Research Paper

Diet affects the redox system in developing Atlantic cod (*Gadus morhua*) larvae

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A B S T R A C T

The growth and development of marine fish larvae fed copepods is superior to those fed rotifers, but the underlying molecular reasons for this are unclear. In the following study we compared the effects of such diets on redox regulation pathways during development of Atlantic cod (*Gadus morhua*) larvae. Cod larvae were fed a control diet of copepods or the typical rotifer/*Artemia* diet commonly used in commercial marine fish hatcheries, from first feeding until after metamorphosis. The oxidised and reduced glutathione levels, the redox potential, and the mRNA expression of 100 genes in redox system pathways were then compared between treatments during larval development. We found that rotifer/*Artemia*-fed cod larvae had lower levels of oxidised glutathione, a more reduced redox potential, and altered expression of approximately half of the redox system genes when compared to copepod-fed larvae. This rotifer/*Artemia* diet-induced differential regulation of the redox system was greatest during periods of suboptimal growth. Upregulation of the oxidative stress response transcription factor, *nrf2*, and NRF2 target genes in rotifer/*Artemia* fed larvae suggest this diet induced an NRF2-mediated oxidative stress response. Overall, the data demonstrate that nutritional intake plays a role in regulating the redox system in developing fish larvae. This may be a factor in dietary-induced differences observed in larval growth.

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Introduction

The growth and development of marine fish larvae is superior when fed zooplankton consisting of their natural diet of copepods rather than the rotifers fed in commercial hatcheries [1–4]. This difference appears to be linked to differences in nutritional composition between the two zooplankton types, with rotifers having low levels of several nutrients, such as zinc, taurine and/or protein, compared to copepods [3,5–9]. The lower levels of certain nutrients in rotifers are probably deficient to sustain the high growth rates and successful metamorphosis of larval fish evolutionary adapted to the elevated levels of nutrients typical of copepods in their natural habitat [10,11]. However, the underlying molecular reasons why the nutrient composition of copepods results in faster growth rates and improved development of fish larvae than that of rotifers remains unclear.

Growth and other developmental processes are a balance between cellular proliferation, differentiation and apoptosis at both temporal and spatial levels [12]. For instance during larval fish ontogeny, cells undergo differentiation during organogenesis, proliferation during growth, and apoptosis when larval type tissue is replaced by adult type tissue [13–15]. These shifts in cell fate are associated with changes in the cellular redox environment, which is generally less reductive (more oxidised) in apoptotic vs differentiating vs proliferating cells [16]. The cellular redox environment affects cell fate since gene expression, protein function and molecular pathways are often sensitive to the reduction potential [17,18]. For example, a more oxidised cellular environment favors the release of the nuclear factor-erythroid 2-related factor 2 (NRF2) transcription factor from a complex with another protein, KEAP1. Once released, NRF2 induces the transcription of at least 50 mammalian genes; many of which code for antioxidants, thiol oxidoreductases and glutathione synthesis/recycling genes; that are involved in maintaining the cellular redox balance and/or are involved in redox signalling (reviewed by [19]).

Cellular redox homeostasis is maintained by redox couples that

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act as electron buffers due to their ability to readily cycle between oxidised and reduced forms. The major cellular redox couples are reduced/oxidised glutathione (2GSH/GSSG), cysteine (2Cys/CySS) and thioredoxin (Trx(SH)$_2$/TrxSS) [20]. The ratios and levels of 2GSH/GSSG is the most important cellular redox couple, and a common measure used to assess the cellular redox environment [16]. The state of the redox environment is important for proper development in mammals [12]. In line with this, many genes coding for proteins that maintain the cellular redox system are dynamically regulated during fish embryonic and larval development [21–23]. Therefore we hypothesise that the nutritional composition of rotifers may differentially regulate the redox system in rotifer fed-fish larvae and subsequently contribute to their suboptimal growth and development in comparison to fish larvae fed copepods.

In the following study, Atlantic cod (*Gadus morhua*) larvae were fed either a standard commercial diet of rotifers or a natural zooplankton diet that consisted primarily of copepods [24] from first feeding. We measured the reduced/oxidised glutathione levels and the mRNA expression of 100 genes involved in redox regulation in whole fish during development. Atlantic cod was utilised as the model as it is an ecologically important apex predator and a key economic species in the fisheries of the North Atlantic region [25,26]. The redox system was focused on due to its importance to overall health. The 100 redox system genes investigated were chosen based on their importance within the redox system and/or because of their membership to gene families that are important within the redox system [12,16,19,21–23]. We found that rotifer-fed larvae had a more reduced cellular environment during development, and had elevated levels of mRNA coding for proteins in glutathione synthesis/recycling, methionine reductase and redox system transcription factor pathways compared to larvae fed copepods. In total, 46% of the genes analysed were differentially regulated in rotifer-fed larvae, and we suggest these changes reflect a rotifer induced NRF2-mediated oxidative stress response in cod larvae. Overall, the data demonstrate that nutrient intake plays a role in regulating the redox system in fish larvae, and suggests that nutrient induced changes in the redox system may contribute to the observed differences in larval fish growth and development.

Materials and methods

Cod larvae husbandry

The fish were maintained in accordance with the Norwegian Animal Welfare Act of 20th December 1974, No. 73, Sections 20–22, amended 19th June 2009. Atlantic cod eggs were incubated and larvae/juveniles reared as previously described [3]. Briefly, cod eggs were obtained from communal spawning of captive raised parents, and incubated in 70 L incubators described by van der Meeren and Lønøy [27], under standard conditions (gentle aeration, continuous lighting and water exchange, temperature between 5.8 and 6.1 °C, and 35 ppt salinity). At 4 days post-hatch (dph), 50,000 larvae were transferred to each of 6 black PEH 500 L start-feeding tanks (100 larvae/L) and reared under standard conditions (gentle aeration, 16L:8D photoperiod; continuous water exchange, temperature increased from 8 to 11.6 °C over the first 10 days of the experiment and then kept constant, 34.7 ± 0.2 ppt salinity, and algae paste (*Nannochloropsis* sp.) additions prior to feeding [3]).

Experimental diets

Cod larvae received either a standard commercial or standard wild type live feed as previously described [3]. Briefly, the standard commercial diet (referred to as the "rotifer" diet hereafter) consisted of enriched rotifers (*Brachionus* sp.) from 4 to 31 dph, enriched rotifers and enriched instar II *Artemia* (SepArt cysts, INVE Aquaculture, Dendermonde, Belgium) between 32 and 35 dph, and solely enriched instar II *Artemia* from 36 to 63 dph. The rotifer/*Artemia* enrichment protocols were designed to give the most nutritionally complete live feed based on current knowledge.

The standard wild type feed was a mix of natural marine zooplankton dominated by copepod species (referred to as the "copepod" diet). The zooplankton was collected from a saltwater pond system. Pond operation, hydrographical and biological monitoring, and copepod filtration system and harvest procedures are detailed in van der Meeren et al. [24]. Larval cod were fed zooplankton from 4 until 44 dph, to match the feeding period of the rotifer diet (4–63 dph) based on development, e.g. both feeding regimes were used in stages 0–4. The size fractions of zooplankton fed to cod larvae increased with age, starting with copepod nauplii and including more copepodid stages in older larvae. The live feed diets were fed to cod larvae three times per day (09:45, 15:15 and 19:00).

