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Aquaculture 593 (2024) 741354

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Vitamin B6 and selenium supplementation induce contrasting effects in the transsulfuration pathway of juvenile rainbow trout (*Oncorhynchus mykiss*) with interactive effects in stressed fish

Pauline Wischhusen [a,*], Cécile Heraud [b], Richard Broughton [a], Anne Surget [b], Anthony Lanuque [b], Frédéric Terrier [b], Stéphanie Fontagné-Dicharry [b], Mónica B. Betancor [a]

[a] Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, FK9 4LA Stirling, Scotland, UK
[b] INRAE, Université de Pau et des Pays de l'Adour, E2S UPPA, NUMEA, 64310 Saint-Pée-sur-Nivelle, France

ARTICLE INFO

Keywords:
Vitamin B6
Selenomethionine
Glutathione
Transsulfuration
Hyperoxia stress

ABSTRACT

This study aimed to investigate the interactive effects between selenium and vitamin B6 supplementation in the transsulfuration pathway and glutathione metabolism of juvenile rainbow trout (*Oncorhynchus mykiss*) and in response to oxidative stress. Four plant protein-based diets (48% crude protein and 23% total lipid) naturally low in selenium and vitamin B6 were designed: CTL, without any selenium or vitamin B6 supplementation; SEL, supplemented with 4 mg selenium per kg diet supplied as selenomethionine (SeMet); PYR, supplemented with 50 mg pyridoxine hydrochloride (PN.HCL) per kg diet and SEPY, co-supplemented with SeMet and PN.HCL. Groups of 50 juvenile rainbow trout (28 ± 3 g) were randomly distributed in a flow-through system in triplicate tanks per treatment and fed on one of the experimental diets two times per day for twelve weeks. In addition, 15 fish per tank were exposed to periodic hyperoxia stress for one week prior to sampling. Therefore, the dissolved oxygen levels in the tanks were increased from 8 mg/l to 13 mg/l during 8 h per day. Dietary SeMet supplementation increased feed intake (1.44 ± 0.03 vs. 1.49 ± 0.02), but without any significant differences in final body weight (180 ± 3 vs 184 ± 3 g) between groups. SeMet supplementation was associated with increased liver and muscle S-adenosylhomocysteine levels. There was a general decrease of transsulfuration metabolites by SeMet supplementation in muscle tissue. On the other hand, PN.HCL supplementation increased the gene expression of the first step transsulfuration pathway enzyme, *cbs*, in liver tissue with similarly higher levels of transsulfuration metabolite homocysteine and total glutathione. Fish subjected to periodic hyperoxia showed lower reduced glutathione levels in liver tissue, which indicates modifications to the cellular redox system of fish in response to the stress. In stressed fish, interactive effects of SeMet and PN.HCL supplementation were detected on transsulfuration metabolites in both liver and muscle tissue. The results show that SeMet and PN.HCL supplementation can induce contrasting effects in the transsulfuration system of fish. The significant interactions in stressed fish between SeMet and PN.HCL supplementation indicate that both nutrients are required to maintain glutathione homeostasis under challenging environmental conditions.

1. Introduction

Modern aquafeeds are increasingly formulated using a mix of alternative raw materials that replace fish meal as the dominant protein source in the feeds (Aas et al., 2022). However, this transition is accompanied by several challenges as fish meal is not only considered a gold standard in terms of amino acid profile, but additionally delivers a range of micronutrients to the diet (Hansen et al., 2015; Hemre et al.,

Abbreviation list: CBS, Cystathionine β-synthase; CGL, Cystathionine γ-lyase; CTL, Treatment without selenium or vitamin B6 supplementation; GPX, Glutathione peroxidase; GSH, Glutathione; GSSG, Oxidized glutathione; hCys, Homocysteine; HO, Periodic hyperoxia stress; NO, Normoxic conditions; PL, Pyridoxal; PM, Pyridoxamine; PN, Pyridoxine; PN.HCL, pyridoxine hydrochloride; PYR, Vitamin B6 supplemented treatment; rGSH, Reduced glutathione; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Se, Selenium; SeGPX, Seleno-dependent glutathione peroxidase; SEL, Selenium supplemented treatment; SeMet, Selenomethionine; SEPY, Selenium and vitamin B6 supplemented treatment; TBARS, Thiobarbituric acid reactive substances.

* Corresponding author at: Institute of Marine Research, P.O. Box 1870, Nordnes, Bergen 5817, Norway.
E-mail address: pauline.wischhusen@hi.no (P. Wischhusen).

https://doi.org/10.1016/j.aquaculture.2024.741354
Received 21 March 2024; Received in revised form 4 July 2024; Accepted 12 July 2024
Available online 15 July 2024

2016). Although mineral and vitamin pre-mixes supplement essential nutrients, adaptations to those formulations are difficult due to the lack of knowledge about the micronutrient requirements in many species (NRC, 2011). In addition, there can be legal limitations for the inclusion of specific vitamins and minerals in animal feeds such as selenium, which is known to be toxic in excess (EFSA, 2013). A better understanding of not only micronutrient requirements, but especially their functional role and how they interact will be necessary to efficiently adapt to changing feed formulations in the future.

Vitamin B6 collectively represents pyridoxine (PN) and all its water-soluble (pyridoxal (PL), and pyridoxamine (PM)) and phosphorylated (pyridoxine 5-phosphate, pyridoxamine 5-phosphate and pyridoxal 5-phosphate) forms (Stover and Field, 2015). In animal tissues, the major vitamin B6 forms are PL and PM, whereas PN can be found mainly in plant products. According to NRC (NRC, 2011), the minimum dietary vitamin B6 requirements in rainbow trout (*Oncorhynchus mykiss*) are 3 mg per kg to support normal growth and health. In Atlantic salmon (*Salmo salar*), beneficial effects of a surplus supplementation in plant-ingredient based diets were suggested (Adam et al., 2022). The pyridoxine vitamers exhibit multifunctional roles in the metabolism of fish (Akhtar and Ciji, 2021), such as in one-carbon metabolism, where vitamin B6 enables the transfer of one-carbon groups and acts as a co-factor for enzymes of the transsulfuration pathway.

Selenium (Se) is a micromineral obtained by fish mainly through the diet in the form of selenomethionine (SeMet) (Wang et al., 2022). Although Se induces toxic effects at high concentrations (Berntssen et al., 2018), a minimum Se requirement of 0.15 mg Se per kg diet in juvenile rainbow trout is defined by NRC (NRC, 2011). Recent studies suggest that this level might not be sufficient, and a dietary supplementation could be warranted in plant-based formulations, naturally low in Se (Antony Jesu Prabhu et al., 2016). The essential requirement of Se relates to selenoproteins that carry out key metabolic functions such as glutathione peroxidases (SeGPX) in the antioxidant system (Steinbrenner et al., 2016). Indeed, almost all selenoproteins with known functionality have been described to contribute towards cellular oxidative homeostasis. To be incorporated into selenoproteins, dietary SeMet is metabolized following the methionine cycle and the vitamin B6 dependent transsulfuration pathway to form selenocysteine (Finkelstein, 1998) (Fig. 1).

During the first step of the methionine metabolism, S-adenosylmethionine (SAM) is biosynthesized, which then acts as a methyl donor in various cellular reactions to form S-adenosylhomocysteine (SAH). In the next step, SAH is hydrolysed to homocysteine (hCys), which can then either be regenerated to methionine or further pushed towards transsulfuration (Dalto and Matte, 2017). In the transsulfuration pathway cystathionine is formed from hCys by action of cystathionine β-synthase (CBS) using vitamin B6 as a co-factor (Aitken et al., 2011). In a second step, cystathionine is then further metabolized to cysteine by the action of a vitamin B6 dependent cystathionine γ-lyase (CGL). It is believed that the enzymes in the methionine cycle and transsulfuration pathway act in a similar way on methionine metabolites and their Se-analogues (Kajander et al., 1991). However, selenocysteine will then be directed towards selenoprotein synthesis by further B6-dependent reactions (Esaki et al., 1982), while cysteine (Cys) contributes towards the biosynthesis of taurine or glutathione (GSH). Glutathione, as a major redox molecule, in turn, serves as the substrate for antioxidant enzymes such as SeGPX (Flohe et al., 1973).

Large stocking densities coupled to space constraints within farmed environments are common stressors in farmed fish. In trout, conditions such as fluctuations in water oxygen concentrations have been associated with oxidative stress (Dong et al., 2011; Kalinowski et al., 2019; Ritola et al., 2002), an imbalance of the production of reactive species (e.g., reactive oxygen species) and the antioxidant system. Such redox challenges have major implications for cellular methionine cycling. Generally, oxidative stress has been associated with an increased flux towards transsulfuration, whereas the recycling of methionine is reduced (Mosharov et al., 2000). This suggests that the fate of Se and vitamin B6 might change under stressful conditions and that the availability of these micronutrients could contribute towards the ability of fish to cope with environmental stressors.

