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► **To cite this version:**

Pauline Wischhusen, Laurence L. Larroquet, Thierry Durand, Camille Oger, Jean-Marie Galano, et al.. Oxidative stress and antioxidant response in rainbow trout fry exposed to acute hypoxia is affected by selenium nutrition of parents and during first exogenous feeding. *Free Radical Biology and Medicine*, 2020, 155, pp.99-113. 10.1016/j.freeradbiomed.2020.05.006 . hal-02861694

HAL Id: hal-02861694

<https://hal.science/hal-02861694>

Submitted on 2 Jun 2021

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Oxidative stress and antioxidant response in rainbow trout fry exposed to acute hypoxia is affected by selenium nutrition of parents and during first exogenous feeding

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ARTICLE INFO

Keywords:

Selenium
Nutritional programming
Acute hypoxia
Isoprostanoids
Antioxidant

ABSTRACT

Selenium (Se) deficiency is a problem widely encountered in humans and terrestrial livestock production with increasing attention also in aquaculture. Se supports the antioxidant system, which becomes especially important during stressful conditions. In the present study, the effect of Se-supplementation in broodstock and fry diets on the performance and antioxidant metabolism of rainbow trout fry under acute hypoxia was investigated. Rainbow trout broodstock were fed plant-ingredient based diets either without any Se-supplementation (Se level: 0.3 mg/kg) or supplemented with Se supplied as sodium selenite or as hydroxy-selenomethionine (Se level: 0.6 mg/kg respectively) for 6 months prior to spawning. The progenies were subdivided into three triplicate feeding groups and fed diets with similar Se levels compared to the parental diets, resulting in a 3x3 factorial design. After 11 weeks of feeding, the fry were either sampled or subjected to a hypoxic stress challenge. One hundred fish were transferred to tanks containing water with a low oxygen level (1.7 ± 0.2 ppm) and monitored closely for 30 min. When a fish started to faint it was recorded and transferred back to normoxic water. Direct fry feeding of the hydroxy-selenomethionine supplemented diet improved the resistance towards the hypoxic stress. On the contrary, fry originating from parents fed Se-supplemented diets showed a lower stress resistance compared to fry originating from parents fed the control diet. Fry subjected to hypoxia showed elevated oxidative stress with reduced glutathione (GSH) levels and increased isoprostanes (IsoP) and phytoprostanes (PhytoP) levels produced by lipid peroxidation of polyunsaturated fatty acids (PUFA), arachidonic and α -linolenic acids respectively. Increased mRNA expression of transcription factors (*nrf2*, *nfkb*, *keap1X2*) and decreased mRNA expression of antioxidant enzymes (*trxr*, *sod*, *gstt*) indicated a transcriptional regulation of the antioxidant response. In stressed fry, the mRNA expression of several antioxidant genes including *gr*, *msr* and *gstt* was found to be higher when fed the control diet compared to the sodium selenite treatment, with a contrary effect for parental and direct Se nutrition on *gpx*. The long-term parental effect becomes of greater importance in stressed fry, where more than half of the genes were significantly higher expressed in the control compared to the selenite supplemented group.

1. Introduction

Selenium (Se) is an important micronutrient to health in humans as well as animals [1]. Selenoproteins, that carry selenocysteine as an integral component, play a major role not only in the immune response,

but especially in the antioxidant defense [2,3]. The glutathione peroxidases (GPX) with the Se-dependent family (SeGPX) help to control the cellular redox state, but other selenoenzymes such as thioredoxin reductase (TrxR) and methionine sulfoxide reductase B (MsrB) can also decrease oxidative damage to lipids and other biomolecules [4]. The

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expression and activity of selenoproteins is closely linked to the Se status and has been used to determine optimal dietary Se levels [5]. Based on the maximum total GPX activity, the optimal Se ingestion in humans was determined to be around 47 µg per day in males and females [6]. However, human Se intake differs strongly between different parts of the world as dietary Se availability is largely dependent on soil concentrations based on plant-soil interactions. Se deficiency is considered to be a concern with 1 in 7 people worldwide [7] and is also known to affect livestock production [8]. The Food Standard Agency (FSA) in UK analyzed 28 food groups in a study of total diet and identified fish to contain the second highest Se concentrations after organ meat [9] making it a main contributor for Se intake in European countries, but given the increasing world-wide fish consumption also in other continents [10]. Today, more and more seafood in the human food basket is coming from aquaculture production where nutrient levels can be more easily monitored and the supply can be modified to ensure sufficient Se levels in fish [11].

The supply of antioxidants like Se is important for fish in wildlife, but also in captivity as they are subjected to various stressors such as changes in oxygen levels that are associated with an increase of reactive oxygen species (ROS) [12]. Even short-term oxidative stress resulting from an imbalance between antioxidants and ROS can induce the peroxidation of long-chain polyunsaturated fatty acids (LC-PUFA) in fish tissues [13]. Traditionally, the main Se source in most intensively farmed fish feeds is fishmeal at levels above the authorized levels of 0.5 mg Se/kg diet in animal feeds [14] meaning that no Se supplementation is allowed in aquafeeds, but today it is widely replaced with plant protein sources containing in general lower Se levels [15]. Recent studies have shown that the supplementation of plant-ingredient based fish diets also above the legal levels with various Se sources can improve the growth performance and health status of cultivated fish species [16–18], which is especially important during sensitive stages. We have earlier shown that dietary Se affects the antioxidant status and reproductive performance of rainbow trout broodstock and its possible effects on the progeny [19]. Dietary supplementation is made either using inorganic salts of selenite or organic Se sources such as Se-enriched yeast or hydroxy-selenomethionine. So, it can be expected that based on the antioxidant role of Se, adequate levels will support the fish to cope with stressors like reduced oxygen levels, but also improve health and product quality. Hence, the objective of the present study was to assess the impact of Se-supplementation in broodstock and fry diets on the performance and antioxidant metabolism of rainbow trout fry exposed to acute hypoxic stress.

2. Material and methods

2.1. Ethical statement

All experimental procedures complied with the European Directive 010/63/EU for the protection of animals used for scientific purposes, and the French Decree no. 2013-118 for animal experimentation. The scientists in charge of the experiment received training and personal authorization.

2.2. Experimental diets

The diets were designed based on plant-derived proteins and manufactured using a twin-screw extruder (BC 45, Clextal, Firminy, France) at the INRAE experimental facilities in Donzacq (Landes, France). The basal Se level in the diet was 0.3 mg Se/kg, which served as a negative control in broodstock (Bnc) and fry diets (Fnc) (Table 1). Broodstock or fry diets supplemented with organic Se (Bso and Fso) had a targeted total dietary concentration of 0.6 mg Se/kg. Similarly, sodium selenite was used as an inorganic Se source in broodstock diet Bss and fry diet Fss at a targeted concentration of 0.6 mg Se/kg. The analyzed Se concentrations in the diets Fnc, Bnc and Fso were as expected,

Table 1

Formulation and composition of experimental diets.

Diet	Broodstock diets			Fry diets		
	Bnc	Bss	Bso	Fnc	Fss	Fso
<i>Ingredients (%)</i>						
Plant meals ^a	74	74	74	74	74	74
Crystalline amino acids and attractant mixture ^b	3	3	3	3	3	3
Soybean lecithin ^c	2	2	2	2	2	2
Fish oil ^c	8	8	8	8	8	8
Vegetable oils ^d	8	8	8	8	8	8
Carophyll® pink ^e	0.04	0.04	0.04	–	–	–
Vitamin and mineral mixture without Se ^f	5	5	5	5	5	5
Sodium selenite (µg/g diet) ^g	–	0.7	–	–	0.7	–
Seliseo® (µg/g diet) ^g	–	–	15	–	–	15
<i>Analytical composition (DM, %)</i>						
Dry matter	96	98	97	95	94	95
Crude protein	49	50	50	51	50	50
Total lipid	23	22	23	20	21	21
Gross energy (kJ/g DM)	25	25	25	25	25	25
Ash	6	6	6	6	6	6
Se (mg/kg)	0.3	0.8	0.7	0.3	0.5	0.6

^a Plant meals (% diet): 20% wheat gluten (Roquette), 18% corn gluten meal (Inzo), 15% soybean protein concentrate Estril®75 (Sopropêche), 6% soybean meal (Sud-Ouest Aliment), 5% rapeseed meal 00 (Sud-Ouest Aliment), 5% white lupin meal Farilup 500 (Terrena), 3% dehulled pea meal Primatex (Sotexpro), 2% whole wheat (Sud-Ouest Aliment).

^b Crystalline amino acids and attractant mixture (% diet): 1.34% L-lysine, 0.3% DL-methionine, 0.5% glucosamine, 0.3% taurine, 0.3% betaine, 0.2% glycine, 0.2% alanine.

^c Soybean lecithin from Louis François and fish oil from Sopropêche.

^d Vegetable oils (% diet): 4% rapeseed oil, 2.4% linseed oil, 1.6% palm oil (Daudry).

^e Contained 10% astaxanthin

^f Vitamin and mineral mixture without Se (per kg diet): retinol acetate, 55,000 IU; cholecalciferol, 2500 IU; DL-α-tocopherol acetate, 50 IU; sodium menadione bisulfate, 10 mg; thiamin-HCl, 1 mg; riboflavin, 4 mg; niacin, 10 mg; D-calcium pantothenate, 20 mg; pyridoxine-HCl, 3 mg; D-biotin, 0.2 mg; folic acid, 1 mg; cyanocobalamin, 10 µg; L-ascorbyl-2-polyphosphate, 50 mg; myo-inositol, 0.3 g; choline, 1 g; CaHPO₄·2H₂O, 33 g; CaCO₃, 2.15 g; Mg(OH)₂, 1.24 g; KCl, 0.9 g; NaCl, 0.4 g; FeSO₄·7H₂O, 0.2 g; ZnSO₄·7H₂O, 40 mg; MnSO₄·H₂O, 30 mg; CuSO₄·5H₂O, 30 mg; NaF, 10 mg; KI, 0.4 mg; CoCl₂·6H₂O, 0.2 mg. All ingredients were diluted with α-cellulose.

^g Sodium selenite contained 42% Se (Sigma-Aldrich) and Seliseo contained 2% Se (Adisseo).

whereas Bss had a Se concentration of 0.8 mg/kg, Bso a concentration of 0.7 mg/kg and Fss had 0.5 mg Se/kg diet (Table 1).

2.3. Experimental design

The progeny of three dietary rainbow trout broodstock groups (Bnc, Bss and Bso) were used in this experiment. The diets were fed to female and male broodstock fish over a 6 month period prior to spawning as previously described [19]. At the beginning of exogenous feeding, swim-up fry from each of the three parental groups were randomly subdivided into one of the three fry feeding groups (Fnc, Fss, Fso) in triplicate, resulting in a 3x3 factorial design. Each triplicate group consisted of 250 fish/tank reared in 50L fiberglass tanks supplied with flow-through spring water at 17 °C and a saturated dissolved oxygen concentration of 9.2 ± 0.1 ppm. The fish were hand fed six times daily during the first three weeks of the trial and four times daily afterwards to apparent satiation. After 11 weeks of feeding, the fish were either sampled or exposed to an acute hypoxic stress challenge following the protocol adapted from Borey et al. [20]. One hundred fish per tank were transferred to new tanks containing water with a low dissolved oxygen concentration of 1.7 ± 0.2 ppm created by the supply of N₂. The fish were monitored closely and once a fish had lost its equilibrium,

Table 2
Oligonucleotide primers used to assay mRNA levels by Fluidigm PCR.

