THÈSE

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Parental selenium and antioxidant status in fish

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Rôle du sélénium sur les fonctions anti-oxydantes des poissons géniteurs

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Abbreviations

AMD	S-adenosylmethionine decarboxylase
BHMT	Betaine homocysteine methyltransferase
Bnc	Experimental treatment: Broodstock control
Bso	Experimental treatment: Broodstock hydroxy-selenomethionine
Bss	Experimental treatment: Broodstock sodium selenite
CBS	Cystathionine-β-synthase
CGL	Cystathionine-γ-lyase
cGPx	Cytosolic GPx
Cu	Copper
Cys	Cysteine
D-A-CH	Germany, Austria and Switzerland
DIO	Iodothyronine deiodinases
DNMT	DNA methyltransferase
EFsec	SeCys-specific elongation factor
FAO	Food and Agriculture Organization of the United States
Fe	Iron
Fep15	Fish 15 kDa selenoprotein
Fnc	Experimental treatment: Fry control
Fso	Experimental treatment: Fry hydroxy-selenomethionine
Fss	Experimental treatment: Fry sodium selenite
GNMT	Glycine N-methyltransferase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
H_2S	Hydrogen selenide
Hcys	homocysteine
I	Iodine
Met	Methionine
Mg	Manganese
mGPx	Mitochondrial GPx
Msr	Methionine sulfoxide reductase
MsrB	Methionine sulfoxide reductase B
nGPx	Nuclear GPx
Nrf2	Nuclear factor erythroid 2 related factor 2
RDA	Recommended Dietary Allowances
ROS	Reactive oxygen species
RS	Reactive species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
Se	Selenium
SeCys	Selenocysteine
SeGPX	Se-dependent GPx
SeHcy	Seleno-homocysteine
SelH, SelI, SelJ, SelK,	Selenoprotein H, Selenoprotein I, Selenoprotein J, Selenoprotein K,
SelL, SelM, SelN, SelO,	Selenoprotein L, Selenoprotein M, Selenoprotein N, Selenoprotein O,
SelP, SelS, SelT, SelV,	Selenoprotein P, Selenoprotein S, Selenoprotein T, Selenoprotein V,
SelW	Selenoprotein W
Sep15	Selenoprotein 15
SeMet	Selenomethionine
SeNP	Nanoparticle selenium
SPS2	Selenophosphate synthetase 2
TGR	Thioredoxin glutathione reductase
Trx	Thioredoxin
TrxR	Thioredoxin reductase
Zn	Zinc

1. GENERAL INTRODUCTION

1.1. Fish to feed the world

According to the Food and Agriculture Organization of the United States (FAO), food security exists, when all people, at all times have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life [1]. The role of fish to reach food security becomes increasingly important as world fish consumption grew with an annual average rate of 1.5% from 1961 to 2015 and is expected to grow also further in the future (Fig. 1).

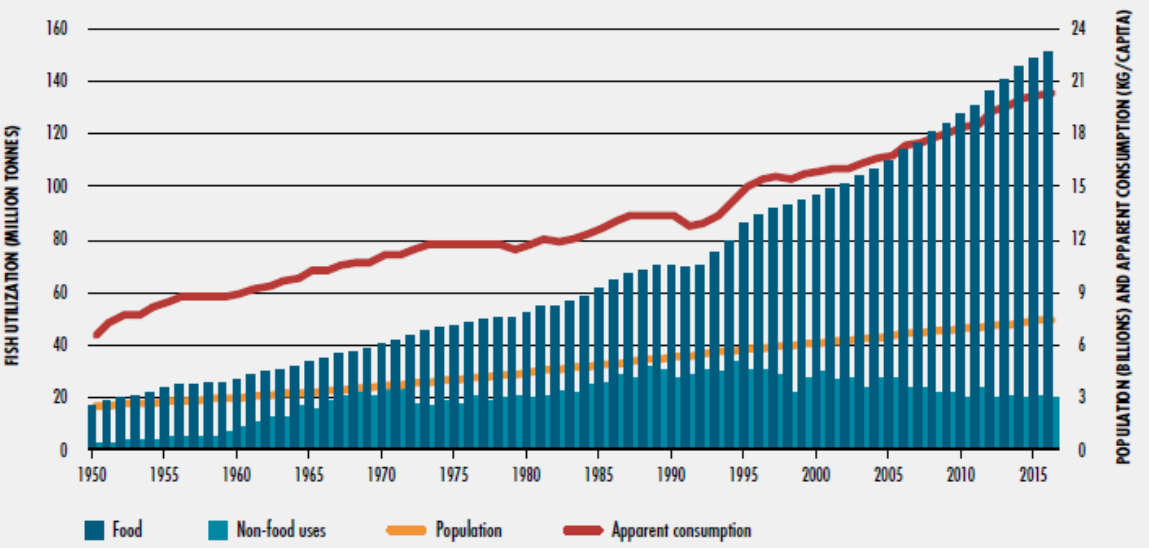

Fig. 1. World fish utilization and apparent consumption. Source: FAO [1]

Increasing fish consumption can bring health benefits in human nutrition due to the high protein content and the high levels of long-chain polyunsaturated fatty acids found in fish and other aquatic products [2]. Additionally, today, it is well recognized that fish contains significant amounts of vitamins and minerals like phosphorous, calcium and selenium that could help to address micronutrient deficiencies often referred to as "hidden hunger" [3,4]. At the world market level, fish supply is covered by two sources, fisheries and aquaculture (Fig. 2). While until the beginning of the 21 century, fish catches clearly dominated the world market, however, with annual growth rates of over 8%, aquaculture production gained more and more importance and since 2015, aquaculture supplies more than 50% of all fish consumed

worldwide [1]. The greatest proportion of fish is directly going to human consumption, but a fraction of global landings is also used for the production of fishmeal and fish oil referred to as "reduction fisheries" [5]. Fishmeal is mainly used by the aquaculture industry to produce compound aquafeeds used to feed direct-fed species which are of high value and estimated to make up 70% of cultivated fish and crustacean species [6]. Therefore, sustainable fish nutrition is one of the main constrains for the further development of the aquaculture industry.

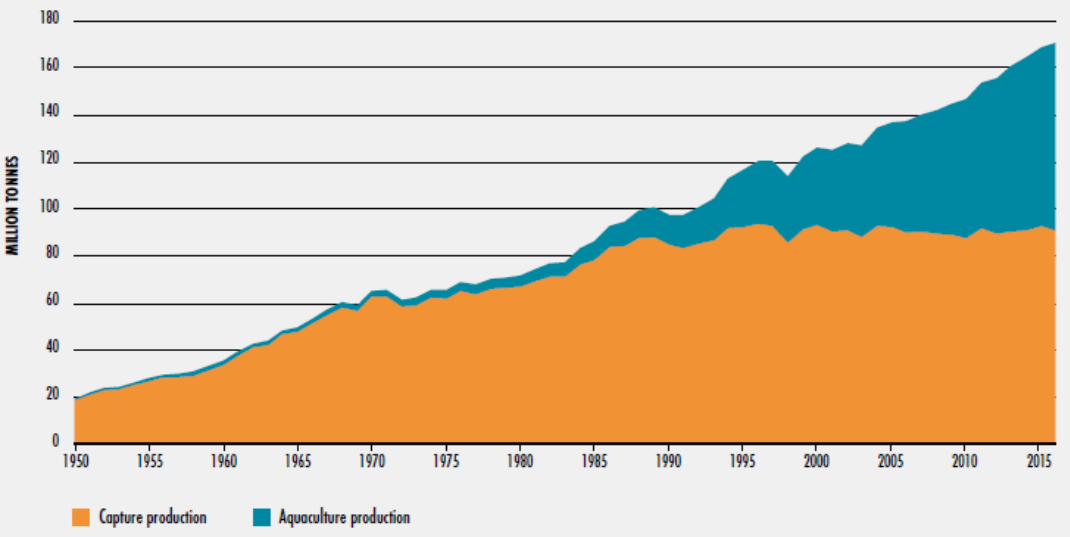

Fig. 2. World Capture Fishery and Aquaculture production. Source: FAO [1]

1.2. Fishmeal and fish oil reduction for more sustainability

The composition of aquafeeds is determined by three main factors: first, the nutritional requirements of fish, which vary not only between species [7], but also at different developmental stages of their life-cycle [8]; second, by the nutritional composition of available ingredients and third, by the price of these ingredients [9]. It can be assumed that drivers for the development of new diet formulations are either the implementation of new species, the updated knowledge about the nutritional requirements of a species or the limitation of availability ingredients, either physical or by price. For several years, the major hot spot in

industrial and scientific aquaculture has dealt with the dependency on fishmeal and fish oil as limiting resources [6,10]. Fishmeal is characterized by a high quality and well balanced protein profile, easy to digest without the presence of anti-nutritional factors, while fish oil is rich in long-chain polyunsaturated fatty acids covering the essential fatty acid requirements explaining the intensive use especially for carnivorous species with high protein needs [7]. The search for alternative ingredients led already to a significant reduction in fishmeal proportion of aquafeeds and projections estimate a further decrease also in the following years [10]. Terrestrial replacements mainly consist of plant products, which however, bring new challenges for finding the optimal nutrient composition. Plant ingredients present several disadvantages especially for carnivorous species, which are not adapted to digest them. Besides the relative low protein content [11], which can be improved by the supplementation of essential amino acids, a major topic of concern, the presence of anti-nutritional factors represents a limiting factor in plant meals, as it can reduce digestibility and absorption and may even induce toxicity [12]. Improvement has been achieved by the application of different processing techniques to either remove or eliminate these compounds [13]. As a consequence, in commercial diets for Atlantic salmon the estimated levels of fishmeal range around 18%, while plant proteins account for about 40% [14].

1.3. Impact on micronutrient levels

With the replacement of the main protein and lipid sources in fish diets, in any case, a careful evaluation for the availability of other nutrient groups, including microminerals should be performed. Common trace elements are iron (Fe), copper (Cu), iodine (I), manganese (Mg), zinc (Zn) and selenium (Se). These microminerals are essential, but required only in small amounts as components in the enzyme and hormone systems [15]. Micromineral concentrations can vary greatly within similar feed categories, but usually they are found to be low in ingredients used for practical fish diets and the addition of mineral pre-mixes are common practice to enhance

their levels without detailed consideration of basal levels. The inclusion of a mineral premix in a plant ingredient-based rainbow trout diet even resulted in supranutritional level for Cu and Mg [16] which should be avoided to exclude negative impacts up to toxicity level as many micronutrients show only narrow ranges between requirements and toxicity. In addition, losses to the environment by urinary excretion should be kept low. On the other hand, Zn and Se have been found to be suboptimal even at high supplementation levels in the mentioned study [16] possibly due to reduced availability connected to interactions with other ingredients, like the presence of anti-nutritional factors. The narrow range between required and toxic levels resulted thus in strict supplementation limits for all animal feeds including aquafeeds given by the authorities in several countries (e.g. European Food Safety Authority, US Food and Drug Administration). This might be problematic for the introduction of new plant ingredient-based diets to the market and further research is required to evaluate the impact of changing micronutrient levels on the metabolism and consequently to define optimal supplementation levels in plant ingredient-based diets for cultivated fish species.

1.4. Risk of selenium deficiency

Reduced Se levels in plant-based aquafeeds create a health risk in fish. The biological function of Se is mainly based on selenoproteins that are involved in different metabolic reactions [17]. With glutathione peroxidase (GPx), thioredoxin reductase (TrxR) and methionine sulfoxide reductase B1 (MsrB1), selenoenzymes play a major role in the antioxidant system. Former studies in fish already showed that the supplementation of Se in plant-based diets can improve the antioxidant status by elevated expression and activity of antioxidant enzymes [18]. However, little knowledge in fish exists about the effect of Se throughout the different life stages, although from other animals it is well established that Se deficiency can severely impair the reproductive performance and Se can deliver antioxidant protection in the progeny through parental Se transfer [15,19,20]. Recent studies also suggested that Se ingestion might induce

epigenetic mechanisms including DNA methylation that can implement physiological changes in the following generations in long-term [21,22]. These processes might differ between dietary Se forms as they follow different metabolic pathways and the choice of the Se feed additive could result in different metabolic responses [23].

Several questions remain to be answered in order to define the optimal strategy exchanging Se sources in plant ingredient-based aquafeeds without risking negative consequences on the performance. Therefore, this PhD project aimed to investigate the effect of parental Se nutrition in fish on reproduction and its metabolism in broodstock and progeny.

2. LITERATURE REVIEW

2.1. Antioxidant system, the basics [24]

Oxygen can cause toxic effects even in aerobic organisms. This is related to the fact that radical species can derive from oxygen. A free radical is defined as any species capable of independent existence that contains one or more unpaired electrons. Oxygen species with higher reactivity compared to O_2 are collectively termed as reactive oxygen species (ROS). By this definition ROS not only include free radicals, but also other oxygen species like H_2O_2 that react fast with selective molecules. Reactive species (RS) can also derive from other agents including chlorine, bromine and nitrogen. RS are constantly generated during metabolic processes and can trigger an oxidation, a reaction where atoms or molecules lose an electron, hydrogen or gain oxygen, or a reduction, which are the counter reactions. These reactions change the cellular redox state, based on thermodynamics determining the kinetics and direction of the reaction. The redox state mediates many metabolic processes and its change cannot be considered as generally harmful as it functions as a signaling process to mediate metabolic processes, but the excess of RS causes oxidative stress, which is an imbalance between RS and antioxidants which control them. Cellular oxidative stress can result in increased proliferation, cell injury, senescence and ultimately cell death. RS can target all types of biomolecules including lipids, DNA, protein and carbohydrates. In healthy organisms, a well-balanced antioxidant system is in place to prevent oxidative damage up-regulated in response to oxidative stress. The antioxidant system is carried by antioxidants, substances that delay, prevent or remove oxidative damage to a target molecule. Antioxidants can be synthesized *in vivo* or taken up from the environment e.g. in the diet. Antioxidants may scavenge, react with and/or remove RS, control RS formation or protect or replace oxidative sensitive biomolecules. Also the reparation of molecules damaged by oxidation can be considered as an antioxidant defense. Major dietary antioxidants include vitamin E (especially α-tocopherol), vitamin C and carotenoids. Selenium, on the other hand, is not an antioxidant itself, but rather functions as a precursor for the *in vivo* antioxidant defense based on enzymes, their substrates and co-factors.

2.2. Discovery of selenium as essential micronutrient

The trace element Se was discovered in 1817 by two Swedish chemists Jöns Jakob Berzelius and Johan Gottlieb Gahn in search for a toxic element causing illness in workers on acid plants producing sulfuric acid [25]. Given the circumstances of this discovery, Se has been considered as a toxin in the following years and it was only in 1957, when Schwartz and Foltz demonstrated that Se can prevent liver necrosis in rats, that Se was classified as an essential micronutrient. Shortly after that, Se was included in the list of essential nutrients and since then Se has been the subject of many scientific studies. Today, its supplementation is a well-accepted practice in terrestrial livestock production where it contributes to the improvement of growth and reproduction and a better health by preventing metabolic diseases related to Se deficiency [26].

2.3. The selenium cycle

2.3.1. SELENIUM IN THE ENVIRONMENT

The element Se is widespread in the environment and continuously recycled in the atmosphere, marine and terrestrial systems [27]. Thereby, the terrestrial system, in linkage with agricultural activities can be considered as the main point of entry by Se into animal and human health [28]. Large differences in Se levels exist between different parts of the world [29], but areas with adequate or deficient content present a larger scale compared to areas with Se excess [30] (Fig. 3).

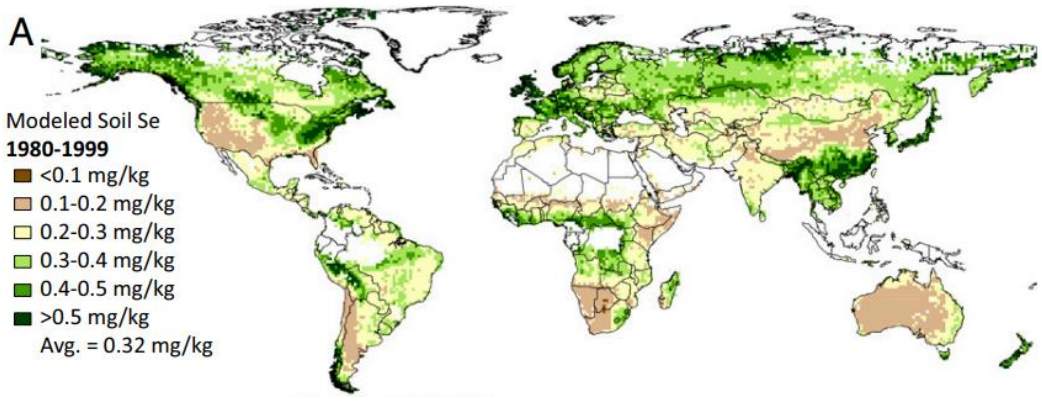

Fig. 3. Geographical representation of modeled soil Se content by Jones et al [30]

Se deposition into soil can occur through different routes (Fig. 4). Natural emission to the atmosphere mainly originates from marine biomethylation, but can also occur through crustal weathering, sea spray and volcanic eruption [31]. Studies have proven that the atmosphere plays an important role for the distribution of Se with an annual cycle of 13,000-19,000t [31] transiting Se to different parts of the world with an average distance range of 2,500-5,000km [32]. Large amounts of atmospheric Se also originates from anthropogenic sources, mainly the fossil fuel combustion, especially coal, and non-ferrous metal production and manufacturing [33]. Due to its high mobility, the extent to which different Se sources contribute to the atmospheric levels are still largely unknown [32].

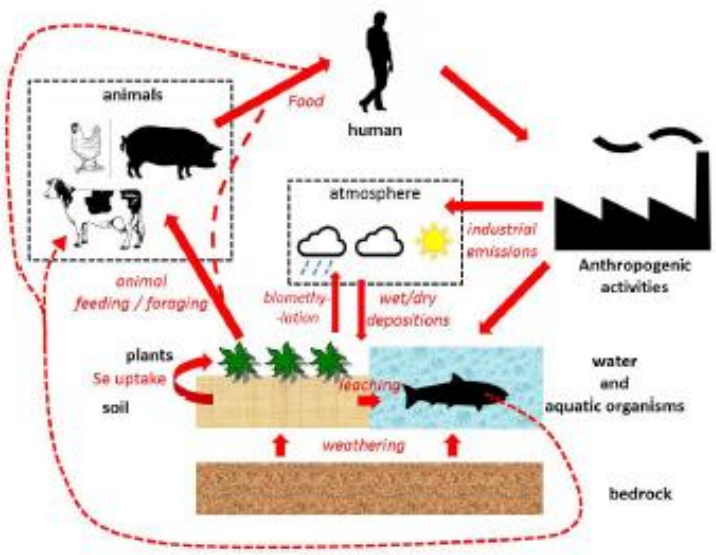

Fig. 4. Schematic selenium cycle. Source: Godin [34]

From the atmosphere, Se is submitted to water bodies and soil, through precipitation. Other natural soil Se sources include mainly Se spread during volcanic eruptions and Se can be also found in rocks, where in most cases it substitutes for sulfur in sulfide minerals due to their high similarity in chemical properties [28]. However, in soil forming rocks, Se levels are low and average only between 0-0.41 mg/kg with the highest concentrations found in sediment rocks [35]. Soil Se is mainly inorganic, existing in four valence states: Se(-II) (=selenide), Se(0) (=elemental Se), Se(IV) (=selenite) and Se(VI) (=selenate) [36]. In general, selenite is the predominant species under acidic and reducing conditions showing lower water solubility and thus bioavailability compared to selenate, the predominant form in alkaline and well aerated soils [37]. Seleno-complexes within organic matter are still largely unidentified, but due to both biotic and abiotic mechanism, retention is high creating low bioavailability for plants.

2.3.2. ENVIRONMENTAL CONTAMINATION BY SELENIUM

In the environment, Se poisoning has been of a concern in aquatic ecosystems impacted by industrial or agricultural activities such as exposure to agriculture drain-water, sewage sludge,

flying ash from coal-fired power plants, oil refineries and mining of phosphate or metal ores [38]. Thereby, the bio-accumulating nature of Se creates a risk along the food web, because even though fish can take up Se through the gills, dietary exposure usually creates the highest risk. Mortality, reduced overall or kidney growth, malformations as well as reduced fertility are typical toxicity symptoms in Se poisoned fish [39–41]. In nature, high waterborne Se levels might also alter the typical fish migration pattern. Also, in terrestrial animals and humans, toxic effects by Se are well known [42,43]. This might be connected to the consumption of accumulator plants, contaminated drinking water and food or wrongly taken dietary supplements (especially in the USA).

2.4. Selenium in plants

2.4.1. UPTAKE

Se levels in plants are rather determined by the Se bioavailability than soil Se concentrations which can lead to Se deficiency even in areas with adequate Se levels. Several bio-physio-chemical parameters including pH, redox state, Se speciation, organic matter content and the presence of competing ions can affect the bioavailability of Se [28]. Transporters in the roots actively mediate the Se uptake [44]. Se concentrations inside and outside the plants define the preferences for the transporters. Organic Se forms were found to be much faster taken up compared to inorganic Se forms, however, the mobility of Se within the plant was higher for selenate compared to the organic form of selenomethionine (SeMet) [45]. Selenate is taken up through sulfate transport channels, explaining the reduced uptake under high sulfur concentrations [46]. Selenite can be actively transported through phosphate transporters and was therefore inhibited by the presence of phosphorous, but selenite can additionally enter the plant through passive transfusion [47]. Se is mainly accumulated in vacuoles and can flux within the plant following sulfate transporters to the leaves. Plants can be divided into three categories based on their ability to store Se [48]. Non-accumulators (e.g. grasses and crops) contain less

than 100 mg Se/kg dry weight (DW) and cannot survive in Se-rich soils, secondary-accumulator (e.g. brown mustard and broccoli) store between 100-1000 mg Se/kg DW, while hyperaccumulator (e.g. Astragalus species, which include some herbs) contain levels above that. Hyperaccumulator contain methylated forms of selenocysteine (SeCys) and SeMet that can be transformed to dimethyldiselenide and subsequently volatized [49].