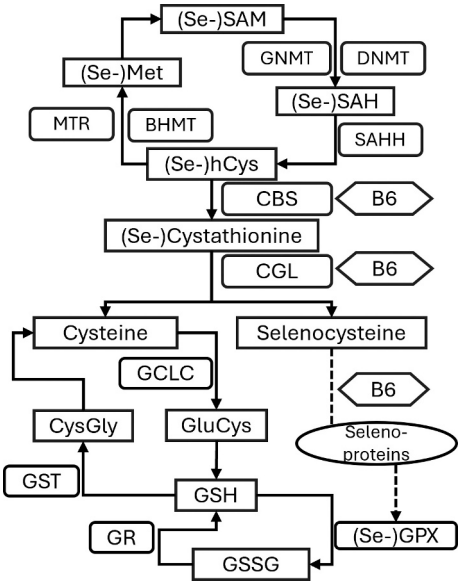

Fig. 1. Selenium (Se) and vitamin B6 (B6) in the methionine cycle, transsulfuration and glutathione pathway.
BHMT, betaine-homocysteine *S*-methyltransferase; CBS, cystathionine-β-synthase; CGL, cystathionine-γ-synthase; CysGly, cysteinyl-glycine; DNMT; DNA methyltransferase; GCLC, glutamate-cysteine ligase; GluCys, γ-glutamyl cysteine; GNMT, glycogen *n*-methyltransferase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; hCys, homocysteine; Met, methionine; MTR, methionine synthase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase.

Therefore, this study was designed to investigate the interactive effect of dietary Se and vitamin B6 in the transsulfuration and GSH metabolism of rainbow trout. In addition, the role of Se and vitamin B6 in rainbow trout under challenging environmental conditions were investigated by exposing fish to fluctuating oxygen conditions prior to sampling.

2. Materials and methods

2.1. Experimental diets

Diets were manufactured at the INRAE experimental facilities in Donzacq, France using a twin-screw extruder (45 BCE, Clextral, Firminy, France). The basal diet (CTL) was formulated based on plant protein and fish and vegetable oils, to meet the minimum dietary requirements of rainbow trout as per (NRC, 2011) (Table 1).

The mineral and vitamin pre-mix in the CTL diet was formulated without Se or vitamin B6 supplementation. In addition, three experimental feeds were prepared either supplemented with Se (SEL), vitamin B6 (PYR) or both Se and vitamin B6 (SEPY). Selenium was supplemented in the form of SeMet (Excential Selenium 4000, Orffa, Breda, Netherlands), which is well bioavailable and, in contrast to inorganic Se, requires vitamin B6 as a co-factor for its biosynthesis to selenocysteine (Soda et al., 1999). The Se supplementation level of 4 mg per kg corresponds to that typically found in fishmeal-based diets and the optimal supplemental level for rainbow trout growth as suggested by (Wang et al., 2022; Wang et al., 2018). Vitamin B6 was supplemented in form of pyridoxine hydrochloride (PN.HCL) at 50 mg per kg, as positive effects on muscle DHA and lipid peroxidation were described in rainbow trout at this level (Maranesi et al., 2005).

Table 1
Formulation and composition of experimental diets.

	CTL	SEL	PYR	SEPY
Ingredients [%]				
Plant meals[1]	72	72	72	72
Crystalline amino acids and attractant mixture[2]	3.3	3.3	3.3	3.3
Marine lecithin	3.6	3.6	3.6	3.6
Fish oil	8	8	8	8
Vegetable oils[3]	8	8	8	8
Mineral and vitamin mixture without Se and pyridoxine[4]	5.1	5.1	5.1	5.1
Se [mg/kg diet] as SeMet[5]	0	4	0	4
PN.HCL [mg/kg diet][6]	0	0	50	50
Analytical composition [DM, %]				
Dry matter	96	96	97	96
Crude protein	48	48	48	48
Total lipid	23	23	23	23
Gross energy [kJ/g DM]	25	25	25	25
Ash	7	7	7	7
Selenium [mg/kg]	0.14	3.85	0.19	3.85
Vitamin B6 [mg/kg]	3.37	3.40	48.8	50.4

[1] Plant meals (% diet): 18% wheat gluten, 16% corn gluten meal, 15% soybean protein concentrate, 5% soybean meal, 5% white lupin meal, 5% rapeseed meal, 5% whole wheat, 3% dehulled pea meal.

[2] Crystalline amino acids and attractant mixture (% diet): 1.5% L-lysine, 0.3% L-methionine, 0.5% glucosamine, 0.3% taurine, 0.3% betaine, 0.2% glycine, 0.2% alanine.

[3] Vegetable oils (% diet): 4% Rapeseed oil, 2.4% Linseed oil, 1.6% Palm oil.

[4] Mineral and vitamin mixture (per kg diet): 5000 IU retinol acetate, 2500 IU cholecalciferol, 100 IU DL-α-tocopherol acetate, 10 mg sodium menadione bisulfate, 1 mg thiamine-HCL, 4 mg riboflavin, 10 mg niacin, 20 mg D-calcium pantothenate, 0.2 mg D-biotin, 1 mg folic acid, 10 μg cyanocobalamin, 50 mg L-ascorbyl-2-polyphosphate, 0.3 g myo-iositol, 1 g choline, 33 g $CaHPO_4{\cdot}2H_2O$, 2.15 g $CaCo_3$, 1.24 g $Mg(OH)_2$, 0.9 g KCl, 0.4 g NaCl, 20 g $FeSO_4{\cdot}H_2O$, 35 mg $ZnSO_4{\cdot}H_2O$, 10 mg $CuSO_4{\cdot}5H_2O$, 10 mg NaF, 3 mg Cal2, 0.05 mg $CoCO_3$, 0.75 mg BHA, 0.75 BHT, 0.15 mg propyl gallate, 200 mg sepiolite. All ingredients were diluted with α-cellulose.

[5] L-Selenomethionine (SeMet), Excential Selenium 4000, Orffa, Breda, Netherlands.

[6] Pyridoxine hydrochloride (PN.HCL). SeMet and PN.HCL diluted with α-cellulose were added in a premix representing 0.3% diet (part of mineral and vitamin mixture).

2.2. Experimental set-up

Twelve 150 l fibreglass tanks were used in this experiment corresponding to triplicate tanks per treatment (Fig. 2). All tanks were supplied with flow-through spring water at 17 °C and a dissolved oxygen concentration of 8 mg/l. Each tank was stocked with 50 fish at an initial stocking density of 9 kg/m^3. Feeding was done twice daily to apparent satiation by hand. The overall feeding trial lasted for 12 weeks, however, the number of fish was reduced to 30 individuals per tank to correct stocking density from 43 kg/m^3 to 26 kg/m^3 according to growth after 9 weeks (Roy et al., 2021). At the end of the twelfth week, 14 fish per tank were sampled at normoxic (NO) conditions, while the remaining fish from each dietary treatment were subjected to an oxygen stress challenge (initial stocking density at stress challenge: 19 kg/m^3). Therefore, the fish were exposed to episodic mild hyperoxia (HO) over a one-week period as previously defined (Kalinowski et al., 2019). The dissolved oxygen levels in the tanks were increased to 13 mg/l (= 163%) during normal working hours of the farm (09:00 to 17:00 h), before returned to normoxic conditions (= 8 mg/l) overnight (8 h HO: 16 h NO). The fish were starved for 24 h before all samplings, the sampling protocols were similar in stressed and non-stressed fish. All fish were killed with an overdose of benzocaine prior to tissue dissection. Four whole fish per tank were collected for nutrient body analysis and stored at −20 °C. Liver and muscle tissue samples were divided in pools of four fish per tank according to planned analysis during the sampling. The tissue samples were snap frozen in liquid nitrogen and stored at −80 °C until analysis.

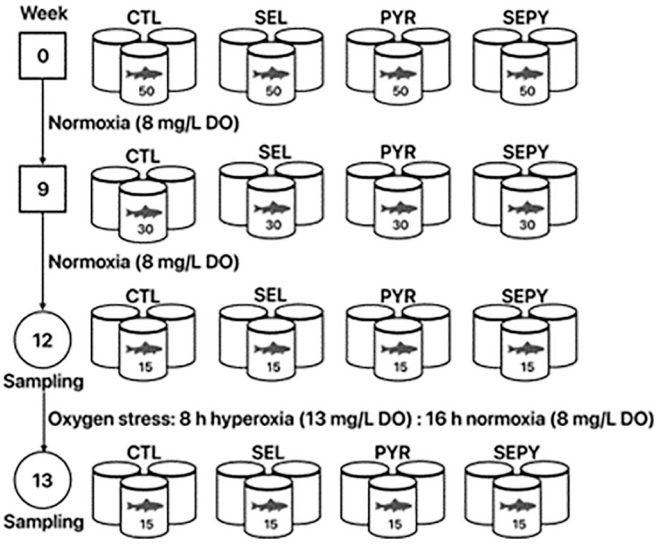

Fig. 2. Experimental set-up: In total 12 tanks were used corresponding to triplicate tanks per treatment (CTL, SEL, PYR, SEPY). The initial number of 50 fish/tank was reduced to 30 fish/tank after 9 weeks to correct stocking density. Sampling of 15 fish/tank was done after 12 weeks of feeding the experimental diets at standard conditions with 8 mg/l dissolved oxygen (DO) level in the tanks. The remaining fish were exposed to periodic hyperoxia prior to sampling. Therefore, the oxygen levels in the tank were elevated to 13 mg/L DO for 8 h/day over a one-week period.