Gene	Accession no.	Forward primer	Reverse primer	Amplification size
<i>selpa1</i>	EE605178	gcccaaacaggaagatgtgt	gggcaggagatatggttagg	100
<i>selpa2</i>	HF969249.1	cagccacctgttggagtat	cctggagtagggccacca	82
<i>selpb</i>	HF969250.2	gtgtcttctcactctgtgaa	ggaacgtcagctcccacat	187
<i>gpx1a</i>	HE687021	aatgtggcgtcactctgagg	caattctctgatggcaca	131
<i>gpx1b1</i>	CA357669.1	cgagctccatgaacggtacg	tgcttcccgttcacatccac	183
<i>gpx1b2</i>	HE687023	tcggacatcaggagaactgc	tccttccattcacatccac	121
<i>gpx4a1</i>	HE687024	gaaaaggcttctgggaaatg	ctccaccacactgggatcat	112
<i>gpx4a2</i>	HE687025	agaaatacaggggcgactgt	gcatctccgcaactgagag	90
<i>gpx4b</i>	CA344428.1	ttggaggtcaggagccaggt	accctttcccttggcctgtt	152
<i>trxr3a</i>	HF969246.1	gagcctcctcaagtgtgac	agtgaactccagaggccaga	158
<i>trxr3b</i>	HF969247.1	acaaaatcaaggcgaccaac	ggcagagagaacaggtctc	148
<i>msrb1a</i>	BX313019.3	ctggcctgcttctactgaga	ttccacatcggacctgaa	87
<i>msrb2</i>	BX311214.3	aggggacagagatgccttc	cccatgagcctcttgaacg	149
<i>msrb3</i>	CX041708.1	tcaggttggccttcttcta	cgaggtgagaaccactgga	118
<i>msra1</i>	BT073399.1	gatggctatgttgggatgg	acctgtgcaggtctctctg	136
<i>sod1</i>	AF469663.1	tggctctggaagctgattg	ttgtcagctctcagtcac	201
<i>sod2</i>	CA352127.1	tccttgactgactactacgac	ggcctcctcattaaacctc	201
<i>cat</i>	BX087110.3	tgatgtcacacaggtgcgta	gtggcctcagttgttgag	195
<i>gr</i>	HF969248.1	ctaagcgcagctcatagtg	acaccctgtctgacgacat	108
<i>gclc1</i>	GSONMT00071788001	aggccagagatggcagcta	cagcctaacttgggaatga	176
<i>gclc2</i>	GSONMT00065033001	aggccagagatggcagcta	ttgggaatgatgtgatggtg	166
<i>gstπ</i>	BX302932.3	tcgctgactggacgaaagga	cgaaggtctctcaacccatc	196
<i>nrf2</i>	CA360709.1	tgagctgcagcaatgtctga	gttgggcaatgggtagaagc	124
<i>keap1X1</i>	GSONMT00034445001	gctacgtgatgtctcccct	ggatcctcatagcgccaggt	116
<i>keap1X2</i>	GSONMT00019673001	actgggaggttatgacggga	ctctcgtctctcatgcttc	179
<i>nfkb</i>	BX880658.3	cagcgtctaccaggctaaagagat	gctgttcgatccatccgcaact	181
<i>Ikb</i>	BT074199.1	agagacagactgcgtccac	cggccttcagtagcctctct	72
<i>Ikb2</i>	CDQ64494.1	agacacgcttctccacttg	gacgtccactatctcagcc	164
<i>tnfa3</i>	HE798146.1	cccccaagcaccctacaacc	gtcttcgggtccgtagcttg	82
<i>hsp70</i>	AB062281.1	cgagcagctgacaaataca	tgcttccaaccaggaaatg	194
<i>hif1ab1</i>	XM_021584966	ctcacccttagctactgat	aacttctcttgcgtgtgag	183
<i>hif1ab2</i>	NM_001124288.1	ccccacccttagagtctc	tggtgagtaaggaagcaggg	183
<i>ahra</i>	AF065137.3	ctctcgtctgacaaactct	gctgttcatccattcatgtt	138
<i>ahrβ</i>	AF065138.4	ccagctctgaatggctttg	tgactgatggaagcccaggt	97
<i>ppara</i>	AY494835.1	ctggagctggatgacagtga	ggcaagtttttcagcagat	192
<i>pparγ</i>	CA345564.1	cccacggaaactcaccgttt	ggatctggatacggcggaag	168
<i>EF1α</i>	AF498320.1	tcctcttggctctttcctg	acccgaggacatctctgtg	159
<i>β-actin</i>	AJ438158.1	gatgggcccgaagacagcta	tcgtcccgtggtgacgat	105

Selp, selenoprotein P; gpx1; glutathione peroxidase 1; gpx4, glutathione peroxidase 4; trxr, thioredoxin reductase; msr, methionine sulfoxide reductase; sod1, superoxide dismutase 1; sod2, superoxide dismutase 2; cat, catalase; gr, glutathione reductase; gclc, glutathione-cysteine ligase catalytic subunit; gstπ, glutathione-s-transferase π; nrf2, nuclear factor erythroid 2-related factor 2; keap1, Kelch-like ECH-associated protein; nfkb, nuclear factor kappa-light chain-enhancer of activated B cells; Ikb, nfkb inhibitor; ahr2, aryl hydrocarbon receptor 2; hsp70, 70 kDa heat shock protein; ppara, peroxisome proliferator activated receptor α; pparγ, peroxisome proliferator activated receptor γ; hif1α, hypoxia inducible factor 1 subunit α; tnfa3, tumor necrosis factor; EF1α, eukaryotic translation elongation factor 1α.

it was removed from the hypoxic tank and immediately transferred back to a tank with saturated dissolved oxygen levels where they returned to normal swimming behavior within a few seconds. The latency period from the beginning of the experiment until the fish lost its equilibrium was recorded. After 30 min, the stress challenge was terminated and all fish were anesthetized and killed with an overdose of benzocaine, weighed, frozen as whole fish in liquid nitrogen and stored at -80°C until further analysis.

2.4. Analytical methods

2.4.1. Proximate analysis and fatty acids

For proximate composition of diets, dry matter was determined after drying at 105°C for 24h, protein (N x 6.25) by Kjeldahl after acid digestion [21], ash after 10 h incineration at 550°C and gross energy in an adiabatic bomb calorimeter. Total lipids were extracted from diets and a pool of five whole fry and quantified gravimetrically following Folch et al. [22], using dichloromethane instead of chloroform. Fatty acid and methyl esters were prepared and analyzed as previously described [23].

2.4.2. Markers of oxidative stress

Oxidative stress markers were analyzed on a pool of three homogenized whole fry per tank. To assess lipid peroxidation of different LC-PUFA, F_2 -isoprostanes (F_2 -IsoP), the product of arachidonic acid (20:4n-6, ARA) peroxidation, was measured in 0.5 g using Cayman Chemical 8-isoprostane enzyme immunoassay kit (Bertin Pharma, Montigny le Bretonneux, France) according to manufacturer's instruction similar to the analysis at swim-up fry stage [19]. In addition, the specific isoprostanes of ARA peroxidation (IsoP ARA) including 15- F_{2t} -IsoP (F_{2t} -IsoP1), 15-*epi*-15- F_{2t} -IsoP (F_{2t} -IsoP2), 5(RS)-5- F_{2t} -IsoP (F_{2t} -IsoP3) and 5- F_{2t} -IsoP (F_{2t} -IsoP4) as well as 15- A_{2t} -IsoP (A_{2t} -IsoP) were assessed and in parallel, isoprostanes from eicosapentaenoic acid (IsoP EPA) including 8-*epi*-8- F_{3t} -IsoP (F_{3t} -IsoP1) and 8- F_{3t} -IsoP (F_{3t} -IsoP2) and phytosterols (PhytoP ALA) and phytofurans (PhytoF ALA) from α-linolenic acid (18:3n-3, ALA) including *ent*-16- F_{1t} -PhytoP (PhytoP1), *ent*-16-*epi*-16- F_{1t} -PhytoP (PhytoP2), 9- F_{1t} -PhytoP (PhytoP3), 9-*epi*-9- F_{1t} -PhytoP (PhytoP4), *ent*-16 B_{1t} -PhytoP (PhytoP5), *ent*-9 L_{1t} -PhytoP (PhytoP6), *ent*-9 L_{1t} -PhytoP (PhytoP7), *ent*-16(RS)-9-*epi*-ST- Δ^{14} -10-PhytoF (PhytoF1) and *ent*-9(RS)-12-*epi*-ST- Δ^{10} -13-PhytoF (PhytoF2) as well as neuroprostanes (NeuroP DHA) from docosaheptaenoic acid (22:6n-3, DHA) including 4(RS)-4- F_{4t} -NeuroP (NeuroP1), 10(S)-10- F_{4t} -

Table 3

Effects of rainbow trout broodstock and fry nutrition on growth performance of offspring fed different level and sources of Se (initial mean body weight: 99.7, 87.1 and 91.6 mg for Bnc, Bss and Bso, respectively).

	Parental effect			Direct feeding effect			p-value		
	Bnc	Bss	Bso	Fnc	Fss	Fso	PE	DF	PE×DF
Survival (%)	97.1 ± 0.7	98.4 ± 0.3	97.2 ± 0.7	98.2 ± 0.3	97.5 ± 0.7	97.0 ± 0.7	0.24	0.39	0.13
Final weight (g)	4.7 ± 0.1	4.5 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	0.09	0.40	0.07
Weight gain (%) ^a	59.7 ± 1.0 ^b	66.2 ± 0.7 ^a	65.6 ± 1.1 ^a	64.8 ± 1.2	63.2 ± 1.4	63.5 ± 1.5	< 0.01	0.29	0.05
Feed efficiency ^b	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	0.43	0.25	0.73

Values are mean ± SEM (n = 9 rearing tanks, three broodstock or three fry groups). Within rows and for each diet-related effect: parental (PE) or direct feeding (DF), means not sharing a common superscript letter are significantly different according to two-way ANOVA.

^a (weight gain (g))*100/(initial weight (g)*days).

^b wt gain (g)/feed intake (g).

NeuroP (NeuroP2), 10-*epi*-10-F_{4t}-NeuroP (NeuroP3), 13A(RS)-13-F_{4t}-NeuroP (NeuroP4), 13B(RS)-13-F_{4t}-NeuroP (NeuroP5), 14(RS)-14-F_{4t}-NeuroP (NeuroP6), 20(R)-20-F_{4t}-NeuroP (NeuroP7) and 20-*epi*-20-F_{4t}-NeuroP (NeuroP8) were analyzed by microLC-MS/MS as previously described [24,25]. Protein oxidation was assessed using spectrometric determination of protein carbonyls on 0.25 g homogenized fry as previously described [19]. Total (GSHT), oxidized (GSSG) and reduced glutathione (GSH) were measured in 0.3 g samples using Cayman glutathione assay kit (Bertin Pharma) according to manufacturer's instructions with protein concentration assessed by the method of Lowry et al. [26] using BSA as a standard. Tocopherols were extracted following Folch et al. [22] and analyzed on 0.1 g samples as previously described [19]. Vitamin C was measured by HPLC [27].

