNE from 25 until 100% epiboly ($p < 0.001$), a stabilization until the 40-somite stage, and another increase until the hatching gland stage ($p < 0.001$). There was no general effect of temperature on the MNE of *nox1* ($p > 0.05$, rm ANOVA), but when using *t* tests, the mRNA level of *nox1* was higher at 6 °C than at 10 °C at the 40-somite stage ($p < 0.05$).

Mnsod and *CuZnsod* had very different mRNA levels during ontogeny (Figs. 4M and 4N, respectively). The mRNA profiles of both enzymes demonstrate relatively stable mRNA level during the cleavage and early blastula stages. *Mnsod* was degraded between early blastula and late blastula ($p < 0.001$), stable during the gastrula stage, and increased sharply between 100% epiboly and the hatching gland stage ($p < 0.001$). The MNE of *Mnsod* was stable during the gastrula stages but increased sharply from 100% epiboly until the hatching gland stage ($p < 0.001$). The *CuZnsod* mRNA level was stable during the cleavage and blastula stages, increased gradually during the gastrula stages ($p < 0.001$), and was stable between 100% epiboly and the hatching gland stage. MNE data show a stable mRNA level for *CuZnsod* during the gastrula stages and an increase in mRNA level between 100% epiboly and the 40-somite stage at 6 °C ($p < 0.01$) and between 100% epiboly and the hatching gland stage at 10 °C ($p < 0.01$). There was a significant general temperature effect ($p < 0.05$, rm ANOVA) and the mRNA level was lower at 10 °C than at 6 °C at all stages ($p < 0.05$, *t* tests) except at the hatching gland stage.

The C_T for *cat* (Fig. 4O) showed a low number of copies in general for this gene and the variation in the data was large. The MNE of *cat* showed an increase in mRNA level from 25% epiboly until the hatching gland stage, both temperatures combined ($p < 0.05$). There were no differences in mRNA level due to temperature.

The C_T for metallothionein (*mt*; Fig. 4P) showed a gradual decrease during the cleavage and blastula stages until early gastrula ($p < 0.001$), then a stabilization from early gastrula until 25% epiboly, with an increase through the rest of the gastrula stages until the 40-somite stage ($p < 0.001$), and then a decrease until the hatching gland stage ($p < 0.001$). The MNE of *mt* at 6 °C increased significantly from 25 to 100% epiboly ($p < 0.05$) and again until the 40-somite stage ($p < 0.001$). At 10 °C the increase was less pronounced and only statistically significant between 25% epiboly and the 40-somite stage ($p < 0.01$). The MNE of *mt* decreased at 6 °C ($p < 0.001$) and stabilized at 10 °C from the 40-somite until the hatching gland stage. There was no general effect of temperature on MNE of *mt*, but mRNA level was significantly lower at 10 °C versus the 6 °C group at the 40-somite stage when using *t* tests at single developmental stages ($p < 0.01$).

Discussion

The high mortality observed in the 10 °C continuous heat-stress group compared to the 6 °C control group demonstrates that 10 °C is above the optimal temperature range for cod embryos. The effect of the temperature stress on survival was most pronounced at the blastula stage but persisted through the 25% epiboly stage at 10 °C (2 days postfertilization). After 25% epiboly and onward the mortality was low in both groups.

We report that all quantified mRNA levels were highly dynamic during embryonic development with gene-specific unique mRNA level patterns indicating the importance of regulating redox genes during early development. Generally the redox system responded to the temperature stress by downregulation (lower MNE) of several mRNA levels and by changing antioxidant enzyme activities and the concentrations of GSH and GSSG. The *nrf2*, *trxred*, *g6pd*, *gclc*, *nox1*, *CuZnsod*, and *mt* genes showed lower MNE at specific stages during embryonic development of Atlantic cod and this is in accordance with a gene-specific reduced mRNA level at 10 °C, compared to 6 °C, even in common reference genes [5]. Only *gpx1* showed higher MNE at 10 °C, whereas the MNE of *gpx3*, *gpx4b*, *glrx2*, *trx*, *gr*, *Mnsod*, and *cat* were not affected by temperature. The reason that *gpx1* showed higher mRNA level at high temperature, opposite to the general trend for the other genes, is not known. In fish it has proven difficult to clearly separate between the *gpx1* and the *gpx2* genes [16]. *gpx2* is to date not identified in the teleost genomes; however, cytosolic *gpx* from fish typically show 78–87% similarity to mammalian *gpx2* [16].