2.3. Nutritional analysis of diets and tissues

Diets and freeze-dried homogenized whole fish samples were used for proximate composition analysis: dry matter was determined after drying at 105 °C for 24 h, ash after a 10 h incineration at 550 °C. The Kjeldahl method after acid digestion (N x 6.25) was used to determine protein. Gross energy was measured using an adiabatic bomb calorimeter. Total Se was analysed in diets, liver, muscle and freeze dried homogenized whole-body fish samples. Briefly, 40–80 mg of sample was digested in triplicate and analysed by Inductively Coupled Plasma Mass Spectrometry (Thermo Scientific, XSeries2 ICP-MS, Waltham, MA, USA) as previously described (Wischhusen et al., 2023). Diets were analysed for total vitamin B6 by LC-FLD standard method at Eurofins laboratory (Vitamin Testing Denmark). The three vitamin B6 derivates, PL, PM and PN, were extracted from pooled muscle and liver tissue as well as from freeze dried whole-body fish. Both liver and whole fish (0.5 g) were blended in 5 ml of aqueous formic acid solution (5% *v*/v), whereas muscle tissue (1 g) was blended in 5 ml of HPLC grade water. Both samples and calibrants were spiked with 25 μl of a 200 ng/ml $^{13}C_3$ pyridoxine solution (Merck). Calibration curves were created for PL, PM and PN to the internal standard ^{13}C pyridoxine. Samples were centrifuged for 10 min at 2490*g* to pellet material. De-lipidation was achieved by the addition of 5 ml methyl tert-butyl ether to both liver and whole-body samples, or 5 ml of isohexane to muscle extracts. The upper, lipid containing extracts, were discarded, with 3.75 ml of ice-cold acetonitrile added to the lower phase to precipitate proteins. After 1 h on ice, the samples were centrifuged, with the supernatant transferred to clean tubes, which were subsequently dried over-night at 30 °C using centrifugal evaporation. Finally, samples were resuspended in 400 μl of water +0.1% formic acid (*v*/v) and centrifuged at 13,148*g* before being transferred to glass vials suitable for LC analysis. Samples were analysed by LC-MS/MS using an Acquity I-class UPLC (Waters) coupled to a Xevo-TQS mass spectrometer (Waters). Analytes were separated on a BEH C18 column (2.1 × 100 mm, 1.7 μm) (Waters), with solvent A comprising

water +0.1% formic acid (v/v) and solvent B 100% methanol. The column was kept at 40 °C with a flow rate of 0.3 ml/min, with 4 µl injected on column. The solvent gradient started with 100% A, which was held for 0.5 min. Solvent B was then ramped to 50% over 3 min and then held for 1.5 min. Solvent A was then returned to 100% over 3 min and then held for 2 min to re-equilibrate the column. The mass spectrometer was run in MRM mode, with the source settings as follows; Capillary voltage 3 kV in positive mode, cone voltage 40 V, source offset 30 V, desolvation temperature 300 °C, desolvation and cone flow rates of 800 and 150 L/h respectively and a nebuliser pressure of 7 bar. The MRM settings for the B6 vitamers were pyridoxal 168.1 > 150.1, cone 20 V, 14 CE V, pyridoxamine 169.1 > 152.1, cone 20 V, 15 CE V, pyridoxine 170.1 > 134.1, cone 40 V, 21 CE V, $^{13}C_3$ pyridoxine 173.1 > 137.1, cone 40 V, 21 CE V.

2.4. Glutathione and metabolites of the one-carbon metabolism

Pen extraction and analysis of the aminothiols hCys, Cys, γ-glutamyl cysteine (GluCys) and cysteinyl-glycine (CysGly) as well as oxidized (GSSG) and reduced glutathione (rGSH) was done by LC-UV or -FL on 3 g homogenized pooled liver or muscle tissue ($n = 6$), respectively, as previously described (Wischhusen et al., 2021) with little modifications. Briefly, tissues were homogenized with a Precellys-like homogeniser in 50 mM phosphate, 1 mM EDTA-Na2 (pH = 6.5) buffer. Part of homogenate was collected for further protein content estimation using colorimetric BCA method. After centrifugation (10,000 *g*. 15 min, 4 °C), deproteinization of the supernatant was performed using equal volume of 10% metaphosphoric acid solution (m/V). Finally, the supernatant was filtered through 0.22 µm PVDF microcolumn. The extract was kept at −20 °C until analysis. Separation, detection, and quantification were done as previously described (Wischhusen et al., 2020).

2.5. Oxidative stress and antioxidant measures

To measure glutathione peroxidase (GPX) activity, samples were homogenized in an eight-time dilution of 20 mM-phosphate buffer (pH 7.4) containing 1 mM EDTA using an Ultra-Turrax. After centrifugation (10 min at 2000 *g*) the supernatant was diluted in 0.5% triton solution and the samples left on ice for 1 h. A solution of 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 2 mM sodium azide, 2 mM GSH, 0.1 mM NADPH and 0.2 mM glutathione reductase was used for the reduction of cumene hydroperoxide (0.2 mM) by SeGPX or H_2O_2 by total GPX. The enzyme activity was read in a plate reader (30 °C and 340 nm). Lactate was measured on 0.3 µl of blood plasma using a hand-held device (Lactate Pro 2, Arkray Europe, NL). Blood plasma cortisol analysis was done using LC-MS/MS. Therefore, 500 µl of 1% KCl and 500 µl of ethyl acetate were added to 100 µl of sample. To the samples and calibrants, 50 µl of a 50 ng/ml d4 cortisol solution was added (Merck). Following centrifugation, the upper ethyl acetate phase was transferred to a clean tube, and a second ethyl acetate extraction was performed, which was pooled with the first, with the extracts dried under nitrogen and resuspended in 100 µl of cold methanol: water (1, 1 *v*/v). After 1 h on ice, the samples were centrifuged (2 min at 14,000 rpm) and the supernatant transferred to glass vials suitable for LC analysis. The analytes were separated on a HSS T3 column (2.1 × 5.0 mm, 1.8 µm) (Waters), with solvent A comprising water +0.1% ammonium formate (*w/v*) and 0.1% formic acid (*v*/v) and solvent B comprising methanol +0.1% ammonium formate (w/v) and 0.1% formic acid (v/v). The column flow rate was set at 0.6 ml/min with the column temperature set at 60 °C, with 10 µl inject on column. The solvent gradient started at 55% A and 45% B, which was held for 0.1 min. Solvent B was ramped to 98% B over 5 min, which was then held for a further 1 min. Solvent A was then ramped back to 55% over 1 min and held for 2 min to re-equilibrate the column. The mass spectrometer settings were the same as previously mentioned, with the analyte MRM settings as follows; cortisol 363.2 > 121.1, cone 30 V, 26 CE V, d4 cortisol 367.2 > 121.1, cone 30 V, 26 CE V. Thiobarbituric acid reactive substances (TBARS) were determined in 1 g of liver tissue homogenized in 7.5% trichloracetic acid solution. The homogenized tissue was filtered through 110 nm filter paper (Whatman No 1) before 1 ml 0.02 M thiobarbituric acid solution was added to 1 ml of filtrate. Then, the samples were heated to 100 °C for 35 min using a water bath and de-gased for 5 min in an ultrasonic bath. Precipitates were removed by centrifugation (6 min at 13,226 g). Finally, the supernatant was read in a spectrophotometer at 532 nm against a blank and concentrations calculated to a standard.

2.6. Molecular analysis

Total RNA extraction was performed on approximately 100 mg liver sample of four individual fish per tank using 1 ml of TRIzol (Invitrogen, Thermo Fisher Scientific, UK) (Wischhusen et al., 2023). Quality and quantity of RNA was confirmed running 200 ng of total RNA on a 1% agarose gel complemented by Nanodrop ND-1000 (Labtech Int., East Sussex, UK) reads. Prior to reverse transcription (High Capacity cDNA Reverse Transcription kit, Applied Biosystems, REF4368813, Warrington, UK with random primers) two pools of 2 ng total RNA were created per tank. Quantitative real-time PCR (qPCR) was used to determine relative RNA expression levels of target genes using the primer sequences provided in Table 2.

The qPCR was done using a qTower^3 G real-time PCR Thermal Cycler (Analytic Jena GmbH, Jena, Germany) at the following temperature profile: 50 °C for 2 min, 95 °C for 10 min, followed by 34 cycles of 95 °C for 15 s, Tm for 30 s and 72 °C for 30 s. The qPCR reaction mix included 5 µl Luminaris HiGreen qPCR Master Mix (Thermo Scientific, Hemel Hempsted, UK), 0.5 µl random primers (10 pmol), 1.5 µl deionised water and 2.5 µl cDNA at 1/20 dilution for target genes and 1/200 dilution for housekeeping genes. The melting curves were systematically screened for quality control.

2.7. Statistical analysis

All data are given as mean ± standard error of means. All statistical analyses were performed using R (R Core Team, 2022). Significant effects of dietary SeMet and PN.HCL supplementations on growth performance were evaluated using two-way ANOVA followed by Tukey's HSD. Otherwise, a three-way repeated measure ANOVA was performed to evaluate the effects of dietary SeMet and PN.HCL supplementations as well as the effect of the periodic hyperoxia challenge (rstatix, version 0.7.2). The data are presented as means of groups receiving SeMet (+Se: SEL, SEPY) or PN.HCL (+B6: PYR, SEPY) supplemented or SeMet (-Se: CTL, PYR) or PN.HCL (-B6: CTL, SEL) non-supplemented diets over the entire 13 weeks experimental period, respectively. Also, means across all treatments in fish before and after hyperoxic stress exposure are presented. Significant main effects without significant interaction were followed by a pairwise comparison to identify differences between groups. Significant interactions were further investigated using simplified two- and one-way ANOVA models on each level of the respective variable. All significant interactions of dietary SeMet and PN.HCL supplementations are presented as individual figs. A significant difference was assumed at $p < 0.05$.

3. Results

3.1. Growth performance

All experimental diets were readily accepted by the fish. Neither final body weight nor specific growth rate was significantly affected by dietary SeMet or PN.HCL supplementation (Table 3 and Fig. 3). Although absolute daily feed intake was not significantly different between groups, feed intake was higher in fish fed diets supplemented with SeMet compared to fish fed diets without SeMet supplementation when expressed by % body weight. However, feed conversion ratio was not

Table 2
Oligonucleotide primers designed to assay mRNA abundance by rt-qPCR.