2.4.3. Antioxidant activity and mRNA levels

Activities of anti-oxidant enzymes such as total GPX, SeGPX, seleno-independent GPX (indGPX), catalase (CAT), glutathione-S-transferase (GST) and superoxide dismutase (SOD) were measured in 0.5 g of pooled and homogenized samples of three whole fry per tank as previously described [19]. Also, mRNA was extracted from three individual fry per tank (= nine individuals per dietary treatment) and for quality control gene expression was measured on some selected genes by reverse transcription PCR (RT qPCR) as previously described [19], before high-throughput qRT-PCR was used on the RNA extracts to quantify gene expression at the GenotoulPlatform (<http://genotoul.fr/>) using the BioMark® 96:96 Dynamic Array (Fluidigm Corporation, San Francisco, CA, USA) according to manufacturer's protocol. Briefly, each cDNA sample was pre-amplified with a pool of primers specific to the target gene by using the following program: 95 °C for 10 min, then 14 cycles of 95 °C for 15 s and 60 °C for 4 min. The pre-amplified products were diluted 1:5 in 10 mM Tris-HCl, pH 8;

0.1 mM EDTA and analyzed by qRT-PCR using the following conditions: 95 °C for 10 min, then 35 cycles of 95 °C for 15 s and 60 °C for 30 s. Some genes were analyzed in duplicate for which an average value was used during further statistical analysis. The output was analyzed with Fluidigm real-time PCR analysis software (Fluidigm Corporation v5.4.2). All primer sequences are presented in Table 2. A geometric mean of the mRNA expression of two house-keeping genes, namely, EF1α and β-actin was used as a reference and the relative expression of the target genes was determined by the ΔΔCT method with a control from the group fed Bnc and Fnc sampled before the stress challenge [28].

2.5. Statistical analysis

The statistical analysis was performed using statistical software R (R Development Core Team, 2008). Results are given as mean ± standard error of means (SEM). Data collected during the hypoxic stress challenge were analyzed by adapting a Kaplan-Meier model used in survival analysis studies (R: survminer, version 0.4.6). Thereby, the time a fish started to lose its equilibrium and consequently has been removed from the tank was considered as the "time of death" within the total experimental period. Direct feeding and parental nutritional history were included as factors possibly influencing the time period a fish was able to resist the hypoxic conditions. When a significant difference was found a Log-Rank test was applied as a post hoc test to identify differences between groups. Data obtained from the analyzed samples were, in a first step, examined by PCA (R: factoextra, version 1.0.6) in search for biological cluster as well as outliers. In the gene expression dataset one individual from the BncFss treatment sampled before stress appeared as an outlier defined as mean ± 6 times standard deviation and was hence removed after a manual verification by boxplot.

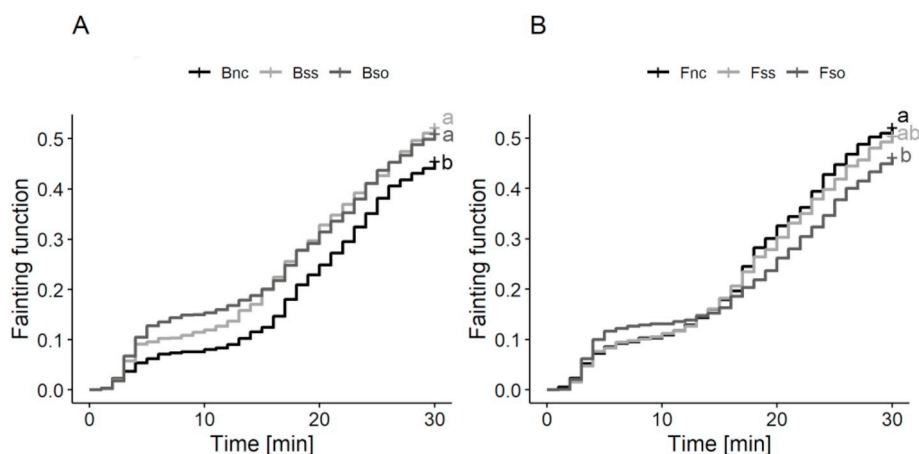


Fig. 1. Empirical fainting function based on Kaplan-Meier [1-(number of surviving fish/total number of fish)] by parental nutritional history (A) or direct Se feeding group (B). Superscript letters represent significant differences between survival curves based on a log rank test.

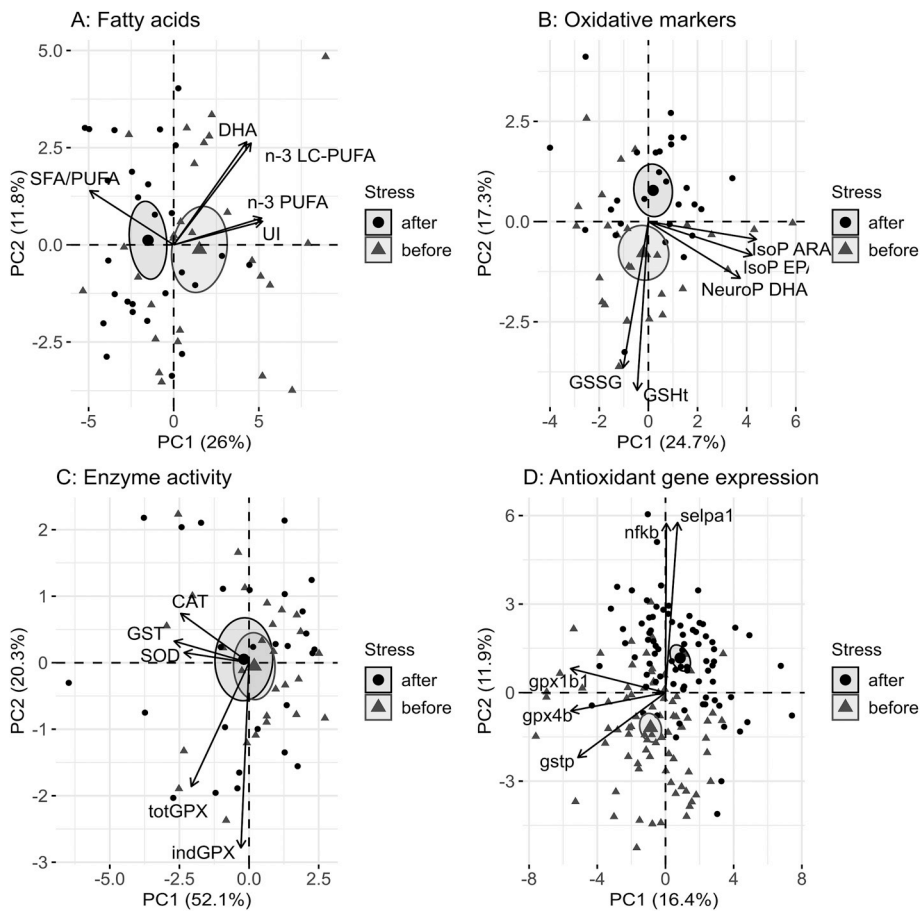


Fig. 2. PCA biplots on fatty acids (A), oxidative markers (B), specific antioxidant enzyme activity (C) and gene expression of antioxidant proteins (D) measured in whole fry before and after the hypoxic stress challenge. Arrows represent the 5 most contributing variables to the model, respectively A: UI, unsaturation index; n-3 PUFA, sum of n-3 polyunsaturated fatty acids; n-3 LC-PUFA, sum of long chain n-3 PUFA; DHA, docosahexaenoic acid and SFA/PUFA, ratio between saturated and polyunsaturated fatty acids; B: GSht, total glutathione; GSSG, oxidized glutathione; NeuroP DHA, sum of neuroprostanes from DHA; IsoP ARA, sum of isoprostanes from arachidonic acid; IsoP EPA, sum of isoprostanes from eicosapentaenoic acid; C: CAT, catalase; GST, glutathione S-transferase; SOD, superoxide dismutase; GPX, total glutathione peroxidase; indGPX, seleno-independent GPX. D: *gpx1b1* and *gpx4*, glutathione peroxidase 1 and 4; *gstp*, glutathione-S-transferase π ; *selpa1*, selenoprotein P; *nfkb*, nuclear factor kappa-light chain-enhancer of activated B cells. Ellipses represent the 95% confidence intervals around a center of 27 rearing tanks (pool of 3 individuals for each tank) in A-C and 81 individuals in D.

Table 4
 Stress effect on selected biomarkers of fatty acids and oxidative stress assessed in rainbow trout fry.

	Before stress	After stress
ARA ^a	0.87 ± 0.01 ^a	0.85 ± 0.01 ^b
EPA ^a	2.41 ± 0.0 ^a	2.30 ± 0.02 ^b
DHA ^a	7.85 ± 0.1 ^a	7.63 ± 0.04 ^b
SFA ^a	22.3 ± 0.1 ^b	22.5 ± 0.1 ^a
MUFA ^a	35.7 ± 0.1 ^b	36.0 ± 0.1 ^a
UI	172 ± 0 ^a	171 ± 0 ^b
GSht ^b	11.9 ± 0.3 ^a	10.4 ± 0.3 ^b
GSSG ^b	1.44 ± 0.07 ^a	1.18 ± 0.06 ^b
GSH ^b	10.4 ± 0.3 ^a	9.3 ± 0.3 ^b
F ₂ -IsoP ^c	0.17 ± 0.02 ^b	0.25 ± 0.03 ^a
IsoP EPA ^c	0.33 ± 0.02	0.33 ± 0.02
A ₂₁ -IsoP ^c	0.33 ± 0.02 ^a	0.25 ± 0.02 ^b
F ₃₁ -IsoP1 ^c	0.16 ± 0.01	0.15 ± 0.01
F ₃₁ -IsoP2 ^c	0.17 ± 0.01	0.17 ± 0.01
PhytoP + F ^c	23.4 ± 1.5 ^b	28.2 ± 1.7 ^a
NeuroP ^c	15.6 ± 0.8 ^a	13.3 ± 0.5 ^b
NeuroP7 ^c	0.79 ± 0.04 ^a	0.69 ± 0.04 ^b
α-tocopherol ^d	1.24 ± 0.06	1.11 ± 0.06
Vitamin C ^e	14.3 ± 0.29	14.1 ± 0.26

Values are mean ± SEM (n = 27 rearing tanks, pool of 3 individuals). Within rows values not sharing the same superscript letter are significantly different according to one-way-ANOVA followed by Tukey's HSD.

^a [% of total fatty acids].

^b [μM/mg protein].

^c [pg/kg].

^d [ng/kg].

^e [mg/kg].

Parental, direct feeding and stress effects were analyzed using three-way ANOVA. After, the model has been simplified to determine the stress effect using one-way ANOVA. The stress effect was found to be similar between three-way and one-way ANOVA as represented here. The parental and direct feeding effects were analyzed using two-way ANOVA, before and after stress respectively. To meet homogeneity and normal distribution, percentages were arc-sin transformed, while gene and enzyme activity data rank transformed before analysis. The significance level was set to $p < 0.05$ in all analysis and Tukey's HSD was used as a post hoc test.

3. Results

3.1. Effect of Se nutrition and acute hypoxic stress on rainbow trout fry performance

During the 11-week feeding trial there was no significant difference in survival between the groups. Neither parental nutritional history nor direct feeding of Se had any effect on feed efficiency or final body weight (Table 3). However, the weight gain was significantly higher in the groups originating from parents fed the Se-supplemented diets. This parental effect was observed when fry were fed directly the respective additional Se source compared to fry originating from parents fed the non-supplemented diet Bnc (67 ± 1 vs $59 \pm 1\%$ in BssFss vs BncFss and 68 ± 1 vs $59 \pm 2\%$ in BsoFso vs BncFso).