2.4.2. METABOLISM IN PLANTS

Selenate can be metabolized in the chloroplasts of the leaves by enzymes involved in the sulfate assimilation. The first step is a reduction by ATP sulfurylase to adenosine phosphoselenate, which can be further reduced non-enzymatically via glutathione (GSH) to GSH-conjugated selenite and further through several intermediates to GSH-conjugated selenide, whereby the last reaction might require glutathione reductase (GR) activity [44,50] (Fig. 5). Uptaken selenite can be directly GSH-conjugated to follow the similar pathway as selenate. GSH-conjugated selenide is thought to be the substrate for cysteine (Cys) synthase to synthesize SeCys.

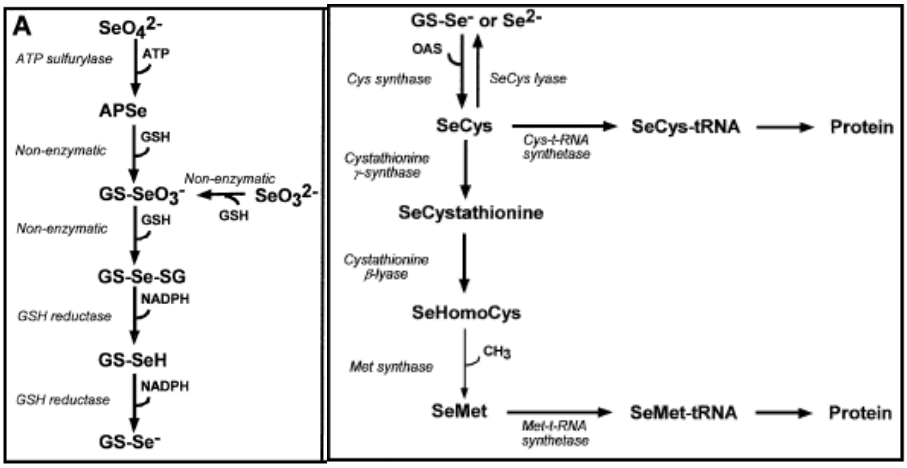

Fig. 5 Selenium metabolism in plants. Source: Terry et al [44]

The main product of the Se metabolism in plants is SeMet. The transformation of SeCys to SeMet by enzymatic mediated reaction occurs by formation of seleno-cystathionine and seleno-

homocysteine (SeHcy) [44,50]. In higher plants (=containing vascular tissue), Se incorporation in proteins might occur on a nonspecific bases as a substitute for Met or in case of SeCys for Cys as proteins containing Se are without any known functional requirements for growth [50].

2.5. Metabolism in humans and animals

Humans and animals can metabolize Se taken up by the diet to biological active compounds. Independently from the dietary form, it is considered that the intestinal absorption of dietary Se sources is high [51,52]. However, bioavailability seems to differ according to dietary Se form which is also connected to fundamentally different metabolic pathways between the Se species [53] (Fig. 6).

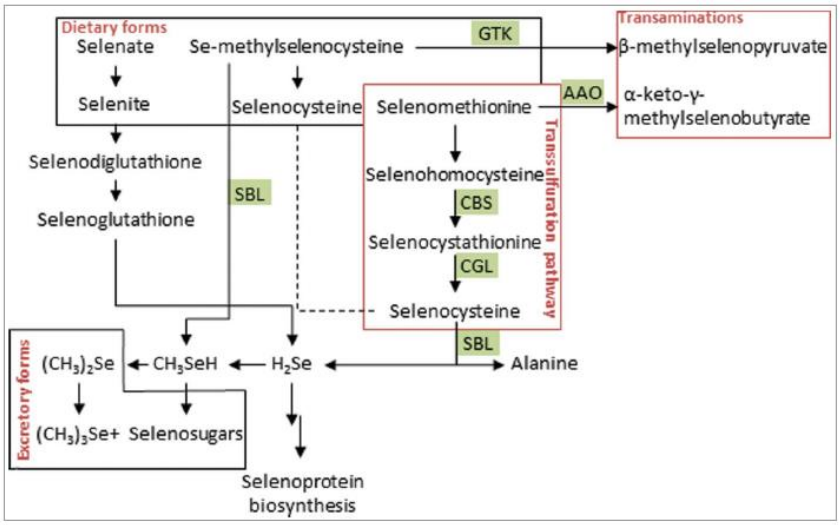

Fig. 6. Metabolism of dietary selenocompounds. Source: Speckmann and Grune [21]

The majority of inorganic selenite is considered to follow a three-step reduction in a GR-dependent pathway to hydrogen selenide (H_2Se). In a first step, selenite is non-enzymatically bound to GSH forming selenodiglutathione through several intermediates. Afterwards, a NADPH-dependent reduction is mediated by GR or TrxR, first to form the unstable product selenoglutathione and finally H_2Se. But, selenite was also found to be a substrate for TrxR equally resulting in the formation of selenide, however, producing oxidized NADPH [54] (Fig. 7).

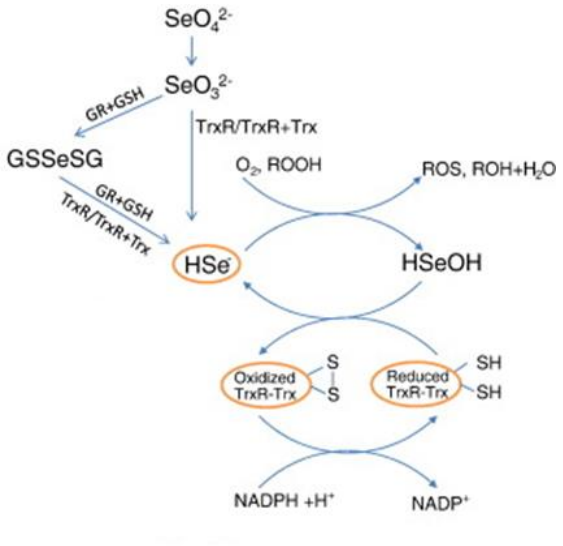

Fig. 7. Metabolism of inorganic seleno-compounds by GSH and TrxR. Adapted from Lu et al. [54]

Similarly, organic Se forms, with SeMet representing the main dietary Se form [23], are synthesized to H_2Se, however, following a different pathway (Fig. 6). SeMet can be co-metabolized with methionine (Met) either through methylation to methyl selenol by methionine-γ-lyase or demethylated following the transsulfuration pathway [22,55]. Today, it is assumed that in metabolism, it cannot be distinguished between SeMet and its sulfur analogue resulting in two consequences: first, chance determines whether SeMet or Met is metabolized and second, the metabolic fate of SeMet is highly dependent on the overall Met pool [56]. Similar to Met, SeMet can yield to the Se homologue SeHcy by Met-adenosyltransferase and methyltransferase reaction. SeHcy can be either re-methylated or converted to SeCys by the regulatory enzymes cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CGL). Substrate specific SeCys lyases in turn then catalyze the synthesis of selenide from SeCys. Independent from its dietary origin, H_2Se is highly cytotoxic as it can get auto-oxidized consuming NADPH and producing ROS, a process mediated by TrxR [54] (Fig. 7). Therefore, H_2Se is either used as the precursor for selenoprotein synthesis or methylated to excretory forms. Main excretion routes are through urine and feces [57].

2.6. Selenium in proteins

Proteins containing Se can be divided into three groups. The first group is composed by Se-containing proteins where SeMet is incorporated non-specifically as a replacement of Met [58]. This process has a higher probability in proteins containing naturally high amounts of Met and the non-specific incorporation is highly dependent on the SeMet/Met ratio and therefore on dietary Met levels as well as on dietary SeMet levels. In contrast, the second group is characterized by the specific incorporation of Se in selenoproteins, that is independent from the ingested Se form [59]. In selenoproteins, Se is incorporated as SeCys according to the genetic code, a process detailed more in the following section. Some evidence is given that in addition also a third group of Se-binding proteins exists, where Se is specifically attached to the protein, but no gene sequence for the incorporation of Se in the protein can be found [58]. Se-binding proteins were first described in mouse tissues with still limited knowledge about their function [60]. A more recent study suggests that Se-binding proteins are involved in the delivery of Se to the selenophosphate synthetase system in selenoprotein production [61].

2.7. Selenoproteins

2.7.1. SYNTHESIS

The process of selenoprotein synthesis described by Xu et al. [62] has been reviewed several times and is summarized in this paragraph [17,63,64]. As mentioned above, selenoproteins are characterized by the presence of SeCys at their active center. In contrast to other amino acids, SeCys needs to be biosynthesized from a specific tRNA encoded in the selenocysteine tRNA[Ser]Sec (*trsp)* gene with H_2Se functioning as the Se donor (Fig. 8A). In a first step, serine is attached as a mediator to SeCys $tRNA^{[Ser]Sec}$ by seryl tRNA synthetase to form Seryl-$tRNA^{[ser]sec}$ which is in a second step phosphorylated by phosphoseryl tRNA kinase to build phosphoseryl-

tRNA[ser]sec the final substrate for SeCys synthesis. Afterwards, selenophosphate synthetase 2 (SPS2) and ATP activate selenide to replace the phosphate resulting in seleno-cysteryl-tRNA[Ser]Sec, which delivers SeCys to the polypeptide chain during translation.

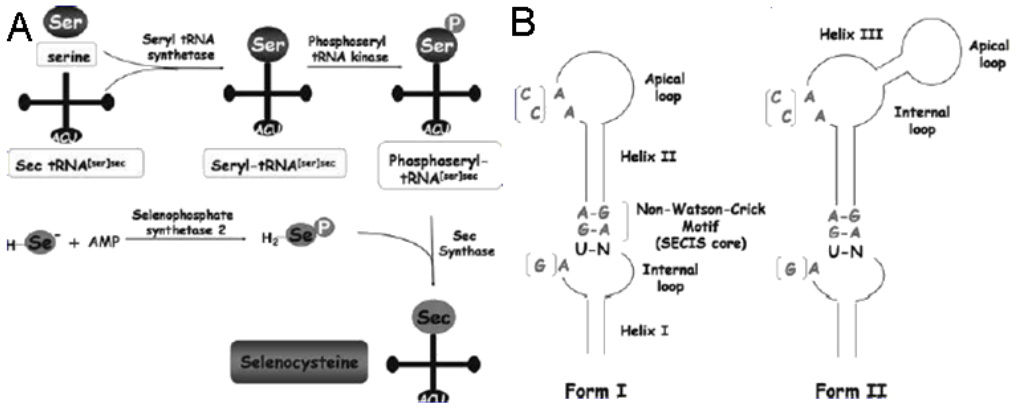

Fig. 8. SeCys biosynthesis pathway in mammalian cells (A) and structure of SECIS element (B). Adapted from Papp et al [17]

The tRNA[Ser]Sec is anticodon complementary to UGA, which redundantly codes also for tryptophan (Trp) in mitochondria and otherwise is serving as a stop codon. To decode UGA as SeCys other conditions, besides the presence of tRNA[Ser]Sec, need to be fulfilled including the presence of a secondary RNA stem-loop structure located up to several kilobases away from the UCA codon in the 3' untranslated region called a SECIS element. The SECIS element always contains a core region with two non-Watson-Crick G-A base pairing and an ampical loop with two consequentially unpaired AA or CC residues (Fig. 8B). In addition, several proteins, especially SPS2 and the SeCys-specific elongation factor (EFsec), are involved in the binding complex during translation; however, their exact role has not yet been resolved [65].

2.7.2. HIERARCHICAL REGULATION

Under limiting Se conditions, two hierarchical principles apply, first the tissue supply and secondarily the transcriptional regulation of the tissue level. Studies in rats using labeled ^{75}Se showed that ingested Se is unequally distributed in the body with priority given to the brain, the

reproductive and endocrine organs [66,67]. Within tissues selenoprotein expression levels adjust differently to decreasing Se levels [68]. The hierarchy of selenoprotein expression has been reviewed by Sunde [69] suggesting the following hierarchy for some well-studied selenoproteins from most to least affected by Se: GPx1 > Iodothyronine deiodinases 1 (DIO1), Selenoprotein P (SelP), TrxR1 > GPx4. Consequently, GPx1 levels have been widely used as an indicator for optimal Se levels [70]. However, the complex mechanisms behind the translational regulation are not yet fully understood. A key component in this regulation might be SPS2 as mutations in the latter are associated with selective disruptions of selenoprotein synthesis [71] and also $tRNA^{[Ser]Sec}$ can exist either as methylated or unmethylated isoform. The two existing isoforms of SeCys $tRNA^{[Ser]Sec}$ exhibit differences in activity, indicating that tRNA methylation might be a regulatory mechanism in selenoprotein expression [72].

2.7.3. SEXUAL DIMORPHISM

None of the 25 human selenoproteins are located on the sex chromosome and silencing of the *trsp* gene that is necessary for the selenoprotein production leads to equal mortality in both males and females [73], but nonetheless, sex specific differences apply, when it comes to Se biology [74]. Already the Se metabolism has been found to be sexually dimorphic due to gender specific expression of enzymes involved [75]. TrxR1 involved in the reduction of selenite to H_2Se for protein incorporation was found to diminish under selenite supplementation in females, while its expression in males increased by supplementation of various Se forms [76]. In addition, CBS and CGL enzymes of the transsselenation pathway of dietary SeMet, can be regulated by sex hormones [77,78]. Also, the hierarchical distribution of Se in the body varies between males and females, possibly in link to the preferential Se supply to the testes in males, where GPx4, but also SelP play an important role for spermatogenesis [79]. Therefore, it seems logical that in males under limited Se conditions, testes and brain compete for Se supply [76] and males are found to be generally more responsive to changing Se levels [74]. Such gender

specific differences in Se uptake and distribution might already link to the reason for sex-specific expression patterns of selenoproteins as no sexual dimorphism in biosynthesis for translation factors such as SPS2 and EFsec were detected, while under regular diet, selenoproteins with DIO1, GPx and SelP were differentially expressed in various female and male mice tissues including liver and kidney [80,81]. In light of these findings, it seems necessary to include a sex specific design for nutritional Se studies when possible.

2.8. Selenoproteome

2.8.1. MAMMALIAN SELENOPROTEOME

The nomenclature of selenoproteins was based on a root symbol followed by a letter consecutively given [82]. However, the root symbols varied (e.g. SEL, SEP, SELENO) and quickly, selenoenzymes were re-named with the discovery of their biological functions. The human genome is known to contain 25 genes coding for selenoproteins [83] (Tab.1). For many selenoproteins, the functional knowledge is incomplete and besides their antioxidant function, studies mainly focused on their role in cancer.

Tab 1. Mammalian and fish selenoproteome. Adapted from Mariotti et al [84]

	Mammals	Fish
Selenophosphate synthetase 2a (SPS2a)	Only in marsupials	YES
Selenophosphate synthetase 2b (SPS2b)	YES	NO
Selenoprotein P (SelP)	YES	YES
Selenoprotein Pb (SelPb)	NO	YES
Thioredoxin reductase 1 (TrxR1)	YES	YES
Thioredoxin reductase 2 (TrxR2)	YES	YES
Thioredoxin glutathione reductase (TGR)	YES	NO
Glutathione peroxidases 1a (GPx1a)	YES	YES
Glutathione peroxidases 1b (GPx1b)	NO	YES
Glutathione peroxidases 2 (GPx2)	YES	YES
Glutathione peroxidases 3 (GPx3)	YES	YES
Glutathione peroxidases 3b (GPx3b)	NO	YES
Glutathione peroxidases 4a (GPx4a)	YES	YES
Glutathione peroxidases 4b (GPx4b)	NO	YES
Glutathione peroxidases 6 (GPx6)	YES	NO
Iodothyronine deiodinases 1 (DIO1)	YES	YES
Iodothyronine deiodinases 2 (DIO2)	YES	YES
Iodothyronine deiodinases 3 (DIO3)	YES	YES
Iodothyronine deiodinases 3b (DIO3b)	NO	YES
Selenoprotein 15 (Sep15)	YES	YES
Fish 15 kDa selenoprotein (Fep15)	NO	YES
Selenoprotein H (SelH)	YES	YES
Selenoprotein I (SelI)	YES	YES
Selenoprotein J (SelJ)	NO	YES
Selenoprotein J2 (SelJ2)	NO	YES
Selenoprotein K (SelK)	YES	YES
Selenoprotein L (SelL)	NO	YES
Selenoprotein M (SelM)	YES	YES
Selenoprotein N (SelN)	YES	YES
Selenoprotein O (SelO)	YES	YES
Selenoprotein O2 (SelO2)	NO	YES
Methionine sulfoxide reductase 1a (SelR/ MsrB1a)	YES	YES
Methionine sulfoxide reductase 1b (SelR/ MsrB1b)	NO	YES
Selenoprotein S (SelS)	YES	YES
Selenoprotein T1a (SelT1a)	YES	YES
Selenoprotein T1b (SelT1b)	NO	YES
Selenoprotein T2 (SelT2)	NO	YES
Selenoprotein U1a (SelU1a)	Only in platypus	YES
Selenoprotein U1b (SelU1b)	NO	YES
Selenoprotein U1c (SelU1c)	NO	YES
Selenoprotein V (SelV)	YES	NO
Selenoprotein W1 (SelW1)	YES	YES
Selenoprotein W2a (SelW2a)	NO	YES
Selenoprotein W2b (SelW2b)	NO	YES
Selenoprotein W2c (SelW2c)	NO	YES
TOTAL	27	41

Selenophosphate synthetase 2 (SPS2)

SPS2 or SEPHS2 is a selenoprotein required for the synthesis of selenoproteins and in this sense also for the synthesis of itself [85]. As earlier described, SPS2 catalyzes the conversion of selenide to selenophosphate. Therefore, SPS2 might be an important protein in the regulation of selenoprotein expression. In eukaryotes, a Cys homologue protein exists with SPS1, that can however, not fully functionally replace SPS2 and selenoprotein synthesis remains strongly impaired in unique presence of SPS1 [86]. The regulatory mechanisms behind SPS2 activity remains to be investigated. Some studies suggest a relationship between SPS2 expression and different forms of cancer, especially breast cancer [87].

Selenoprotein P (SelP)

The unique structure of SelP serves as an indicator for the function of the protein (Fig. 9). SelP can be divided into two main domains, first, the N-terminus, which consists of one SeCys and a redox motif, commonly found in selenoproteins using thioredoxin (Trx), and second, a C-terminus that contains a varying number of SeCys making it the only identified mammalian selenoprotein that incorporates more than one SeCys [88].

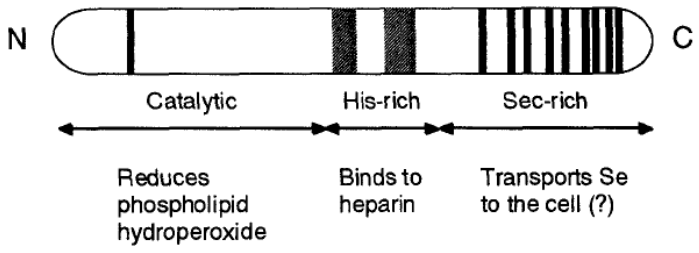

Fig. 9. Domain structure of SelP and its function. Source: Saito and Takahashi [89]

The insertion of SeCys in SelP is provided by two SECIS elements in the 3' untranslated region, which were found to be unequally efficient in SeCys incorporation possibly connected to a control mechanism that checks the availability of SeCys for SelP synthesis and differentiates the

four different isoforms, first identified in rats, that contain 1, 2, 6 or 10 SeCys, but further studies are required to verify this hypothesis [90,91]. The high numbers of SeCys make SelP a suitable transport and storage protein for Se. The main SelP synthesis takes place in the liver from where it is released to the bloodstream and accounts for the highest Se levels found in plasma. Hence, the liver is responsible for the regulation of whole-body Se levels that decreased in SelP knockout mice with a parallel increase of urinary Se concentrations indicating that Se for body selenoprotein synthesis is either incorporated in SelP or other liver selenoproteins or otherwise excreted as excess Se by the liver [92]. Optimal Se supply is the pre-requisite for selenoprotein synthesis and therefore plasma SelP levels are often used as a biomarker for dietary Se requirements. Until today, the mechanism of Se release from SelP remains unclear. Besides the transport function, the redox motif indicates that SelP can also conduct antioxidant function as *in vitro* it was found to protect low-density lipoproteins against oxidation [93].

Thioredoxin reductases (TrxR)

TrxR are named according to their ability to reduce oxidized Trx. Trx is is a class of small redox proteins widely spread in animal tissues [94]. On one hand, Trx can act as reducing agents for antioxidant enzymes like MsrB, but importantly they can reduce a wide range of proteins by formation of a disulfide bridge between the two –SH groups they contain. Thereby, Trx get oxidized themselves and need to be reduced by Se-containing enzymes of the TrxR family using NADPH [95] (Fig. 10).