Following the live feed period, larvae from both treatments were weaned onto a formulated diet (AgloNorse®, Tromsø Fiskeindustri AS, Tromsø, Norway). Weaning occurred when the larvae had reached the same sizes and corresponded to 58–63 dph in the rotifer treatment, and 36–44 dph in the copepod treatment. During these weaning periods, live feed were fed to larvae once a day at 15:00. After the weaning periods, both treatments were maintained on formulated diets for the remainder of the trial.

Sampling procedure

Whole larvae were sampled 2 h after the morning feeding as previously described [3]. Due to the anticipated, and actual large differences in larval growth between treatments, larvae were sampled at specific developmental stages, based on standard lengths as previously described [3]. Briefly, the average standard length of fish at each developmental stage were; *stage 0*: 4.5 mm, *stage 1*: 5.2 mm, *stage 2*: 7.0 mm, *stage 3*: 9.3 mm, *stage 4*: 13.9 mm, *stage 5*: 25.1 mm. The ages (dph) of the fish sampled in each treatment (rotifer/copepod) relative to developmental stage were; *stage 0*: 4/4, *stage 1*: 11/11, *stage 2*: 22/22, *stage 3*: 31/29, *stage 4*: 54/37, *stage 5*: 71/53. In relation to feed type, in the rotifer treatment only rotifers were fed to cod larvae between stages 0 and 3. Except for an initial short period of co-feeding with rotifers, *Artemia* were fed between stages 3 and 4. In the copepod treatment, copepods were fed between stages 0 and 4. Except for an initial short co-feeding period with live feed, formulated diet was fed from stage 4 in both treatments [3].

RNA extraction and RNA sequencing

From cod larvae samples stored in RNA later (Ambion, Austin, Texas, USA) total RNA was extracted using Qiagen E21 universal tissue kits for stage 0, RNeasy microkits for stages 1–3 and mini kits for stages 4 and 5 (QIAGEN, Valencia, CA, US) according to procedures provided by the manufacturer. The amount of RNA was quantified using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and quality checked using a 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA). RNA was extracted from a pooled sample of larvae at stage 0, while equal amounts of total RNA from five individuals from each biological triplicate in stages 1–5 were pooled for sequencing. cDNA sequencing libraries preparation and sequencing were performed by the Norwegian Sequence Centre (Oslo, Norway) using Illumina TruSeq RNA Sample Preparation Kits. Using the multiplexing

strategy of the TruSeq protocol six paired end libraries were performed per one lane of the Illumina HiSeq 2000. The RNASeq data were mapped to the annotated cod genome assembly [28] ATL-COD1C (http://codgenome.no/data/ATLCOD1C/) using the Burrows–Wheeler aligner [29]. Reads not mapping with at least 90% identity, or not mapping to exons, or with both pairs mapping on the same strand were removed. On average, 47 million 100 bp paired end reads were mapped for each group. The reads were normalised by the total number of mapped sequences. The raw data have been deposited and can be found at The Sequence Read Archive (SRA) at NCBI (Accession ID: SRP056073).

GSSG/2GSH concentration analysis

For the analysis of total (tGSH) and oxidised (GSSG) glutathione, supernatants were prepared from samples using a commercial kit (Prod. No. GT40, Oxford Biomedical Research, Oxford, UK) and then analysed for absorbance at 405 nm in a microplate reader (iEMS Reader Ms, Labsystems, Finland) as previously described [21].

Calculations

The two electron half-cell reduction potential of the 2GSH/GSSG redox couple was calculated according to the Nernst equation:

$$E_h = E_0' - RT/nF \ln([GSH]^2/[GSSG])$$

where the GSH and GSSG concentrations are in M and E_h is given in volts. E_0' is the standard reduction potential at pH 7 and 25 °C and was assumed to be -0.240 V. The measurements are the average of whole larvae and do not take into account that the reduction potential varies between organs, and between organelles within the cells [16,30,31].

Statistics

Data were analysed with Statistica (Statsoft, Tulsa, OK, USA, Ver. 12) using factorial ANOVA (two factors; feed type and developmental stage) followed by Tukey's HSD post hoc test. Equality of variances were analysed using Levene's test, and data failing this test were Box–Cox transformed [32] to reduce heteroscedasticity. Data are presented as the mean $\pm$ SEM, $n=3$, with the exception of GSH data which were $n=4$ of analytical replicates at development stage 0, and $n=6$ (biological replicates) for developmental stages 1 and 2. RNA-Seq data are presented as normalised reads, and this analysis was not performed at development stage 0. Differences among means were considered significant when $p < 0.05$.

Results

Growth and survival

These results are given by Karlsen et al. [3]. Briefly, the growth was similar in rotifer- and copepod-fed larvae in stages 0–2. During stage 2–4, the daily length increase in copepod-fed larvae was almost 2 fold that of the rotifer-fed larvae, while after the larvae were weaned onto the same formulated diet at stage 4, the growth rates were again similar. There were no differences in survival between the groups.

Glutathione levels

The reduced (GSH) and oxidised (GSSG) glutathione levels were measured in developing cod larvae fed either rotifers or copepods. There were no differences in the levels of GSH between treatments
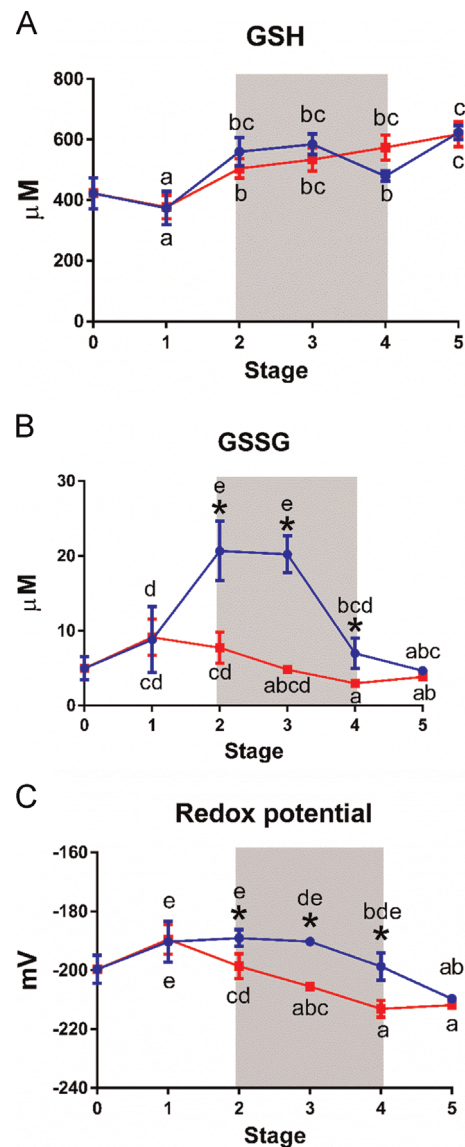