During the hypoxic stress challenge, fry originating from parental groups fed the control diet (Bnc) showed a significantly better

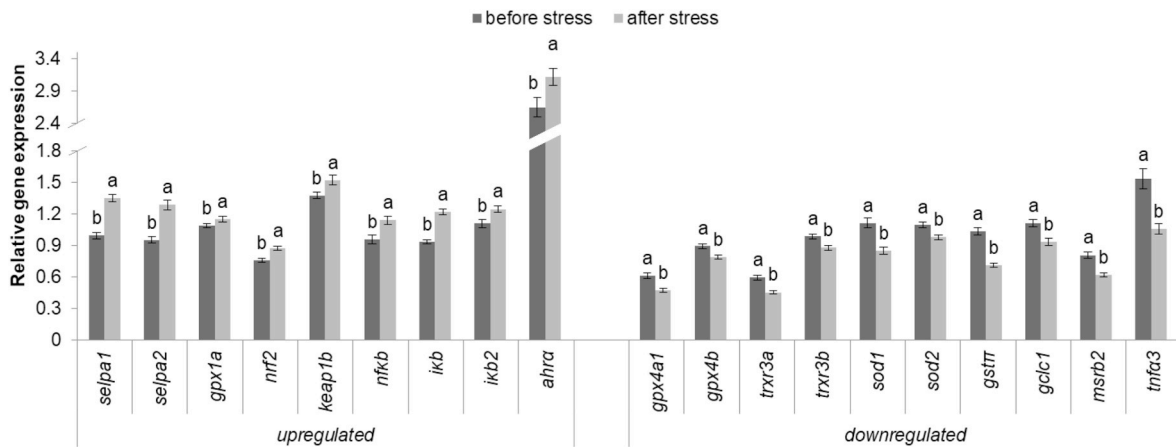


Fig. 3. Relative gene expression of significantly different expressed genes in whole fry before or after they were exposed to an acute hypoxic stress challenge for maximum 30 min. Data were normalized to a geometric mean of *ef1a* and β -*actin* and expressed as fold-changes of mRNA abundance compared to a sample from before stress in the BncFnc group. Bars represent means \pm SEM (n = 81 individuals representing 3 individuals per tank on a total of 27 tanks). Differences in superscript letters are showing significant differences according to one-way ANOVA followed by Tukey's HSD.

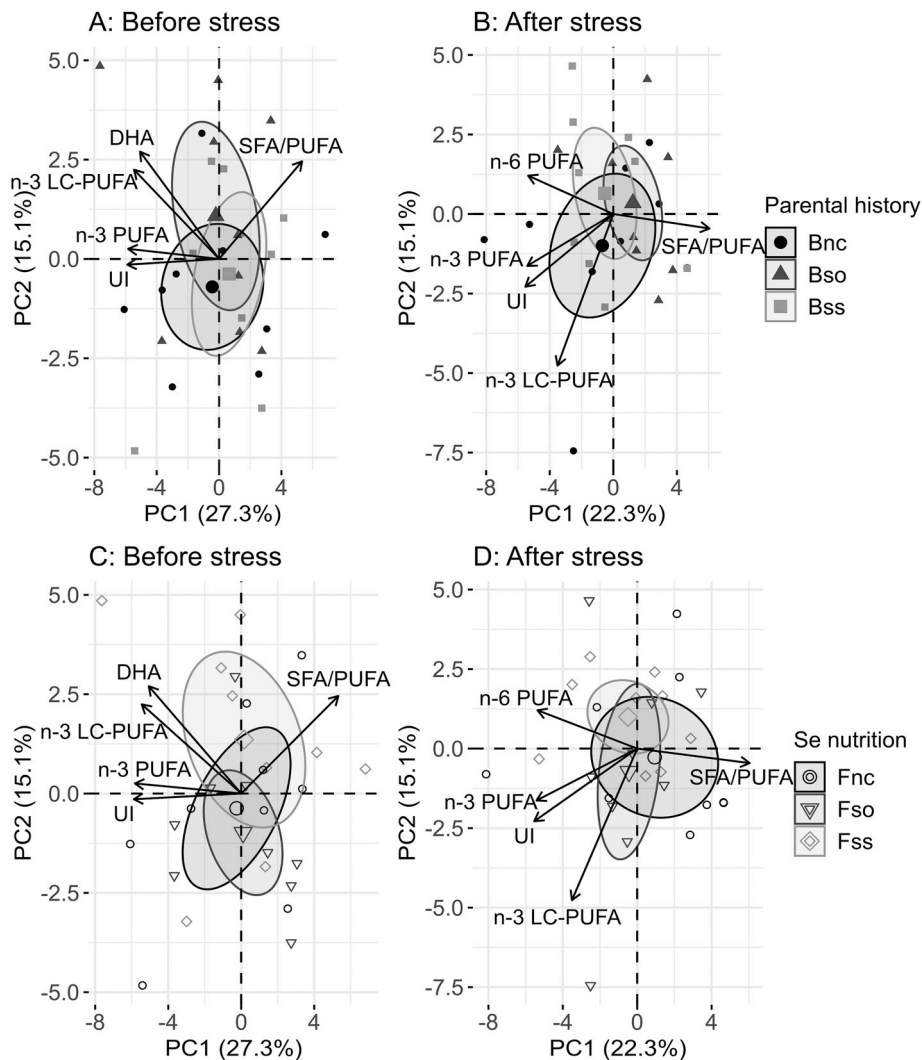


Fig. 4. PCA biplots on fatty acids grouped by either their parental nutritional history (A and B) or direct Se feeding (C and D) measured in whole fry before (A and C) and after (B and D) hypoxic stress. Arrows represent the 5 most contributing variables to the model, respectively (UI, unsaturation index; n-3 PUFA, sum of n-3 polyunsaturated fatty acids; n-6PUFA, sum of n-6 polyunsaturated fatty acids; n-3 LC-PUFA, sum of n-3 long chain PUFA; DHA, docosahexaenoic acid and SFA/PUFA, ratio between saturated and polyunsaturated fatty acids). Ellipses represent the 95% confidence intervals around a center of 9 rearing tanks (pool of 3 individuals).

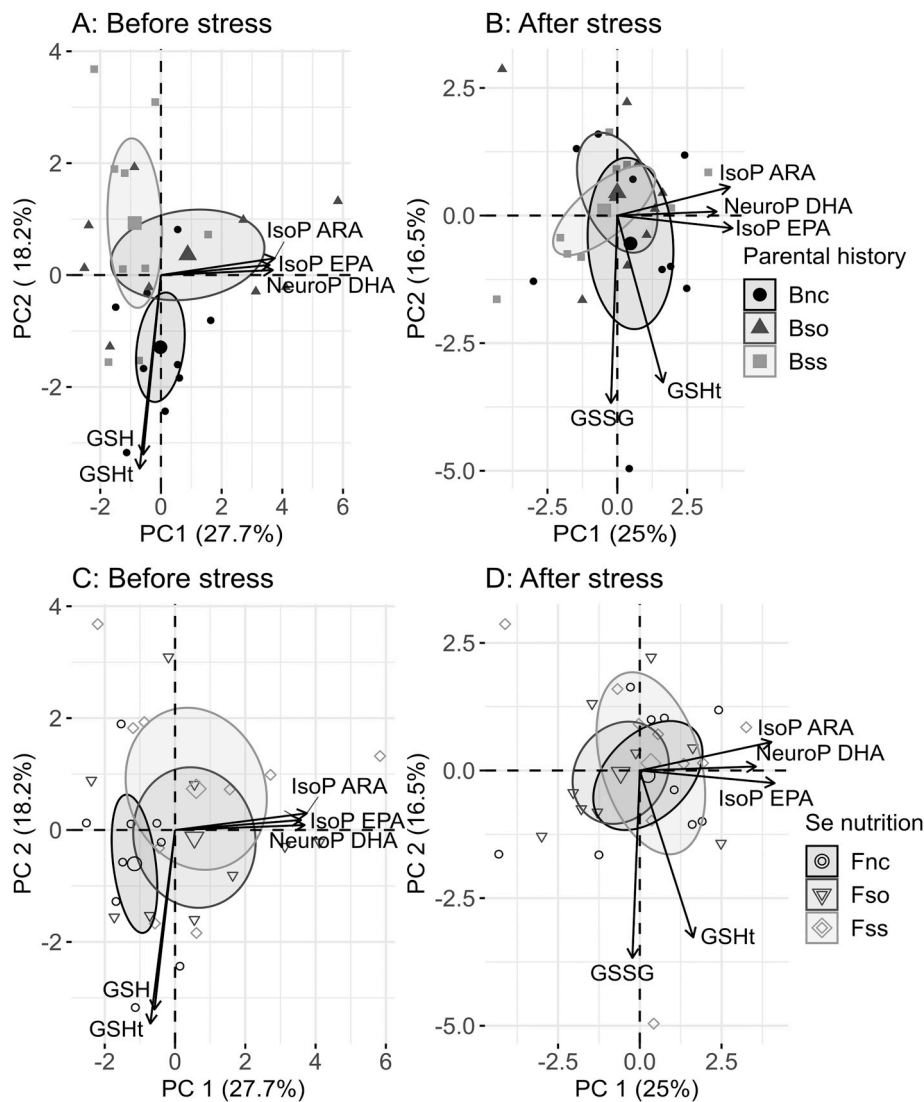


Fig. 5. PCA biplots on marker for oxidative stress grouped by either their parental nutritional history (A and B) or direct Se feeding (C and D) measured in whole fry before (A and C) and after (B and D) hypoxic stress. Arrows represent the 5 most contributing variables to the model, respectively: IsoP ARA, sum of isoprostanes from arachidonic acid; IsoP EPA, sum of isoprostanes from eicosapentaenoic acid; NeuroP DHA, sum of neuroprostanes from docosahexaenoic acid; GSHt, total glutathione; GSSG, oxidized glutathione. Ellipses represent the 95% confidence intervals around a center of 9 rearing tanks (pool of 3 individuals).

resistance towards the hypoxic stress compared to fry originating from one of the Se-supplemented groups (Fig. 1). Similarly, during the first 5 min of the experimental period fry directly fed the control diet (Fnc) showed a significantly better stress resistance compared to fry fed the diet supplemented with organic Se (Fso). However, considering the overall period of 30 min Fnc had a significantly lower stress resistance compared to Fso. The stress resistance of fry fed the inorganic selenium source (Fss) was not significantly different from the two other groups, neither within the first 5 min nor for the whole stress period.

3.2. Effect of acute hypoxia on oxidative status and antioxidant system of rainbow trout fry

Clustering of fatty acids in PCA between normoxic and hypoxic stressed fry indicated significant changes in the fatty acid profile by the hypoxic stress challenge (Fig. 2A). The main contributing variables were n-3 PUFA with DHA. The significant decrease of the PUFA: ARA, EPA and DHA was at the expense of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) that increased, leading to a reduced unsaturation index (UI) after the stress challenge (Table 4), whereas ALA levels were not significantly modified (6.8 ± 0.1 vs

$6.7 \pm 0.1\%$ of total fatty acids).