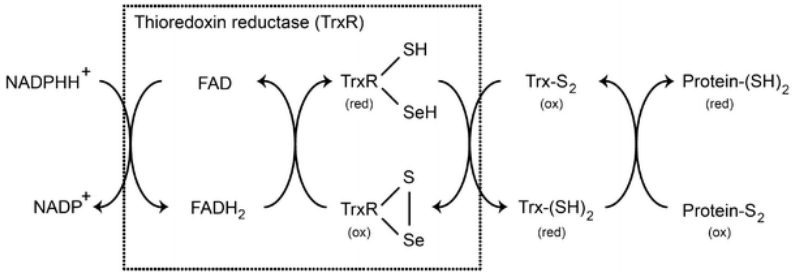

Fig. 10. Reaction sequence of TrxR-mediated reduction of protein disulfides. Source: Tinkov et al. [96]

In mammals, three TrxR are known: cytosolic TrxR1, mitochrondrial TrxR2 and thioredoxin glutathione reductase (TGR), an enzyme containing both a Txr and GSH motif possibly evolved from the TR1 gene in mammals as it cannot be found in fish [84]. TrxR are the only enzymes to reduce oxidized Trx and therefore they regulate the activities of Trx, but TrxR can also act on other substrates like lipid hydroperoxides or lipoic acid and even some Se compounds such as selenite and selenodiglutathione [54]. The activity of TrxR is dependent on the Se status and decreases under Se deficiency [97].

Glutathione peroxidases (GPx)

GPx catalyze the reduction of H_2O_2 or organic peroxides to water or their corresponding alcohols using GSH as a reductant [94]. In humans so far, eight GPx have been identified from which, five are selenoproteins and defined thus as Se-dependent GPx (SeGPX) with GPx1-4 and GPx6 [98]. However in mouse, rat and fish, GPx6 has been shown to contain Cys instead of SeCys [98]. It is understood that the identified GPx in mammals originate from one ancestral gene, however, in broader context genetic analysis found over 700 CysGPx homologue sequences over all domains and due to this complex evolutionary history it is still under debate whether the ancestral protein contained SeCys or Cys [99–101]. In general, CysGPx seem to be less substrate specific, while SeGPx showed enhanced enzymatic efficiency [102]. Se-dependent GPx1 was the first to be described for enzyme kinetics (Fig. 11), however, the mechanism appears to be similar for GPx2 and GPx3 [103]. The enzymatic activity is based on a ping-pong mechanism, where in the first peroxidation part of the reaction, selenic acid is formed as an intermediate, that is, in the second reducing part with two GSH, reduced back, releasing oxidized GSH (GSSG) and H_2O. Thereby, the constant rate of the two-step reaction with GSH is lower compared with the constant in the peroxidation part, allowing an efficient reaction circle based on cellular

concentration differences between GSH (usually 1-10 mM) and peroxides (usually < 1 μM) [98,104,105]. The SeGPx are differently distributed in tissues and cellular compartments.

$$\text{GPx-Se}^- + \text{H}_2\text{O}_2 \rightarrow \text{GPx-SeOH} + \text{OH}^- \quad (1)$$

$$\text{GPx-SeO}^- + \text{H}^+ + \text{GSH} \rightarrow \text{GPx-Se-SG} + \text{H}_2\text{O} \quad (2)$$

$$\text{GPx-Se-SG} + \text{GSH} \rightarrow \text{GPx-Se}^- + \text{H}^+ + \text{GSSG} \quad (3)$$

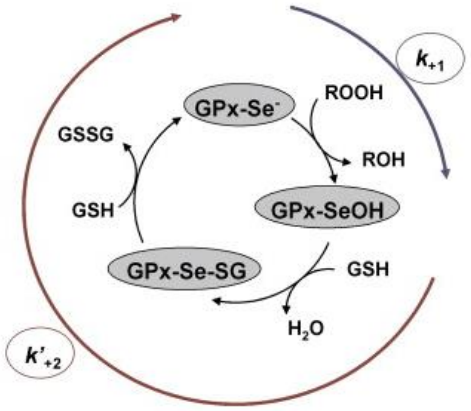

Fig. 11. Catalytic cycle of glutathione peroxidases. Source: Brigelius-Flohé and Maiorino [98]

GPx1 was the first selenoprotein to be discovered and it is the most abundant GPx form, present in the cytosol of all types of cells [98,106]. However, it ranks low in hierarchy and its levels drop under Se deficiency. Under normal physiological conditions, mice deficient in GPx1 developed normally and appeared healthy and fertile [107], but knockout mice died when exposed to H_2O_2 highlighting the essential role of GPx1 in the oxidative stress response [108]. GPx1 overexpression resulted in beneficial antioxidant response [109]. However, overexpression of GPx1 is associated with hyperglycemia and hyperinsulinemia producing the type 2 diabetes-like phenotype [110]. Free radicals like H_2O_2 have been shown to mediate signaling function in cells with a suppression of excessive inflammatory response to acute lung injuries. Thus, it is not surprising to find GPx1 to support the counter reaction with enhanced pro-inflammatory prostaglandin production [111].

Under Se deficiency the expression of GPx2 remains relatively constant showing a preferential level of expression relative to other selenoproteins [105]. GPx2 is an enzyme identified in the

gastrointestinal tract that is expressed in almost all epithelia including lung, breast, gall bladder and urinary bladder [112]. Likewise to other GPx it is expected to reduce hydroperoxides. In the lung, GPx2 has been identified as a stress-inducible GPx isoform taking part in the pulmonary antioxidant defense system regulated by the nuclear factor erythroid 2 related factor 2 (Nrf2) [113]. In the intestine it was also suggested that GPx2 represents a primary defense against hydroperoxides produced during food digestion [114], which is in line with its reduced expression during fasting [115]. In the intestine the expression levels of GPx1 and GPx2 appear complementary with high expression for GPx2 in proliferating cells and high expression of GPx1 in differentiated cells [112]. The role of GPx2 in intestinal stem cells for growth and differentiation might be the reason why GPx2 overexpression was associated with colorectal cancer [116,117].

The main source for GPx3 is the kidney from where it is released to the plasma [118]. Its expression changes under oxidative stress through regulation by the hypoxia-inducible-factor 1 [119]. When purified, GPx3 catalyzes the reduction of not only H_2O_2, but also low weight organic peroxides and phospholipid hydroperoxides [120,121]. Hypermethylation of the GPx3 promotor and gene silencing has been correlated to the occurrence of different cancer forms [122,123].

Encoded by the same gene, GPx4 is present in cytosol (cGPx4), mitochondria (mGPx4) and nucleus (nGPx4) as three different isoforms. GPx4 can directly reduce phospholipid- and cholesterol-hydroperoxides using GSH and also thiols [124]. cGPx4 is essential for life as its absence during embryonic development [125] or a reduction in its activity of about 80% in adult mice [126] has been shown to be lethal. The impaired adult mice showed mitochondrial damage, reduced ATP production in liver, neuronal loss and elevated apoptosis. The essentiality of GPx4 might be a result of its ability to maintain lipid homeostasis, which prevents the iron-dependent formation of toxic ROS resulting in the induction of ferroptosis [127]. Although, the replacement of SeCys with Cys in GPx4 allowed normal embryogenesis in mice, they were still

sensitive towards peroxide-induced ferroptosis indicating the vital role of Se in GPx4 [128]. While cGPx4 is ubiquitously expressed in all tissues, mGPx4 and nGPx4 are primary expressed in testes with essential role for sperm maturation and male fertility [129].

While GPx6 is a selenoprotein in humans, in other species including rodents, it only contains a 'fossil' SECIS element and SeCys is replaced by Cys [98]. GPx6 has not been purified so far and knowledge of its function is scarce, except for its location in the olfactory system with high expression in the Bowman's gland suggesting a role in detoxification of odorants during olfactory signal transduction [130].

Iodothyronine deiodinases (DIO)

There are three DIO (DIO1, DIO2 and DIO3) that exist that can either add or remove ions to thyroid hormones T3 and T4 to activate or inactivate them [131] (Fig. 12). Thyroid hormones increase metabolic function including heart rate, O_2 consumption, gluconeogenesis, glycolysis, protein turnover and cardiac output [132]. They are produced in the thyroid gland mainly as low active T4 and then transported through the blood to different tissues where they are converted to the highly active T3 form or to an inactive T3 form (rT3) or inactive T2 by DIO.

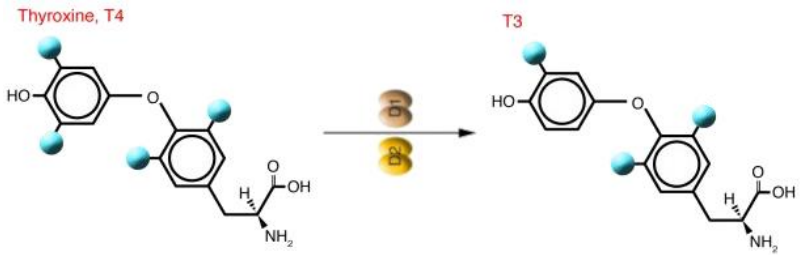

Fig. 12. Basic deiodinase reaction for the activation of T4 to T3. Modified from Bianco and Kim [133]

In vitro, DIO activity has been shown to decrease under oxidative stress and could not be restored by Se supplementation [134]. The DIO activity was found to be highly conserved under Se-deficiency in almost all tissues of fetal and neonatal rats with exception of liver and skin

[135]. In DIO1 and DIO2-knockout mice, thyroid hormone homeostasis was impaired, while general health seemed unaffected [136]. DIO3-deficient mice developed abnormalities including partial perinatal mortality, growth retardation and impaired fertility [137]. However, humans seem to be less tolerable to DIO loss compared to mice [138].

Selenoprotein 15 (Sep15)

Sep15 is a Trx-like protein [139] and expressed in a wide range of tissues with the highest levels in kidney, liver, testes, thyroid and prostate, including a prominent expression during embryonic lens development in the eye [140,141]. The expression can be regulated by the Se status. In correlation to its location in the endoplasmic reticulum, expression levels change in response to mild stress provoked by the accumulation of unfolded proteins, but the absence of Sep15 did not result in acute endoplasmic stress [142] and related to this, a functional role was found in the quality control of protein folding [143]. Sep15 is also a target in cancer studies because decreased expression was detected in colon cancer cells [144] and also single nuclear polymorphism in the Sep15 gene was associated with several forms of cancer [145].

Selenoprotein H (SelH)

SelH has been recently described as a Trx fold-like protein suggesting it might have redox functions [146]. The amino acid sequence showed a DNA binding site probably allowing the regulation of gene expression levels in response to changing redox status as in murine HT22 cells, SelH overexpression led to higher levels of GSH, GPX activity and higher overall antioxidant capacity [147]. In human MRC-5 diploid fibroblast cell lines, SelH could protect against cellular senescence under oxidative stress [148] and the overexpression of SelH in mitochondria provided neuroprotection against UVB-induced cell death [149]. However, the underlying mechanisms and detailed function of SelH still remains to be investigated.

Selenoprotein I (SelI)

SelI was found to be expressed in various tissues with especially high concentrations in brain, placenta, liver and pancreas. Intracellularly it is present in the nucleus, cytoplasm and endoplasmic reticulum, but absent in the nucleolus [150]. First, SelI was described as EPT1 to be involved in the formation of the glycerophospholipid phosphatidylethanolamine, which constitutes for more than half of the phospholipids in eukaryotic membranes [151].

Selenoprotein K (SelK)

SelK is localized in the endoplasmic reticulum and found in high concentrations in human heart [152]. It showed high redox potential with low peroxidase activity to reduce phospholipid hydroperoxides [153]. Expression levels have been shown to be regulated by Se levels and high expression was found in T-cells of mice [154]. As a transmembrane protein, it is involved in the regulation of Ca^{2+} flux in T-cells, neutrophils and macrophages. The functional regulation of Ca^{2+} flux in melanoma cells by SelK was associated with tumor growth regulation [155]. The accumulation of misfolded proteins increased SelK expression [156] and the importance in the immune system was also shown in knockout mice that displayed lower survival when exposed to the West Nile virus [154].

Selenoprotein M (SelM)

SelM is located within the endoplasmic reticulum and expressed mainly in the brain where it is involved in antioxidant neuroprotection and calcium regulation [157]. Due to this neuroprotective function, SelM has been studied in connection to Alzheimer's disease and found to block the aggregation of the amyloid-β peptide suggesting its involvement in the disease, possibly through improved resistance to oxidative stress generated during the formation of

amyloid-β oligomers [158,159]. However, in SelM knockout mice, no cognitive deficits could be detected, but they exhibited increased adiposity indicating that SelM might be also involved in energy metabolism and body weight control [160].

Selenoprotein N (SelN)

SelN is a glycoprotein localized in the endoplasmic reticulum, ubiquitously expressed in all tissues in humans, with high expression levels in fetal tissue, while lower expressed in adults indicating an involvement in early development [161]. At least two gene isoforms of SelN exist and mutations on the SelN gene have been associated with spine muscular dystrophy making SelN the first selenoprotein that was linked to a genetic disorder [162,163]. In mouse models, SelN deficiency was associated with abnormal lung development [164].

Selenoprotein O (SelO)

SelO is localized in the mitochondria and expressed throughout all mouse tissues [165]. The lack of sensitivity to low Se levels suggests a conserved role of SelO, but its function still remains unclear. SelO was found to be redox active and in ATDC5 cells, SelO deficiency was associated with inhibited sox9, col II and aggrecan gene expression [166]. Thereby, SelO presence was essential for chondrocyte viability, proliferation and chondrogenic differentiation.

Methionine sulfoxide reductase B1/ Selenoprotein R (MsrB1/ SelR)

SelR is a zinc Se homologue of the sulfur containing methionine sulfoxide reductase (Msr) commonly found in many organisms [167]. Met in proteins can be readily oxidized by reactive oxygen species (ROS) to produce methionine sulfoxide, which is more hydrophilic and can thus disrupt the protein structure. Methionine sulfoxide exists either as S-isomer or R-isomer

targeted by specific Msr (Fig. 13). One sulfur containing MsrA exists, which specifically reduces the S-isomer, while at least three MsrB (MsrB1, MsrB2 and MsrB3) in mammals exist targeting the R-isomer. The most common MsrB is MsrB1 (SelR) located in cytosol and nucleus, which therefore represents an important redox selenoenzyme [94].

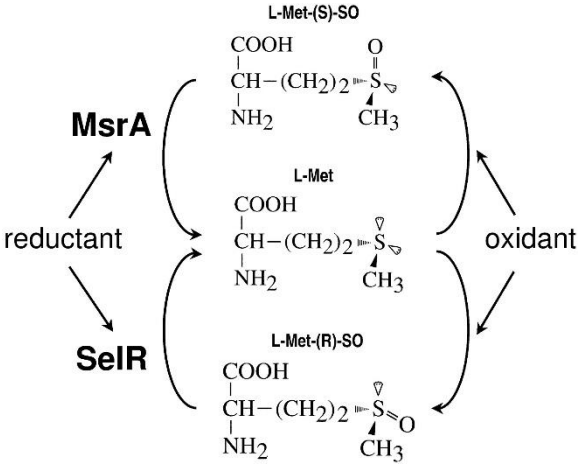

Fig. 13. A model pathway of methionine sulfoxide reductase. Source: Kryukov et al. [167]

Based on its ability to recover oxidized Met, MsrB1 has been shown to both protect transepithelial Mg^{2+} transporter during oxidative stress [168] and inhibit peroxynitrite-induced apoptosis in human lens epithelium [169]. In addition, it was suggested that the reduction of oxidized Met by MsrB1 might have regulatory function by controlling the immune response through the promotion of anti-inflammatory cytokine expression in macrophages [170].

Selenoprotein S (SelS)

SelS, also called SEPS1 and originally described as Tanis, can be regulated by glucose and was described to act dysfunctionally in a diabetic state [171]. Due to its interaction with amyloid A, an acute phase inflammatory-response protein, it was suggested to be a potential link between diabetes and inflammation [172]. Effectively, a single nucleotide polymorphism of the SelS gene was associated with the increase proinflammatory cytokine production commonly observed in

various disorders [173]. Therefore, the impact of genetic variation of SelS was studied in relation to several diseases including coronary heart disease [174], colorectal cancer [175] and autoimmune inflammatory disease [176] and the question was raised if it might be a therapeutic agent [177]. However, the function of SelS still remains poorly understood. Located in the membrane, it seems to mediate intracellular membrane transport [178]. *In vitro* isolated SelS has shown the Trx-dependent ability to reduce H_2O_2 [179]. However, in mice intestinal epithelial cells, the depletion of SelS did not cause or modulate endoplasmic stress, but it still showed increased expression in response to endoplasmic stress, suggesting it might not be a regulator, but a marker for it [180].

Selenoprotein T (SelT)

During embryonic development SelT seems to be an essential protective protein for neuronal cells. In mice, SelT deficiency during brain development resulted in long-lasting cerebral malfunction [181] and under Parkinson's disease it could prevent neurodegeneration caused by oxidative stress [182]. Thereby, SelT was suggested to function as a target gene of neuropeptide pituitary adenylate cyclase-activating polypeptide [183]. Localized in the endoplasmic reticulum, it is involved in the regulation of the Ca^{2+} flow. While ubiquitously expressed during embryonic development, in adult rats it was only found in endocrine tissue where it helped to adapt to stressful conditions of high hormone levels [184].

Selenoprotein V (SelV)

SelV is not well characterized yet. It was described as a globular protein to occur in the seminiferous tubules of the testes [185]. In mice, it has been shown to be expressed in all postnatal stages, especially high during puberty and gradually decreased in adulthood [186].

Based on substrate specificity, it performed both GPx and TrxR activity, highlighting thus its potential antioxidant properties.

Selenoprotein W (SelW)

SelW was early discovered as a selenoprotein by isolation from rat muscle tissue [187]. Following studies that showed that SelW can be found in all animal tissues, with high concentrations in skeletal muscle, heart, tongue and brain, while liver concentrations were comparably low without any correlation between tissue Se concentrations and SelW concentrations [188]. Nevertheless, SelW expression could be modified by dietary Se levels [189,190] and in addition gender-related differences in expression were found [191]. In developing mice, SelW was highly expressed in nervous system, skeletal muscle and heart, showing immediate response to oxidative stress [192]. In chicken embryos, SelW was shown to have antioxidant properties to protect from H_2O_2-mediated apoptosis [193]. According to studies performed on cells, the reduction of H_2O_2 was dependent on GSH [194] making it a target for methylmercury [195]. Depletion of SelW in skeletal muscle was associated with Ca^{2+} leakage found in muscle injury indicating that its antioxidant role might be to regulate the intercellular Ca^{2+} flow [196].

2.8.2. SPECIFICITIES OF THE FISH SELENOPROTEOME

In general, aquatic life has been associated with larger selenoproteome than terrestrial life [197] and evolutionary studies indicate that the mammalian selenoproteome trends towards a reduction [198,199]. Therefore, it is not surprising to find bony fish to have the largest known selenoproteome under vertebrates. However, the number of analyzed fish species is fairly limited and mainly includes well-characterized model species. In a comparative study of the vertebrate selenoproteome by Mariotti et al [84], of the 45 identified selenoproteins, 21 were

commonly found in all vertebrates (Tab.1). The largest selenoproteome was found in bony fish including pufferfish, medaka, stickleback and zebrafish. Zebrafish had the largest selenoproteome with 38 selenoproteins. In all bony fish, evolutionary studies indicated that gene duplication generated GPx1b, GPx3b, GPx4b, DIO3b, SelT2, MsrB1b and SelU1c. In zebrafish there were additional copies for SelO (SelO2), SelT1 (SelT1b) and SelW2 (SelW2b). The selenoproteins SelL and SelJ are, so far, only found in fish, while Fep15, the fish 15 kDa selenoprotein, originally exclusively described in bony fish [200] was also found in elephant shark and has a Cys homologue in frogs [84].

Selenoprotein P (SelP) in fish

With the identification of SelPb in zebrafish and without the presence in placental mammals, SelPb was first thought to be evolved in fish, but recent phylogenetic analysis found similar gene sequences also in other mammals [84,201]. However, in salmonids it was found that gene duplication resulted in four SelP genes (SelPa1, SelPa2, SelPb1 and SelPb2) [202]. In addition, the number of SeCys at the C-terminus of SelP was found to be especially high in fish species (Fig. 14; e.g. 10 in humans and rats vs 17 in zebrafish and rainbow trout and 16 in Atlantic salmon) and might be related to the well-developed selenoproteome in these species creating a high Se demand for the selenoprotein synthesis.