Fig. 1. The effect of diet on the redox environment in cod larvae. The reduced (GSH; graph A) and oxidised (GSSG; graph B) glutathione levels (μM), and the 2GSH/GSSG reduction potential (E_{GSH}, mV; graph C) in developing cod larvae fed either rotifers ($\square$ red line) or copepods ($\circ$ blue line). Cod larvae were sampled based on life stages, starting with first-feeding (Stage 0, 4 dph; 4.5 mm length), through metamorphosis (Stage 4, between 37 and 54 dph; $\approx$ 14 mm length) until the early juvenile phase (stage 5, between 53 and 71 dph; $\approx$ 25 mm length). Shaded areas indicate development stages where copepod-fed larvae had higher growth rates than those fed rotifers [3]. Letters indicate statistical relationships between data across both stage and treatment, while * is used to highlight statistical differences occurring at specific developmental stages between treatments (factorial ANOVA, $p < 0.05$). Figures with p values indicate statistically significant main effects of diet where no factorial effects at specific stages were identified (main effect ANOVA, $p < 0.05$). Data were not compared against stage 0, which represents the mean $\pm$ SEM of analytical quadruplicates. All other data are mean $\pm$ SEM $n=3$ of biological replicates.

during development (Fig. 1A). In contrast, GSSG levels were 3, 4 and 2 fold lower, and the redox potentials were 5%, 8% and 7% lower, at stages 2, 3 and 4 respectively, in rotifer compared to copepod-fed cod larvae (Fig. 1B and C). The differences in GSSG observed between treatments were due to an increase in GSSG levels in copepod-fed cod larvae at stage 2 ($p < 0.05$) that was absent in cod larvae fed rotifers (Fig. 1B). In relation to development, this GSSG increase resulted in a constant redox potential from stages 1 to 4 in copepod-fed larvae, compared to the decreasing (more reduced) redox potential observed between stages

1 and 4 in rotifer-fed cod larvae ($p < 0.05$) (Fig. 1C).

Gene expression

A total of 100 genes involved in the redox system, covering antioxidant, thiol oxidoreductases, heat shock proteins, glutathione synthesis/recycling, methionine sulfoxide reductase and redox response transcription families were analysed for expression levels in developing cod larvae fed either rotifers or copepods (See supplementary material for full list of genes analysed). The response of these genes to diet type were compared as a main effect which included grouped data from all of the five stages from first feeding until after metamorphosis (4.5–25 mm in length) and as a factorial effect where the effect of feed type on gene expression levels were assessed at each developmental stage. Results are presented with focus on the difference in gene expression between treatments, but changes in gene expression with development are considered briefly.

Overall, the expression of 46% (46 out of the 100) of the analysed genes was affected by the diets. Around half (56%) of the affected genes were upregulated in rotifer compared to copepod-fed cod larvae (Fig. 2A). The majority (88%) of the differential gene regulation between treatments occurred during developmental stages 2–4, and very few genes were differentially regulated at stages 1 (5%) or 5 (7%) (Fig. 2B).

All the analysed genes coding for proteins in the GSH synthesis/recycling pathway; *gclc*, *gclm*, *gss*, *gsr*, *g6pda* and *g6pdb*; were upregulated ($p < 0.05$) in rotifer compared to copepod-fed cod larvae at one or more development stages (Fig. 3 and Table 1). In relation to key developmental stages, rotifer-fed cod larvae had stable, while copepod-fed cod larvae had decreasing, expression of *gclm* and *gss* (development stages 2–4; Fig. 3B and C). Both treatments had decreased expression of *gclc* between stages 2 and 4 (Fig. 3A, $p < 0.05$), while development stage did not affect *g6pd* or *gsr* gene expression ($p > 0.05$, Fig. 3E F).

In line with the large number of redox system genes affected by the dietary treatments, key regulators of redox system gene expression, the transcription factors *keap1a*, *keap1b* and *nrf2*, were upregulated ($p < 0.01$) in cod larvae fed rotifers compared to those fed copepods (Fig. 4). These three genes represent around 40% (3 out of 7) of the analysed redox system gene regulators (Table 1). In relation to development stage, copepod-fed cod larvae had stable expressions of these genes from stage 2 to 4; while in the same period rotifer-fed cod larvae had increased ($p < 0.05$) *nrf2* expression (Fig. 4).

Around half (53%, Table 1) of the analysed genes from antioxidant pathways were affected by the treatments, but results were mixed in terms of regulation direction, even for closely related genes (Fig. 5). For example, in the glutathione peroxidase family both *gpx1* and *gpx4b* were up-regulated, while *gpx7* was down-regulated in rotifer compared to copepod-fed cod larvae (Fig. 5B, C and E); similar contrasts were observed within the peroxiredoxin family (F–H). Much of the diet induced differential regulation of the antioxidant coding genes occurred in the early development stages (i.e. stage 1, *sepp1*; stage 2, *cat*, *gpx4b*, 7, *prdx2* and 3, Fig. 5). However several genes, such as *gpx3* and *prdx3* were differentially regulated at later stages, and thus overall the antioxidant gene family was both an early and persistent responder to dietary differences (Fig. 5). Diet also affected which antioxidant genes were most dynamically expressed during development. For example, up then down-regulation (or vice versa) of gene expression during development occurred for *cat*, *gpx1a*, *prdx2* in copepod-fed larvae, but for *gpx4b* and *sepp1a* in rotifer-fed larvae (Fig. 5).

Over half (57%, Table 1) of the analysed genes coding for proteins that reduce oxidised cysteine residues (thiol oxidoreductases), or interact directly with these enzymes (thioredoxin interacting proteins; *txnip*'s) were affected by the diets (Fig. 6). Of these, the *txnrd*'s (1 and 3), *txnipa* and *srxn1* were upregulated, while *glrx*, *txnl1*, *txnl4* and *txnipb* were downregulated at either a single development stage or as a main effect in rotifer-fed larvae (Fig. 6). The largest number of differences induced by the diets was observed at development stage 3 (3 out of 5 factorial effects, Fig. 6). Only *txnl1* in copepod-fed larvae (Fig. 6B) was dynamically expressed (up then down regulated) during development. With this exception, any changes in gene regulation that occurred in either treatment with development generally persisted until the last stage analysed. For example *txnrd3* was upregulated at stage 2 in rotifer fed larvae ($p < 0.05$), from where it remained upregulated until stage 5 (Fig. 6G). The expression of several genes; *txnrd1*, *srxn1* did not change due to development ($p > 0.05$) in either diet group (Fig. 6F and H), or changed late in development, for example *txnipa* and *b* in copepod-fed fish, and *glrx* for both diets (A, D and E).