In line with the clustering of the data in PCA, also other oxidative markers were found to be affected by the hypoxic stress challenge (Fig. 2B). Significant changes include an increase of F₂-IsoP, produced from peroxidation of ARA, and an increase of PhytoP and PhytoF produced from peroxidation of ALA (Table 4). On the other hand, a significant decrease of NeuroP produced from peroxidation of DHA, was found in stressed fry. In addition, in stressed fry, GSHt, GSH and GSSG were significantly decreased compared to normoxic fry. Other markers for oxidative stress including tocopherol (2.3 ± 0.1 vs 2.1 ± 0.1 µg/g), vitamin C (14 ± 0 vs 14 ± 0 mg/kg) and protein carbonyl (21 ± 1 vs 21 ± 1 nmol/mg prot) levels were not significantly affected by hypoxic conditions.

The antioxidant enzyme activity was not clustered for hypoxic stress and accordingly, none of the analyzed enzymes significantly changed in activity before and after stress (Fig. 2C). In contrast, the expression of genes coding antioxidant enzymes, but also other proteins involved in the antioxidant system was strongly clustered by the hypoxic stress challenge (Fig. 2D). The main contributing variables included *gpx1b1*, *gpx4b*, *gstπ* as genes coding for antioxidant enzymes, but also the selenoprotein *selp1* and the transcription factor *nfxb*. In general,

Table 5

Parental and direct feeding effect on selected biomarkers of fatty acids and oxidative stress assessed in rainbow trout fry before and after the hypoxic stress challenge.

	Parental effect (PE)			Direct feeding effect (DF)			p-value		
	Bnc	Bss	Bso	Fnc	Fss	Fso	PE	DF	PE×DF
Before stress									
ARA ^a	0.88 ± 0.01	0.86 ± 0.02	0.87 ± 0.01	0.87 ± 0.01	0.86 ± 0.01	0.87 ± 0.02	0.68	0.84	0.73
EPA ^a	2.40 ± 0.03	2.36 ± 0.03	2.47 ± 0.04	2.38 ± 0.03	2.44 ± 0.03	2.40 ± 0.05	0.09	0.44	0.31
DHA ^a	7.80 ± 0.09	7.97 ± 0.13	7.76 ± 0.41	7.88 ± 0.10	7.75 ± 0.09	7.90 ± 0.13	0.41	0.62	0.78
SFA ^a	22.1 ± 0.2	22.5 ± 0.1	22.2 ± 0.2	22.2 ± 0.1	22.3 ± 0.2	22.4 ± 0.1	0.17	0.62	0.63
MUFA ^a	35.7 ± 0.1	35.5 ± 0.2	35.7 ± 0.1	35.7 ± 0.1	35.8 ± 0.1	35.5 ± 0.2	0.57	0.37	0.43
UI	170 ± 1	171 ± 0	171 ± 0	171 ± 1	171 ± 0	170 ± 0.4	0.34	0.59	0.82
GSHt ^b	13.2 ± 0.5 ^a	11.0 ± 0.6 ^b	11.5 ± 0.5 ^b	12.6 ± 0.5	11.3 ± 0.5	11.8 ± 0.6	0.01	0.19	0.59
GSSG ^b	1.68 ± 0.10	1.33 ± 0.15	1.33 ± 0.12	1.56 ± 0.10	1.32 ± 0.16	1.45 ± 0.12	0.10	0.45	0.88
GSH ^b	11.5 ± 0.5 ^a	9.7 ± 0.5 ^b	10.1 ± 0.5 ^{ab}	11.0 ± 0.5	10.0 ± 0.5	10.3 ± 0.5	0.04	0.34	0.70
F ₂ -IsoP ³	0.17 ± 0.04	0.15 ± 0.02	0.21 ± 0.03	0.12 ± 0.02	0.23 ± 0.04	0.16 ± 0.03	0.37	0.06	0.55
IsoP EPA ³	0.35 ± 0.02	0.29 ± 0.03	0.36 ± 0.05	0.26 ± 0.02 ^b	0.38 ± 0.03 ^a	0.37 ± 0.03 ^{ab}	0.27	0.02	0.70
A _{2t} -IsoP ³	0.33 ± 0.02	0.29 ± 0.03	0.36 ± 0.06	0.28 ± 0.02	0.34 ± 0.05	0.37 ± 0.05	0.23	0.09	0.05
F _{3t} -IsoP1 ³	0.17 ± 0.01	0.15 ± 0.01	0.17 ± 0.03	0.13 ± 0.01 ^b	0.19 ± 0.02 ^a	0.18 ± 0.02 ^{ab}	0.70	0.04	0.77
F _{3t} -IsoP2 ³	0.18 ± 0.01	0.14 ± 0.01	0.19 ± 0.03	0.13 ± 0.02 ^b	0.19 ± 0.02 ^a	0.19 ± 0.02 ^a	0.08	0.02	0.66
PhytoP + F ³	24.0 ± 2.5	19.9 ± 2.2	26.4 ± 2.8	22.1 ± 2.7	23.0 ± 2.8	25.2 ± 2.5	0.21	0.67	0.38
NeuroP ³	15.6 ± 0.6	14.6 ± 1.0	16.7 ± 2.0	13.0 ± 0.6	17.0 ± 1.5	16.8 ± 1.2	0.50	0.06	0.61
NeuroP7 ³	0.79 ± 0.05	0.70 ± 0.04	0.88 ± 0.01	0.63 ± 0.03 ^b	0.83 ± 0.08 ^{ab}	0.89 ± 0.07 ^a	0.11	0.02	0.29
α-tocopherol ^d	1.08 ± 0.08 ^b	1.51 ± 0.01 ^a	1.14 ± 0.09 ^b	1.33 ± 0.01	1.17 ± 0.08	1.22 ± 0.01	0.01	0.48	0.50
Vitamin C ^e	14.6 ± 0.2	14.4 ± 0.3	13.8 ± 0.7	14.3 ± 0.6	14.1 ± 0.5	14.4 ± 0.5	0.48	0.94	0.12
After stress									
ARA ^a	0.84 ± 0.01	0.83 ± 0.01	0.86 ± 0.01	0.86 ± 0.01	0.83 ± 0.01	0.84 ± 0.01	0.06	0.15	0.49
EPA ^a	2.31 ± 0.03	2.26 ± 0.03	2.34 ± 0.02	2.29 ± 0.02	2.35 ± 0.02	2.27 ± 0.04	0.18	0.16	0.76
DHA ^a	7.55 ± 0.07	7.67 ± 0.07	7.68 ± 0.07	7.70 ± 0.08	7.61 ± 0.06	7.59 ± 0.07	0.33	0.54	0.57
SFA ^a	22.5 ± 0.2	22.5 ± 0.1	22.4 ± 0.1	22.4 ± 0.1	22.5 ± 0.1	22.6 ± 0.1	0.78	0.44	0.96
MUFA ^a	36.1 ± 0.1	35.8 ± 0.1	35.9 ± 0.2	36.0 ± 0.1	35.9 ± 0.1	36.0 ± 0.1	0.21	0.76	0.83
UI	170 ± 1	171 ± 0	171 ± 0	171 ± 1	171 ± 0	170 ± 0	0.34	0.59	0.82
GSHt ^b	11.4 ± 0.7	10.0 ± 0.4	9.9 ± 0.4	10.6 ± 0.5	10.1 ± 0.8	10.6 ± 0.3	0.15	0.80	0.88
GSSG ^b	1.27 ± 0.14	1.18 ± 0.07	1.11 ± 0.12	1.17 ± 0.11	1.18 ± 0.15	1.22 ± 0.10	0.66	0.96	0.84
GSH ^b	10.1 ± 0.6	8.8 ± 0.5	8.8 ± 0.4	9.4 ± 0.5	9.0 ± 0.7	9.4 ± 0.3	0.16	0.78	0.88
F ₂ -IsoP ^c	0.26 ± 0.05	0.22 ± 0.04	0.28 ± 0.04	0.30 ± 0.05	0.25 ± 0.03	0.21 ± 0.04	0.74	0.46	0.75
IsoP EPA ^c	0.35 ± 0.03	0.32 ± 0.03	0.34 ± 0.02	0.24 ± 0.03 ^a	0.24 ± 0.03 ^a	0.29 ± 0.03 ^b	0.73	0.01	< 0.01
A _{2t} -IsoP ^c	0.22 ± 0.06	0.24 ± 0.06	0.22 ± 0.03	0.23 ± 0.04 ^{ab}	0.26 ± 0.06 ^a	0.19 ± 0.04 ^b	0.31	< 0.01	< 0.01
F _{3t} -IsoP1 ^c	0.17 ± 0.02	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.01 ^a	0.16 ± 0.01 ^a	0.14 ± 0.02 ^b	0.38	0.01	< 0.01
F _{3t} -IsoP2 ^c	0.18 ± 0.01	0.18 ± 0.02	0.17 ± 0.01	0.18 ± 0.01	0.19 ± 0.02	0.16 ± 0.02	0.70	0.24	0.03
PhytoP + F ^c	29.8 ± 2.6	28.1 ± 3.1	26.8 ± 3.1	29.2 ± 3.2	29.2 ± 3.4	26.3 ± 2.1	0.66	0.60	0.01
NeuroP ^c	13.9 ± 0.9	12.2 ± 0.7	13.6 ± 0.7	12.8 ± 0.8	14.0 ± 0.7	12.9 ± 0.9	0.29	0.50	0.29
NeuroP7 ^c	0.75 ± 0.07	0.62 ± 0.06	0.72 ± 0.06	0.64 ± 0.07	0.75 ± 0.06	0.69 ± 0.06	0.30	0.45	0.39
α-tocopherol ^d	1.01 ± 0.09 ^b	1.33 ± 0.09 ^a	1.00 ± 0.10 ^b	1.18 ± 0.11	1.21 ± 0.09	0.96 ± 0.10	0.02	0.09	0.19
Vitamin C ^e	14.3 ± 0.23	13.7 ± 0.64	14.2 ± 0.51	13.3 ± 0.39 ^b	13.9 ± 0.49 ^{ab}	15.0 ± 0.28 ^a	0.52	0.03	0.70

Values are mean ± SEM (n = 9 rearing tanks, three broodstock or three fry treatments and each is a pool of three individuals). Within rows and for each diet related effect: parental (PE) or direct feeding (DF), means not sharing a common superscript letter are significantly different according to two-way ANOVA followed by Tukey's HSD.

^a [% of total fatty acids].

^b [μM/mg protein].

^c [pg/kg].

^d [ng/kg].

^e [mg/kg].

selenoproteins were found to be both significantly up- and down-regulated by the hypoxic stress (Fig. 3). Isoforms for *selpa* and *gpx1a* were found to be upregulated after the stress, while isoforms for *gpx4* and *trxr3* genes were found to be downregulated. Also the expression of other genes coding antioxidant enzymes, including *sod1*, *sod2*, *gstv*, *gclc1* and *msrb2* was reduced after the hypoxic stress challenge. On the other hand, transcription factors including *keap1b*, *nfbk* with its inhibitors *ikb* and *ikb2* were higher expressed after the stress challenge, while *tnfa3* significantly decreased in stressed fry. A significantly higher mRNA expression in stressed compared to normoxic fry was also found for *ahra*.