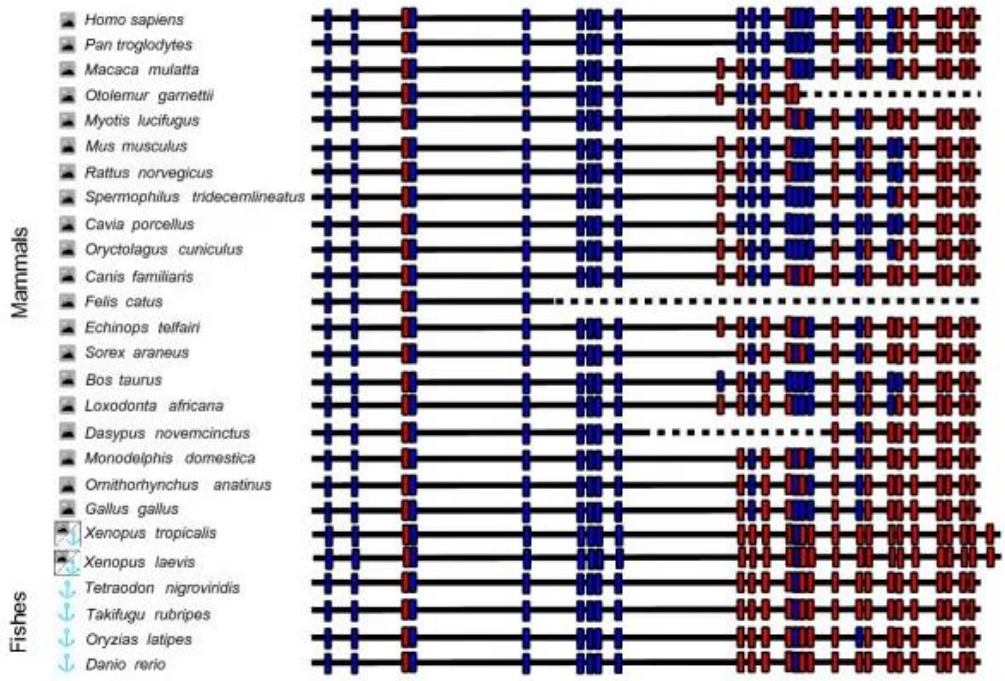

Fig. 14. SeCys (red) and Cys (blue) content of SelPs in vertebrates. Dotted lines correspond to undetected sequences in genomes with low sequence coverage. Adapted from Lobanov et al [199]

Fish 15 kDa selenoprotein (Fep15)

Fep15 likely evolved by gene duplication of the Sep15 gene [200]. In contrast to Sep15, where the active site consists of SeCys and cysteine, in Fep15 it only contains SeCys.

Selenoprotein J (SelJ)

A Cys homologue of SelJ was found in jellyfish where it functions as a structural crystallin [203]. Consistent with a potentially similar role to the jellyfish Cys homologue, zebrafish SelJ has preferential and homogeneous expression in the eye lens of developing embryos. Its exact role remains to be characterized in fish.

Selenoprotein L (SelL)

SelL is located in the cytosol and showed low redox potential [204]. In zebrafish, SelL incorporated two SeCys that were found to form a diselenide bond in bacteria. SelL can be also found in other aquatic organisms where one or both SeCys might be replaced by Cys.

2.9. Selenium, seleno-compounds and the antioxidant system

It can be seen from the previous section, that the function of selenoproteins is diverse in terms of the antioxidant metabolism, but no selenoprotein has been described to directly scavenge radicals and thus could be termed as a true antioxidant [205,206]. Selenoproteins rather seem to act in the biological antioxidant defense system by their enzymatic function. Among them, are key enzymes like GPx and TrxR where the unique redox property of Se was suggested to compensate for the complex and costly synthesis process [207,208]. The advantage may result from the stronger oxidative properties of selenate compared to sulfate. In this way, selenoenzymes catalyze redox reactions counteracting oxidative stress. A question that has not been answered so far is how other Se metabolites and low-molecular weight species act in the antioxidant system. Although, the majority of biological Se can be found in selenoproteins during their metabolism consecutively Se-metabolites such as H_2Se, dimethyl selenide, monoselenophosphate or methyl selenide emerge, which were found to scavenge ROS *in vitro* thus acting as antioxidants [209,210]. However, their study *in vivo* brings challenges due to their short life span and their activity was found to range between antioxidant and prooxidant depending on physiological condition [206]. In humans, selenometabolites and low molecular weight species like selenoneine were found to only account for a small fraction in blood and urine [211,212]. On the other hand, in fish, the role of low molecular weight species seems more developed as depending on species, high levels of the body Se were identified in the low-molecular range [213]. In cod, salmon, rainbow trout and eel, 76-88% of Se was in high molecular range, but especially in flat fish, about half of the Se was found in low-molecular

weight range. In tuna, selenoneine was even isolated as the predominant organic Se form by Yamashita and Yamashita [214]. Selenoneine is a seleno analogue of ergothioneine and was shown to be a strong antioxidant with scavenging behavior on radicals resulting in seleninic acid [215,216] (Fig. 15).

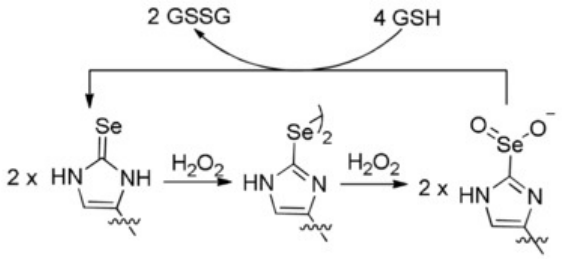

Fig. 15. Reaction of selenoneine with H_2O_2 to seleninic acid and its reformation with GSH. Adapted from Lim et al. [216]

In addition, evidence has been found that selenoneine accelerates the detoxification of methyl mercury in fish [217,218]. Also, other Se antioxidants were found to prevent metal-mediated oxidative damage highlighting that not only ROS scavenging, but especially a metal interaction in the antioxidant mechanism of Se could be of importance [219].

2.10. Selenium and reproduction

Evidence for a role of Se in reproduction became obvious shortly after its recognition as an essential micronutrient and introduction in livestock production where it sustainably improved reproductive success.

In males, Se is transported to the testes by SelP, where its uptake is regulated by apolipoprotein E receptor 2 to Sertoli cells where it is further processed [220]. Sufficient Se levels in the male reproduction organs in form of GPx4 are indispensable for the production of sperm [221]. Encoded by the same gene, in total three forms of GPx4 exist with cGPx as the most abundant, but mGPx4 and nGPx4 are predominantly expressed in testes during spermatogenesis. In

spermatids, GPx4 can function as an enzyme to reduce lipid peroxides and protect sperm against oxidative damage [222]. During spermatogenesis, however, GPx4 crosslinks to other proteins and becomes an integral part in the mid-piece of spermatozoa, where Se is mainly located [223]. Consequently, in mammals, Se deficiency has been associated with impaired sperm motility [224], morphological alterations [225,226] up until infertility [227]. Spermatogenesis is mediated through a variety of sex hormones, especially testosterone synthesized in Leydig cells. It was shown *in vitro* that the testosterone production in sheep Leydig cells can be regulated by the activation of the extracellular-signal-regulated kinase in a dose-related manner [228] and explains the increase of testosterone observed in relation to dietary Se supplementation *in vivo* [229,230]. Behne et al [230] studied the effect of dietary Se deficiency on reproduction in rats over four generations and found that from the second generation onwards, Se deficiency decreased testis mass for more than 50%. In goats, the morphological development of testis progeny was linked to decreased testosterone levels by maternal Se nutrition [231] showing that adequate dietary Se levels are not only relevant for males, but also for females for proper testis development and spermatogenesis of their male progeny.

The evidence to date on the relationship between Se and female reproduction is sparser, however, in a cross-sectional study in human female family members, life-cycle differences in plasma Se levels were found with lower concentrations in daughters and grandmothers compared to mothers, which correlated to their plasma estrogen levels [232]. This might be related to a regulatory role of Se on estradiol production in granulosa cells. In goat granulosa cells, Se treatment was associated with an increased expression of genes involved in steroid synthesis [233]. In bovine granulosa cells, the inhibition of nitric oxide, a free radical produced in granulosa cells, was obtained by Se supplementation [234]. In both studies, Se treatment was additionally found to support cell proliferation in the follicles. Follicular fluid contains SeGPx, which seems to support the oocyte-sperm fusion, as an increased GPx activity in follicular fluid was associated with lower failure in oocyte fertilization [235]. However, models of Se levels in

adolescent girls found no correlation between the plasma Se and plasma estradiol indicating differences in Se metabolism throughout the life cycle [236]. In the female mice brain, Se status increased from adolescence to pregnancy to lactation, suggesting increasing Se requirements during these phases. Estrogen seems to be a regulator for the distribution of Se to different body tissues as, even though the absorption of dietary Se into the liver was not affected by estrogen, higher estrogen levels were associated with a higher percentage of incorporation in SelP compared to GPx, nevertheless, plasma GPx activity increased simultaneously with estrogen treatment [237]. In general, Se requirements are thought to be higher during pregnancy as even normal pregnancy raises oxidative stress levels and creates a systemic inflammatory response and might explain why Se deficiency is associated with pregnancy complications [238]. Special care must be taken during pregnancy as maternal Se levels decrease also due to a placental transfer of SelP. Thereby, low Se levels can in turn induce negative effects on the fetal ovarian development and overall fetal growth [239]. The parental Se nutrition has been shown to provide an antioxidant system for the progeny in place at the time of birth. In the progeny postnatal Se nutrition will determine the further Se status required for a healthy development [240].

2.11. The concept of nutritional programming

Metabolic programming describes a concept, where the exposure to a stimulus, including nutritional challenges, encountered during critical windows of development, induce long-term metabolic and phenotypical changes in an organism [241]. Numerous studies in mammals suggest that stimuli received during prenatal stages correlate with the occurrence of disorders and chronic disease later in adulthood [242,243]. However, such adaptive changes may remain silent until exposed to new environmental stimuli, which then reveal the metabolic adaptations. Although the grounding principles in nutritional programming might be similar between mammals and fish it should be remembered that the early development in mammals is directly

mediated by the maternal nutrient circulation, both in the uterus and during lactation, while in fish the maternal connection ends before fertilization [244]. Several studies in fish have suggested that the nutrient composition in the eggs and also the first feeding event can have long-term consequences on growth, neuronal development, stress tolerance, inflammation as well as the lipid and carbohydrate metabolism [245–249]. Consequently, nutritional programming has been suggested as a new breeding strategy in aquaculture to adapt and direct the livestock plasticity through environmental intervention [250]. However, a careful selection of the stimuli and timing as well as detailed understanding of underlying mechanisms will be necessary for such directed modulations. One of the most likely mechanisms of programming effects is an epigenetic modification, known to be sensitive to environmental factors [251].

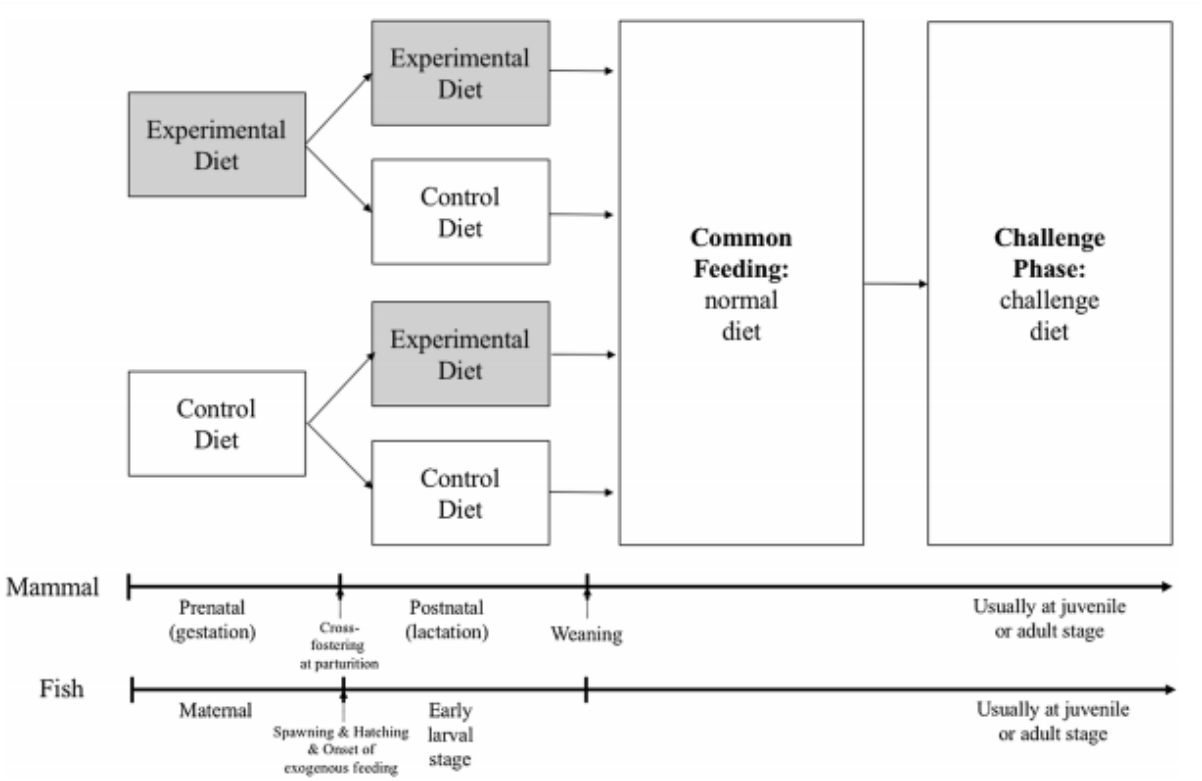

Fig. 16. Schematic figure of classical crossover experimental design used in nutritional programming studies. Source: Hou and Fuiman (2020) [244]

2.12. Selenium and epigenetics

An epigenetic trait is a stably heritable phenotype, resulting from changes in a chromosome without alterations in the DNA sequence [252]. The regulatory mechanisms include DNA methylation, histone modification, nucleosome remodeling and non-coding RNAs. Thereby, DNA methylation might represent the most extensively studied epigenetic mechanism. Diet can play a regulatory role in the makeup of the epigenome not only in the present generation, but also impacts the further performance of the progeny [253]. Essential micronutrients including folate, choline, betaine and other B vitamins have been popular targets in epigenetic studies as they serve as co-factors in one-carbon metabolism providing S-adenosylmethionine (SAM), the general donor for methyl groups (Fig. 16). Nutritional epigenetics is the study of epigenetic mechanisms related to gene-diet interaction and could be used in livestock production to modify the phenotype of cultivated species [254]. Also other nutrients including Se are targets for epigenetic studies [21].

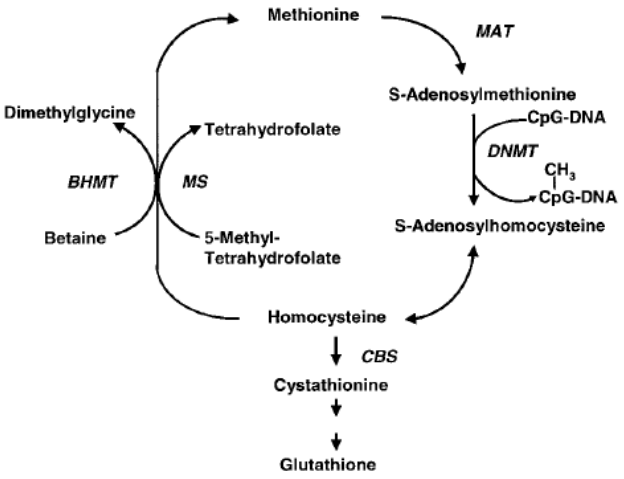

Fig. 17. Simplified version of one-carbon metabolism involving DNA methylation. CH_3, methyl group; CpG, cytosine-guanine dinucleotide sequence. Enzymes: BHMT, betaine homocysteine methyltransferase; CBS, cystathione synthase; DNMT, DNA methyltransferase; MAT, methionine adenosyltransferase; MS, methionine synthase (also called MTR). Source: Davis and Uthus [255]

In mice liver, global DNA methylation was increased by Se supplementation together with the overall methylation potential indicated through the ratio between SAM, which acts as universal

methyl donor, and S-adenosylhomocysteine (SAH) [256]. However, contrary effects were found in another study on mice, where the introduction of Se supplementation in deficient diets induced decreased methylation potential and DNA methylation [257]. Differences might be related to the use of different dietary Se supplementation forms as SeMet was used in the first study whereas sodium selenite was used in the second one. The processing of SeMet through the transsulfuration pathway suggests that SeMet might be an important factor in the one-carbon metabolism. Usually, liver represents a suitable organ to determine the methylation status of an individual as it is the main organ for methyl group production via the one-carbon metabolism. Also, in several chicken tissues, Se deficiency was associated with decreased DNA methylation, however, to a different degree starting from liver > brain > leg muscle > thalamus > wing muscle, from the lowest to the highest decrease [258]. The decreased DNA methylation was associated with a decrease in DNA methyltransferase (DNMT) mRNA levels, but increase in methyl-DpG-binding domain protein 2. In rats, hepatic DNMT levels decreased under Se deficiency, but without any changes in global DNA methylation in liver, but only in colon [255,259]. Changes in DNA methylation were also detected under supranutritional Se levels, but Se deficiency seemed to appear more important concerning changes in the methylation status. Interactive effects were found for Se and folate, where a combined deficiency reinforced effects observed under folate deficiency and on the other hand, Se supplementation could decrease effects of folate deficiency [255,259]. The authors suggested that Se might support the formation of GSH from homocysteine (Hcys) that usually builds up under folate deficiency. Epigenetic modifications by Se are not limited to adults. In a human study on newborns, placental Se concentration was inversely correlated to the hypermethylation of the cytosine-phosphate-puanine site GFI1 associated to muscle tone status at birth [260].

2.13. Selenium in human nutrition and health

Maximum plasma selenoprotein synthesis can be used as an easily accessible value to define the optimal Se intake levels. The USA Recommended Dietary Allowances (RDA) for Se are given by the National Institutes of Health (Tab. 2) based on maximum plasma GPx synthesis [261]. The RDA in adults is 55 µg/day, but are higher in pregnant or lactating woman.

Tab 2. Adequate Intake levels for Se given by D-A-CH [262] and Recommended Dietary Allowance for Se and Tolerable Upper Intake Levels given by the National Institutes of Health (USA) [261] given in µg/day

	Adequate intake (D-A-CH)	Recommended dietary Allowance (USA)	Tolerable Upper Intake Levels (USA)
Infants	10-15	15	45
Children	15-45	20-40	60-280
Adolescence	45-70	40-55	400
Adult male	70	55	400
Adult female	60	55	400
Pregnant woman	60	60	400
Lactating woman	75	70	400

The reference intake value given together by Germany, Austria and Switzerland (D-A-CH) was recently revised to be based now on plasma SelP saturation, which gives higher values compared to GPx activity [262,263]. This change was due to a study on Chinese people, where plasma SelP was found to be maximal at a dietary Se intake of 49 µg/day [264]. Taking weight differences into account, D-A-CH calculated a recommended intake value of 70 µg/day in men and 60 µg/day in woman, which accounts for a plasma Se level of 100-120 µg/l. In many parts of the world, the estimated Se intake does not meet the recommended levels [265]. Se deficiency was associated with an increased risk of mortality, poor immune function and cognitive decline [266].To increase the Se intake, when required, it was suggested to use agronomic and genetic biofortification [267]. Cereals, fish and meat are considered to be dominant food sources for Se in human nutrition [268–270]. In Finland, with the decision to introduce nation-wide Se into crop fertilizers, the mean human plasma Se concentration could be significantly elevated to meet recommended levels [271]. In various other products, Se supplementation can be used to

increase dietary levels like it is done in the poultry industry to produce Se-enriched eggs [272] and could be taken under consideration by the aquaculture industry.

However, care must be taken as excess Se can quickly cause toxic effects. In humans, acute toxicity symptoms include diarrhea, fatigue, hair loss, joint pain, nail discoloration and nausea [43]. The mechanisms behind Se toxicity seem to relate to its association with GSH and an increase in free radical production [273]. To prevent adverse effects, maximum Se levels are given in drinking water and to animal diets by governmental authorities in different countries [274]. The upper tolerable Se intake level given by the US National Institutes of health is 400 µg/day in adult men and women [261].

2.14. Selenium supplementation in terrestrial agriculture

2.14.1. SUPPLEMENTATION FORM

Severe Se deficiency has been associated with several metabolic disorders in livestock production. To meet Se requirements, modern animal production systems deliver Se via trace-mineral premixes (usually around 0.2-0.3 mg/kg) [275]. As earlier mentioned, Se supplementation in animal feeds is regulated and maximum supplementation levels within the EU are given at 0.2 µg/g according to the European Food Safety Authority and in the US of 0.3 µg/g according to the US Food and Drug Administration for both organic and inorganic Se products available to the market [276,277]. Organic Se forms of SeMet appear less toxic, more efficiently stored in the body and transferred to the progeny via eggs, placenta and milk compared to inorganic selenite [278,279]. The crucial factor for the development of organic seleno-products in animal nutrition is to stabilize SeMet, which is easily oxidized in pure form. Commercial products with organic Se sources can come from Se-yeast with a total Se content of 69.2-94% from which 62.5-89.1% was identified as SeMet [280]. More recently, also hydroxy-selenomethionine, as a purified form of SeMet, has been introduced to the market [281] and

found to have higher bioavailability compared to Se-yeast in studies on chicken [282]. In addition, in the scientific community, the use of nanoparticle Se (SeNP) as a possible food additive has attracted some attention. In this scenario, Se undergoes physical, chemical or biological treatment to create stable particles of less than 100 nm size [283]. These particles are considered to penetrate the mucus membrane in the gastrointestinal tract allowing a high bioavailability. In addition, the lower surface to volume ratio in the crystalline hexagonal might allow a slower release of the Se in SeNP and therefore less interaction with other particles and lower toxicity [284].

2.14.2. SELENIUM IN POULTRY PRODUCTION

In poultry production, Se supplementation is used to increase animal Se levels throughout all developmental stages. In breeders, dietary Se supplementation is not only beneficial to improve egg production, but rather to transfer adequate amounts to the eggs to support the developing embryo during the especially stressful period of hatching, where redox balance is in risk [20]. However, the Se transfer was found to be poor with inorganic Se sources, as SeMet was the predominant form in egg yolk and embryo [279]. Se-enriched eggs can be also considered as an important Se source for human consumption promoting the supplementation in laying hens. The same is true for the nutrition in broilers, where Se supplementation, especially in organic form, increases Se meat content [285] and adequate Se levels also support animal health under stressful conditions by elevated expression of selenoproteins.