Around one third (36%, Table 1) of the genes coding for proteins in the heat shock protein family were affected by the diets (Fig. 7). Like the antioxidant genes, there was no clear direction of regulation, with around half the genes up-regulated and the other half down-regulated in response to the rotifer diet (Fig. 7). Diets induced differential regulation of heat shock protein genes only within the stages with large growth differences between treatments, with four, seven and four genes differentially regulated due
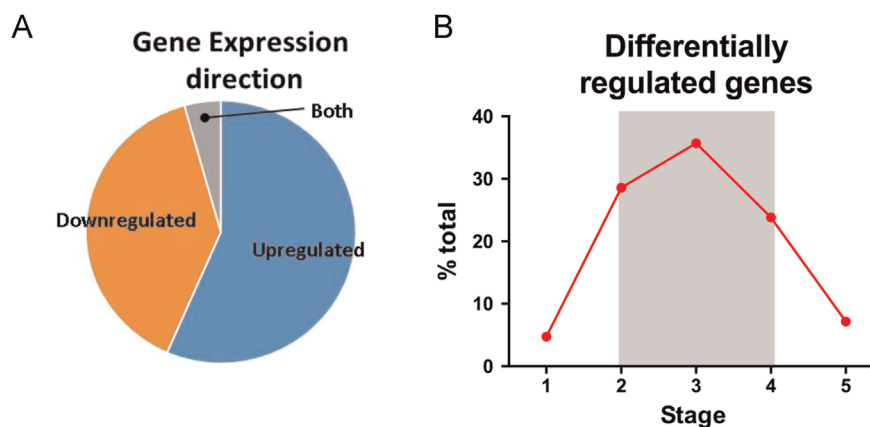

Fig. 2. The effect of diet on the redox system transcriptome of cod larvae. The main effect of the rotifer diet (up or down-regulation in comparison to those fed copepods) on the affected redox system related genes (% of total affected genes, graph A), and the number of redox system genes differentially regulated between treatments at each larval development stage (% of total number, graph B). Further information on stages and shaded areas can be found in the legend of Fig. 1.
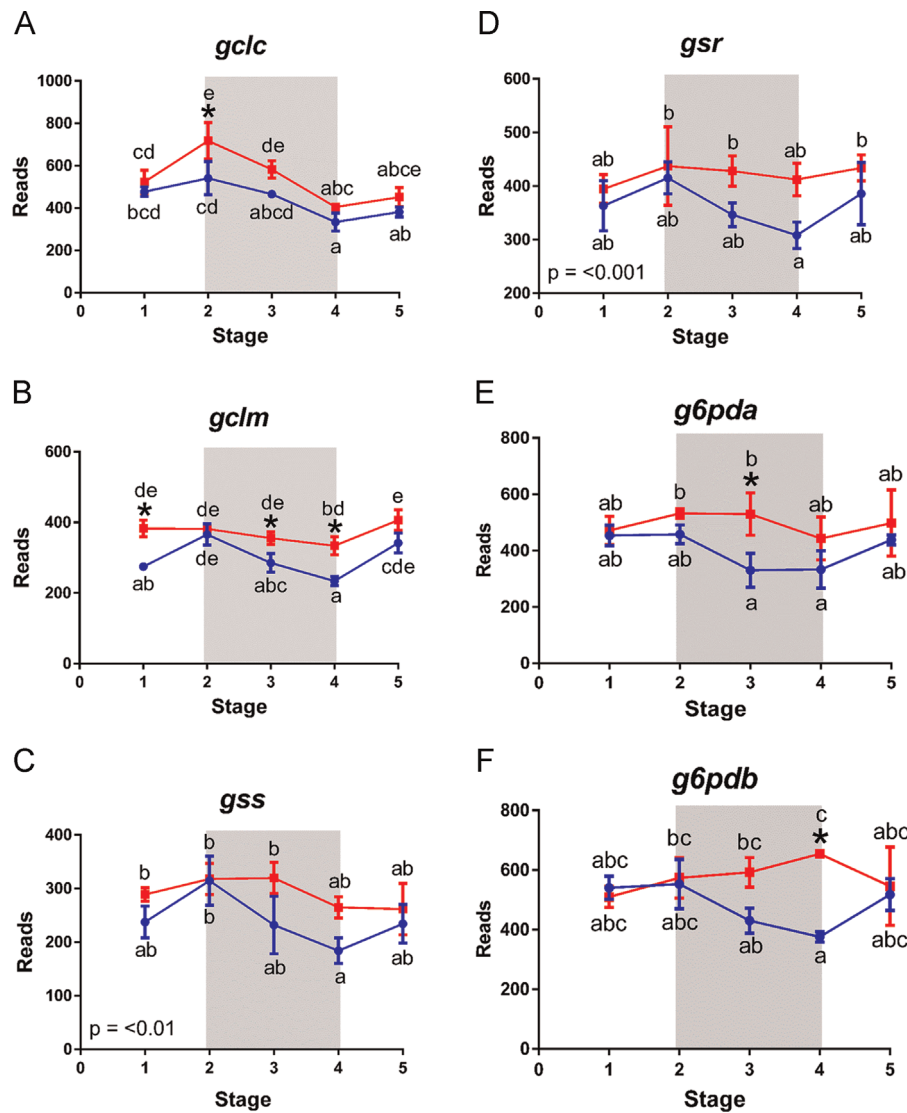
Fig. 3. The effect of diet on gene expression in glutathione synthesis/recycling pathways in cod larvae. The mRNA expression of *glutamate–cysteine ligase catalytic subunit* (A) and *modifier subunit* (B), *glutathione synthetase* (C), *glutathione reductase* (D), *glucose-6-phosphate dehydrogenase a* (E) and *b* (F) genes in developing cod larvae fed either rotifers (□ red line) or copepods (○ blue line). Shaded areas cover life stages that copepod-fed larvae had elevated growth rates compared to those fed rotifers. Letters indicate statistical relationships between all data points, with * and *p* values indicating statistical factorial and main effects, respectively, of diet ($p < 0.05$). See Fig. 1 for more details. Data are mean $\pm$ SEM, $n = 3$.

to diet at stages 2, 3 and 4, respectively (Fig. 7). The heat shock protein also contained members that had the largest fold change within the redox system genes. For example the rotifer diet inducing a 14–15 fold higher expression level of *hsp70la* and *b* than the copepod diet at stage 4 (Fig. 7E and F). In general, many heat shock family genes were highly expressed, for example *hspa5*,

hsp90a.1 and *hsp90ab1* all obtained expression levels of > 10,000 reads during development in one or both treatments (Fig. 7C, G and H). In reference to development, in contrast to the rotifer diet, the copepod diet induced the upregulation of heat shock protein family members earlier in development (for example at stage 2, *hsp90b1a*, *hsp90b1b* and *hsp70a*; stage 3, *hspa14*, *hsp90a.1*, *asha1b*,

Table 1
A summary of the changes in expression of genes involved in redox system regulation in rotifer compared to copepod-fed Atlantic cod larvae.