Gene	Accession no.	Forward primer	Reverse primer	Amplification size	Tm °C
selpa1	EE605178	gcccaaacaggaagatgtgt	gggcagggagatatggtagg	100	60
selpa2	HF969249.1	cagccacctggttggagtat	cctggagtagggccacca	82	61
gpx1a	HE687021	aatgtggcgtcactctgagg	caattctcctgatggccaaa	131	61
gpx1b1	CA357669.1	cgagctccatgaacggtacg	tgcttcccgttcacatccac	183	64
gpx1b2	HE687023	tcggacatcaggagaactgc	tccttcccattcacatccac	121	61
gpx4a1	HE687024	gaaaggcttcctgggaaatg	ctccaccacactgggatcat	112	61
gpx4a2	HE687025	agaaatacaggggcgacgtt	gcatctccgcaaactgagag	90	61
cbs	NM_001124686.1	ccacctcaggcaatacaggt	aacatccaccttctccatgc	107	60
dnmt1	XM_021557911.1	ttgccagaagaggagatgcc	cccaggtcagcttgccatta	152	60
gnmt	XM_021585680.1	ctcaagtacgcgctgaagga	cactctggtccccttttgaagt	187	60
cgl	EU315111.1	caccaaccccaccatgaaag	gcgctggaagtaggctgaca	118	64
sahh	XM_021609053.1	atcaaacgggccacagatgt	tcgtaccttccatggcagc	167	60
gclc1	GSONMT00071788001	aggccagagtatggcagcta	cagcctaaccttgggaatga	176	60
bhmt	FR908041.1	cagagaagcacggtaactgg	ttctttgtgctgcatcaggt	188	60
mtr	XM_021576690.1	aatgcaggtctgcccaatac	ctgatgtgtgcaggagtcgt	137	60
gstp	BX302932.3	tcgctgactggacgaaagga	cgaaggtcctcaacgccatc	196	64
gr	HF969248.1	ctaagcgcagcgtcatagtg	acaccctgtctgacgacat	108	60
ef1α	AF498320.1	tcctcttggtcgtttcgctg	acccgagggacatcctgtg	159	60
β-actin	AJ438158.1	gatgggccgaaagacagcta	tcgtcccgtggtgacgat	105	60

Selp, selenoprotein P; gpx, glutathione peroxidase; cbs, cystathionine-β-synthase; dnmt; DNA methyltransferase; gnmt, glycogen *n*-methyltransferase; cgl, cystathionine-γ-synthase; sahh, S-adenosylhomocysteine hydrolase; gclc, glutamate-cysteine ligase catalytic subunit; bhmt, betaine–homocysteine *S*-methyltransferase; mtr, methionine synthase; gstp, glutathione S-transferase; gr, glutathione reductase; ef1α, alpha subunit of the elongation factor-1; β-actin, beta-actin.

significantly different between groups. The hepato-somatic index was higher in fish fed diets supplemented with PN.HCL compared to fish fed diets without PN.HCL supplementation. Stressed fish, sampled after exposure to one week of periodic hyperoxia, were significantly bigger than fish sampled at the end of feeding trial (192 ± 3 vs. 179 ± 3 g, $p < 0.01$), however, without any significant differences between the dietary treatments (Table 3).

Proximate analysis of whole fish revealed that the dry matter, total lipid, energy, and ash content of the fish remained unaffected by either SeMet or PN.HCL supplementation (Table 4). However, the crude protein content decreased in stressed fish when fed diets supplemented with SeMet compared to fish fed diets without SeMet supplementation. In addition, stressed fish showed significantly higher dry matter, energy and ash content compared to the non-stressed fish.

3.2. Tissue selenium and vitamin B6 levels

Dietary SeMet supplementation significantly increased tissue Se levels including liver and muscle (Table 4). The total Se retention was higher in fish fed diets without SeMet supplementation compared to the fish fed diets supplemented with SeMet (Table 3). A significant interaction between dietary SeMet and PN.HCL supplementation was detected on total Se levels in muscle tissue, which could not be confirmed by two-way ANOVA ($p = 0.06$). In liver tissue, all three vitamin B6 derivates, PL, PM and PN, were significantly higher in fish fed diets supplemented with PN.HCL (Table 4). In muscle tissue a similar increase could only be detected for PL. Muscle PN levels were below the detection limit in some samples.

Periodic hyperoxia reduced liver PL and PM levels in the fish (Table 4). In addition, fish subjected to hyperoxic stress showed higher muscle PM levels if fed SEPY compared to PYR (Fig. 4).

3.3. Metabolites of the methionine cycle and glutathione metabolism

At the end of the feeding trial, the levels of SAM, the first step metabolite of the methionine cycle, were not significantly affected by either SeMet or PN.HCL supplementation in liver or muscle tissue of fish (Table 5). However, the levels of the second step metabolite, SAH, were increased by SeMet supplementation in both liver and muscle resulting in a reduced SAM/SAH ratio, an indicator of the cellular methylation potential. Liver hCys levels were unaffected by SeMet supplementation, but significantly increased due to PN.HCL supplementation. On the contrary, muscle hCys levels were decreased by both SeMet and PN.HCL supplementation.

Dietary SeMet supplementation was generally associated with lower levels of metabolites within the GSH metabolism including Cys and CysGly in liver tissue, as well as Cys, GluCys, CysGly and total GSH in muscle tissue. On the other hand, PN.HCL supplementation significantly increased total liver GSH levels, however with significantly reduced CysGly levels (Table 5). A similar decrease by PN.HCL supplementation on CysGly was also observed in muscle tissue, however, Cys and GluCys levels were significantly increased. Liver GluCys in non-stressed fish showed a significant interaction between SeMet and PN.HCL supplementation. Fish fed PYR had significantly higher liver GluCys levels compared to fish from any of the other treatments (Fig. 5).

The hyperoxic stress challenge resulted in a general decrease of metabolites associated with the methionine cycle and GSH metabolism, with the exception of hCys in liver tissue and total GSH in muscle tissue, which remained unaffected by the oxygen stress (Table 5). In addition, an interactive effect within stressed fish between SeMet and PN.HCL supplementation on several metabolites were detected. SAM was significantly higher in SEL compared to CTL with SEPY in-between (Fig. 6A). Also, liver hCys levels were higher in fish fed PYR compared to SEPY (Fig. 6B), while muscle hCys levels were intermediate for fish fed SEPY compared to PYR or SEL (Fig. 6C). In addition, liver Cys levels were higher in fish fed CTL compared to SEL, but not SEPY (Fig. 6D).

3.4. Oxidative stress and antioxidant markers

While rGSH levels in liver or muscle tissue remained unaffected by SeMet or PN.HCL supplementation, GSSG levels were significantly elevated in liver and muscle tissue by both SeMet and PN.HCL supplementation (Table 6). This resulted in a lower rGSH/GSSG ratio by PN.HCL supplementation in liver tissue and by SeMet supplementation in muscle tissue. The activity of the antioxidant enzyme GPX increased with SeMet supplementation in liver tissue, which relates to both, SeGPX and the non-Se-dependent GPX forms. Liver TBARS levels were significantly lower in fish fed SeMet supplemented diets but remained unaffected by PN.HCL supplementation. The blood plasma stress markers cortisol and lactate remained unaffected by both dietary SeMet or PN.HCL supplementation.

The hyperoxic stress challenge resulted in decreased liver rGSH levels of fish, which simultaneously increased in muscle tissue (Table 6). Associated, a higher rGSH/GSSG ratio in muscle tissue of stressed fish

Table 3
Performance of fish after feeding the experimental diets for 12 weeks at standard rearing conditions.

	Dietary supplementation				Two-way ANOVA		
	-Se	+Se	-B6	+B6	Se	B6	Int
	(CTL, PYR)	(SEL, SEPY)	(CTL, SEL)	(PYR, SEPY)			
Initial weight [g]	28.0 ± 0.3	27.5 ± 0.2	27.7 ± 0.3	27.8 ± 0.3	ns	ns	ns
Body weight, before stress [g]	184 ± 3	180 ± 3	179 ± 2	185 ± 3	ns	ns	ns
Body weight, after stress [g]	190 ± 6	196 ± 4	189 ± 5	196 ± 4	ns	ns	ns
Specific growth rate[1]	2.24 ± 0.05	2.24 ± 0.04	2.22 ± 0.04	2.25 ± 0.05	ns	ns	ns
Absolute feed intake[2]	1.53 ± 0.04	1.54 ± 0.05	1.52 ± 0.05	1.55 ± 0.03	ns	ns	ns
Feed intake [% BW][3]	1.44 ± 0.03[b]	1.49 ± 0.02[a]	1.47 ± 0.02	1.46 ± 0.04	**0.02**	ns	ns
Feed conversion ratio[4]	0.94 ± 0.22	0.87 ± 0.01	0.95 ± 0.21	0.86 ± 0.02	ns	ns	ns
HSI[5]	1.24 ± 0.05	1.25 ± 0.03	1.19 ± 0.03[b]	1.30 ± 0.04[a]	ns	**0.05**	ns
Se retention[6]	68 ± 2[a]	55 ± 1[b]	60 ± 4	63 ± 3	**<0.01**	ns	ns
B6 retention[6]	1.87 ± 0.58	1.10 ± 0.16	2.04 ± 0.56	1.09 ± 0.35	ns	ns	ns

Mean ± standard error of means ($n = 3$ tanks). Unlike superscript letters indicate significant differences between groups (+Se, supplemented with SeMet; —Se, no SeMet-supplementation; +B6, supplemented with PN.HCL; —B6, no PN.HCL-supplementation) and ns = non-significant according to two-way ANOVA.