2.14.3. SELENIUM IN MAMMALIAN FARM ANIMALS

In young pigs, 0.5 μg/g Se were required to support optimal GPx expression [286]. Feeding Se-deficient diets was associated with histological lesions in hepatic and muscular tissue and increased mortality [287]. In pigs, low hepatic Se levels were associated with hepatosis dietetica and nutritional myopathy [288]. Regulatory mechanisms of selenoproteins by Se deficiency

were tissue specific [289]. Se supplementation was found to support boar reproduction [19] and maternal SeMet supplementation could increase milk Se content with beneficial effects in the progeny seen by improved growth, antioxidant state (GPx activity) and energy production (ratio of thyroid hormones T3/T4) [290].

Also in dairy cattle, Se supplementation was shown to have beneficial effects for the calves with improved antioxidant status, increased antibody levels after vaccination, reduced occurrence of bovine respiratory heat disease [291] and improved immunity also for the dairy cattle itself [292]. Thereby, dietary requirements in dairy cattle are higher compared to those in beef cattle [293]. However, oxidative stability of meat could not be improved by Se supplementation [294].

2.15. Selenium in fish nutrition

Compared to other livestock production systems, Se nutrition gained only recently attention in the aquaculture sector that was related to the exchange of protein source in major diet formulations that gradually reduced fishmeal as the main Se source in traditional aquafeeds. In fish, physiological symptoms for Se deficiency are growth depression, impaired stress and immune response, reduced meat quality and increased mortality [295–298]. At a cellular level, Se is associated with the expression and activity of selenoproteins affecting the antioxidant metabolism in fish [18]. According to the meta-analysis of Antony Jesu Prabhu et al. [299] dealing with 25 studies about Se requirements in fish, the requirement levels vary according to species and hepatic GPx activity has been identified to be the most appropriate criterion to determine them. However, studies on Se nutritional requirements are not available for all cultivated fish species. Requirement levels for some selected species are given in Table 3.

Tab 3. Selenium requirements for different fish species

Species and initial body weight	Requirement [µg/g feed]	Se source	Criterion	Reference
Rainbow trout (~1.3g)	0.15 – 0.38	Na_2SeO_3	Maximal GPx3	Hilton et al. [300]
Rainbow trout (~100g)	1.5 – 4.9	Se-yeast	selenoprotein expression	Pacitti et al. [301]
Fingerling channel catfish (~65g)	0.1 - 0.5	Na_2SeO_3	Maximal GPx3	Gatlin & Wilson [302]
Juvenile grouper (~12.2g)	0.7	SeMet	Weight gain and Se retention	Lin & Shiau [303]
Yellowtail kingfish (~19 g)	5.56	Se-yeast	Growth rate	Le & Fotedar [304]
Juvenile Nile tilapia (~1.85g)	1.06 – 2.06	SeMet	Weight gain	Lee et al. [305]
Juvenile gibel carp (~2.74g)	1.18	SeMet	Weight gain, GPx activity, tissue levels	Han et al. [306]

Hilton et al. [300] defined optimal dietary Se levels in rainbow trout to range between 0.15 and 0.38 µg/g dry feed based on maximal plasma GPx levels in a 20-week feeding trial with the minimum level to prevent deficiency symptoms at 0.07 µg/g and possibly toxic bioaccumulation to occur at levels around 3 µg/g. In that study, sodium selenite was used as a feed supplement. However, Pacitti et al. [301] detected no toxic effects supplementing a basal diet of 0.9 µg/g with 4 µg/g Se-yeast over a 10-week feeding period, but only at the highest supplementation level of 8 µg/g, defining the optimal Se level at 1.5 µg/g based on hepatic GPx expression. The differences in safe limits and optimal dietary levels between the two studies might be connected to higher toxicity of inorganic Se supplements [307] and in parallel, inorganic Se was, likewise to other animals, also in fish found to be less bioavailable compared to organic Se. Organic Se increased muscle Se content more efficiently [308] and was mobilized under stressful conditions resulting in better stress response [309]. But, in general higher Se requirements were found in stressed trout under crowding conditions showing beneficial effects of both organic and inorganic Se supplementation related to an increase of cellular oxidative stress in stressed trout [310]. Indeed, Se has strong effects in the antioxidant system and the presence of other antioxidants like vitamin E in the diet might mitigate deficiency effects of dietary Se, when sufficiently supplied. For example, a significant interaction between vitamin E and Se was found

on lipid peroxidation levels with reduced weight gain in double deficient and Se deficient treatments, but not in the low vitamin E treatment in rainbow trout [311]. In addition, under stressful conditions, dietary vitamin E was found to remove adverse effects related to high-stocking density [312]. In another study on Atlantic salmon, supplemental Se was found to maintain hepatic tocopherol levels and the combined deficiency of both nutrients resulted in muscular dystrophy [313]. In chinook salmon, a combined deficiency even increased mortality levels by 31% [314]. These results show that Se requirements can change in relation to the basal diet composition and Se in plant ingredient-based diets might exert different bioavailability compared to Se from fishmeal-based diets [16,315]. In rainbow trout fry, a plant ingredient-based diet was found to be suboptimal even at the maximal allowed dietary Se level of 0.5 μg/g [18] showing that the topic of Se supplementation in fish diets will remain important also in up-coming years.

While knowledge on the role of Se and selenoproteins in the metabolism of mammals is incomplete, studies in fish with a rich selenoproteome, raise many questions in order to be able to define optimal Se levels by supplementation of plant ingredient-based diets. Most of the studies investigating the effect of dietary Se on the metabolism in fish were performed in juveniles or at the early stages that are often considered as sensitive during the life cycle. From the experience in other animals, it could be assumed that the Se requirements are changing during the life cycle and that broodstock fish could represent a key stage in the metabolism of Se in these animals. In zebrafish, sex specific expression patterns for selenoproteins were found [76] and Se supplementation was shown to increase reproductive success [316]. In addition, in rainbow trout, SeMet could increase gonadal steroidogenesis [317]. These results point also in fish towards a role of Se in the reproductive system. In female fish, the oogenesis represents an additional drain for essential nutrients like Se that are provided to the progeny during the embryonic development [8]. In zebrafish embryos, SeNP was found to reduce apoptosis and cell death provoked by ROS [318], which indicates that Se also supports the antioxidant system in the progeny. However, while these first studies point towards an important role of selenium

nutrition in broodstock fish, extensive studies are missing, which represents the basis of this PhD.

3. OBJECTIVES OF THE PHD

Based on this context, the present PhD aimed to investigate the impact of low Se levels in plant ingredient-based broodstock diets on reproductive performance and the metabolism in the progeny. The work was done based on the following hypotheses:

- **In fish, at physiological relevant dietary levels parental selenium is transferred to the progeny resulting in beneficial effects in their antioxidant system**
- **Parental selenium induces a nutritional programming effect in the progeny**
- **Stressful conditions will impact the effect of selenium supplementation**
- **Organic and inorganic selenium supplements in parental diets will result in different metabolic responses**

To test the hypotheses a feeding trial was designed using rainbow trout broodstock and their offspring investigating the following aspects: the reproductive performance, changes in oxidative status of broodstock and progeny, the transfer and distribution of Se in the progeny and the mechanisms behind the parental effects (epigenetic modification). In addition, the use of two different market available feed additives in either inorganic (sodium selenite) or organic (hydroxy-selenomethionine) form should testify effects related to differences in the metabolic pathway of dietary seleno-compounds. The given chapers represent the different aspects of this work:

3.1. Reproductive performance and parental selenium transfer (Paper 1)

The objective of this study was to evaluate the effect of different Se sources on the antioxidant metabolism and reproductive performance of broodstock fish. In addition, the question should be answered, if parental selenium might improve the antioxidant status in the progeny before first-feeding.

3.2. Selenium imaging in the progeny (Paper 2)

The metabolism of inorganic and organic Se forms is fundamentally different. It raises the question, if hierarchical differences in Se distribution to different organs might apply for both Se

provided by parental transfer and direct feeding, such differences in tissue Se levels might relate to Se form-specific effects observed in the study. The work done in this chapter was based on the hypothesis:

3.3. Long-term parental effects on fry performance and stress resistance after first feeding (Paper 3)

The aim of the study was to evaluate, if parental effects caused by Se nutrition on the antioxidant metabolism will persist in the progeny after their first-feeding. Hypoxia was applied to create a stressful environment and evaluate how parental and direct Se nutrition can influence the physiological stress response.

3.4. Interactions between selenium and the one-carbon metabolism (Paper 4 and Paper 5)

The study aimed to investigate the influence of Se nutrition on the one-carbon metabolism and if parental nutrition might alter the one-carbon metabolism of the progeny causing epigenetic changes with persisting modifications in their phenotype.

4. MATERIAL AND METHODS

This PhD was based on a transgenerational feeding trial with rainbow trout, which were fed experimental diets containing different levels and source of Se.

4.1. Experimental diets

The rainbow trout diets for both broodstock fish and fry were designed to meet their nutritional requirements according to NRC [7]. Diets were manufactured at the INRAE experimental facilities in Donzacq (Landes, France). All diets were based on plant-derived proteins at a crude protein level of 50% and a total lipid content of 22% with 8% fish oil inclusion (Tab.4). The composition of broodstock and fry diets differed by the inclusion of 0.04% Carophyll pink® for the broodstock diets. The basal dietary composition resulted in a dietary Se level of 0.3 µg Se/g dry feed in the broodstock and fry negative control diet Bnc and Fnc, respectively. The basal diet was supplemented with 0.3 µg Se/g dry feed either as inorganic sodium selenite in Bss and Fss or as hydroxy-selenomethionine in Bso and Fso (Fig. 17) to achieve a total dietary Se level close to the legal limit of 0.5 µg total Se per g feed in supplemented diets [262].

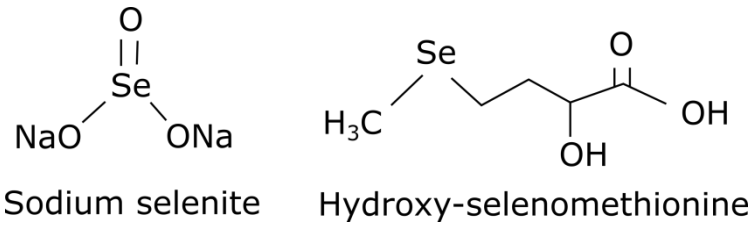

Fig. 18. Structure of sodium selenite and hydroxy-selenomethionine used as dietary feed supplements

The analyzed Se concentration in the Fso diet was as expected at 0.6 µg Se/ g feed, whereas Bss and Bso with 0.8 and 0.7 Se/g feed a slightly higher concentration as expected and Fss had a final Se concentration of 0.5 µg/g diet.

Tab 4. Formulation and composition of experimental diets (g/100g wet weight)

	Broodstock			Fry		
Diet	**Bnc**	**Bss**	**Bso**	**Fnc**	**Fss**	**Fso**
Ingredients						
Plant meals[1]	74	74	74	74	74	74
Crystalline amino acids and attractant mixture[2]	3	3	3	3	3	3
Soybean lecithin[3]	2	2	2	2	2	2
Fish oil[3]	8	8	8	8	8	8
Vegetable oils[4]	8	8	8	8	8	8
Carophyll® pink[5]	0.04	0.04	0.04	-	-	-
Vitamin and mineral mixture without Se[6]	5	5	5	5	5	5
Sodium selenite (µg/g diet)[7]	-	0.7	-		0.7	
Selisseo® (µg/g diet)[7]	-	-	15			15
Analytical composition						
Dry matter (DM, %)	96	98	97	95	94	95
Crude protein (% DM)	49	50	50	51	50	50
Total lipid (% DM)	23	22	23	20	21	21
Gross energy (kJ/g DM)	25	25	25	25	25	25
Ash (% DM)	6	6	6	6	6	6
Se (mg/kg)	0.3	0.8	0.7	0.3	0.5	0.6
Fatty acid profile (% total fatty acids)						
14:0	5.1	4.6	5.0	5.6	5.5	5.5
16:0	25.5	24.4	25.6	25.4	24.2	24.5
18:0	3.1	3.2	3.3	2.9	2.8	2.8
Total saturates[8]	35.1	33.6	35.3	35.6	34.2	34.5
16:1	3.8	3.5	3.7	4.1	4.0	4.0
18:1	28.0	28.8	27.5	27.0	28.1	29.0
Total monoenes[8]	32.5	33.0	31.8	31.7	32.7	33.6
18:2 n-6	16.7	17.3	17.0	17.9	16.4	16.8
20:4 n-6	0.5	0.4	0.5	0.3	0.5	0.3
Total n-6	17.2	17.7	17.4	18.5	17.9	17.8
18:3 n-3	9.9	10.1	9.7	9.6	9.5	9.1
18:4 n-3	0.6	0.5	0.5	0.4	0.4	0.4
20:5 n-3	2.3	2.1	2.2	2.4	2.3	2.2
22:6 n-3	1.5	1.8	2.1	1.8	2.3	2.2
Total n-3	14.3	14.6	14.6	14.2	14.5	13.8

[1]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).

[2]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.

[3]Soybean lecithin from Louis François and fish oil from Sopropêche.

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

[5]Contained 10% astaxanthin (DSM).

[6]Vitamin and mineral mixture without Se (per kg diet): retinol acetate, 55,000 IU; cholecalciferol, 2,500 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; $CaHPO_4 \cdot 2H_2O$, 33 g; $CaCo_3$, 2.15 g; $Mg(OH)_2$, 1.24 g; KCl, 0.9 g; NaCl, 0.4 g; $FeSO_4 \cdot 7H_2O$, 0.2 g; $ZnSO_4 \cdot 7H_2O$, 40 mg; $MnSO_4 \cdot H_2O$, 30 mg; $CuSO_4 \cdot 5H_2O$, 30 mg; NaF, 10 mg; KI, 0.4 mg; $CoCl_2 \cdot 6H_2O$, 0.2 mg. All ingredients were diluted with α-cellulose.

[7]Sodium selenite contained 42% Se (Sigma-Aldrich) and Selisseo contained 2% Se (Adisseo).

[8]Total saturates include 12:0, 15:0, 17:0 and 20:0 and total monoenes include 14:1 and 20:1.

4.2. Experimental design

The experimental design is illustrated in Fig. 18. Three year old male and female rainbow trout at the second reproduction season from the INRAE experimental fish farm of Lées-Athas (Pyrénées-Atlantiques, France) were used as broodstock. Each group consisted of 25 females and 15 males individually tagged and randomly allocated to three circular 2-m diameter tanks supplied with flow through spring water (8 ± 1°C). The experimental diets were given once daily until visual satiation for a period of 6 months prior to spawning. The growth was checked regularly and at spawning period the fish were monitored closely to determine ovulation. Oocytes of each spawning female were collected and fertilized with a pool of sperm collected from males of the same dietary treatment. To obtain individual hatching data the eggs from each female were reared separately in small trays until swim-up fry stage in flow-through spring water at 8 ± 1°C.

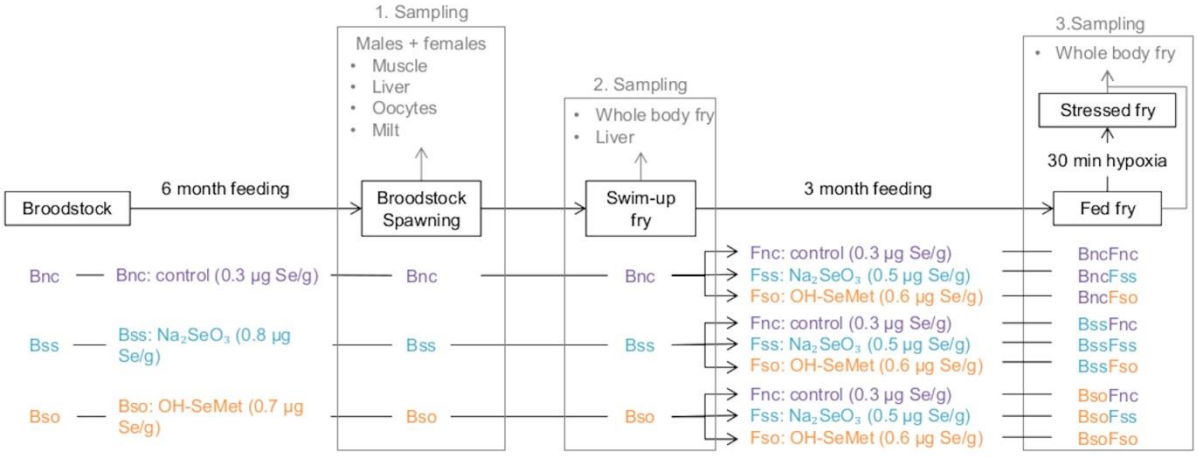

Fig. 19. The experimental design of the rainbow trout feeding trial

In addition, at the second spawning date a pool of offspring for each dietary group was created using 5 females respectively. The pool was co-cultivated to the individual trays to serve as the F1 generation in the fry feeding trial. Therefore, at swim-up stage the pooled fry from each of the parental groups were randomly sub-divided into one of the three fry feeding groups (Fig. 18) in triplicate. Each triplicate group consisted of 250 fry per tank reared in 50L fiberglass

tanks supplied with flow through water at 17°C. The fish were hand fed 4-6 times daily to apparent satiation for a total period of 11 weeks.

After 11 weeks of feeding, the fish were either sampled or subjected to an acute hypoxic stress challenge (Fig. 19). One hundred fish per tank were transferred from tanks with normally oxygenated water (9.2 ± 0.2 ppm dissolved oxygen) to tanks with hypoxic conditions (1.7 ± 0.2 ppm dissolved oxygen) created by N_2 supply. The fish were monitored for signs of discomfort and the latency period was recorded. Fainting fish were immediately removed from the hypoxic water and kept at normoxic conditions until termination of the hypoxic stress after 30 min for sampling of stressed fry.

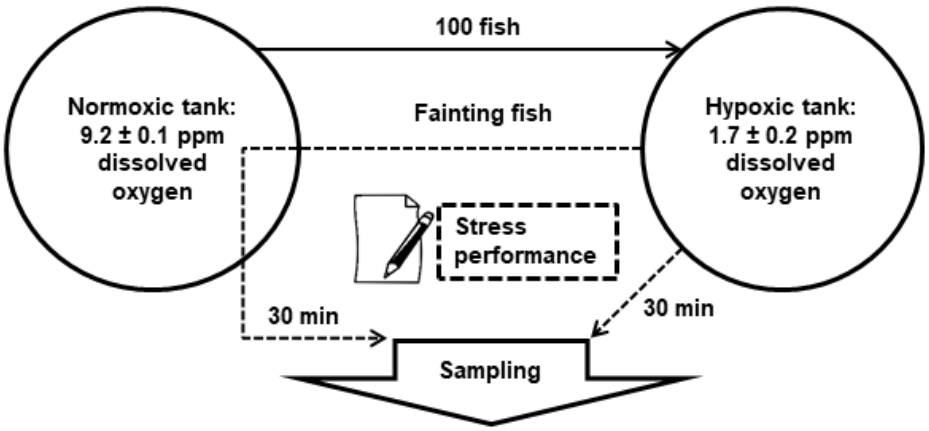

Fig. 20. Protocol of the acute hypoxic stress challenge in 11-week fed fry

4.3. Sampling

Broodstock fish were sampled at spawning for liver, muscle, oocytes and milt (Fig. 18). Progeny was sampled at swim-up fry stage and after 11 weeks of feeding before and after subjected to the hypoxic stress challenge as whole fry. Swim-up fry were additionally dissected to obtain the liver. All samples were frozen in liquid nitrogen and stored at -80°C until further analysis.

4.4. Sample analysis

The analyses performed in the different tissues are listed in Tab 4. Detailed information on the experimental procedure can be obtained from the mentioned research articles.

Tab 5. Summary of tissue analysis performed on rainbow trout samples

Analysis	Tissue	Publication
Total selenium	Broodstock: *liver, muscle, oocyte, sperm* Progeny: *swim-up fry, 11-week fed fry, stressed fry*	1 + 2
Selenium speciation	Broodstock: *oocytes* Progeny: *swim-up fry, 11-week fed fry*	1 + 2
Se + micromineral mapping	Progeny: *swim-up fry, 11-week fed fry*	2
Histological staining	Progeny: *swim-up fry, 11-week fed fry*	2
Antioxidant enzyme activity	Broodstock: *liver* Progeny: *swim-up fry, 11-week fed fry, stressed fry*	1 + 3
Gene expression: antioxidant, selenoproteins	Broodstock: *liver* Progeny: *swim-up fry, 11-week fed fry, stressed fry*	1 + 3
Vitamin E and C	Progeny: *swim-up fry, 11-week fed fry, stressed fry*	1 + 3
Glutathione levels	Broodstock: *liver* Progeny: *swim-up fry, 11-week fed fry, stressed fry*	1 + 3
Protein carbonyls	Progeny: *swim-up fry, 11-week fed fry, stressed fry*	1 + 3
8-Isoprostanes	Progeny: *swim-up fry, 11-week fed fry, stressed fry*	1 + 3
Isoprostanoids	Progeny: *11-week fed fry, stressed fry*	3
Fatty acid composition	Progeny: *11-week fed fry, stressed fry*	3
Free amino acids	Progeny: *swim-up fry, 11-week fed fry, stressed fry*	4 + 5
Aminothiols	Broodstock: *liver, oocytes* Progeny: *swim-up fry, 11-week fed fry, stressed fry*	4 + 5
SAM/SAH	Broodstock: *liver, oocytes* Progeny: *swim-up fry, 11-week fed fry, stressed fry*	4 + 5
Gene expression: 1C proteins	Broodstock: *liver* Progeny: *swim-up fry, 11-week fed fry, stressed fry*	4 + 5
DNA methylation pattern (RRBS)	Progeny: *liver of swim-up fry*	4

5. PUBLICATIONS

The following articles were prepared in line of this PhD work:

[1] Effect of dietary selenium in rainbow trout (*Oncorhynchus mykiss*) broodstock on antioxidant status, its parental transfer and oxidative status in the progeny

[2] Tissue localization of selenium of parental or dietary origin in rainbow trout (*Oncorhynchus mykiss*) fry using LA-ICP MS bioimaging

[2] 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

[3] Parental selenium affects the one carbon metabolism and hepatic DNA methylation pattern in rainbow trout (*Oncorhynchus mykiss*) fry

[5] Long-term effect of parental selenium on the one-carbon metabolism in rainbow trout (*Oncorhynchus mykiss*) fry exposed to hypoxic stress

5.1. Publication 1

Effect of dietary selenium in rainbow (*Oncorhynchus mykiss*) broodstock on antioxidant status, its parental transfer and oxidative status in the progeny

Objective:

Investigate the effect of different selenium sources in broodstock diets on the antioxidant metabolism and reproductive performance of rainbow trout. The underlying hypothesis is that parental selenium can improve the antioxidant status in the progeny before first feeding.