3.3. Effect of selenium nutrition on the fatty acid profile

In accordance with the lack of cluster for direct or parental Se nutrition (Fig. 4 A and B), only few fatty acids showed significant differences according to parental nutritional history of Se. In normoxic fry, 18:4n-3 was significantly lower, when originating from parents fed

sodium selenite (Bss) compared to the control (Bnc), while 20:2n-6 decreased in Bss compared to both other parental groups and 18:3n-6 was significantly higher in fry originating from parents fed organic Se-supplemented diets (Bso) compared to the two other groups. On the other hand, the mono-unsaturated fatty acid 20:1 was significantly lower in Bss compared to Bnc, both in normoxic and stressed fry. In stressed fry, 14:0 and 15:0 were both significantly reduced when originating from parents fed Bnc compared to Bss, while 18:0 was found to be significantly higher in Bnc compared to Bss (data not shown: 2-w ANOVA, p < 0.05). Similar to parental feeding, fatty acids showed no clustering according to direct Se nutrition before and after stress (Fig. 4C and D), but in normoxic fry, 22:4n-6 was significantly higher when fed organic Se-supplemented diets (Fso) compared to fry fed the control diet (Fnc), while 22:5n-6 was significantly higher in the group fed Fso compared to fry fed inorganic selenium supplemented diets (Fss). In addition, in stressed fry 12:0 and 14:0 were both significantly lower in Fnc compared to the supplemented groups and 15:0 significantly higher in Fso compared to Fss and Fnc (data not shown: 2-w

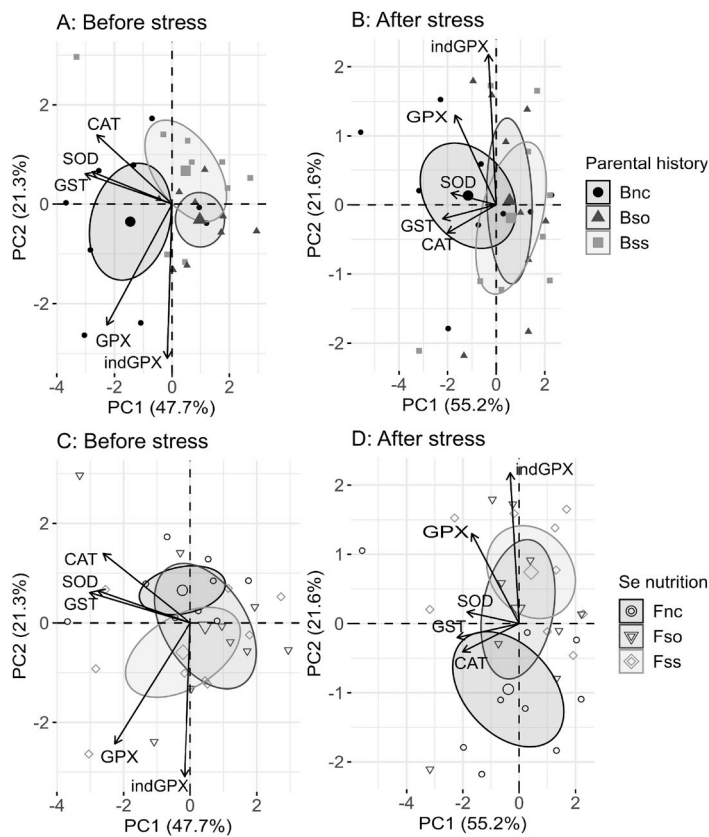


Fig. 6. PCA biplots on specific antioxidant enzyme activity grouped by either their parental nutritional history (A and B) or direct Se feeding (C and D) measured in whole fry before (A and C) and after (B and D) hypoxic stress. Arrows represent the 5 most contributing variables to the model, respectively (CAT, catalase; SOD, superoxide dismutase; GST, glutathione S-transferase; GPX, total glutathione peroxidase; indGPX, seleno-independent GPX). Ellipses represent the 95% confidence intervals around a center of 9 rearing tanks (pool of 3 individuals).

Table 6

Changes in specific antioxidant enzyme activity by parental history and direct Se nutrition measured in rainbow fry before and after subjected to a hypoxic stress challenge.

	Parental effect (PE)			Direct feeding effect (DF)			p-value		
	Bnc	Bss	Bso	Fnc	Fss	Fso	PE	DF	PE×DF
Before stress									
GPX ^a	28 ± 2 ^a	23 ± 1 ^b	24 ± 1 ^{ab}	23 ± 1 ^b	27 ± 2 ^a	25 ± 1 ^{ab}	0.03	0.03	0.89
indGPX ^a	9 ± 1	6 ± 1	8 ± 1	7 ± 1	9 ± 1	8 ± 1	0.15	0.28	0.11
GR ^a	14 ± 1	11 ± 1	12 ± 1	13 ± 1	13 ± 1	11 ± 1	0.13	0.43	0.43
CAT ^b	61 ± 4 ^a	51 ± 6 ^{ab}	44 ± 2 ^b	54 ± 5	51 ± 4	52 ± 5	0.02	0.82	0.51
GST ^a	125 ± 7 ^a	111 ± 7 ^{ab}	97 ± 7 ^b	121 ± 5 ^a	109 ± 9 ^{ab}	102 ± 7 ^b	0.01	0.05	0.03
SOD ^b	9 ± 1	7 ± 1	7 ± 0	8 ± 1	7 ± 1	8 ± 1	0.25	0.66	0.63
After stress									
GPX ^a	28 ± 2	24 ± 2	25 ± 2	23 ± 2	27 ± 2	27 ± 2	0.39	0.28	0.76
indGPX ^a	9 ± 1	7 ± 1	7 ± 2	5 ± 1 ^b	10 ± 1 ^a	8 ± 1 ^{ab}	0.53	0.03	0.90
GR ^a	15 ± 1	14 ± 2	13 ± 1	15 ± 1	12 ± 2	14 ± 1	0.19	0.11	0.31
CAT ^b	71 ± 9	51 ± 7	52 ± 3	65 ± 9	51 ± 5	58 ± 5	0.08	0.45	0.72
GST ^a	125 ± 11	106 ± 8	103 ± 5	118 ± 11	106 ± 7	110 ± 8	0.18	0.57	0.86
SOD ^b	9 ± 2	7 ± 1	7 ± 0	9 ± 2	7 ± 1	7 ± 1	0.26	0.83	0.22

Values represent mean ± SEM (n = 9 rearing tanks, three broodstock or three fry treatments, three pooled fry). Within rows and for each diet related effect: parental (PE) or direct feeding (DF), means not sharing a common superscript letter are significantly different according to two-way ANOVA followed by Tukey's HSD.

^a [mU/mg prot].

^b [U/mg prot].

ANOVA, p < 0.05).

3.4. Effect of selenium nutrition on oxidative stress markers

In normoxic fry, oxidative markers showed a clustering by parental dietary history in PCA with separate groups for fry originating from parents fed the control diet (Bnc) and fry originating from parents fed the Se-supplemented diets, however, with a small overlap between Bnc and fry originating from parents fed the hydroxy-selenomethionine supplemented diet Bso (Fig. 5A). The main contributing variables were

GSht and GSH as well as IsoP EPA and ARA and NeuroP DHA, but none of these variables showed a significant main effect for parental nutrition by two-way ANOVA, where only α-tocopherol levels were significantly higher in fry originating from parents fed sodium selenite (Bss) compared to both other groups (Table 5).

In stressed fry, the analyzed markers for oxidative stress were not clustered according to parental nutritional history anymore (Fig. 5B). Nonetheless, a similar parental effect for α-tocopherol compared to normoxic fry was detected (Table 5). In addition, a significant interaction, for the parental nutritional history in the sodium selenite

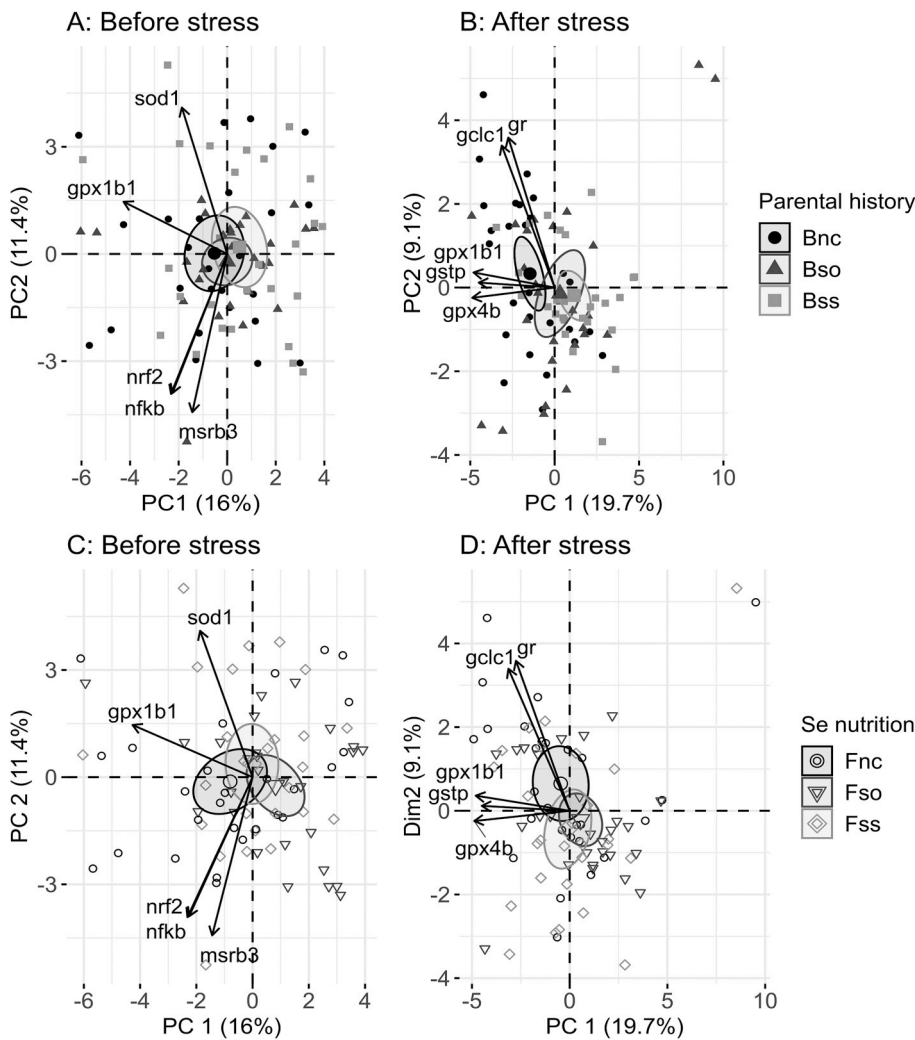


Fig. 7. PCA biplots on gene expression of antioxidant proteins grouped by either their parental nutritional history (A and B) or direct Se feeding (C and D) measured in whole fry before (A and C) and after (B and D) subjected to hypoxic stress. Arrows represent the 5 most contributing variables to the model, respectively (*gpx*, glutathione peroxidase with *gpx1b1* and *gpx4b*; *sod1*, superoxide dismutase; *gr*, glutathione reductase; *gclc*, glutathione-cysteine ligase catalytic subunit; *gstp*, glutathione-s-transferase π ; *nrf2*, nuclear factor erythroid 2-related factor 2; *nfkb*, nuclear factor kappa-light chain-enhancer of activated B cells). Ellipses represent the 95% confidence intervals around a center of 27 individuals.

treatment was found for all PhytoF and all PhytoP, except PhytoP5, with the highest concentration in the BssFss treatment and the lowest concentration in the BsoFss treatment (39 ± 3 vs 17 ± 1 pg/g sample). No clustering by PCA according to oxidative stress markers was found for direct Se nutrition either in normoxic or hypoxic stressed fry (Fig. 5C and 5D). Nonetheless, stressed fry fed hydroxy-selenomethionine (Fso) had significantly higher vitamin C levels compared to fry fed the control diet (Fnc). IsoP EPA showed a significant interaction between parental and direct Se nutrition for fry originating from parents fed Bss with significantly higher IsoP EPA in BssFss compared to BssFnc and BssFso (42 ± 2 vs 28 ± 1 and 26 ± 1 pg/g sample).