The redox environment is important in cell signaling and the GSH/GSSG redox couple is considered the most important cellular redox buffer, present in most eukaryotic cells in millimolar concentrations. The relative and absolute concentrations of GSH and GSSG are strictly regulated [7–9,17]. The average redox potential of the cell (E) can be calculated using the Nernst equation: $E = E_0' - \mathrm{RT/nF}\ \ln([\mathrm{GSH}]^2/[\mathrm{GSSG}])$. E_0' is the standard potential of the redox couple at pH 7 and 25 °C [18]. Increasing concentrations of GSH in relation to GSSG result in an increasingly negative E, which approaches negative infinity when the GSSG concentration approaches 0. In this study, the GSSG concentration was below the detection limit of the method (corresponding to $E = -220$ mV) at 6 °C in the earliest embryonic stages until 50–70% epiboly. Therefore, the overall cellular environment seems to have been more reduced in the earliest versus the later stages of cod embryology. In the 10 °C group, and the 6 °C group from 100% epiboly, the GSSG concentration was detectable. Assuming an E_0' of −240 mV for the GSH/GSSG couple, the redox potentials of the embryos in this period were in the range of −220 to −190 mV, e.g., slightly more oxidized than the normal range of cell potentials (−260 to −200 mV) [8,18]. At the 40-somite and hatching gland stages, E was significantly higher in embryos held at 6 °C compared to 10 °C (−200 and −213 mV, respectively, $p < 0.01$, at the hatching gland stage). Furthermore, the total GSH concentration was significantly higher at 10 °C than at 6 °C at 100% epiboly, indicating a more reduced state in the 10 °C group at this stage. The redox potential is important in determining the activity of a range of signaling molecules, including enzyme systems, transcription factors, DNA methylation, and posttranscriptional control mechanisms of gene expression [1,7–9,18]. The magnitude of the cellular changes can be large, because a change in potential from −260 to −200 mV can give a 100-fold change in protein phosphorylation [8]. Therefore, the different redox states of embryos at the two temperatures may explain some of the differences in MNE and enzyme activities observed.

A reduced cellular environment occurs during growth stimulation (mitosis), whereas less reduced cell environments (more oxidized) stimulate cellular differentiation, and cell apoptosis occurs under the least reduced/most oxidized cellular conditions [1,8,18,19]. All three processes are integopment. The development of the redox potential of the embryo in this study appears to fit the pattern of ontogeny, a reduced cell environment during the cleavage and blastula stages dominated by proliferation and more oxidized conditions from late gastrulation when cellular differentiation and eventually organogenesis starts.

The mRNA levels of redox-associated genes were highly regulated and stage dependent during embryonic development. There are two major patterns of mRNA profiles during embryo development; the first is for genes that are strictly of maternal origin and are degraded during the cleavage and blastula stages, the second is for the zygotic genes, which increase their mRNA level during the period of zygotic gene activation [20], as previously described for Atlantic cod [5,21]. None of the redox-associated mRNA's were solely maternal, but some mRNA's were clearly maternally deposited and were degraded before gastrulation (*glrx2*, *Mnsod*) or during the first half of gastrulation (*gclc*, *gr*, *nox1*, *cat*, *mt*). Other mRNA's seem to have been maternally deposited, but were not degraded before the onset of zygotic mRNA production (*nrf2*, *gpx1*, *glrx3*, *trx*, *trxred*, *CuZnsod*). Both groups indicated increased mRNA levels during the zygotic gene activation.