Approach:

Rainbow trout broodstock were divided into three groups and fed either a control diet or a diet supplemented with selenium either in organic or inorganic form. Broodstock performance was monitored during the spawning period. Different tissues in males and females were sampled for selenium analysis. Antioxidant parameters were measured in broodstock liver.
Collected oocytes were fertilized with sperm from males of the same dietary treatment and offspring performance was monitored until swim-up fry stage. At swim-up fry stage the progeny was sampled and analyzed for whole-body selenium as well as antioxidant and oxidative stress parameters.

Major findings:

- Dietary selenium increased the number of spawning females in rainbow trout
- Earlier spawning of femeales was achieved in those fish fed hydroxy-selenomethionine
- Broodstock fed Se-supplemented diets transferred more selenium to the progeny, especially with hydroxy-selenomethionine
- Higher GPX activity and mRNA levels of selenoproteins was observed in offspring of broodstock fed organic selenium
- Improved vitamin E and C levels were observed in the progeny from the organic selenium treatment
- Parental selenium decreased GSH to GSSG ratio in swim-up fry

Conclusion:

Selenium supplementation in broodstock diets can be beneficial for the reproductive performance of rainbow trout females, when given plant-based diets with low basal selenium level. At physiological relevant concentrations, the parental selenium can support the antioxidant metabolism of the progeny before first feeding, but the impact on the glutathione metabolism deserves further investigation

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Effect of dietary Se in rainbow trout (*Oncorhynchus mykiss*) broodstock on antioxidant status, its parental transfer and oxidative status in the progeny

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ARTICLE INFO

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ABSTRACT

We studied the effects of supplementing a plant-ingredient based rainbow trout broodstock diet with different forms of selenium (Se) on reproductive performance, parental Se transfer and antioxidant metabolism in their progeny before first-feeding. Three groups of rainbow trout were fed a diet either without selenium supplementation (NC, basal Se level: 0.3 ppm) or supplemented with 0.3 ppm Se either in the form of sodium selenite (SS) or as hydroxy-selenomethionine (SO, dietary Se level: 0.7 ppm) over a 6-month period prior to spawning. In Se-supplemented groups, the total number of females spawning was significantly higher compared to the negative control group and the females fed SO began to spawn earlier compared to females fed SS or NC. Total Se concentrations were significantly higher in female muscle of SO group. Higher Se concentrations in the oocytes of both Se-supplemented groups confirmed a maternal transfer of Se, while total Se concentrations in milt samples were not significantly different between dietary groups. There was no effect on glutathione peroxidase (GPX) or other antioxidant enzyme activity in the liver of female or male broodstock, whereas in the swim-up fry, GPX activity was significantly higher in both Se-supplemented treatments with the highest activity in the SO group. Se-supplementation enhanced the expression of hepatic SelPa in broodstock males and females along with MsrB1, GPx1a, GPx4a2, CAT, Gclc, Keap1 and MsrB2 in male liver. In swim-up fry, only organic Se-supplementation led to a higher gene expression for SelPa, GPX1a, GPX1b2, CAT and MsrB2. An improvement of the oxidative status in whole-body of swim-up fry could not be confirmed in this study as the GSH/GSSG was even lower in swim-up fry of Se-supplemented groups, whereas the 8-isoprostane levels were lower in the SS group and no effect on protein carbonyl levels could be detected. Organic Se-supplementation led to a significant increase in the α-tocopherol and vitamin C levels in the progeny. These results show that dietary Se in broodstock nutrition influences spawning occurrence and along with the parental transfer, several traits in the progeny are affected.

1. Introduction

While fishmeal based diets naturally contain high amounts of selenium (Se) covering the nutritional requirements (Hilton et al., 1980), plant based diets are generally lower and vary more widely in their Se content (Combs, 2001). With the ongoing replacement of fish meal and fish oil in aquafeeds, there is a need to better understand the metabolic role of Se, which is complex and not yet fully explored. The Se requirements in fish are reported to vary between 0.15 and 0.70 mg/kg diet (Antony Jesu Prabhu et al., 2016), depending on species (Watanabe et al., 1997), life stage (Bell et al., 1985) and bioavailability of the Se form (Wang and Lovell, 1997; Penglase et al., 2014a). Thereby, organic Se forms have been described to have a higher bioavailability compared to inorganic forms, leading to increased tissue Se content (Le and Fotedar, 2014; Fontagné-Dicharry et al., 2015; Antony Jesu Prabhu et al., 2016). Dietary Se, both in surplus and deficiency, can have severe

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consequences for the fish. Already a dietary Se level of 3 mg/kg, slightly above the recommended level, can in the long term induce adverse effects (Berntssen et al., 2018). On the other hand, Se deficiency can reduce the expression and activity of selenoproteins (Hesketh, 2008; Elia et al., 2011; Penglase et al., 2014a), of which many are involved in the antioxidant protection of the cell (Papp et al., 2007). In a previous study, working with low Se levels in rainbow trout (*Oncorhynchus mykiss*) fry, the positive impact of Se supplementation on Se-dependent GPX activity and gene expression was demonstrated, especially when an organic Se supplement was used (Fontagné-Dicharry et al., 2015). Se supplementation was found to increase the expression of selenoprotein P not only in rainbow trout, but also in zebrafish (*Danio rerio*) (Penglase et al., 2014a; Fontagné-Dicharry et al., 2015; Pacitti et al., 2015; Wang et al., 2018). Under a dietary Se deficiency, besides the effects at the cellular level, a reduced growth and an increased mortality in fish are also reported (Wang et al., 2013). In rainbow trout, the negative impact of Se deficiency on body weight could not be detected (Bell et al., 1986), but liver vitamin E levels were significantly reduced and the combined effect of vitamin E and Se deficiency led to a reduced weight gain (Bell et al., 1985). Most of the studies investigating the effect of dietary Se on the metabolism in fish have been undertaken with juveniles or early stages and there is little information on the effects of Se in broodstock nutrition, except studies investigating the impact of Se-supplementation at high levels (Hardy et al., 2010; Penglase et al., 2014a,b). However, the reproduction period is substantially different to other life stages as energy is not used for growth, but mobilized to the reproductive organs; especially in females where nutrients are accumulated in the ovarian oocytes during vitellogenesis. Therefore, Se deficiency effects observed at early stages might be different in broodstock fish and might affect spawn quality. Male fertility and reproduction have been shown to be reduced in several animal species fed Se-deficient diets (Hosnedlova et al., 2017) and could be linked to the presence of the selenoprotein GPx4 in male sperm (Imai et al., 2009). A reduced antioxidant capacity of offspring by maternal Se deficiency have already been described in broilers (Pappas et al., 2006; Zhang et al., 2014; Xiao et al., 2016). Furthermore, the dietary supplementation of Se in poultry breeders led to an increased fertility of males and maternal transfer of Se, improving the oxidative status in the progeny (Słowińska et al., 2011; Wang et al., 2011; Zhang et al., 2014). Se toxicity trials undertaken with zebrafish (Thomas and Janz, 2015) and brown trout (*Salmo trutta*) (Covington et al., 2018) suggest that a parental transfer of Se in fish exists at toxic levels. The current study was undertaken to address whether similar effects are detected in broodstock fish fed nutritionally relevant dietary levels of Se in different forms.

2. Material and methods

2.1. Experimental setup

Three-year-old rainbow trout (*Oncorhynchus mykiss*) females and males of the same genetic origin from the INRA experimental fish farm of Lées-Athas (Pyrénées-Atlantiques, France) were used as broodstock. Rainbow trout broodstock were randomly allocated to three circular 2-m diameter tanks supplied with flow-through (1 L per *sec*) spring water at 8 ± 1 °C with 25 females (initial mean weight: 1.1 ± 0.2 kg, second reproduction season) and 15 males (0.9 ± 0.3 kg) per tank (stocking density: 40 kg/m^3). The fish were hand fed once a day to visual satiation for a period of 6 months prior to spawning. Each fish was tagged in the dorsal muscle with a passive integrated transponder (PIT, 12 × 2 mm, ISO 11784/11785, IER, Suresnes, France) and the individual growth was followed until spawning. During the spawning period, fish were regularly checked for ovulation and at spawning, oocytes of each female were collected through stripping. At the first spawning date, one female from the SS-group and two females from the SO-group were over-mature (with oocytes exhibiting polar condensation of vitellus and lipid goblets) and they were excluded from the trial as well as one NC female during the second spawning date due to low spawning quantity and quality. Otherwise, two pools of 50 oocytes from each female were lined up to measure total length and afterwards weighed to calculate the average diameter and weight of oocytes, respectively. All other eggs were fertilized with sperm collected on the same day from a pool of males of the same dietary group. Eggs from each female were reared separately in small trays supplied with flow-through spring water at 8 ± 1 °C to follow individual hatching data. The embryonic development was followed until swim-up fry stage. The survival was monitored and dead eggs were removed every two days. Survival rates were calculated at eyed stage (33 dpf), hatching (46 dpf) and swim-up stage (65 dpf).

2.2. Sample collection

Broodstock fish were sampled at spawning. First, fish were anaesthetized with benzocaine (30 mg/L) for stripping and afterwards killed by a sharp blow on the head for liver and muscle sampling. Muscle tissue was sampled in duplicate at the dorsal fin as approximately 15 g of fillet. The weights of whole fish, spawn (total eggs collected from each female after stripping and removal of coelomic fluid) and liver were recorded to calculate absolute and relative fecundity (number of eggs/female and number of eggs/kg female, respectively), gonado-somatic index (GSI, percentage of spawn weight to weight of female) and hepatosomatic index (HSI, percentage of liver weight to weight of fish). Tissue samples and oocytes (2 × 50 units per female) were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. The progeny was sampled at swim-up fry stage. Pooled fry, for every female separately, were euthanized with an overdose of benzocaine (60 mg/L), weighed and afterwards subdivided in different sample size according to planned analysis, before freezing as whole-body fry in liquid nitrogen and stored at −80 °C until further analysis.

All procedures were performed in compliance with the European Directive 2010/63/EU for the protection of animals used for scientific purposes and the French Decree no. 2013-118 for animal experimentation.

2.3. Experimental diets

Diets were manufactured using a twin-screw extruder (BC 45, Clextral, Firminy, France) at the INRA experimental facilities in Donzacq (Landes, France). The three diets (NC, SS and SO) were based on plant-derived proteins and had similar levels of crude protein (50%) and total lipid (22%) and differed only in Se content (Table 1). The NC-diet containing 0.3 mg Se/kg feed was not supplemented with Se and served as negative control. The SS and SO diets were supplemented with either sodium selenite (SS-diet) or hydroxy-selenomethionine (Selisseo®, SO-diet). In both supplemented diets, the targeted Se concentration was 0.6 mg Se/kg, which remained lower to concentrations usually found in fish meal based diets (Fontagné-Dicharry et al., 2015), but feed analyses gave a final dietary Se content of 0.8 mg Se/kg in SS diet and 0.7 mg Se/kg in SO diet.

2.4. Proximate, mineral and fatty acid analysis

For proximate composition of diets, dry matter (DM) was determined after drying at 105 °C for 24 h, protein (N × 6.25) by Kjeldahl method after acid digestion (ISO, 5983-1:2005), ash after 10 h incineration at 550 °C and gross energy in an adiabatic bomb calorimeter. Total lipid in diets and broodstock muscle was extracted and quantified gravimetrically following Folch et al. (1957), using dichloromethane instead of chloroform. Fatty acid methyl esters were prepared and analyzed as previously described (Fontagné et al., 2008). Total phosphorus was determined by the molybdate blue/ascorbic acid method at 820 nm after mineralization and acid digestion (AFNOR, 1992). Total

Table 1
Formulation and composition of experimental diets (g/100 g dry weight).

Diet	NC	SS	SO
Ingredients			
Plant meals[1]	74	74	74
Crystalline amino acids and attractant mixture[2]	3.14	3.14	3.14
Soybean lecithin[3]	2	2	2
Fish oil[3]	8	8	8
Vegetable oils[4]	8	8	8
Carophyll® pink[5]	0.04	0.04	0.04
Vitamin and mineral mixture without Se[6]	4.82	4.82	4.82
Sodium selenite (μg/g diet)[7]	–	0.7	–
Selisseo® (μg/g diet)[7]	–	–	15
Analytical composition			
Dry matter (DM, %)	96	98	97
Crude protein (% DM)	49	50	50
Total lipid (% DM)	23	22	23
Gross energy (kJ/g DM)	25	25	25
Ash (% DM)	6	6	6
Phosphorus (% DM)	1.2	1.1	1.2
Se (mg/kg)	0.3	0.8	0.7
Fatty acid profile (% total fatty acids)			
14:0	5.1	4.6	5.0
16:0	25.5	24.4	25.6
18:0	3.1	3.2	3.3
Total saturates[8]	35.1	33.6	35.3
16:1	3.8	3.5	3.7
18:1	28.0	28.8	27.5
Total monoenes[8]	32.5	33.0	31.8
18:2 n-6	16.7	17.3	17.0
20:4 n-6	0.5	0.4	0.5
Total n-6	17.2	17.7	17.4
18:3 n-3	9.9	10.1	9.7
18:4 n-3	0.6	0.5	0.5
20:5 n-3	2.3	2.1	2.2
22:6 n-3	1.5	1.8	2.1
Total n-3	14.3	14.6	14.6

[1] 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).

[2] 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.

[3] Soybean lecithin from Louis François and fish oil from Sopropêche.

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

[5] Contained 10% astaxanthin (DSM).

[6] 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; $CaHPO_4{\cdot}2H_2O$, 33 g; $CaCo_3$, 2.15 g; $Mg(OH)_2$, 1.24 g; KCl, 0.9 g; NaCl, 0.4 g; $FeSO_4{\cdot}7H_2O$, 0.2 g; $ZnSO_4{\cdot}7H_2O$, 40 mg; $MnSO_4{\cdot}H_2O$, 30 mg; $CuSO_4{\cdot}5H_2O$, 30 mg; NaF, 10 mg; KI, 0.4 mg; $CoCl_2{\cdot}6H_2O$, 0.2 mg. All ingredients were diluted with α-cellulose.

[7] Sodium selenite contained 42% Se (Sigma-Aldrich) and Selisseo contained 2% Se (Adisseo).

[8] Total saturates include 12:0, 15:0, 17:0 and 20:0 and total monoenes include 14:1 and 20:1.

Se was measured using inductively coupled plasma mass spectrometry (ICP-MS, Agilent series 7500cx) by Ultra Trace Analyses Aquitaine (UT2A, Pau, France) for diets, broodstock tissues and oocytes according to Vacchina and Dumont (2018) and by the Institut des Sciences Analytiques et de Physico-Chimie pour l'Environnement et les Matériaux (IPREM, Pau, France) for swim-up fry as described by Godin et al. (2015). Selenomethionine (SeMet) and selenocysteine (SeCys) were analyzed by liquid chromatography (HPLC, Agilent series 1200) coupled to ICP-MS by UT2A for oocytes according to Vacchina et al. (2018) and by IPREM for swim-up fry according to Godin et al. (2015).

2.5. Antioxidant enzyme activity and mRNA levels

Antioxidant enzyme activity was measured as previously described by Fontagné-Dicharry et al. (2014) in 0.3 g samples of broodstock liver and pooled whole-body fry. Briefly, all samples were first homogenized with an Ultra-turrax in an eight-time dilution of 20 mM-phosphate buffer (pH 7.4) containing 1 mM EDTA, afterwards centrifuged at 2000*g* for 10 min, the supernatant diluted in 0.5% triton solution and incubated for 1 h before using the different enzyme assays. The glutathione peroxidase (GPX, EC 1.11.1.9) activity was measured in 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 (GR) following the reduction of cumene hydroperoxide (0.2 mM) for Se-dependent GPX (SeGPX) and H_2O_2 (50 μM) for total GPX (totGPX) at 30 °C and 340 nm. Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by monitoring the inhibition of nitrotetrazolium reduction at 550 nm, and was expressed as the amount of enzyme required to inhibit the rate of nitrotetrazolium reduction by 50% per mg protein at 37 °C. The catalase (CAT, EC 1.11.1.6) activity was measured by following the reduction of H_2O_2 in a solution of 67 mM phosphate buffer, 1 mM EDTA and 20 mM H_2O_2 (pH 7.4) at 30 °C and 240 nm. GR (EC 1.8.1.7) activity was determined measuring the reduction of NADPH in a solution containing 100 mM phosphate buffer (pH 7.5), 1 mM GSSG and 50 μM NADPH at 30 °C and 340 nm. Glutathione-S-transferase (GST, EC 2.5.1.18) activity was measured in a solution of 100 mM phosphate buffer (pH 6.5), 1 mM EDTA, 1 mM GSH and 1 mM CDNB at 30 °C and 340 nm, using the extinction coefficient of 9.61 mM^{-1}/cm. One unit of enzyme is defined as the amount needed to catalyze the reaction of 1 μM substrate per minute. The protein concentration was determined according to Lowry et al. (1951), using bovine serum albumin (BSA) as a standard.

Total RNA was isolated from 100 mg samples of broodstock liver and a pool of three whole-body swim-up fry by using Trizol reagent (Invitrogen, Cergy-Pontoise, France). For quantitative RT-PCR, complementary DNA was generated from 1 mg total RNA using SuperScript III RT (Invitrogen) and a mix of oligo (dT)15 and random primers (Promega, Charbonnières, France). For each sample, RT was performed in duplicate and quantitative PCR analyses were performed in LightCycler® 480 Instrument II (Roche Diagnostics, Meylan, France) using LightCycler® 480 SYBR Green I Master mix (Roche Diagnostics). Total reaction volume was 6 μL, with 2 μL of the diluted RT reaction mixture (dilution 40) and 4 μL of master mix added with 0.4 mM of each primer (Table 2). The PCR protocol was initiated at 95 °C for 10 min for initial denaturation of the cDNA and hot-start Taq-polymerase activation, followed by 45 cycles of a three-step amplification program (15 s at 95 °C, 10 s at the melting temperature of 60 °C, 4.8 s at 72 °C). Melting curves were systematically monitored (5 s at 95 °C, 1 min at 65 °C, temperature slope at 0.11 °C/s from 65 to 97 °C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Relative quantification of target gene transcripts was performed using β-actin as the reference gene and NC as the reference group using the ΔΔCt method (Pfaffl, 2001).

2.6. Glutathione and oxidative status in the progeny

Total (GSHt), oxidized (GSSG) and reduced glutathione (GSH) were measured in 0.3 g homogenized liver tissues and 1 g of pooled oocyte and fry samples using Cayman glutathione assay kit (Bertin Pharma, Montigny-le-Bretonneux, France) according to the manufacturer's instructions with protein concentration assessed by the method of Lowry et al. (1951) using BSA as a standard. Lipid peroxidation was assessed by measuring concentration of 8-isoprostanes (8-isoPT), the product of non-enzymatic peroxidation of arachidonic acid by reactive oxygen species in 0.5 g of pooled fry as described by Fontagné et al. (2006)

Table 2
Oligonucleotide primers used to assay mRNA levels by real-time quantitative RT-PCR.