3.5. Effect of selenium nutrition on the antioxidant system

The antioxidant enzyme activity clustered according to parental nutritional history in normoxic, but not in hypoxic stressed fry (Fig. 6A and 6B). Separate clusters were found for fry originating from parents of the control group (Bnc) compared to fry originating from parents of the hydroxy-selenomethionine group (Bso), while Bnc and fry from parents of the sodium selenite group (Bss) showed a small overlap. Accordingly, the enzyme activity in normoxic fry was significantly increased in Bnc for total GPX compared to Bss and for CAT compared to Bso. The activity of GST had an interactive effect between parental and direct Se nutrition with the highest concentration in the control group and the lowest concentration in the group fed organic Se, respectively (133 ± 8 mU/mg prot in BncFnc vs 86 ± 10 mU/mg prot in BsoFso). In stressed fry there was no significant difference between the groups

according to parental nutritional history for any of the measured enzymes (Table 6).

PCA of antioxidant enzymes grouped by direct Se nutrition gave two separate clusters: fry fed the control diet (Fnc) and fry fed the sodium selenite supplemented diet (Fss), both, before and after stress (Fig. 6). The two main contributing variables in the models were GPX and indGPX (Fig. 6) that were significantly lower in Fnc compared to Fss in normoxic fry for GPX and in stressed fry for indGPX (Table 6).

Despite the lack of clusters by parental nutritional history of gene expression data in normoxic fry (Fig. 7A), the control group Bnc displayed higher mRNA levels of *gr* compared to both Se-supplemented groups, of *msra1* compared to the sodium selenite supplemented group Bss and of *ppara* compared to the hydroxy-selenomethionine supplemented group Bso. In stressed fry, 12 genes in total were significantly higher expressed in Bnc compared to Bss leading to a strong clustering between these two groups in PCA (Fig. 7B). Thereby, mRNA levels for *msra1*, *msrb3*, *keap1b*, *hifab2*, *ahra* and *ppara* were also significantly higher in Bso compared to Bss (Fig. 8A). For the genes *selpa2*, *gpx1b1* and *gpx1b2* Bso was in-between. In both Se-supplemented groups *gstp*, *gclc1* and *ixb* mRNA levels were reduced compared to Bnc. The gene *tfa3* was lower only in Bso compared to Bnc.

Both, in normoxic and hypoxic stressed fry, gene expression data were not clustered according to direct Se nutrition (Fig. 7C and 7D). Nevertheless, in normoxic fry *gpx1b1* was higher expressed in the group Fss fed diet supplemented with sodium selenite compared to fry fed diet Fso supplemented with hydroxy-selenomethionine and *msrb1a* was lower in both Se-supplemented groups compared to the control group

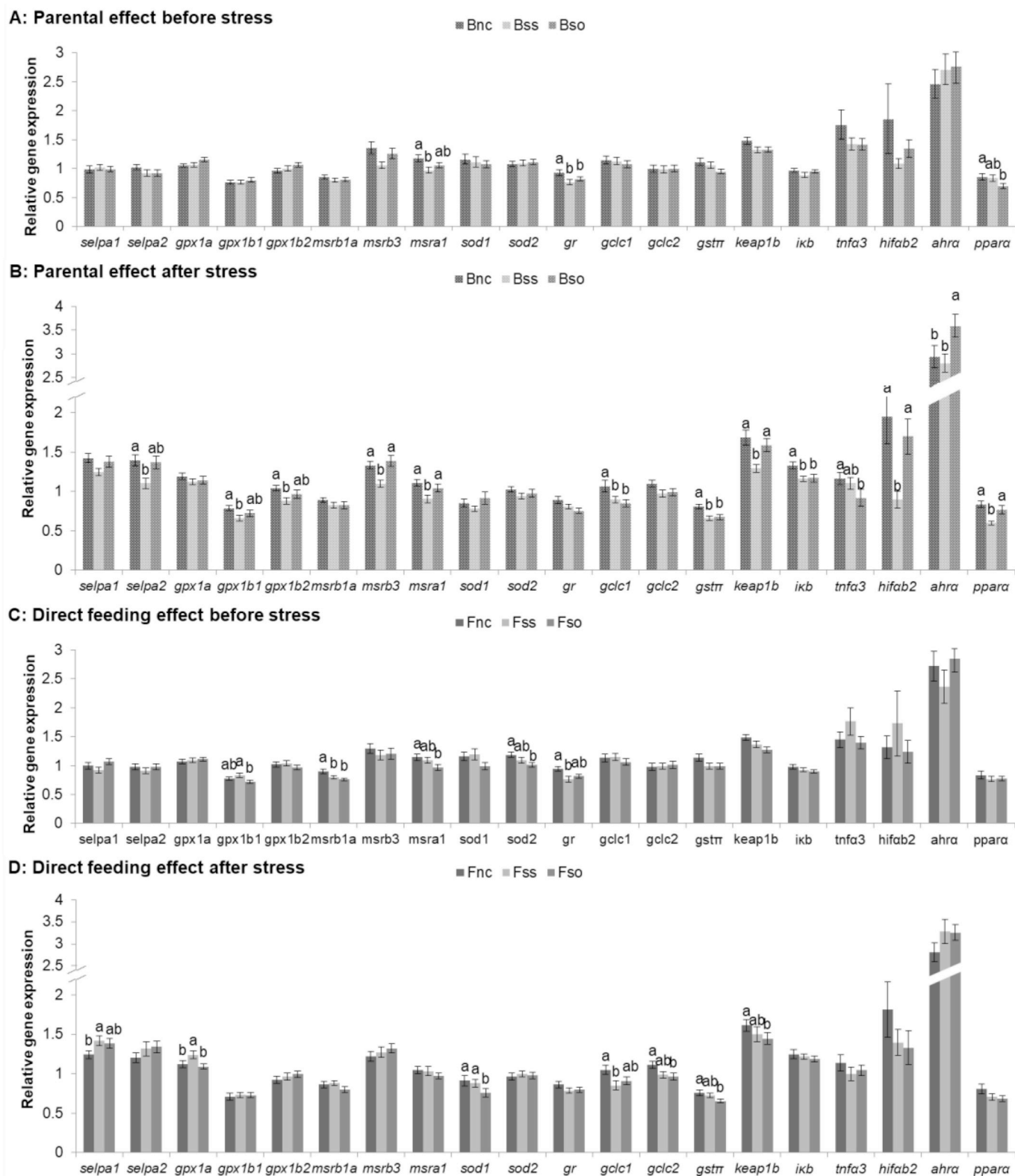


Fig. 8. Genes showing a significant main effect for parental (A + B) or direct feeding (C + D) analyzed in fry before (A + C) and after (B + D) the hypoxic stress challenge. Relative gene expression normalized to a geometric mean of EF1 α and β -actin and expressed as fold-changes of mRNA abundance compared to a sample from before stress in the Bnc/Fnc control group. Bars represent means \pm SEM ($n = 27$ rearing tanks representing 3 individuals from 9 rearing tanks per dietary group). Differences in superscript letters are showing significant differences according to two-way ANOVA followed by Tukey's HSD.

Fnc (Fig. 8B). In addition, *sod2* and *msra1* were higher expressed in Fnc compared to Fso and *gr* was higher expressed in Fnc compared to Fss. In stressed fry, the selenoproteins *selpa1* and *gpx1a* were significantly

decreased in Fnc compared to Fss and in contrast *gclc1* was lower expressed in Fss compared to the Fnc. Other non-selenoproteins affected include *sod1*, *gstrr*, *gclc2* and *keap1b* that were higher expressed in Fnc

compared to Fso.

4. Discussion

Hypoxic stress challenge increased oxidative stress in rainbow trout leading to changes in the fatty acid profile and transcriptional regulation of antioxidant proteins.

We found that the hypoxic stress challenge led to significant changes in the oxidative status. In goldfish, lipid peroxidation was found to be the most rapid response to hypoxic conditions [29], which is consistent with the present findings that F₂-IsoP, but also PhytoP and PhytoF production was enhanced by the hypoxic stress challenge. In parallel, the fatty acid composition of the fish changed with the expected decrease of PUFA including the LC-PUFA such as ARA, EPA and DHA. As NeuroP levels are a byproduct inversely correlated to DHA [30], we expected to find a parallel increase also for NeuroP levels, but on the contrary, NeuroP decreased in stressed fish. This could indicate an increased excretion of isoprostanooids, possibly through the activation of the antioxidant system as GST can catalyze the conjugation of lipid peroxidation products with glutathione thus playing a major role in the isoprostanooid detoxification [31,32]. In the present study, neither GST, nor the activity of other antioxidant enzymes was affected by hypoxic stress, similar to results from another study in rainbow trout exposed to hypoxia [33]. The authors of this latter study proposed that either there might be a non-systematic relationship between the activity levels and the antioxidant defense or fish use other endogenous antioxidants, not usually determined to protect themselves from oxidative stress. However, the antioxidant response might differ on tissue level as found in common carp where CAT activity increased in brain, but not in liver, kidney or muscle and GPX activity increased in brain, but decreased in liver and muscle tissue when exposed to hypoxia [34,35]. In the present study, GSHt and GSSG both decreased during the hypoxic stress challenge, which is different from findings in common carp where the glutathione levels remained stable [34]. Lushchak and Bagnyukova [12] postulated that the maintenance of the GSH/GSSG ratio during stressful conditions seems to be an important protective mechanism for fish undergoing oxidative stress, but mainly referring to studies on periodic changes between normoxic and hyperoxic conditions. In some

mammalian cells and also in several fish species, hypoxia has been linked to an increase in mitochondrial ROS production and therefore oxidative stress leading to an activation of the antioxidant metabolism [33,36]. Our data suggest a transcriptional regulation of the antioxidant defense in rainbow trout exposed to hypoxia through the modulation of the transcription factors Nrf2 and NF-κB leading to a downregulation of antioxidant enzyme mRNA expression. These findings differ from effects observed in cell cultures, where the mRNA expression of *gpx4* was upregulated, while *gpx1a* and *selp* were downregulated in hypoxic conditions [37] and might be connected to a more complex antioxidant response to acute hypoxia in fish that possibly differ on a time and tissue specific manner. The regulation of Nrf2 activity is mainly known to take place on posttranscriptional level mediated by *keap1*, which was similarly upregulated by hypoxia in the present study. However, also transcriptional regulation has been described before in human cells [38] and rainbow trout [39,40] where Nrf2 mRNA expression increased in first feeding rainbow trout fry exposed to oxidative stress possibly related to an incomplete development of the antioxidant system as such effect was not found at later stages in fingerlings or juveniles, where decreasing cytosolic Nrf2 levels suggested a nuclear translocation of Nrf2. It seems contradictory that parallel to the increased mRNA expression of transcriptional factors the antioxidant enzyme mRNA expression was downregulated by hypoxia, but it should be considered that the antioxidant response is a dynamic process where the energetic cost for the biosynthesis of antioxidants can impose a drain on the reductant pool that can only be maintained by the increased activity of glutathione reductase or glutathione production tightly regulated by feedback mechanisms [41]. Therefore, metabolic active organs might give a more differentiated picture compared to whole-body levels and repeated measurements allow a better insight in the time-dependent reaction process.