[1] Specific growth rate = (ln(final body weight)-ln(initial body weight)) x 100 / number of days.

[2] Absolute feed intake = total feed intake / number of fish / number of days.

[3] Feed intake [% BW] = absolute feed intake / average fish weight x 100.

[4] Feed conversion ratio = total feed intake / ((final tank bulk weight + dead fish weight) – initial tank bulk weight).

[5] Hepato somatic index (HSI) = (liver weight / body weight) x 100.

[6] Nutrient retention = (final body nutrient concentration – initial body nutrient concentration) / (average feed uptake per fish x nutrient concentration diet).

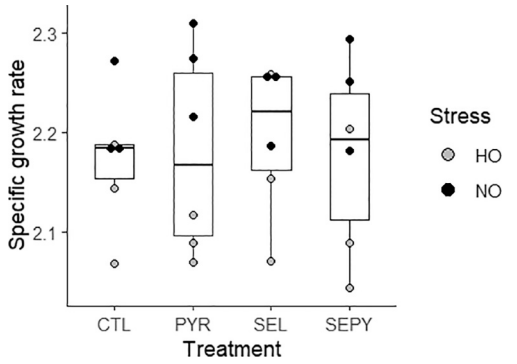

Fig. 3. Specific growth rate of fish per treatment. Specific growth rate = (ln (final body weight)-ln(initial body weight)) x 100 / number of days. Number of days were 84 in fish sampled before (NO) and 91 in fish sampled after (HO) exposure to an hyperoxic stress challenge.

was detected. The activity of antioxidant enzyme GPX, liver TBARS levels as well as oxidative stress markers in blood plasma including cortisol and lactate were not significantly different in fish before and after being subjected to periodic hyperoxia stress.

3.5. Gene expression

The expression of genes encoding enzymes of the methionine cycle including *dnmt1*, *sahh*, *bhmt* and *mtr* remained unaffected by either SeMet or PN.HCL supplementation (Table 7). However, the first step transsulfuration enzyme *cbs* was upregulated in fish fed diets supplemented with PN.HCL, while the second step enzyme *cgl* was downregulated in fish fed diets supplemented with SeMet. Other genes encoding proteins involved in the GSH metabolism including *gclc1*, *gnmt*, *gstp* or *gr* remained unaffected by both SeMet and PN.HCL supplementation. The expression of selenoproteins including *gpx1a*, *gpx1b2*, *gpx4a2* and *selpa1* increased by SeMet supplementation, while PN.HCL had no significant effect on any of the targeted selenoproteins.

Fish subjected to the hyperoxic stress challenge showed a significant increase in the gene expression of *mtr*, while the expression of all other genes analysed related to the methionine cycle or transsulfuration pathway remained unaffected by the stress challenge (Table 7). On the other hand, *gstp*, a GSH antioxidant enzyme, was lower expressed in stressed compared to non-stressed fish. Except for *selpa2*, which was higher expressed in stressed compared to non-stressed fish, none of the other targeted selenoproteins showed any significant difference in gene expression due to the hyperoxic stress challenge.

4. Discussion

4.1. Impact of dietary se and B6 supplementation on growth and tissue nutrient levels

The basal diet in the present study ranged within the minimum dietary requirements of rainbow trout for Se (0.14 mg/kg) and vitamin B6 (3 mg/kg) as stated by NRC (NRC, 2011). However, other studies report the vitamin B6 requirements in trout and other salmonids to range as high as 10–30 mg/kg (Akhtar and Ciji, 2021), while Se requirements have been reported higher at sensitive life stages or under stressful conditions (Antony Jesu Prabhu et al., 2020; Rider et al., 2009). Supranutritional Se levels (4.5 mg Se/kg diet) may also improve flesh quality in rainbow trout (Wang et al., 2018, 2021). On the other hand, both, Se and vitamin B6 deficiency can be associated with reduced growth (Bell et al., 1987; Bordoni et al., 2006; Espe et al., 2020). In the present study, there were no significant differences in growth performance between the treatments, however, stressed fish showed significantly lower crude protein content in whole body when fed SeMet supplemented diets compared to fish fed diets without SeMet supplementation, irrespective of vitamin B6 content. Muscle protein deposition is an essential determinant for fish growth and previous studies indicate that dietary Se can support this process through accelerated postprandial protein synthesis (Wang et al., 2021). In the present study, the feed intake but not absolute feed intake was significantly increased by SeMet supplementation, although a positive effect of Se supplementation on feed intake has not been reported in other studies (Berntssen et al., 2018; Fontagné-Dicharry et al., 2020; Wang et al., 2022), unless the fish were subjected to elevated environmental stress (Küçükbay et al., 2009). Together these results show that further research on the role of dietary selenium in muscle growth of stressed fish might be warranted, especially when the fish are fed plant protein-based diets. The increased feed intake observed in the present study came with lower Se retention in fish fed SeMet supplemented diets. Dietary organic Se sources are generally considered highly bioavailable, though SeMet retention can be lower in animals which are vitamin B6 deficient (Jin and Yin, 2005). Nevertheless, PN.HCL supplementation had no significant impact on Se retention in the present study. Similarly, the liver and muscle Se content remained unaffected by PN.HCL supplementation in this study, while it was impaired in vitamin B6 deficient rats (Beilstein and Whanger, 1989). Overall, the micronutrient levels in the basal diet appear to have been sufficient to

Table 4
Whole body composition (dry matter, DM; crude protein, CP; crude lipid, CL; gross energy [kJ/g]; ash content, AC) [%] and liver and muscle selenium (Se) [μg/g], pyridoxal (PL), pyridoxamine (PM) and pyridoxine (PN) levels [ng/g] of rainbow trout at the end of the 12-week feeding trial before and after subjected to periodic hyperoxic stress.

	Dietary supplementation				Hyperoxic stress		Repeated three-way ANOVA			
	-Se	+Se	-B6	+B6	NO	HO	Se	B6	Stress	Int
	(CTL, PYR)	(SEL, SEPY)	(CTL, SEL)	(PYR, SEPY)						
Body										
DM	32.0 ± 0.08	32.5 ± 0.07	32.2 ± 0.08	32.3 ± 0.08	31.9 ± 0.08^{b}	32.6 ± 0.07^{a}	ns	ns	**0.03**	ns
CP	16.1 ± 0.05^{a}	15.7 ± 0.04^{b}	15.9 ± 0.05	15.9 ± 0.05	15.8 ± 0.04	16.0 ± 0.05	**<0.01**	ns	ns	ns
CL	13.3 ± 0.07	14.2 ± 0.08	13.7 ± 0.08	13.8 ± 0.08	13.5 ± 0.08	14.0 ± 0.08	ns	ns	ns	ns
Energy	9.16 ± 0.05	9.42 ± 0.05	9.29 ± 0.05	9.29 ± 0.05	9.15 ± 0.05^{b}	9.43 ± 0.05^{a}	ns	ns	**0.03**	ns
AC	2.12 ± 0.03	2.05 ± 0.03	2.10 ± 0.03	2.06 ± 0.03	2.02 ± 0.02^{b}	2.14 ± 0.03^{a}	ns	ns	**0.04**	ns
Liver										
Se	0.47 ± 0.01^{b}	20.2 ± 1.07^{a}	10.7 ± 3.2	9.9 ± 3.0	11.5 ± 3.4	9.2 ± 2.7	**<0.01**	ns	ns	ns
PL	1970 ± 432	1722 ± 292	988 ± 116^{b}	2704 ± 356^{a}	2497 ± 407^{a}	1195 ± 177^{b}	ns	**<0.01**	**<0.01**	**B6:Stress**
PM	811 ± 176	632 ± 112	508 ± 78^{b}	935 ± 175^{a}	997 ± 168^{a}	446 ± 57^{b}	ns	**0.02**	**0.01**	ns
PN	206 ± 69	213 ± 68	7 ± 1^{b}	412 ± 45^{a}	229 ± 75	190 ± 62	ns	**<0.01**	ns	ns
Muscle										
Se	0.09 ± 0.01^{b}	1.97 ± 0.04^{a}	1.05 ± 0.29	1.01 ± 0.27	1.06 ± 0.29	1.00 ± 0.28	**<0.01**	ns	ns	**B6:Se**
PL	249 ± 51	355 ± 90	113 ± 10^{b}	491 ± 67^{a}	327 ± 74	276 ± 74	ns	**<0.01**	ns	ns
PM	52 ± 4	55 ± 4	52 ± 4	55 ± 4	55 ± 4	52 ± 4	ns	ns	ns	**B6:Se**
PN	2.62 ± 094	2.68 ± 1.04	1.46 ± 0.47	3.84 ± 1.22	2.18 ± 0.98	3.12 ± 0.98	ns	ns	ns	ns

Mean ± standard error of means (n = 6 tanks). Unlike superscript letters indicate significant differences between groups (+Se, supplemented with SeMet; —Se, no SeMet-supplementation; +B6, supplemented with PN.HCL; —B6, no PN.HCL-supplementation; NO, fish at normoxic conditions; HO, fish subjected to hyperoxic stress) and ns = non-significant according to repeated three-way ANOVA.