Gene	Accession no.	Forward primer sequence	Reverse primer sequence	Amplicon size
SelPa	HF969249.1	cagccacctggttggagtat	cctggagtagggccacca	82
SelPa2	MH085054.1	accttgctgagccagaaact	cagacgaccacacctgtcat	129
SelPb	HF969250.2	gtgtcgttcctcatcgtgaa	ggaacgtcagtctcccacat	187
GPX1a	HE687021	aatgtggcgtcactctgagg	caattctcctgatggccaaa	131
GPX1b1	CA357669.1	cgagctccatgaacggtacg	tgcttcccgttcacatccac	183
GPX1b2	HE687023	tcggacatcaggagaactgc	tccttcccattcacatccac	121
GPX4a1	HE687024	gaaaggcttcctgggaaatg	ctccaccacactgggatcat	112
GPX4a2	HE687025	agaaatacaggggcgacgtt	gcatctccgcaaactgagag	90
GPX4b	CA344428.1	ttggaggtcaggagccaggt	acccttttcccttgggctgtt	152
TR	HF969247.1	acaaaatcaaggcgaccaac	ggcagagagaacaggtcgtc	148
MsrB1	BX313019.3	ctggcctgccttcactgaga	ttcccacatcggaccttgaa	87
MsrB2	BX311214.3	aggggacagagatgcccttc	cccatgagcctctttgaacg	149
MsrB3	CX041708.1	tcaggttggccttccttcta	cgaggtgagaaccacactga	118
MsrA	BT073595.1	agatggctatgcttgggatg	acctgtgcaggtctcttcgt	137
SOD1	AF469663.1	tggtcctgtgaagctgattg	ttgtcagctcctgcagtcac	201
SOD2	CA352127.1	tccctgacctgacctacgac	ggcctcctccattaaacctc	201
CAT	BX087110.3	tgatgtcacacaggtgcgta	gtgggctcagtgttgttgag	195
GR	HF969248.1	ctaagcgcagcgtcatagtg	acaccctgtctgacgacat	108
Gclc	GSONMT00065033001	caaccaactggcagacaatg	cctttgacaaggggatgaga	189
GSTπ	BX302932.3	tcgctgactggacgaaagga	cgaaggtcctcaacgccatc	196
Nrf2	CA360709.1	tgagctgcagcaatgtctga	gttgggcaatgggtagaagc	124
Keap1	GSONMT00034445001	gctacgtgatgtctgcccct	ggtacctcatagcggccagt	116
Keap1X	GSONMT00019673001	actgggaggttatgacggga	cctccgtcctctcatgcttc	179
NF-κB	BX880658.3	cagcgtcctaccaggctaaagagat	gctgttcgatccatccgcactat	181
IκBα	BT074199.1	agagacagactgcgctccac	cggccttcagtagcctctct	72
β-Actin	AJ438158.1	gatgggccagaaagacagcta	tcgtcccagttggtgacgat	105

SelP, selenoprotein P; GPX1, glutathione peroxidase 1; GPX4, glutathione peroxidase 4; TR, thioredoxin reductase; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; CAT, catalase; GR, glutathione reductase; Gclc, glutamate-cysteine ligase catalytic subunit; GSTπ, glutathione S-transferase π; Nrf2, nuclear factor erythroid-2 related factor 2; Keap1, Kelch-like ECH-associated protein 1; NF-κB, nuclear factor kappa-light chain-enhancer of activated B cells; IκBα, NF-κB inhibitor α.

using Cayman Chemical 8-isoprostane enzyme immunoassay kit (Spi-Bio, Massy, France) according to the manufacturer's instructions. Protein oxidation was assessed using the spectrophotometric determination of protein carbonyls by derivatization with 2,4-dinitrophenylhydrazine (DNPH) at 370 nm according to Armenteros et al. (2009) with protein concentration calculated from absorption at 280 nm in the blank sample using bovine serum albumin (BSA) as standard. HPLC was used for determination of vitamin C according to the method described by Mæland and Waagbø (1998). Tocopherols were extracted from a pool of six oocytes and three whole-body fry according to Folch et al. (1957) using dichloromethane instead of chloroform. They were analyzed by HPLC on a Phenomenex Luna® PFP(2) column (100 Å, 3 µm, 100 × 2 mm), with an isocratic mobile phase of methanol/water (93:7 *v*/v) at a flow rate of 0.5 mL/min and column oven temperature of 40 °C using a Waters Alliance 2695 separation module with a Waters 2475 multi wavelength fluorescence detector set at 295/330 nm (excitation/emission) according to Górnaś et al. (2014). The concentration of tocopherol per individual was calculated using the average weight of oocyte or whole-body fry.

2.7. Statistical analysis

Results are given as mean ± standard error (SEM). Differences between dietary groups were analyzed in statistical software R (R Development Core Team, 2008) or SigmaStat 3 (SPSS, Chicago, IL, USA) using one-way ANOVA. Tocopherol data were analyzed using two-way ANOVA to compare data from oocytes with data at swim-up fry stage. All data were tested for normal distribution and homogeneity and only in case the assumptions were not respected, the ANOVA was performed instead on ranks. The binary spawning data were analyzed pairwise using χ^2 test. Percentages were arc-sin transformed before analysis. A Newman-Keuls post-hoc test was used to compare means, when a significant difference ($P < 0.05$) was found.

3. Results

3.1. Growth and reproductive performance in broodstock

All three diets were readily accepted by rainbow trout broodstock. In males and females, no significant difference in initial or final body weight was detected between the three dietary groups (Table 3). In females, no significant difference in daily growth between the control group and the supplemented groups was found. However, the daily growth index was higher in females fed SO compared to SS. In the groups NC and SS, six males and in SO, one female and four males died during the feeding trial before spawning. HSI, muscle lipid content and muscle fatty acid profile (data not shown) were not significantly different between groups for both males and females. At the end of the spawning period in January, the total number of fish spawning was significantly higher in the two Se-supplemented groups SS and SO compared to the control group NC. Also, females fed the organic Se-supplemented diet SO began to spawn significantly earlier compared to females fed the inorganic Se-supplemented diet SS (Fig. 1). No significant difference in reproductive performance, including GSI, fecundity or egg size could be detected (Table 3). During embryonic development, there was no significant difference in survival between NC and the supplemented groups. However, the survival rate was significantly lower in the SS-group than in SO from hatching onwards.

3.2. Se and its transfer from broodstock to progeny

Total Se content was significantly higher in muscle of females fed SO, compared to the ones fed NC or SS (Fig. 2A). The same tendency was noticed in female liver, but no significant difference between the three treatments was detected (Fig. 2B). Total Se content in milt was not significantly different between groups (Fig. 2C). On the other hand, total Se content in oocytes was significantly higher for females fed SO and lower in NC group compared to the SS treatment (Fig. 2D).

The majority of Se in oocytes was present in the form of SeMet,

Table 3
Growth, spawning parameters and offspring performance of rainbow trout broodstock fed different Se sources over 6 months at a constant water temperature of 8 ± 1 °C.

Diet	NC	SS	SO
Male performance			
Initial body weight (kg, n = 15)	0.88 ± 0.06	0.90 ± 0.09	0.82 ± 0.07
Body weight after 6 month (kg, n = 9–11)	1.20 ± 0.12	1.04 ± 0.09	1.03 ± 0.13
DGI[1] (%, n = 9–11)	0.34 ± 0.08	0.28 ± 0.14	0.29 ± 0.12
HSI[1] (%, n = 5)	1.1 ± 0.1	1.1 ± 1.1	1.5 ± 0.2
Muscle lipid content (%, n = 5)	6.8 ± 0.8	6.7 ± 1.1	6.3 ± 0.6
Female performance			
Initial mean body weight (kg, n = 25)	1.13 ± 0.05	1.11 ± 0.03	1.10 ± 0.04
Final mean body weight (kg, n = 24–25)	1.30 ± 0.07	1.21 ± 0.04	1.32 ± 0.05
DGI[1] (%, n = 10–21)	0.27 ± 0.05^{ab}	0.17 ± 0.04^{b}	0.32 ± 0.05^{a}
HSI[1] (%, n = 10–21)	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.2
Muscle lipid content (%, n = 8–18)	7.9 ± 0.6	6.2 ± 0.5	6.9 ± 0.4
GSI[1] (%, n = 13–24)	15.6 ± 1.1	14.3 ± 1.0	15.6 ± 0.7
Spawn weight (g, n = 13–24)	220 ± 16	168 ± 12	205 ± 13
Absolute fecundity (eggs/female, n = 13–24)	3378 ± 209	2687 ± 266	3119 ± 136
Relative fecundity (eggs/kg female, n = 13–24)	2381 ± 148	2269 ± 213	2418 ± 114
Offspring performance			
Egg diameter (mm, n = 13–24)	4.1 ± 0.1	3.9 ± 0.1	4.1 ± 0.0
Egg mean weight (mg, n = 13–24)	54 ± 3	50 ± 2	54 ± 1
Survival at eyed stage (%, n = 12–21)	91 ± 2	79 ± 7	94 ± 1
Hatching rate (%, n = 12–21)	89 ± 2^{ab}	76 ± 6^{b}	92 ± 1^{a}
Survival at swim-up fry stage (%, n = 12–21)	87 ± 2^{ab}	75 ± 6^{b}	91 ± 1^{a}
Mean weight of swim-up fry (mg, n = 12–21)	85 ± 3	82 ± 3	85 ± 2

Values are means ± SEM. Within rows, means not sharing a common superscript letter are significantly different (P < .05) according to one-way ANOVA followed by a Newman-Keuls test.

[1] DGI, daily growth index = 100 x (final body weight$^{1/3}$ – initial body weight$^{1/3}$)/duration (182 days); HSI, hepato-somatic index = 100 x (wet liver weight/fish weight); GSI, gonado-somatic index = 100 x (wet spawn weight/female weight); Spawn weight = total egg weight per female after stripping and removal of cœlomic fluid; absolute fecundity = egg number per female; relative fecundity = egg number/kg female.

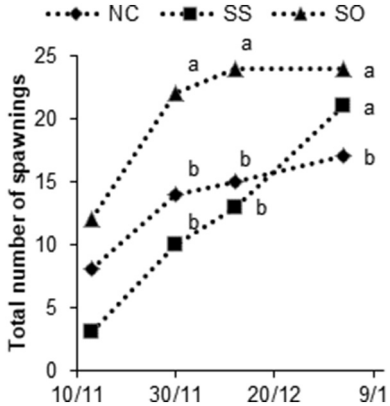

Fig. 1. Total numbers of females spawning in rainbow trout broodstock fed different Se sources over a six months period prior to spawning at a constant water temperature of 8 ± 1 °C. For each date, total numbers of females spawning, not sharing the same superscript letter, are significantly different (P < 0.05) by pairwise comparison of the χ^2.

which was significantly higher in Se-supplemented groups compared to the control group NC with the highest concentration in the organic Se treatment, while the SeCys content was not significantly different between the groups (Table 4). As for oocytes, the total Se content in swim-up fry before first-feeding was the lowest in NC, followed by SS and highest in SO (Fig. 2E). However, with smaller sample size for Se speciation analyses (n = 3), no significant difference between the groups SS and SO were found (Table 4). SeCys content was not significantly different between the three dietary groups of swim-up fry. Only swim-up fry from the organic Se-supplemented group SO showed an increase in SeMet concentration compared to the two other groups (Table 4). The total Se content per individual decreased significantly from oocyte to swim-up fry stage in SS (18 ± 2 to 15 ± 1 ng Se per individual) and SO (29 ± 3 to 23 ± 1 ng Se per individual) groups whereas no significant change in Se content was noticed for NC (11 ± 1 ng Se per individual).

3.3. Changes in selenoproteins and other antioxidant enzyme activity and mRNA levels in broodstock and progeny

There was no effect of broodstock nutrition on GPX (Fig. 3A and B) or other antioxidant enzyme activity in liver of female or male (Table 5). In whole-body swim-up fry, totGPX activity was significantly higher in both Se-supplemented treatments with the highest activity in the SO group (Fig. 3C). This was associated with higher SeGPX activity in SO-group compared to the two other groups. The activity of other antioxidant enzymes was not significantly affected by Se supplementation (Table 5).

The mRNA expression of selenoprotein genes including the different GPX genes was not significantly affected by dietary Se supplementation in broodstock female liver except for SelPa and SelPa2 that were higher expressed in liver of broodstock females fed the inorganic Se-supplemented diet SS compared to the two other groups (Fig. 4A). The other antioxidant genes not coding for selenoproteins were not significantly affected by dietary Se supplementation in female liver (Fig. 4B). In male liver, all the three selenoproteins P were found to be higher expressed in the Se-supplemented treatments SS and SO while GPx1a was significantly higher expressed only in the inorganic Se treatment SS and MsrB1 only in the organic Se treatment SO (Fig. 5A). GPX4a2 mRNA levels were significantly higher in liver of broodstock male fed the organic Se-supplemented diet SO than in the inorganic Se-supplemented group SS. Regarding non-selenoprotein genes, CAT, Gclc and MsrB2 mRNA levels were significantly higher in liver of broodstock males fed the two Se-supplemented groups while Keap1 mRNA levels were only higher in male livers from the inorganic Se supplemented treatment SS compared to the control group NC (Fig. 5B). In whole-body swim-up fry, gene expression of the SO group with organic Se supplementation was enhanced not only for the two selenoproteins Pa and Pa2, but also for the glutathione peroxidases GPx1a and GPx1b2 (Fig. 6A). For other non-selenoproteins involved in antioxidant protection, no significant difference in mRNA levels could be detected between the swim-up fry groups except for CAT and MsrB2 that were higher expressed in the organic Se-supplemented group SO (Fig. 6B).

3.4. Glutathione levels in rainbow trout broodstock and offspring

GSHt, GSSG and GSH contents in male or female liver were not significantly different between the three dietary groups (Fig. 7A and B). In whole-body swim-up fry, GSH was significantly lower in the organic Se-supplemented group SO, while GSSG was higher in both Se-supplemented groups compared to the control group NC (Fig. 7C). Glutathione content was also measured in oocytes, but GSSG was not detectable, while no significant difference in total glutathione was detected between the three dietary groups of oocytes (0.68 ± 0.04 µmol/mg protein).

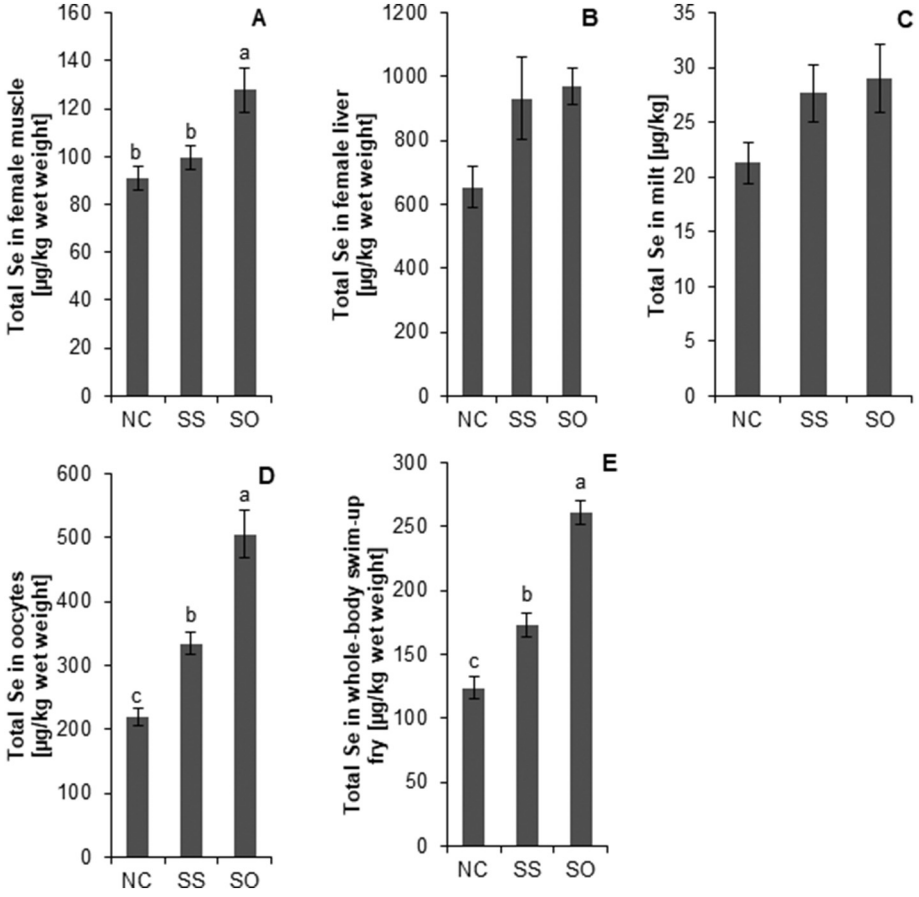

Fig. 2. Total Se content in female muscle (A), female liver (B) and in male milt (C) as well as in oocytes (D) and whole-body swim-up fry (E) from rainbow trout broodstock fed different Se sources over 6 months at a constant water temperature of 8 ± 1 °C. Given as means ± SEM (n = 8, except Se2 milt: $n = 4$ and Se3 milt: $n = 6$). Means not sharing a common superscript letter are significantly different ($P < 0.05$) according to one-way ANOVA followed by Newman-Keuls post-hoc test.

Table 4
Total Se, SeMet and SeCys concentrations (µg Se/kg wet weight), ratio of SeMet and SeCys over total Se, SeMet to SeCys ratio and percentage of total Se in oocytes and whole-body swim-up fry.

	Setot	SeMet	SeCys	SeMet/Setot	SeCys/Setot	SeMet/SeCys	%Setot
Oocytes							
NC	214 ± 12^{c}	118 ± 18^{c}	89 ± 8	0.55 ± 0.06	0.42 ± 0.04^{a}	1.35 ± 0.25^{b}	97 ± 3
SS	321 ± 14^{b}	220 ± 24^{b}	102 ± 14	0.69 ± 0.06	0.32 ± 0.03^{ab}	2.24 ± 0.38^{ab}	100 ± 3
SO	521 ± 19^{a}	373 ± 5^{a}	116 ± 19	0.72 ± 0.02	0.22 ± 0.03^{b}	3.40 ± 0.56^{a}	94 ± 1
Swim-up fry							
NC	197 ± 17^{b}	47 ± 9^{b}	13 ± 2	0.24 ± 0.06	0.07 ± 0.01	3.68 ± 0.77^{b}	31 ± 6
SS	276 ± 11^{a}	40 ± 5^{b}	13 ± 1	0.15 ± 0.03	0.05 ± 0.01	2.95 ± 0.03^{b}	19 ± 4
SO	312 ± 10^{a}	84 ± 6^{a}	12 ± 2	0.27 ± 0.01	0.04 ± 0.00	6.89 ± 0.78^{a}	31 ± 1

Values are mean ± SEM ($n = 3$). Within tissue and column values not sharing a common superscript letter are significantly different ($P < 0.05$) according to one-way ANOVA followed by Newman-Keuls post-hoc test.

3.5. Oxidative status in rainbow trout offspring

At whole-body swim-up fry stage, the GSH/GSSG ratio was significantly lower in the offspring from broodstock fed the Se-supplemented diets SS and SO compared to the control group NC (Table 6). The concentration of 8-isoprostanes was significantly lower in whole-body swim-up fry in the inorganic Se treatment SS compared to the control group NC and organic Se-supplemented group SO. Parental Se-supplementation had no significant effect on the concentration of protein carbonyls in whole-body swim-up fry. However, there was a tendency for lower protein carbonyl content in the organic Se treatment SO, indicative of an improved antioxidant status. This was confirmed with vitamin C that displayed significantly higher amounts in whole-body swim-up fry from the organic Se-supplemented SO treatment compared to the two other groups.

3.6. Tocopherol content in rainbow trout offspring

Organic Se supplementation in rainbow trout broodstock diet significantly increased the α-tocopherol concentration per individual in the offspring. The concentrations of γ- and δ-tocopherols were not significantly affected by dietary broodstock Se supplementation. A significant decrease between oocyte and swim-up fry stage was noticed for δ-tocopherol (Table 7).

4. Discussion

The recent edition of NRC, National Research Council (2011) stated that micronutrient requirements for farmed fish and shrimp are another area where knowledge is lacking; whether dietary micronutrient requirements should be based on minimal levels required to prevent clinical deficiency signs or impaired growth or based on other criteria is

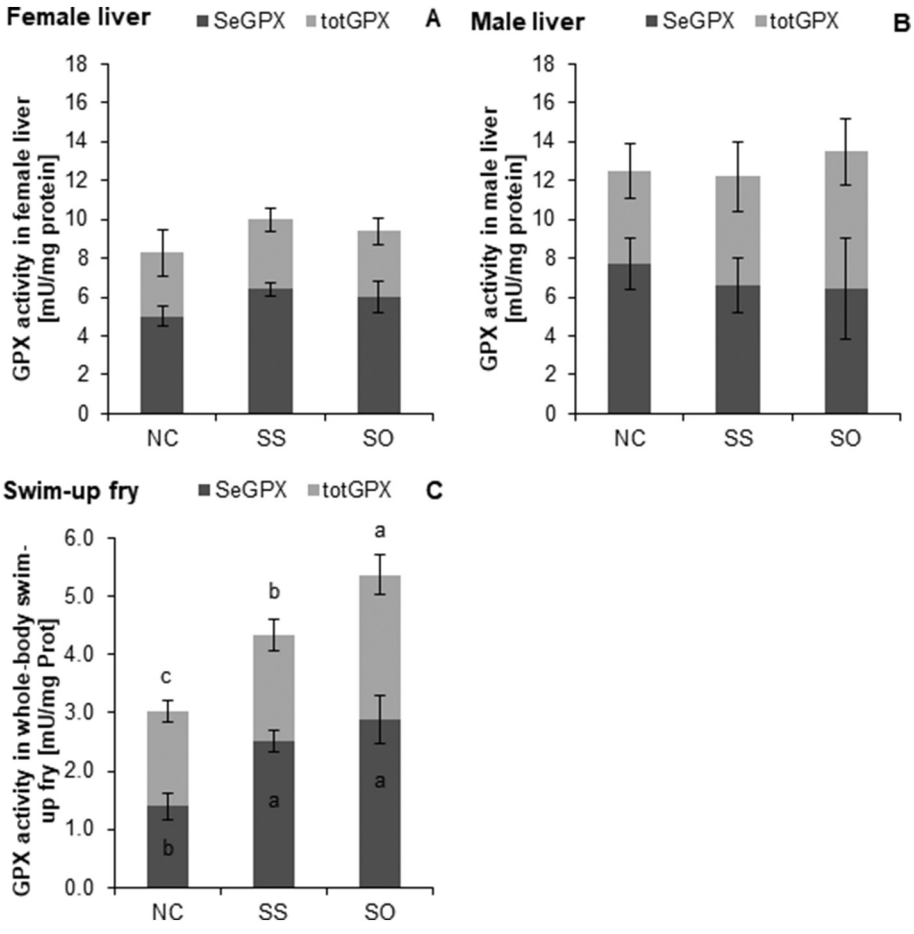

Fig. 3. Total and Se-GPX activity in female liver (A), male liver (B) and whole-body swim-up fry (C) from rainbow trout broodstock fed different Se sources over 6 months at a constant water temperature of 8 ± 1 °C. Bars represent means ± SEM (n = 8, except for male liver: n = 5). Means not sharing the same superscript letter are significantly different ($P < 0.05$) according to one-way ANOVA followed by Newman-Keuls post-hoc test.