Gene family	% Affected	Upregulated	Downregulated	Gene family regulation direction
Antioxidant	53 (10/19)	gpx1a, gpx4b, prdx3*, prdx5, cat	sepp1a, gpx3, gpx7, prdx2, prdx3*, sod2	↑↓
Thiol oxidoreductase	50 (7/14)	txnrd1, txnrd3, txnipa, srxn1	txnl1, txnl4, txnipb, glrx	↑↓
Heat shock protein	36 (17/47)	hsp70a*, b, hsp70la, b, hsp90ab1, ser-pinh1a, stub1, hrsp12	hsp70a*, hspa5, 14, hsp90a.1, hsp90b1a, b, hspb1, 8, serpinh1b, ahsa1b	↑↓
GSH synthesis/recycling	100 (6/6)	gclc, gclm, gss, gsr, g6pda, g6pdb		↑
Methionine sulfoxide reductase	40 (2/5)	msrb1a, msrb2		↑
Redox system transcription factors	43 (3/7)	nrf2, keap1a, keap1b		↑

* Genes that were both up and down regulated during development in rotifer compared to copepod fed cod larvae.
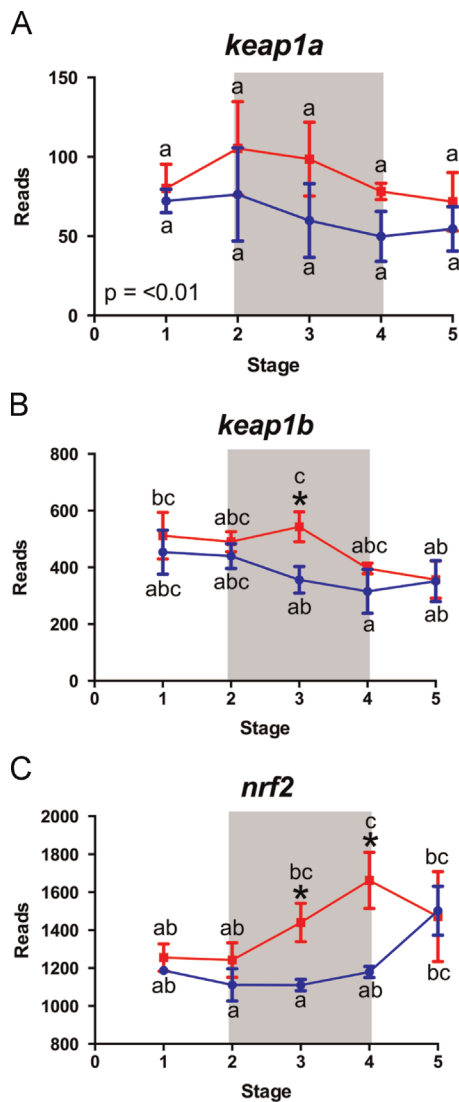
Fig. 4. The effect of diet on gene expression of redox system transcription factors in cod larvae.The mRNA expression of *kelch-like ECH-associated protein 1a* (A), *1b* (B) and *nuclear factor, erythroid 2-like 2* (C) genes in developing cod larvae fed either rotifers (□ red line) or copepods (○ blue line). Shaded areas cover life stages that copepod-fed larvae had elevated growth rates compared to rotifer-fed larvae. Letters indicate statistical relationships between all data points, with * and *p* values indicating statistical factorial and main effects, respectively, of diet ($p < 0.05$). See Fig. 1 for more details. Data are mean ± SEM, $n = 3$.

hspb1 and *hspa5* (Fig. 7)).

The rotifer diet increased the expression of 2 out of 5 genes (40%, Table 1) coding for proteins that reduce oxidised methionine (methionine sulfoxide reductases); *msrb1a* and *msrb2* (Fig. 8). In relation to development stage, *msrb1a* had stable expression, while *msrb2* was upregulated in copepod-fed larvae at stage 5 compared to developmental stages 2–4 (Fig. 8).

Discussion

Copepods are the natural diet of cod larvae [33], and result in superior larval growth and development compared to when rotifers are fed [1,3]. Thus, in the current study we hypothesise that the redox regulation in copepod-fed larvae during development is an optimal state, and that differential regulation from this optimum induced by the rotifer diet represents dysregulation of this system. Overall, we found that the expression of 46% of the analysed redox system related genes, the GSSG concentrations and the redox potential were

differentially, and thus may be dys-regulated in rotifer-fed cod larvae. This demonstrates that nutritional intake plays a major role in regulating the redox system in developing fish larvae.

A rotifer diet disrupts glutathione homeostasis and redox potentials in cod larvae

The differential regulation of the glutathione homeostasis pathways, and subsequently the redox potential in rotifer-fed larvae was a prominent finding in this study. Glutathione is the main cellular redox buffer because of its high cellular concentrations (1–10 mM) and reactive thiol group. Among others, enzyme mediated processes oxidise two GSH to GSSG to supply reducing equivalents to maintain many cellular components in a reduced state (reviewed by [34]). Rotifer-fed larvae had increased mRNA expression of glutathione reductase (*gsr*) and glucose-6-phosphate dehydrogenase (*g6pd*), lower levels of GSSG and a more reduced redox potential, but similar levels of GSH, as larvae fed copepods. The GSR enzyme regenerates GSH by reducing GSSG, using NADPH as the electron source. In turn G6PD is the rate-limiting enzyme in the pentose phosphate pathway for NADPH regeneration (reviewed by [35]), linking this enzyme to the GSH recycling pathway. As in the current study, GSSG levels remained constant and the redox potential became more reduced during the early developmental stages (from 7 to 14 dph) in rotifer-fed larvae in our previous study [21]. Thus the upregulation of the glutathione recycling pathway appears to be a common event associated with the differential regulation of the cellular redox environment in cod larvae fed rotifers.

The implications of this differential regulation are unclear, as the more reduced cellular environment found from developmental stages 2–4 in rotifer-fed larvae (from 7 to 15 mm in length) is thought to favor cellular proliferation, and hence growth [16]. However, opposite to this, the more reduced cellular environment found in rotifer-fed larvae occurred at the same developmental stage that growth rates failed to accelerate in line with copepod-fed larvae [3]. The expression of many redox system genes, including those for proteins from antioxidant, heat shock protein and thiol oxidoreductase families, mirrored changes in GSSG levels in copepod-fed larvae. For example, the GSSG spike at stage two in copepod-fed larvae was accompanied by spikes ($p < 0.05$) in *prdx2*, *prdx3*, *gpx1a*, *gpx7* (antioxidant), *txnl1* (thiol oxidoreductase), *hsp70*, *hsp90b1a*, *hsp90b1b*, and *serpinh1b* (heat shock protein) expression. Whether the GSSG spike precedes, occurs simultaneously, or occurs after the changes in expression of some or all of the responsive redox system genes is unclear. The GSSG spike is likely to be a product of the total balance of enzymatic reactions that are involved in catalysing the production and removal of GSSG [36], and thus may highlight underlying deficiencies in the enzymes that produce GSSG, such as GPX, in rotifer-fed larvae. Perhaps this spike is important for the regulatory role GSSG plays as a substrate for protein glutathionylation, a post-translational modification of exposed cysteine residues that affects protein function [37].

As we assayed whole fish, it is unknown to what degree rotifer-induced differences in gene expression, GSSG or redox potential represent changes occurring over the whole body, or in specific cell types. Increasing evidence suggests the subcellular partitioning of GSH and GSSG levels are of central importance for redox environment homeostasis specifically within organelles and the cytosol [30].