The onset of the zygotic increase was mRNA specific and varied within the frame of gastrulation. Nrf2 acts as a transcription factor for several antioxidant genes and glutathione-metabolizing enzymes by binding to the ARE in the genes' promoter region [22]. The mRNA profile of *nrf2* was similar to those of *gpx1* and *gpx3*, *glrx2*, *glrx3*, *trx1*, and *trxred*, but different from the early zygotic increased mRNA levels of *gpx4b* and *g6pd*, starting between late blastula and early gastrula. It was also different from the late zygotic increased mRNA levels of the GSH supplementing enzymes *gclc* and *gr* and the superoxide anion producer *nox1*, starting after 25% epiboly. *Mnsod* had even later increased zygotic mRNA levels, starting after 100% epiboly. The ontogenic mRNA level for *Mnsod* found in this study was similar to that found in zebrafish [23].

Most of the genes analyzed in this study were upregulated compared to the reference genes during the gastrula stages, as shown by increasing MNEs in this period (*nrf2*, *gpx1*, *glrx3*, *trx*, *g6pd*, *gclc*, *gr*, *nox1*, *cat*, *mt*). Furthermore, many genes involved in the redox balance also showed increased mRNA levels, as judged from the MNE data, during the somite and hatching stages (*gpx1*, *gpx3*, *gpx4b*, *glrx2*, *glrx3*, *g6pd, gr*, *Mnsod*). For another set of mRNA's, increased mRNA levels occurred until the 40-somite stage and then either stabilized (*nrf2*, *gclc*, *nox1*, *CuZnsod*, and *mt*) or decreased until the hatching gland stage (*trx* and *trxred*). These findings clearly illustrate the importance of both anti- and pro-oxidative redox processes, which reflects essential stage-specific functions. One could speculate that the main trend observed, of increasing antioxidant mRNA levels and GSH concentration as the embryo approached hatching, may reflect the increased oxygen consumption and metabolic rate [3], combined with the preparation of the embryo for the higher oxygen tension present in the free-swimming larval stages [19].

There were low correlations between the mRNA levels and the enzyme activities of both MnSOD and CuZnSOD, except from the 100% epiboly stage for CuZnSOD at which both gene and enzyme were lower in the 10°C group. This was also the case with the mRNA levels of the different *gpx* mRNA's and the activity of the GPx enzyme. Taken together the study highlights that thermal stress changes the redox equilibrium of glutathione; however, a consistent link to the lower mRNA levels of both *nrf2*, which is a regulator of glutathione synthesis, and Nrf2 target genes, like the rate-limiting enzyme of glutathione synthesis (*gclc*), was not found. Interestingly, the lower mRNA level at 10°C compared to 6°C of both *nrf2* and *gclc* coincided with the lower mRNA level of genes related to cell growth and mitosis (*pcna*; unpublished data, K.H. Skjærven et al., 2012).

Data simulations predict the surface seawater temperature in the North Atlantic Ocean to become from 1.5 to 4 °C warmer by the end of the 21st century [6]. For the North Atlantic cod stocks, which are distributed over a wide area with temperatures ranging

from 1 to 10 °C [24], an anthropogenic temperature increase is expected to severely change the abundance of the cod stocks [25]. Thus, for stocks spawning below 5 °C the recruitment is expected to increase with increasing sea surface temperature, whereas for those spawning over 8 °C the recruitment will decrease with increasing temperature [25,26]. Several studies have implicated embryonic incubation temperature as an important determinant of subsequent muscle phenotypes and/or deformities within the spinal chord [27,28]. In this study we indicate a thermal effect on the GSH/GSSG redox couple and activities and mRNA levels of redox-related enzymes. As the embryo needs a tightly regulated redox environment to balance between growth and differentiation, these findings suggest that temperature changes might provoke an environmentally induced phenotypic change and eventually change the cod stock propagation.

Conclusion

Atlantic cod embryos were highly affected by an increase in temperature from 6 to 10 °C, as shown by increased mortality and changes in GSSG amount, antioxidant enzyme activities, and mRNA levels, and taken together this might be coupled to changes in the embryo redox potential, which became more reduced at the higher temperature. This indicates that a similar anthropogenic temperature increase in the ocean will challenge the embryo development by changing the delicate redox equilibrium. For both temperature groups, we observed that the GSH concentrations increased and the redox potential became more oxidized in late, compared to early, embryos, coinciding with the onset of organ differentiation. The study highlights that the redox regulation at normal development was highly dynamic and complex.

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