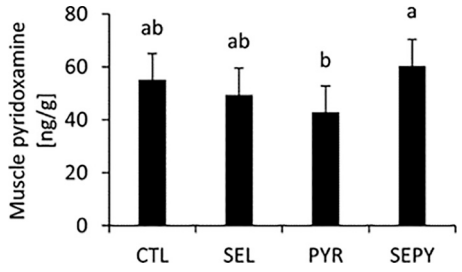

Fig. 4. Significant interaction between dietary SeMet and PN.HCL supplementation on pyridoxamine levels in muscle tissue of fish after 12 weeks of feeding the experimental diets and exposure to a one-week periodic hyperoxia stress challenge. CTL, control without SeMet or PN.HCL supplementation; SEL, with SeMet supplementation (4 mg Se/kg); PYR, with 50 mg/kg PN.HCL supplementation; SEPY, with both SeMet and PN.HCL supplementation. Unlike superscript letters indicate significant differences between treatments.

support normal growth and development of fish in this trial.

4.2. Contrasting effects of se and B6 supplementation on transsulfuration metabolite levels in rainbow trout tissue

In the present study, dietary supplementations of SeMet and PN.HCL induced contrasting effects on transsulfuration metabolite levels. Vitamin B6 is an essential co-factor in transsulfuration. Indeed, a reduction in the activity of the transsulfuration enzyme CGL is one of the primary indicators of suboptimal vitamin B6 status (Lima et al., 2006), while the activity of secondary step transsulfuration enzymes CBS has been reported to be more stable under vitamin B6 restrictions (Lima et al., 2006; Martinez et al., 2000). Nevertheless, the present results show a higher *cbs* gene expression by PN.HCL supplementation. However, the CBS enzyme activity was not measured, and modifications might have been posttranslational. In addition, in the present study higher total GSH levels were detected in liver with vitamin B6 supplementation, which aligns to results obtained in other studies (Davis, 2006; Taysi, 2005). In contrast, the present study showed a decrease in transsulfuration metabolites by SeMet supplementation. At moderate Se levels varying effects of Se on the transsulfuration pathway were reported, which differed according to species (Uthus and Ross, 2007) and utilized Se form (Yin et al., 1992). SeMet is metabolized through the transsulfuration pathway alongside dietary methionine, while sodium selenite is non-enzymatically converted to selenocysteine, for protein biosynthesis. Therefore, effects on this specific pathway are hardly comparable between studies using different Se forms (Dalto et al., 2016). In the methionine cycle, in the first step, SAM is synthesized, which then serves as a methyl donor in cellular reactions to form SAH (Dalto and Matte, 2017). Then, SAH is transformed into hCys by adenosylhomocysteinase. From there hCys can either be recycled to methionine, closing the cycle, or be directed towards the transsulfuration pathway for taurine and GSH biosynthesis (Fig. 1). The present data show that SeMet supplementation resulted in a significant increase of tissue SAH levels. Studies in yeast have shown that Se-SAH tends to accumulate in response to SeMet supplementation (Rao et al., 2010). Possibly this relates to the harmful effects of excessive SeMet. In excess, SeMet can have adverse effects leading to oxidative stress including increased GSSG levels similar to the results in the present study (Misra et al., 2012; Palace et al., 2004). However, the toxic effect of SeMet is believed to not be caused by SeMet itself or Se-SAH, but rather by Se metabolites further down the transsulfuration pathway such as Se-GluCys or Se-GSH (Lazard et al., 2015). Under normal conditions, the majority of SeMet entering the transsulfuration pathway will be directed towards the biosynthesis of selenoproteins through selenocysteine. However, at unfavourable methionine to SeMet ratios, increasing levels of SeMet could be co-metabolized alongside methionine downstream towards Se-GluCys and Se-GSH (Rao et al., 2010). This increase of SAH by Se supplementation has not been observed in an earlier study with rainbow trout fry using a lower dietary Se supplementation level of 0.3 mg/kg (Wischhusen et al., 2021). This suggests that the downregulation of transsulfuration at increasing SeMet supplementation might represent a cellular mechanism to prevent the accumulation of toxic Se-metabolite products in fish. In the present study, this was accompanied with the downregulation of second step transsulfuration enzyme *cgl* in the SeMet supplemented treatments.

Table 5
Concentrations of metabolites of the methionine cycle and glutathione metabolism [pg/mg protein] in liver and muscle tissues of rainbow trout fed a diet supplemented or not with selenomethionine and/or pyridoxamine hydrochloride and sampled before (NO) or after (HO) subjected to periodic hyperoxia stress.

	Dietary supplementation				Hyperoxic stress		Repeated three-way ANOVA			
	-Se	+Se	-B6	+B6	NO	HO	Se	B6	Stress	Int
	(CTL, PYR)	(SEL, SEPY)	(CTL, SEL)	(PYR, SEPY)						
Liver										
SAM	16 ± 1	19 ± 1	17 ± 1	17 ± 1	19 ± 1^{a}	15 ± 1^{b}	ns	ns	**0.01**	**Se:B6**
SAH	0.60 ± 0.04^{b}	0.83 ± 0.07^{a}	0.69 ± 0.08	0.73 ± 0.06	0.82 ± 0.07^{a}	0.61 ± 0.05^{b}	**0.01**	ns	**0.01**	**Se:Stress**
SAM/SAH	27 ± 1^{a}	23 ± 1^{b}	26 ± 1	25 ± 1	24 ± 1	26 ± 1	**0.02**	ns	ns	ns
hCys	8.7 ± 0.9	9.3 ± 0.8	8.1 ± 0.7^{b}	9.9 ± 0.9^{a}	9.3 ± 0.8	8.7 ± 0.8	ns	**<0.01**	ns	**B6:Se; Se:Stress; B6:Se:Stress**
Cys	1522 ± 67^{a}	1112 ± 57^{b}	1357 ± 117	1278 ± 37	1388 ± 77^{a}	1247 ± 92^{b}	**<0.01**	ns	**<0.01**	**B6:Se; B6:Stress; Se:Stress**
total GSH	1099 ± 79	1179 ± 103	1003 ± 66^{b}	1275 ± 97^{a}	1351 ± 76^{a}	927 ± 57^{b}	ns	**0.01**	**<0.01**	**B6:Se:Stress**
GluCys	96 ± 9	93 ± 5	89 ± 5	101 ± 8	111 ± 7^{a}	79 ± 3^{b}	ns	ns	**<0.01**	**B6:Se; B6:Stress; Se:Stress; B6:Se:Stress**
CysGly	63 ± 3^{a}	54 ± 4^{b}	62 ± 4^{a}	55 ± 3^{b}	64 ± 3^{a}	53 ± 3^{b}	**0.02**	**0.05**	**<0.01**	**B6:Stress**
Muscle										
SAM	49 ± 4	47 ± 1	51 ± 4	46 ± 2	53 ± 3^{a}	43 ± 2^{b}	ns	ns	**0.01**	**Se:Stress; B6:Se:Stress**
SAH	0.19 ± 0.02^{b}	0.26 ± 0.02^{a}	0.22 ± 0.02	0.22 ± 0.02	0.25 ± 0.02^{a}	0.19 ± 0.02^{b}	**<0.01**	ns	**0.01**	**B6:Se:Stress**
SAM/SAH	268 ± 16^{a}	205 ± 16^{b}	248 ± 21	225 ± 16	225 ± 17	248 ± 20	**0.02**	ns	ns	ns
hCys	11.0 ± 0.8^{a}	6.1 ± 0.4^{b}	9.7 ± 1.2^{a}	7.4 ± 0.5^{b}	9.1 ± 0.8^{a}	8.0 ± 1.1^{b}	**<0.01**	**<0.01**	**0.03**	**B6:Se; Pyr:Se:Stress**
Cys	55 ± 4^{a}	38 ± 4^{b}	42 ± 4^{b}	52 ± 4^{a}	53 ± 4^{a}	40 ± 4^{b}	**<0.01**	**0.02**	**0.01**	**B6:Se:Stress**
total GSH	62 ± 3^{a}	47 ± 5^{b}	57 ± 6	53 ± 3	54 ± 6	56 ± 3	**0.02**	ns	ns	**B6:Stress**
GluCys	3.7 ± 0.3^{a}	2.7 ± 0.4^{b}	3.1 ± 0.4^{b}	3.4 ± 0.2^{a}	3.3 ± 0.4	3.1 ± 0.3	**<0.01**	**0.02**	ns	**B6:Se; Se:Stress**
CysGly	8.0 ± 0.7^{a}	6.5 ± 0.5^{b}	8.0 ± 0.6^{a}	6.4 ± 0.6^{b}	8.1 ± 0.6^{a}	6.3 ± 0.5^{b}	**<0.01**	**<0.01**	**<0.01**	**B6:Stress; Se:Stress**

Mean ± standard error of means (n = 6 tanks). Unlike superscript letters indicate significant differences between groups (+Se, supplemented with SeMet; —Se, no SeMet-supplementation; +B6, supplemented with PN.HCL; —B6, no PN.HCL-supplementation; NO, fish at normoxic conditions; HO, fish subjected to hyperoxic stress) and ns = non-significant according to repeated three-way ANOVA; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Cys, cysteine; hCys, homocysteine; GSH, glutathione; GluCys, γ-glutamyl cysteine; CysGly, cysteinyl-glycine.

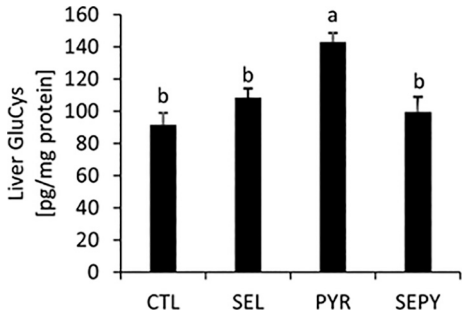

Fig. 5. Significant interaction between dietary SeMet and PN.HCL supplementation on GluCys levels in liver tissue of fish after 11 weeks of feeding the experimental diets. CTL, control without SeMet or PN.HCL supplementation; SEL, with SeMet supplementation (4 mg Se/kg); PYR, with 50 mg/kg PN.HCL supplementation; SEPY, with both SeMet and PN.HCL supplementation. Unlike superscript letters indicate significant differences between treatments.