Overall, it appears that the maintenance of oxidative conditions in cod larvae from first feeding (stage 0) up until metamorphosis (stage 4) is a normal developmental process that may prepare and then maintain the larval system for the normal exponential growth phase (stages 2–4). After stage 4, the redox potential decreased (became more reduced) in copepod-fed larvae to similar levels as in rotifer-fed larvae by stage 5. This could be a normal developmental process, at least in aquaculture, associated with the
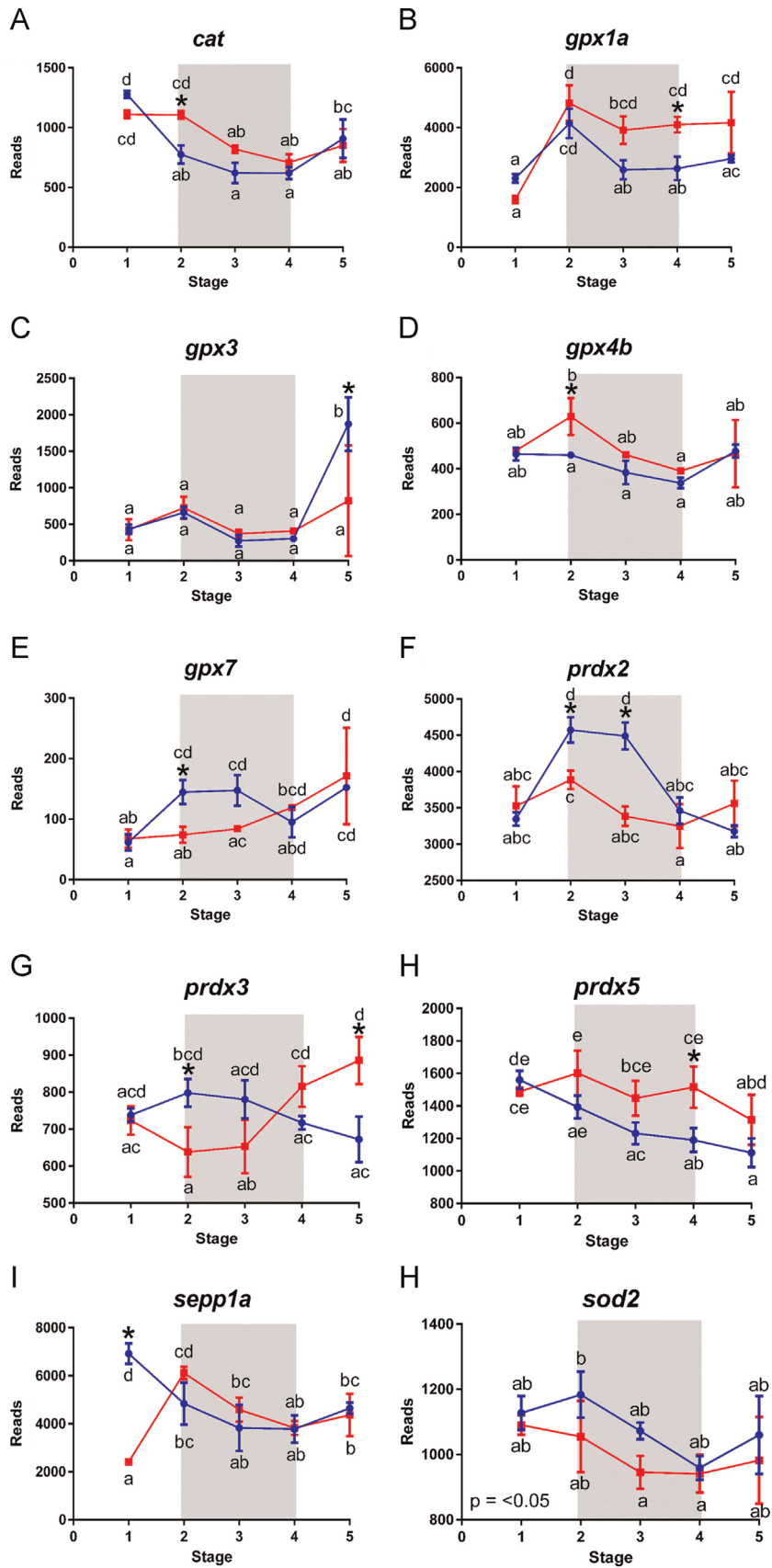
Fig. 5. The effect of diet on the expression of antioxidant coding genes in cod larvae. The mRNA expression of *catalase* (A), *glutathione peroxidase 1a* (B), *3* (C), *4b* (D), *7* (E), *peroxiredoxin 2* (F), *3* (G), *5* (H), *selenoprotein p1a* (I) and *superoxide dismutase 2* (J) in developing cod larvae fed either rotifers (□ red line) or copepods (○ blue line). Shaded areas cover life stages that copepod-fed larvae had elevated growth rates compared to rotifer-fed larvae. Letters indicate statistical relationships between all data points, with * and p values indicating statistical factorial and main effects, respectively, of diet ($p < 0.05$). See Fig. 1 for more details. Data are mean ± SEM, $n = 3$.
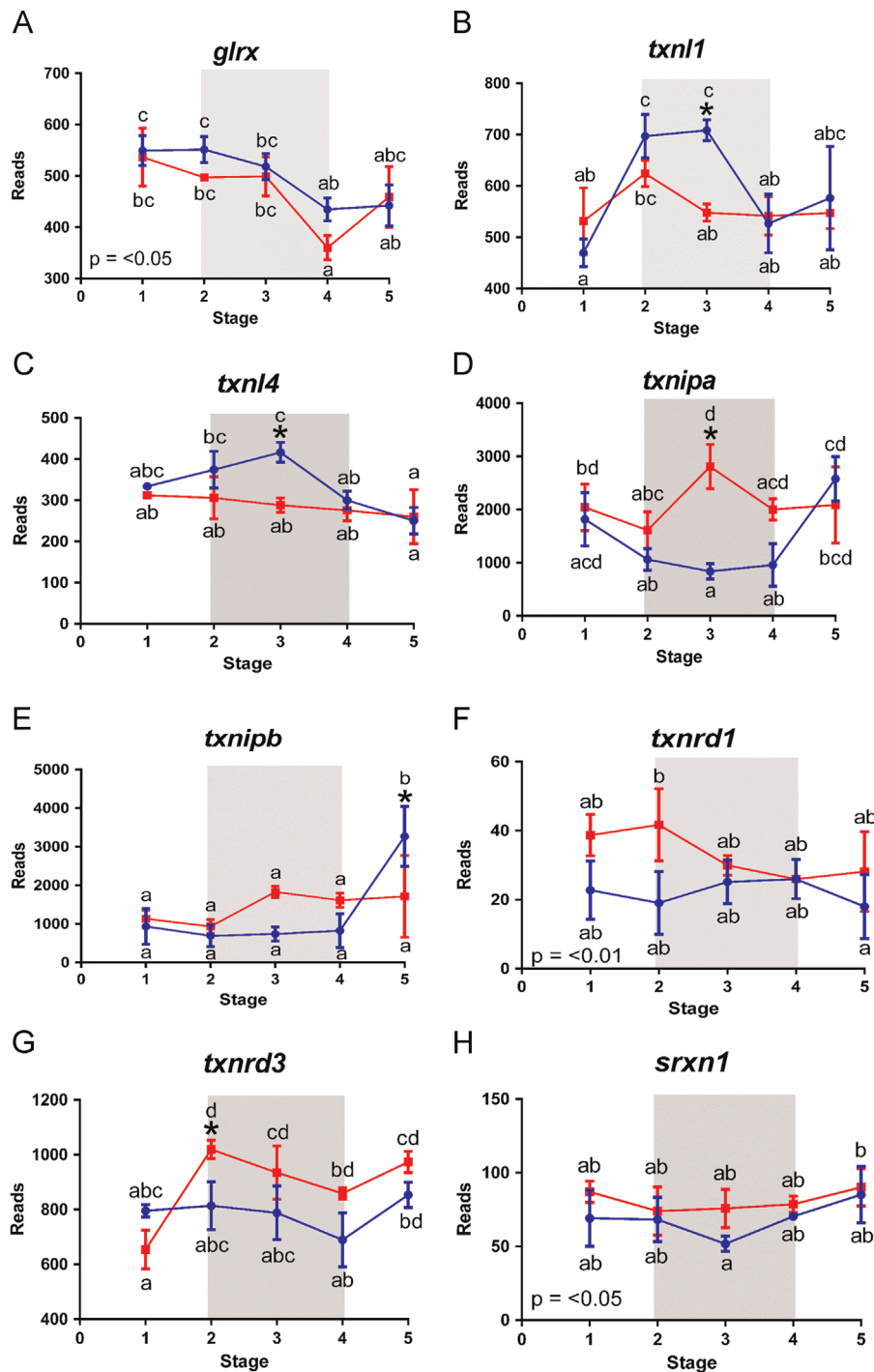
Fig. 6. The effect of diet on the expression of genes in thiol oxidoreductase pathways in cod larvae.The mRNA expression of *glutaredoxin* (A), *thioredoxin-like 1* (B), *4* (C), *thioredoxin interacting protein a* (D), *b* (E), *thioredoxin reductase 1* (F), *3* (G) and *sulfiredoxin 1* (H) in developing cod larvae fed either rotifers (□ red line) or copepods (○ blue line). Shaded areas cover life stages that copepod-fed larvae had elevated growth rates compared to rotifer-fed larvae. Letters indicate statistical relationships between all data points, with * and *p* values indicating statistical factorial and main effects, respectively, of diet (*p* < 0.05). See Fig. 1 for more details. Data are mean ± SEM, *n*=3.