4.1. Parental Se nutrition can cause long-term changes in the antioxidant metabolism of the progeny

No significant difference in survival and final body weight was found in the present study using fry feeds with Se levels ranging between 0.3 and 0.6 mg/kg in accordance with the minimal Se

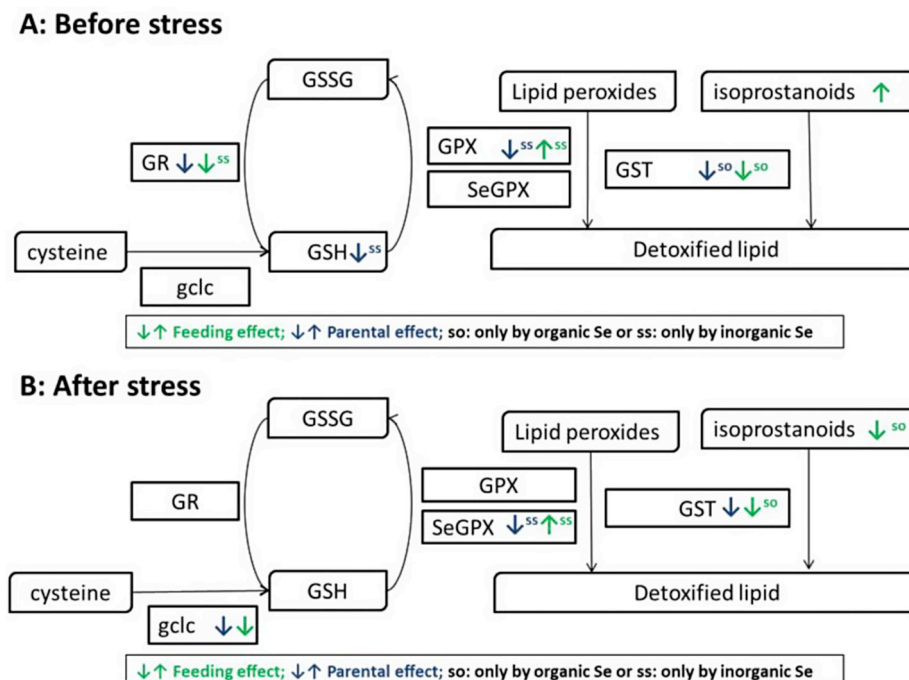


Fig. 9. Effect of parental and direct Se nutrition on the glutathione metabolism in rainbow trout fry before (A) and after (B) subjected to a hypoxic stress challenge.

requirements of 0.15 mg/kg in rainbow trout given by NRC [42]. However, significantly higher weight gain was found by interactive effects between the direct and parental Se nutrition. As the final body weight was similar between groups the higher growth could be a compensatory effect to the lower body weight of swim-up fry in the sodium selenite treatment. On the other hand, it could be also connected to the higher α -tocopherol levels in fry originating from the sodium selenite treatment Bss as Se requirements can vary according to dietary α -tocopherol levels [43]. A positive effect by combined vitamin E and Se-supplementation on the growth rate of rainbow trout have been described in double deficient diets [44]. However, the levels of dietary vitamin E supplied were presumed to meet the requirements of rainbow trout according to NRC [42] and the weight gain was not elevated in the sodium selenite group fed hydroxy-selenomethionine. As part of the deiodinase enzyme system, Se can also regulate thyroid hormones essential for the stimulation of growth hormones in fish [45,46]. We find here that the oxidative status is modified by Se-supplementation with a lower consume of α -tocopherol by parental Se nutrition already detected at swim-up fry stage [19]. On the other hand, IsoP EPA and NeuroP7 levels indicate increased lipid peroxidation in fry fed Se-supplemented diets, which is contrary to other studies were malondialdehyde and F₂-isoP levels linearly decreased with increasing serum Se levels [47] and might be connected to an increased antioxidant metabolism in the control group as indicated by higher GST. Surprisingly, we did not detect an effect of direct dietary Se-supplementation on the activity or mRNA expression of SeGPX whereas in a previous study with rainbow trout fry, the supplementation of 0.3 mg Se/kg to a plant-based diet with a basal Se level of 0.5 mg/kg led to increased whole-body SeGPX activity associated with increased SeGPX gene *gpx1b1*, *gpx1b2* and *gpx4a1* isoform mRNA expression [18]. The differences between the mentioned study and our present results might be related to the lower dietary Se levels used (0.9 vs 0.5 mg/kg) and raises the question, if the Se levels in the present study might have been deficient. For total GPX activity, a contrary effect between direct and parental Se nutrition was found in the sodium selenite supplemented group, which could also explain why, in the present study, no effect on total or SeGPX by organic Se-supplementation in fry diet was detected, although organic Se is known to be better bioavailable and therefore to have stronger effects on SeGPX [18,48] (Fig. 9). This contrary effect was not found for non-selenoprotein antioxidant enzymes that were more active and higher expressed in the control group by both parental and direct feeding. Elevated antioxidant enzyme activity could be an indicator for oxidative stress in the control group due to Se deficiency, as also found in Se-deficient mouse exhibiting increased GST levels [49]. The positive impact of the hydroxy-selenomethionine supplementation in fry diets on the antioxidant protection became visible during the hypoxic stress challenge where these fish could resist the stressful conditions longer compared to fish fed the non-supplemented diet. Similar to GPX, a contrary parental effect on hypoxic stress resistance was found for fry originating from broodstock fish fed Se-supplemented diets suggesting a compensatory effect in fry originating from parents fed Se-supplemented diets. Our results, summarized in Fig. 9, suggest long-lasting modifications of the glutathione metabolism by parental Se nutrition with reduced *gr* mRNA expression by Se-supplementation and lower GSH levels in this group, which is similar to findings at swim-up fry stage where parental Se nutrition decreased GSH levels [19]. However, the observed differences between the two Se-supplemented treatments deserve further investigation as they might be connected to the Se metabolism were organic and inorganic dietary sources result in different Se compounds and tissue deposition [50].

In hypoxic conditions, direct and parental Se nutrition show interactive effects on isoprostanoid levels and both modify antioxidant enzyme mRNA expression.

In hypoxic conditions, higher vitamin C levels remained in fry fed hydroxy-selenomethionine compared to fry fed the non-supplemented diet. At swim-up fry stage, higher vitamin C concentrations in the

hydroxy-selenomethionine treatment were found possibly related to a higher demand for antioxidants in treatments with lower Se levels [19]. Accordingly, the levels of A_{2t}-IsoP and F_{3t}-IsoP in the hydroxy-selenomethionine group were lower, which is similar to findings in albino rats where Se-supplementation inhibited lipid peroxidation, when exposed to hypoxic conditions [51]. However in the present study, the impact of sodium selenite supplementation on lipid peroxidation in hypoxic conditions was less clear, as interactive effects between parental and direct Se nutrition for PhytoP, PhytoF and IsoP EPA were detected. Interactive effects between parental history and Se nutrition could relate to a nutritional programming by the Se stimulus in broodstock fish that correlates to effects by dietary Se in fry and reveals a physiological modification under stressful conditions as seen for other nutritional stimuli [52,53]. On the other hand, comparable higher α -tocopherol levels were found in fry originating from the parental group fed a sodium selenite supplemented diet and studies on rats showed that lipid peroxidation was not associated to Se deficiency alone, but rather in combination with vitamin E deficiency [54]. Nonetheless, other oxidative stress markers were not improved by the Se feeding in hypoxic stressed fry, including the GSH content, contrary to observations of increase by Se-supplementation in neuroblastoma cells exposed to hypoxic conditions [55]. Several studies have reported a positive effect of Se-supplementation on the activity of antioxidant enzymes under hypoxic conditions [51,55,56]. However, in the present study, the dietary effect of Se on the activity of antioxidant enzymes, with the exception of indGPX, was only found in normoxic, but not hypoxic fish, which could indicate interactive effects between the hypoxic stress and the Se nutrition on the antioxidant enzyme activity. Indeed as hypoxic stress was found to reduce mRNA GPX expression, the effect of Se deficiency, that is similar, should have been masked. Another explanation could be the low Se levels in the present study that were possibly deficient even in Se-supplemented groups under hypoxic conditions and highlights the necessity to quantify the Se requirement in rainbow trout fry. Sarada et al. [55] also described that Se-supplementation could stabilize HIF1 α levels *in vitro* in neuroblastoma cells. In the present study two results can be noted, first, the mRNA expression of *hif1a* was not significantly different before and after stress and second, the *hif1ab2* isoform was only found to be significantly reduced in fry originating from parents fed sodium selenite, but not hydroxy-selenomethionine. The expression of this gene showed high variability and it could be concluded that the 30 min stress challenge in this trial was not sufficient to trigger the mRNA *hif1a* expression in all stressed fish. Also, in first feeding rainbow trout fry, an acute hypoxic stimulus for 24h was not found to effect the mRNA levels of *hif1a* [57], possibly because *hif1a* is regulated on a post-transcriptional level to be degraded in the presence of oxygen, but not under hypoxic conditions [58]. However, in hypoxic fish, several of the genes affected by the hypoxic stress were found to be affected also by parental or direct Se nutrition, which highlights that genes affected by Se nutrition are relevant to the antioxidant response in hypoxic conditions in rainbow trout. Our results suggest that under hypoxic conditions parental Se nutrition is especially important not only for the transcriptional regulation of antioxidants including selenoproteins, but also in link to other metabolic pathways including inflammatory responses by *tnfa3* and the xenobiotic metabolism by the *ahra* gene.

5. Conclusion

We found in rainbow trout fry that a hypoxic stress challenge increased the oxidative stress, which activated the antioxidant system though the transcriptional regulation of antioxidant proteins. The dietary Se levels used in the diets of fry might have been too low to sustainably improve the antioxidant system in normoxic rainbow trout. In this context, the connection between Se and other cellular antioxidant components like the Keap1-Nrf2 pathway might require a more detailed look on the cellular level. The increase of parental effects detected in stressed fry indicates that Se nutrition can have long-lasting

effects on the antioxidant metabolism even if parental Se supplementation resulted in reduced hypoxic stress resistance of the progeny. Thus, the origin of these long-lasting effects of parental Se supplementation on the antioxidant system in rainbow trout deserves further investigation.

Authors contribution

S.F.-D., P.A.J.P., M.B., S.J.K. and P.W. were involved in the idea and design of the study. P.W., L.L., T.D., C.O., J.-M.G., A.R., C.V., V.V., J.R., B.F. and S.F.-D. were involved in acquisition, analysis and interpretation of data. P.W. drafted the manuscript, while the other authors revised it critically for important intellectual content. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

Funding

This present study was supported by I-site E2S: Energy and Environment Solutions from the University of Pau and Pays de l'Adour, UPPA contract 2017-17 (P.W., PhD fellowship) and Institute of Marine Research (IMR) (P.A.J.P., ParSel Project no. 15329).

Acknowledgement

The authors wish to thank F. Terrier, F. Sandres and A. Lanuque for the preparation of the diets, care of the fish and assistance during the hypoxic stress challenge. The authors are also grateful to C. Heraud for tocopherol analysis, E. Blanchandin for mRNA expression and V. Bultel-Poncé, A. Guy and G. Reversat for the chemical synthesis of isoprostanoids. The helpful advice of W. Massimino concerning data processing using Fluidigm software was also greatly appreciated.

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