4.3. Selenium supplementation supports selenoprotein synthesis and increases antioxidant parameters

Selenocysteine links the transsulfuration pathway to the selenoprotein translational machinery (Soda et al., 1999). In a first B6-dependent reaction, inorganic selenide will be formed, by action of Se specific selenocysteine lyase. Due to its high cytotoxicity, selenide is then either quickly methylated, consuming SAM, or phosphorylated. The phosphorylated selenide is then incorporated into tRNA Se-cysteinyl by another B6-dependent reaction for further translation into selenoproteins such SeGPX (Flohe et al., 1973). Vitamin B6 deficient animals therefore show an impaired selenoprotein synthesis, which can be supported by B6-supplementation (Lapointe et al., 2012). However, in the present study, using nutrient levels covering minimum requirements, no effect of PN.HCL supplementation on selenoprotein gene expression could be observed. This suggests that the vitamin B6 levels in the non-supplemented diet might have been sufficient to support selenoprotein synthesis in both groups supplemented or not with SeMet. However, SeMet supplementation increased the expression of specific selenoproteins including *gpx1a*, *gpx1b2*, *gpx4a2* and *selpa1*. All these genes are known to be easily regulated by dietary Se levels in plasma or liver and are often used as indictors to determine optimal dietary Se levels (Antony Jesu Prabhu et al., 2020). In the present study, the increase in *gpx* expression levels by SeMet supplementation was also associated with an increase in GPX enzyme activity. Glutathione peroxidases are important antioxidant enzymes that catalyse the reduction of various hydroperoxides via the oxidation of rGSH, which can then be recovered by enzymatic activity of glutathione reductase (GR) (Flohe et al., 1973). In the present study, the increase in GPX activity by SeMet supplementation might account for the higher GSSG levels observed in these treatments, as no effect on *gr* gene expression could be observed. In this relation, the lower TBARS levels in the liver indicate that increased antioxidant activity through SeMet supplementation might have prevented or contributed to the removal of lipid peroxidation products. Indeed, GSH represents a major redox buffer in cells (Halliwell and Gutteridge, 2015). The ratio between reduced and oxidized GSH is an important indicator for the cellular redox status. However, it remains unclear why in the present study similarly supplemented PN.HCL increased tissue GSSG levels. Although studies in mice reported that the biopotency of GPX is B6-dependent (Beilstein and Whanger, 1992), the present data indicate no significant effect of PN.HCL supplementation on the GPX activity in rainbow trout at the tested nutrient levels.

4.4. Selenium and B6 are required to maintain glutathione homeostasis in fish under periodic hyperoxia stress

Animals subjected to stressful environmental conditions metabolically generate high numbers of reactive species such as reactive oxygen species that in excess cause oxidation of essential cellular components including lipids and proteins (Halliwell and Gutteridge, 2015). As detailed above, the biosynthesis of GSH from amino acids through the methionine cycle and transsulfuration is an important pathway to maintain cellular redox homeostasis even under stressful conditions.

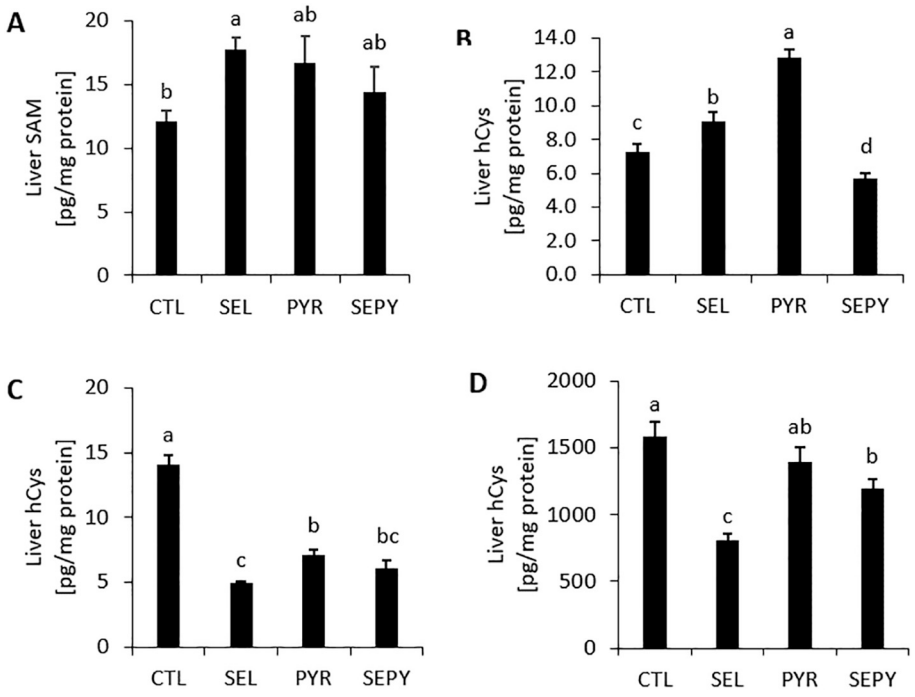

Fig. 6. Significant interaction between dietary SeMet and PN.HCL supplementation on liver SAM (A), liver hCys (B), muscle hCys (C) and liver Cys (D) levels in tissue of fish collected after 12 weeks of feeding the experimental diets and exposure to a one-week periodic hyperoxia stress challenge. CTL, control without SeMet or PN.HCL supplementation; SEL, with SeMet supplementation (4 mg Se/kg); PYR, with 50 mg/kg PN.HCL supplementation; SEPY, with both SeMet and PN.HCL supplementation. Unlike superscript letters indicate significant differences between treatments.

Table 6
Reduced (rGSH) and oxidized (GSSG) glutathione [pg/mg protein], GPX activity [mU/mg protein] and plasma lactate and cortisol levels [g/l] measured in liver or muscle tissue of rainbow trout fed a diet supplemented or not with selenium and/or vitamin B6 and sampled before (NO) or after (HO) subjected to periodic hyperoxia stress.

	Dietary supplementation				Hyperoxic stress		Repeated three-way ANOVA			
	-Se	+Se	-B6	+B6	NO	HO	Se	B6	Stress	Int
	(CTL, PYR)	(SEL, SEPY)	(CTL, SEL)	(PYR, SEPY)						
Liver										
rGSH	385 ± 20	344 ± 26	359 ± 17	371 ± 29	412 ± 23^{a}	318 ± 14^{b}	ns	ns	**0.01**	ns
GSSG	4.1 ± 0.4^{b}	5.2 ± 0.4^{a}	4.0 ± 0.3^{b}	5.2 ± 0.4^{a}	5.2 ± 0.4	4.0 ± 0.3	**0.02**	**0.02**	ns	ns
rGSH/GSSG	104 ± 6^{ns}	75 ± 3^{ns}	97 ± 7^{a}	81 ± 5^{b}	91 ± 7	87 ± 6	**0.05**	**<0.01**	ns	ns
GPX	17 ± 1^{b}	42 ± 1^{a}	30 ± 4	29 ± 4	30 ± 4	28 ± 4	**<0.01**	ns	ns	ns
SeGPX	3.9 ± 0.5^{b}	17 ± 1.9^{a}	11 ± 2.7	9.6 ± 2.0	9.8 ± 2.1	11 ± 2.6	**<0.01**	ns	ns	ns
indGPX	13 ± 1^{b}	25 ± 2^{a}	18 ± 3	19 ± 3	20 ± 3	17 ± 2	**<0.01**	ns	ns	ns
TBARS	0.94 ± 0.06^{a}	0.83 ± 0.04^{b}	0.87 ± 0.06	0.90 ± 0.09	0.90 ± 0.08	0.87 ± 0.07	**<0.01**	ns	ns	**B6:Stress**
Muscle										
rGSH	74 ± 4	64 ± 6	75 ± 6	64 ± 3	64 ± 5^{b}	75 ± 4^{a}	ns	ns	**0.05**	ns
GSSG	0.57 ± 0.05^{b}	0.60 ± 0.06^{a}	0.57 ± 0.03^{b}	0.60 ± 0.08^{a}	0.69 ± 0.06	0.48 ± 0.04	**0.02**	**0.02**	ns	ns
rGSH/GSSG	148 ± 15^{a}	115 ± 11^{b}	135 ± 11	127 ± 17	95 ± 7^{b}	167 ± 10^{a}	**0.01**	ns	**<0.01**	ns
Plasma										
Cortisol	25 ± 3	26 ± 4	26 ± 5	25 ± 2	20 ± 2	30 ± 4	ns	ns	ns	ns
Lactate	0.57 ± 0.07	0.41 ± 0.04	0.44 ± 0.04	0.55 ± 0.07	0.54 ± 0.07	0.45 ± 0.04	ns	ns	ns	ns

Mean ± standard error of means (n = 6 tanks). Unlike superscript letters indicate significant differences between groups (+Se, supplemented with SeMet; —Se, no SeMet-supplementation; +B6, supplemented with PN.HCL; —B6, no PN.HCL-supplementation; NO, fish at normoxic conditions; HO, fish subjected to hyperoxic stress) and ns = non-significant according to repeated three-way ANOVA.
rGSH, reduced glutathione; GSSG, oxidized glutathione; GPX, glutathione peroxidase; SeGPX, seleno-dependent GPX; indGPX, seleno-independent GPX; TBARS, thiobarbituric acid reactive substances.