shift to a formulated diet that occurred after the sampling at developmental stage 4 in both treatments. Whether the same shift occurs in nature remains to be shown.

The rotifer diet appears to induce an NRF2-mediated oxidative response in cod larvae

Genes coding for the key redox response regulating transcription factors; *nrf2*, *keap1a* and *keap1b*; were upregulated in rotifer-fed larvae. The *nrf2* (aka *nfe2l2*) gene codes for a transcription factor (TF) that

upregulates the transcription of cytoprotective genes, including many from antioxidant, heat shock protein, thioredoxin and glutathione synthesis pathways, in response to oxidative stress [38]. Like in zebrafish *Danio rerio* [39], we identified the mRNA of two *keap1*; Kelch-like ECH-associated protein 1; genes (*keap1a* and *1b*) in cod. The single KEAP1 ortholog found in mammals is a cysteine rich protein that suppresses NRF2 function until oxidative conditions occur [38]. The upregulation of *nrf2* and many NRF2 cytoprotective target genes in rotifer-fed larvae suggests that the rotifer diet induced an NRF2-mediated oxidative stress response. The upregulation of several targets
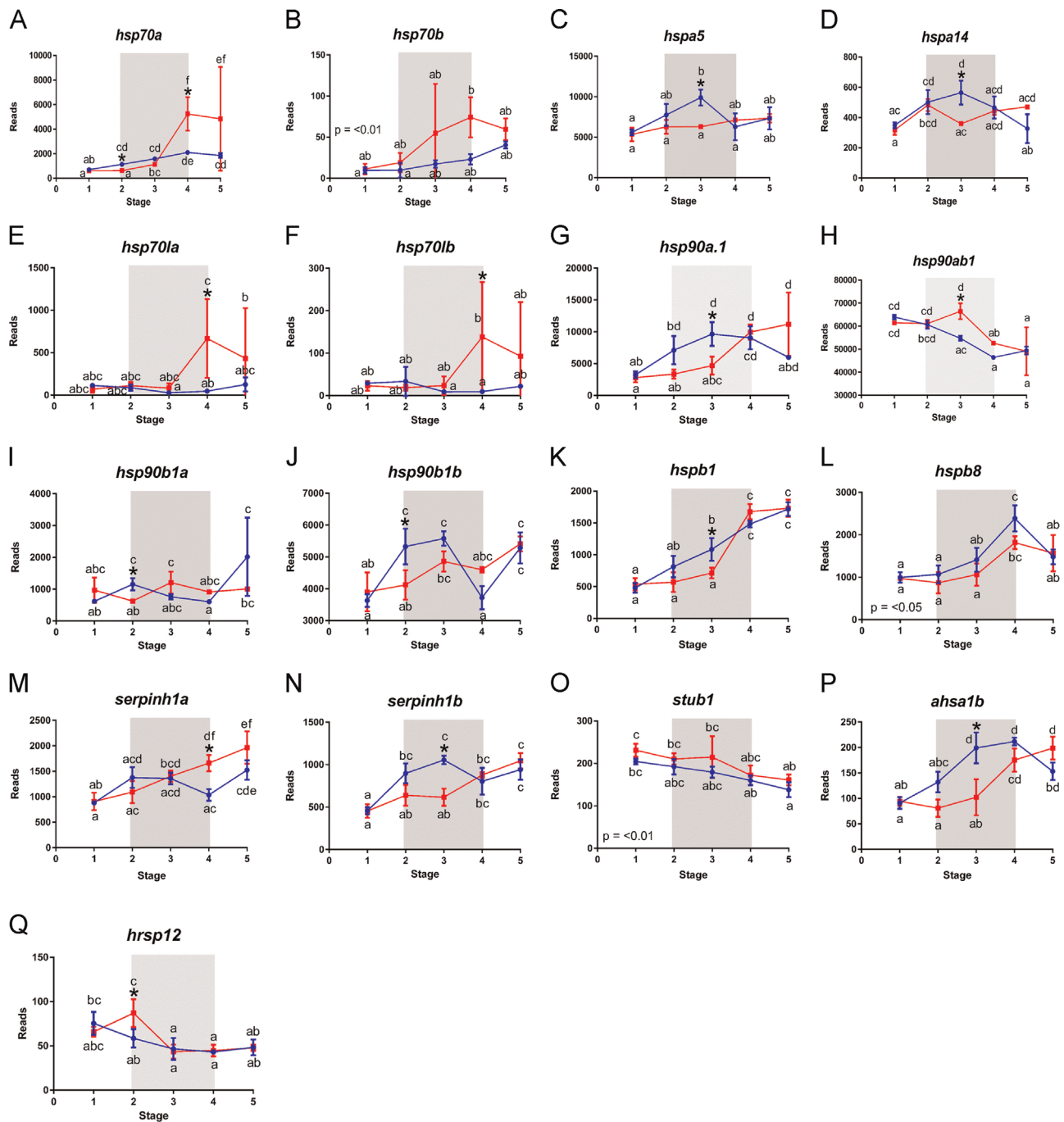
Fig. 7. The effect of diet on the expression of heat shock protein (hsp) system genes in cod larvae.The mRNA expression of **hsp 70 kDa** a (A), b (B) 5 (C) 14 (D) -like a (E), -like b (F); **hsp 90 kDa** a.1 (G), ab1 (H), b1a (I) b1b (J); **hsp 27 kDa** 1 (K); **hsp 22 kDa** 8 (L); serpin peptidase inhibitor, clade H (**hsp 47 kDa**), member 1a (M), 1b (N); stip1 homology and U-Box containing protein 1, E3 ubiquitin protein ligase (O); activator of hsp 90 kDa ATPase homolog 1b (P) and heat shock responsive protein 12 (Q) in developing cod larvae fed either rotifers (□ red line) or copepods (○ blue line). Shaded areas cover life stages that copepod-fed larvae had elevated growth rates compared to rotifer-fed larvae. Letters indicate statistical relationships between all data points, with * and p values indicating statistical factorial and main effects, respectively, of diet ($p < 0.05$). See Fig. 1 for more details. Data are mean ± SEM, $n = 3$.

of NRF2 induced upregulation, such as txnrd3, gclc and gclm [38] occurred earlier in development than nrf2 upregulation. This does not rule out NRF2 induced regulation, as the protein is omnipresent in a KEAP1 associated form to allow a rapid response to oxidative stress, where after it also upregulates its own transcription [38].

Imbalance in regulation between different areas of the redox response may contribute to the negative effects of a rotifer diet.

Several major gene families – the antioxidants, thiol oxidoreductases and heat shock proteins – did not have an overall direction of regulation as a near even divide between the number of genes up- or downregulated in rotifer-compared to copepod-fed cod larvae occurred. However, of greater importance may be imbalances in cellular ROS levels as a result of imbalances in enzyme activities within related pathways. For instance, increased superoxide dismutase 1 (SOD1) activity increases the dismutation of superoxide radicals ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2). Without simultaneous increases in the activities of H_2O_2 reducing enzymes, such as the GPX's, this can lead to H_2O_2 induced oxidative stress
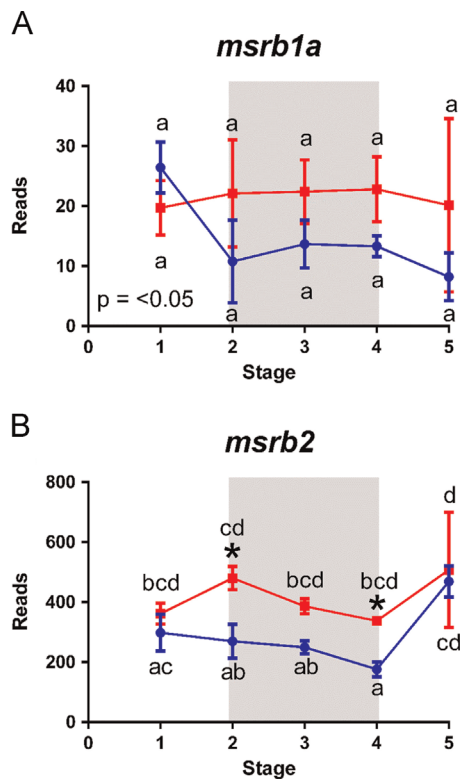
Fig. 8. The effect of diet on the expression of methionine sulfoxide reductase coding genes in cod larvae.The mRNA expression of *methionine sulfoxide reductase 1a* (A) and *2* (B) in developing cod larvae fed either rotifers (□ red line) or copepods (○ blue line). Shaded areas cover life stages that copepod-fed larvae had elevated growth rates compared to rotifer-fed larvae. Letters indicate statistical relationships between all data points, with * and *p* values indicating statistical factorial and main effects, respectively, of diet ($p < 0.05$). See Fig. 1 for more details. Data are mean $\pm$ SEM, $n=3$.

[40]. The more reduced cellular redox environment caused by the rotifer diet could also contribute to redox system enzyme mismatches. For instance, under normoxic conditions PRDX1 reduces H_2O_2, and in the process oxidises TXN [41]. However, under oxidative conditions, PRDX1 can utilise H_2O_2 to oxidise and deactivate, while GSR and GLRX1 can reduce and reactivate, TXNRD1 [42], a possible redox signalling mechanism. It is likely that a multitude of such redox environment induced interactions occur, and their spatial and temporal presence plays a role in proper development of fish larvae.

The nutritional composition differences between rotifers/ Artemia and copepods with potential links to the underlying changes to the redox system.

The rotifer-induced differential regulation of the redox system is most likely linked to the bioavailable levels of one or more nutrients in rotifers that differ to that of copepods [2,3]. In the current study, rotifers were enriched using best practice methodology, but were still deficient in zinc, taurine and protein in comparison to copepods [3]. Of these nutrients, only taurine was lower (≈ 20 fold) in rotifer- vs copepod-fed cod larvae [3]. Thus a rotifer-induced taurine deficiency is a likely candidate for the observed redox system differential regulation. Taurine is a conditionally essential nutrient for many marine fish species, whereby it can be synthesised in adequate amounts to meet requirements in adult but not larval stages [6,43]. Elevated concentrations of taurine are required in the mitochondria where its pH buffering capacity maintains optimum enzyme activity [44]. Taurine is also present in mitochondrial tRNA and thus is important for protein

synthesis [44,45]. Taurine deficiency can result in mitochondrial oxidative stress *in vitro* [45], and rotifer-fed cod larvae have abnormal mitochondria in liver and intestinal cells (Elin Kjørsvik, personal communication). We found that many genes important for maintaining the mitochondrial redox environment – *nrf2*, *gpx1*, *sod2*, *prdx3*, *prdx5* and *msrb2* [19,46,47] – were differentially regulated in rotifer-fed larvae. The mitochondria are a major site of cellular ROS production, metabolism, and cellular redox signalling molecules [46,48,49]. Thus, we suggest ROS induced changes in the mitochondria due to taurine deficiency in rotifer-fed larvae may have a downstream effect that contributes to redox system dysregulation throughout the cell and that this could be one of the factors underlying the inferior growth in rotifer fed fish larvae.

Conclusion

A rotifer diet induced the differential regulation of the redox system transcriptome, glutathione redox couple and cellular redox environment in cod larvae. As opposed to rotifers, a copepod diet resulted in a spike in GSSG concentration and the maintenance of an oxidised cellular environment in cod larvae. The maintenance of a relatively oxidised cellular environment, as observed in copepod fed larvae, may be critical to initiate the molecular response/s underlying the onset of the exponential growth phase that occurs in fish larvae under optimum nutritional conditions.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2015.06.003.

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