Therefore, in the present study a cohort of the animals were subjected to periodic hyperoxia to create oxygen stress before sampling. Fish before or after a periodic hyperoxia showed no significant differences in plasma cortisol. This contrasts an earlier study using a similar stress protocol where increased cortisol levels in hyperoxia stressed fish were reported (Kalinowski et al., 2019). Nevertheless, the presented data show a tendency for higher cortisol levels in stressed fish although not significant ($p = 0.07$, repeated three-way ANOVA). Liver TBARS levels did not increase in stressed fish neither, which indicates that the periodic hyperoxia did not inflict lasting damage to cellular lipids. Nevertheless, the levels of rGSH significantly decreased in stressed fish indicating changes to the cellular redox system by the applied stress protocol. In

Table 7
Relative mRNA abundance in liver tissue of rainbow trout fed a diet supplemented or not with selenium and/or vitamin B6 and sampled before (NO) or after (HO) subjected to periodic hyperoxia stress.

	Dietary supplementation				Hyperoxic stress		Repeated three-way ANOVA			
	-Se	+Se	-B6	+B6	NO	HO	Se	B6	Stress	Int
	(CTL, PYR)	(SEL, SEPY)	(CTL, SEL)	(PYR, SEPY)						
dnmt1	1.61 ± 0.18	1.65 ± 0.22	1.63 ± 0.20	1.64 ± 0.20	1.57 ± 0.23	1.70 ± 0.17	ns	ns	ns	ns
sahh	1.23 ± 0.11	1.35 ± 0.08	1.21 ± 0.10	1.37 ± 0.09	1.36 ± 0.11	1.22 ± 0.09	ns	ns	ns	ns
bhmt	1.64 ± 0.31	1.73 ± 0.51	1.24 ± 0.28	2.13 ± 0.49	2.18 ± 0.52	1.19 ± 0.20	ns	ns	ns	ns
mtr	2.05 ± 0.24	1.75 ± 0.27	1.89 ± 0.28	1.91 ± 0.24	1.45 ± 0.26^{b}	2.35 ± 0.17^{a}	ns	ns	**0.01**	ns
cbs	1.53 ± 0.16	1.32 ± 0.09	1.19 ± 0.10^{b}	1.66 ± 0.13^{a}	1.47 ± 0.15	1.38 ± 0.12	ns	**0.02**	ns	ns
cgl	1.54 ± 0.12^{a}	1.23 ± 0.12^{b}	1.38 ± 0.16	1.39 ± 0.10	1.14 ± 0.08	1.63 ± 0.13	**0.05**	ns	ns	ns
gclc1	1.58 ± 0.18	1.79 ± 0.18	1.50 ± 0.17	1.86 ± 0.17	1.58 ± 0.15	1.79 ± 0.20	ns	ns	ns	ns
gnmt	1.14 ± 0.09	1.25 ± 0.10	1.26 ± 0.11	1.13 ± 0.08	1.10 ± 0.06	1.29 ± 0.12	ns	ns	ns	ns
gpx1a	0.86 ± 0.05^{b}	1.38 ± 0.13^{a}	1.18 ± 0.11	1.06 ± 0.13	1.18 ± 0.13	1.05 ± 0.11	**0.02**	ns	ns	ns
gpx1b1	1.34 ± 0.18	1.75 ± 0.17	1.44 ± 0.17	1.65 ± 0.20	1.67 ± 0.19	1.42 ± 0.18	ns	ns	ns	ns
gpx1b2	1.10 ± 0.10^{b}	3.06 ± 0.26^{a}	1.93 ± 0.30	2.23 ± 0.40	2.05 ± 0.38	2.11 ± 0.33	**<0.01**	ns	ns	ns
gpx4a1	1.07 ± 0.12	1.28 ± 0.19	1.04 ± 0.07	1.31 ± 0.21	1.30 ± 0.19	1.05 ± 0.12	ns	ns	ns	ns
gpx4a2	0.87 ± 0.13^{b}	1.85 ± 0.31^{a}	1.33 ± 0.22	1.38 ± 0.33	1.64 ± 0.35	1.07 ± 0.13	**0.02**	ns	ns	ns
selpa1	1.42 ± 0.12^{b}	2.06 ± 0.18^{a}	1.86 ± 0.21	1.63 ± 0.14	1.53 ± 0.16	1.95 ± 0.18	**0.01**	ns	ns	ns
selpa2	1.43 ± 0.15	1.96 ± 0.18	1.79 ± 0.21	1.60 ± 0.16	1.42 ± 0.15^{b}	1.96 ± 0.18^{a}	ns	ns	**0.02**	ns
gstp	0.84 ± 0.09	0.85 ± 0.09	0.84 ± 0.10	0.85 ± 0.08	1.04 ± 0.07^{a}	0.66 ± 0.07^{b}	ns	ns	**0.02**	ns
gr	1.46 ± 0.13	1.64 ± 0.20	1.47 ± 0.21	1.63 ± 0.12	1.49 ± 0.13	1.61 ± 0.21	ns	ns	ns	ns

Mean ± standard error of means (n = 6 tanks). Unlike superscript letters indicate significant differences between (+Se, supplemented with SeMet; —Se, no SeMet-supplementation; +B6, supplemented with PN.HCL; —B6, no PN.HCL-supplementation; NO, fish at normoxic conditions; HO, fish subjected to hyperoxic stress) and ns = non-significant according to repeated three-way ANOVA.
Selp, selenoprotein P; gpx, glutathione peroxidase; cbs, cystathionine-β-synthase; dnmt; DNA methyltransferase; gnmt, glycogen *n*-methyltransferase; cgl, cystathionine-γ-synthase; sahh, S-adenosylhomocysteine hydrolase; gclc, glutamate-cysteine ligase catalytic subunit; bhmt, betaine–homocysteine *S*-methyltransferase; mtr, methionine synthase; gstp, glutathione S-transferase; gr, glutathione reductase.

this relation, the periodic hyperoxia revealed some interactive effects of SeMet and PN.HCL supplementation on the targeted pathways not detected in fish under normoxic conditions. Muscle PM levels were highest in fish fed the diet supplemented with both SeMet and PN.HCL, while lowest in fish fed the diet with singular PN.HCL supplementation. Studies in mammals have shown that the transport and mobilization of muscular Se is B6-dependent (Yin et al., 1991). Such action could be relevant during stressful conditions where Se requirements increase (Küçükbay et al., 2009), and Se might be mobilized from the muscle into the blood stream. Although no significant effect of the hyperoxic stress on liver or muscle Se levels was detected, the expression of the *selpa2* gene encoding for selenoprotein P, an important plasma transport protein (Seale et al., 2018), was higher in stressed compared to normoxic fish. In addition, SeMet and PN.HCL supplementation had interactive effects on the levels of tissue transsulfuration metabolites. Under oxidative conditions GSH is consumed. This increases the demand for GSH and has been reported to favour its biosynthesis through transsulfuration (Dalto et al., 2015). The present data show that in stressed fish the singular supplementation of SeMet resulted in highest liver SAM levels. This is opposite to an earlier study where fish subjected to hypoxia stress showed lower SAM levels (Wischhusen et al., 2021). The observed differences could be due to the higher selenium supplementation level used in the present study, or the nature of the stress. High SAM is known to activate CBS and therefore associates with increased transsulfuration (Ereño-Orbea et al., 2014). In the present study, lower liver hCys levels in the PN.HCL and SeMet co-supplemented treatment compared to singular SeMet or PN.HCL supplementation indicate that the co-supplementation of these micronutrients supported the flow towards transsulfuration in stressed fish. These complex interactive effects show that both Se and B6 are important factors to maintain glutathione redox homeostasis in fish under challenging oxygen conditions.

5. Conclusion

A deeper understanding of the functional role of specific micronutrients and their inter-relation in metabolic pathways can help us to better adapt to changing feed formulations in the future. This study suggests that the supplementation of PN.HCL and SeMet can induce contrasting effects on the transsulfuration pathway. However, vitamin B6 levels just above minimum requirements were sufficient to support the SeMet metabolism and selenoprotein synthesis. However, interactive effects between Se and vitamin B6 on transsulfuration metabolites in stressed fish highlight the importance of both micronutrients to maintain glutathione redox homeostasis under challenging environmental conditions in fish.

Ethics approval

This trial has been approved by the AWERB ethical committee at the University of Stirling, UK (ID SEPYH 7098). It was performed in compliance with the European Directive 2010/63/EU for the protection of animals used for scientific purposes. All procedures on fish were performed by trained personnel at the INRAE experimental fish farm in Donzacq, France under the national French Decree no. 2013–118.

Funding

The project leading to these results has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 871108 (AQUAEXCEL3.0). The work was performed at INRAE-STPEE research infrastructure, under grant agreement PID17737 (TNA programme). This output reflects only the author's view, and the European Union cannot be held responsible for any use that may be made of the information contained therein.

CRediT authorship contribution statement

Pauline Wischhusen: Writing – original draft, Visualization, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Cécile Heraud:** Writing – original draft, Methodology. **Richard Broughton:** Writing – original draft, Validation, Methodology. **Anne Surget:** Methodology. **Anthony Lanuque:** Validation, Methodology. **Frédéric Terrier:** Validation, Methodology, Investigation. **Stéphanie Fontagné-Dicharry:** Writing – review & editing, Supervision, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Mónica B. Betancor:** Writing – review &

editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors are grateful for all technical assistance in sample analysis at INRAE-STPEE laboratory and at the nutritional analytical service (NAS) at the University of Stirling.

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