Table 5
Antioxidant enzyme activity in liver and whole-body swim-up fry from rainbow trout fed different Se sources over 6 months at a constant water temperature of 8 ± 1 °C.

	Female liver			Male liver			Swim-up fry		
	NC	SS	SO	NC	SS	SO	NC	SS	SO
Enzyme activity									
SOD[1]	14 ± 1	14 ± 1	14 ± 1	19 ± 2	24 ± 2	18 ± 2	5.0 ± 0.2	4.9 ± 0.2	5.1 ± 0.3
CAT[1]	409 ± 32	373 ± 45	391 ± 50	502 ± 60	574 ± 52	424 ± 49	11 ± 1	11 ± 1	11 ± 1
GR[2]	8 ± 1	9 ± 1	9 ± 1	12 ± 2	7 ± 1	11 ± 1	8 ± 1	8 ± 1	8 ± 1
GST[2]	145 ± 10	154 ± 22	145 ± 17	232 ± 19	229 ± 35	220 ± 33	54 ± 4	57 ± 3	55 ± 4

Mean values ± SEM ($n = 8$, except for male liver: $n = 5$). Within rows and for each tissue, means not sharing a common superscript letter are significant different ($P < .05$) according to one-way ANOVA followed by Newman-Keuls post-hoc test.
[1] Expressed in U/mg protein.
[2] Expressed in mU/mg protein.

an open question. Studies on the determination of nutrient requirements should rely on more than one response criterion to test the effect of increasing levels of a nutrient and eventually estimate minimal dietary inclusion levels for each criterion tested, whenever possible.

4.1. Organic and inorganic Se supplementation affects spawning rate and timing

We did not find any positive effect of Se-supplementation to a plant-ingredient based diet on the growth performance of rainbow trout broodstock. It could indicate that the Se levels used in the control diet did not provoke a severe deficiency situation, as was also shown, in an earlier study with rainbow trout, that a Se level of 0.025 mg Se/kg did not significantly affect growth rates (Bell et al., 1986). On the other hand, the growth performance in all treatments of this experiment was low, which can be attributed in part to low water temperatures and the use of plant-based diets or it might be related to the increased demand of energy in reproductive organs during spawning period. These factors might have masked the impact of Se-supplementation on broodstock growth. We did not also find any significant effect of the form of Se-supplementation on reproductive performance parameters, including absolute and relative fecundity, GSI and egg size and weight. These results are in line with observations in zebrafish, where no significant difference in number of eggs and mating success could be found between groups fed deficient (0.09 mg Se/kg) vs. replete (0.65 mg Se/kg) diets (Penglase et al., 2014a). Also, similar to the mentioned zebrafish trial, the survival of the progeny was not significantly different between the control group and fish fed Se supplemented diets, however we found a slightly lower survival in the inorganic supplemented group compared to the control group. But even without a detectable effect on

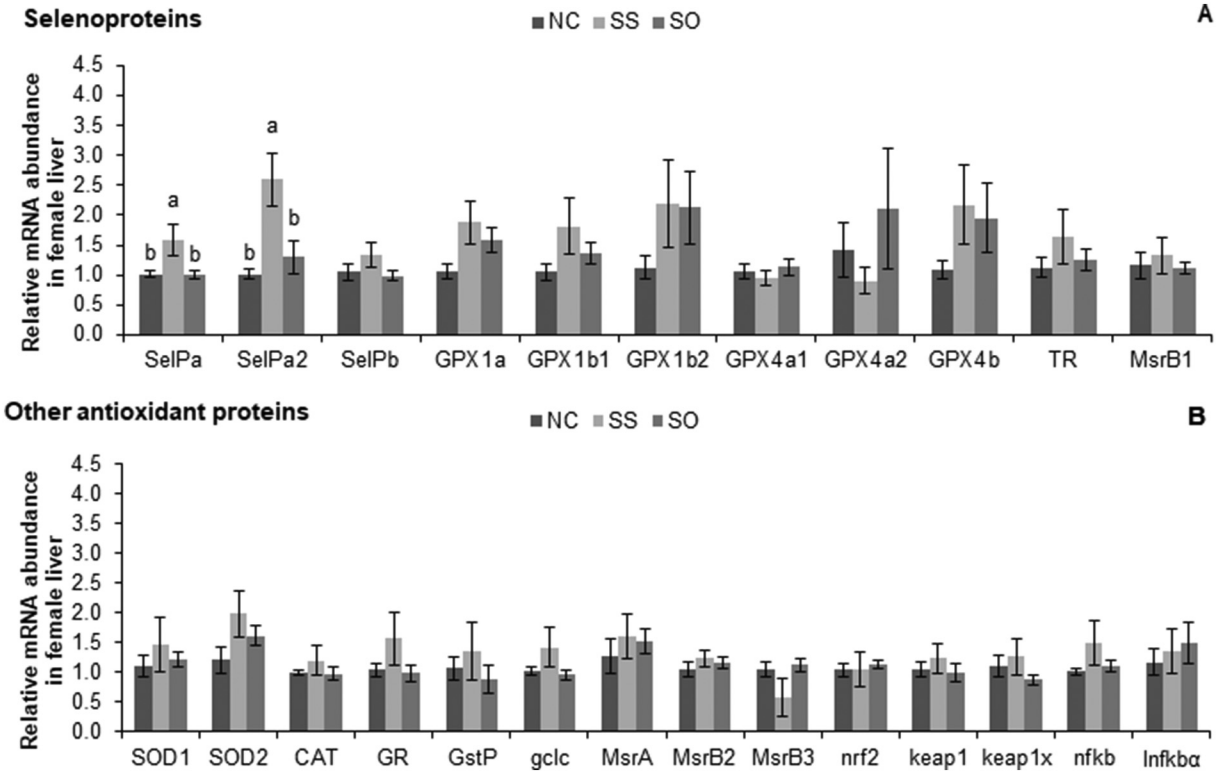

Fig. 4. Relative mRNA abundance of selenoproteins (A) and other proteins involved in antioxidant metabolism (B) in female liver from rainbow trout broodstock fed different Se sources over 6 months at a constant water temperature of 8 ± 1 °C. Data are normalized to ß-actin and expressed as fold-changes of mRNA abundance compared with the control group Se1. Bars represent means ± SEM (n = 8). Means not sharing a common superscript letter are significantly different ($P < 0.05$) according to one-way ANOVA followed by Newman-Keuls test.

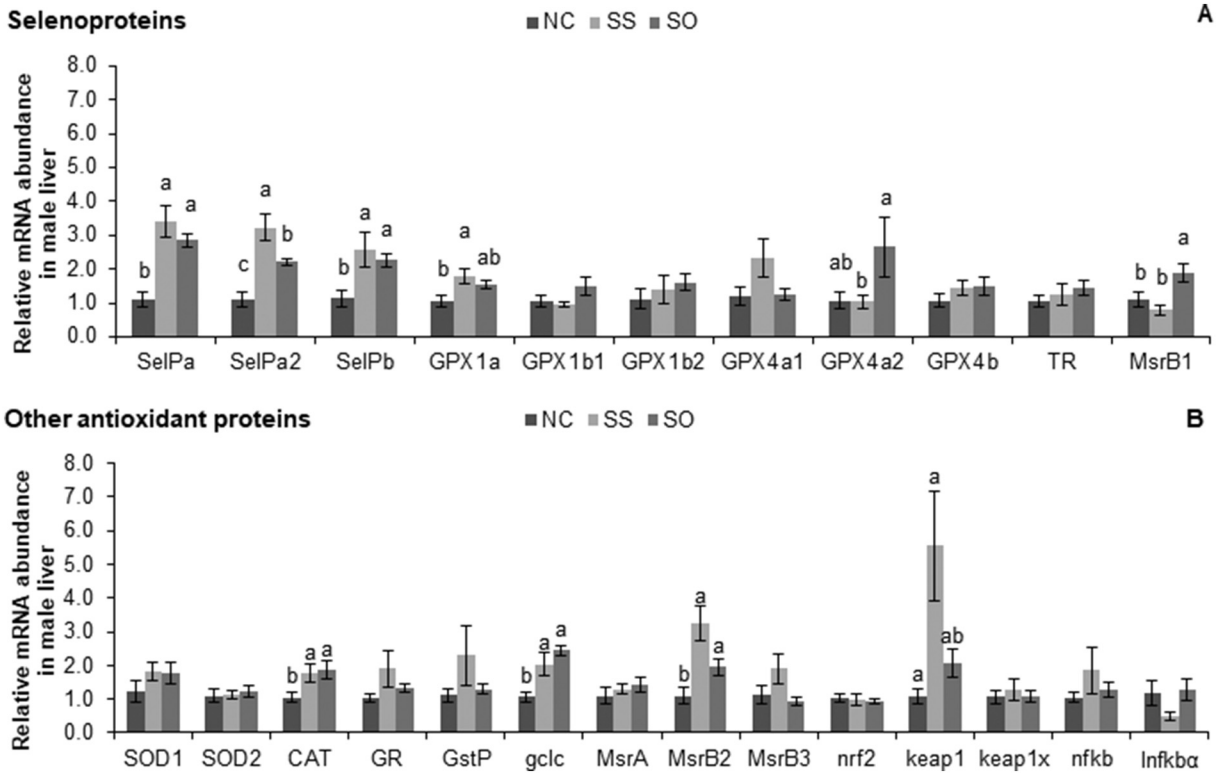

Fig. 5. Relative mRNA abundance of selenoproteins (A) and other proteins involved in antioxidant metabolism (B) in male liver from rainbow trout broodstock fed different Se sources over 6 months at a constant water temperature of 8 ± 1 °C. Data are normalized to ß-actin and expressed as fold-changes of mRNA abundance compared with the control group Se1. Bars represent means ± SEM (n = 5). Means not sharing a common superscript letter are significantly different ($P < 0.05$) according to one-way ANOVA followed by Newman-Keuls test.

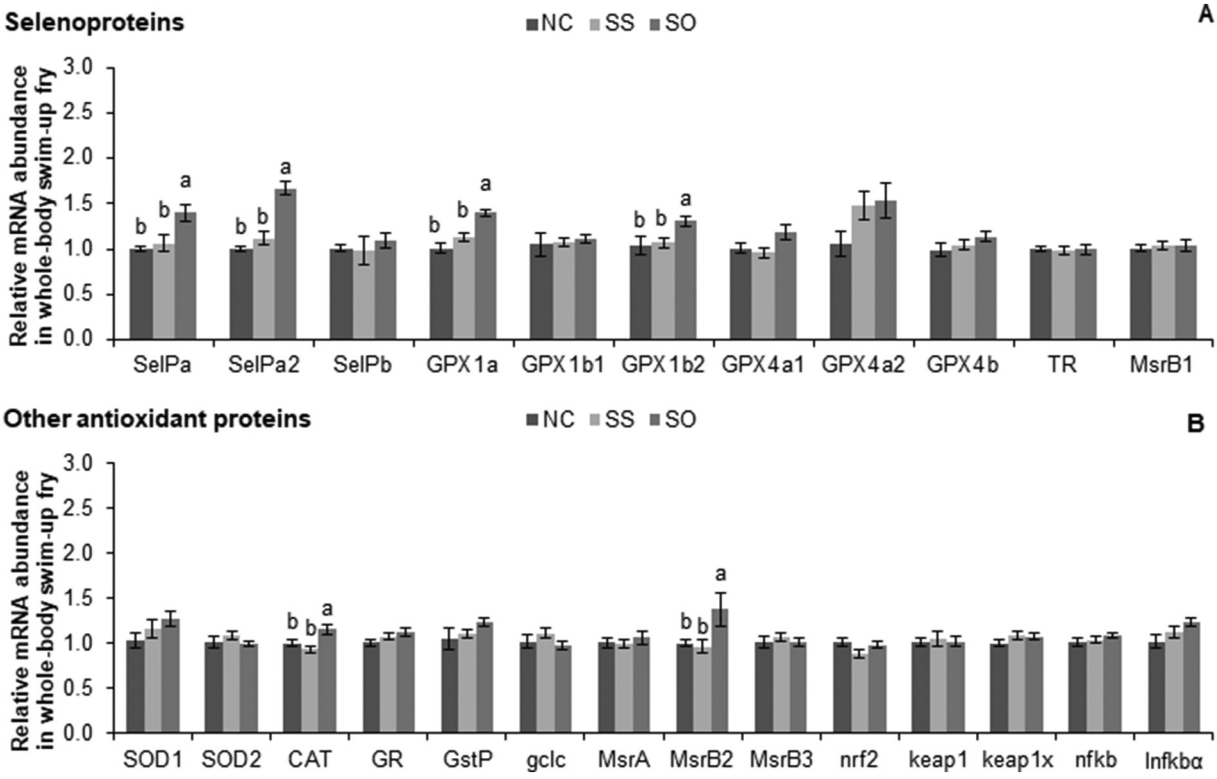

Fig. 6. Relative mRNA abundance of selenoproteins (A) and other proteins involved in antioxidant metabolism (B) in whole-body swim-up fry stage from rainbow trout broodstock fed different Se sources over 6 months at a constant water temperature of 8 ± 1 °C. Data are normalized to ß-actin and expressed as fold-changes of mRNA abundance compared with the control group Se1. Bars represent means ± SEM (n = 8). Means not sharing a common superscript letter are significantly different ($P < 0.05$) according to one-way ANOVA followed by Newman-Keuls test.

growth, the number of females spawning was significantly higher in both Se-supplemented groups and females fed organic Se in form of hydroxy-selenomethionine spawned significantly earlier compared to females receiving the sodium selenite diet. The low survival in progeny of selenite group can be traced back to given ascending females, spawning at the early spawning dates, while progeny of the majority of females in this group did not show any elevated mortality. The differences in spawning pattern and survival between the groups receiving different forms of dietary Se supplementation could be connected to modified vitellogenesis and blood steroid concentrations as these parameters were described to be affected in rainbow trout females fed dietary SeMet at a high level of 4.54 mg/kg (Wiseman et al., 2011). It might have been interesting to also analyze plasma vitellogenin levels in the present study.

The results indicate that a plant-protein based diet with a basal Se level of 0.3 ppm was not sufficiently deficient to affect growth of rainbow trout broodstock, but might negatively affect reproduction by decreasing the number of females spawning, which can be improved by supplementing Se, especially as organic form.

4.2. Effective Se transfer from broodstock to progeny

Tissue analysis revealed elevated female liver and muscle tissue Se concentrations in supplemented groups, compared to the control group, and the concentration in fish fed diets with organic Se supplementation were even higher compared to fish fed inorganic Se diets. The increase of Se in muscle tissue of fish fed Se-supplemented diets was also recently reported in Atlantic salmon (*Salmo salar*), finding higher Se retention for organic Se supplementation in the form of SeMet compared to inorganic supplementation (Sele et al., 2018). The higher retention for organic Se (SeMet), compared to inorganic Se (selenite) was previously described also in rainbow trout fry (Rider et al., 2009; Godin et al., 2015). However, this effect is not systematic, as in some other studies, no effect on tissue Se accumulation was described, when working with similar amounts of Se as studied here (Han et al., 2011). We found elevated Se concentrations in the progeny at oocyte and swim-up fry stage before first feeding in Se-supplemented groups with higher transfer in the group supplemented with hydroxy-selenomethionine. These results are in line with results obtained in hens that describe the parental transfer of Se to the progeny (Pappas et al., 2006; Wang et al., 2011). The Se speciation in oocytes and swim-up fry could show that the SeMet can be found in tissues in higher concentrations compared to SeCys. Thereby, Se supplementation increased the SeMet levels, while SeCys was not affected. The elevated levels of SeMet in oocytes of the selenite treatment are surprising, especially as they are not found any more at swim-up fry stage. Indeed selenite in the Se metabolism is known to be metabolized to selenide and not transformed to SeMet (Burk and Hill, 2015). The elevated status of SeMet in the oocytes of the inorganic Se supplemented group found in this study might be linked to a transfer of SeMet from broodstock to the progeny, a process which could have been limited in the control group compared to the Se-supplemented groups. In a study with rainbow trout fry fed a diet supplemented with sodium selenite or SeMet, SeMet was found to be higher in the SeMet treatment compared to control group and selenite treatment, whereas in comparison to SeMet, the SeCys concentration was not affected by the supplementation at all (Godin et al., 2015). However, there were huge differences in the amount of Se identified as SeMet and SeCys between the two tissues analyzed in this study, which could be also connected to different extraction methods used. In this case, the absolute concentration values should be considered carefully and preferably trends at each stage regarded. However, it cannot be excluded that other seleno-components are present in fry as other Se-containing peaks were observed in chromatograms.

These results showed that Se-supplementation to diets with low

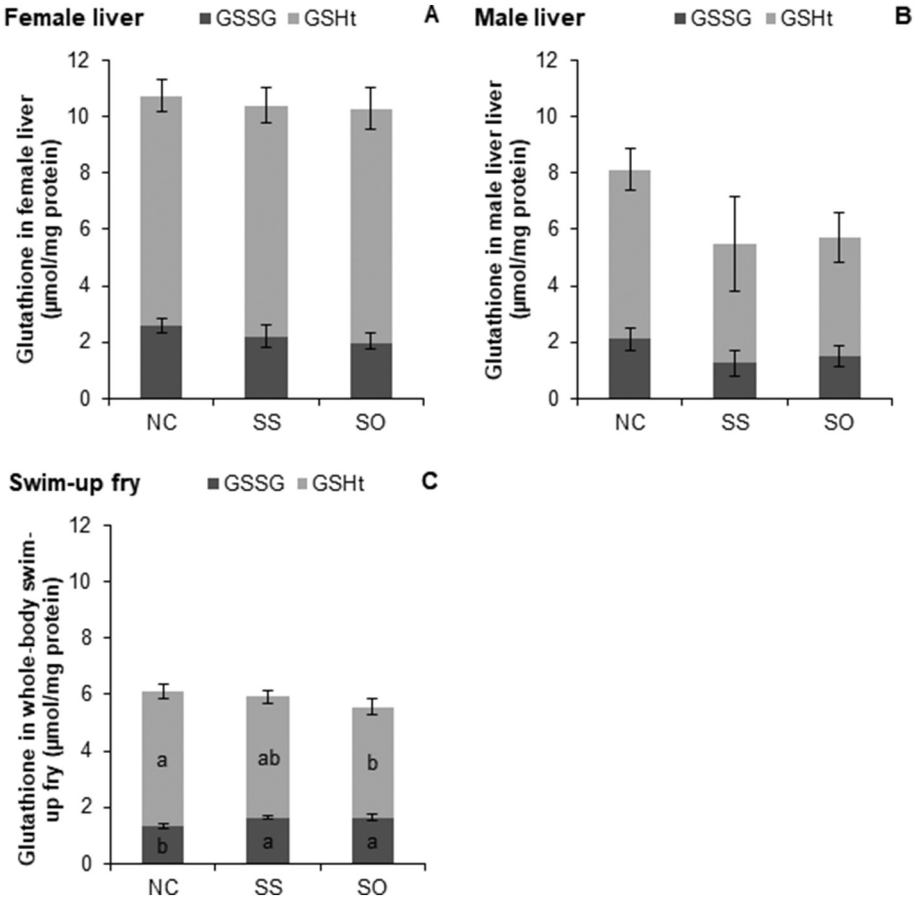

Fig. 7. Glutathione contents in female liver (A), male liver (B), and whole-body swim-up fry (C) from rainbow trout broodstock fed different Se sources over 6 months at a constant water temperature of 8 ± 1 °C. Bars represent means ± SEM (n = 8, except for male liver: n = 5). Difference between total (GSHt) and oxidized glutathione (GSSG) represents reduced glutathione (GSH). Means not sharing a common superscript letter are significantly different ($P < 0.05$) according to one-way ANOVA followed by Newman-Keuls post-hoc test.

Table 6
Oxidative status assessed by GSH/GSSG ratio, 8-Isoprostanes, protein carbonyls content and vitamin C in whole-body swim-up fry from rainbow trout broodstock fed different Se sources over 6 months at a constant temperature of 8 ± 1 °C.

	NC	SS	SO
GSH:GSSG	3.7 ± 0.3[a]	2.7 ± 0.2[b]	2.4 ± 0.1[b]
8-Isoprostanes (pg/g)	44 ± 2[a]	35 ± 3[b]	50 ± 5[a]
Protein carbonyls (nmol/mg protein)	19 ± 1	19 ± 1	16 ± 1
Vitamin C (mg/kg)	29 ± 1[b]	30 ± 2[b]	37 ± 2[a]

Within rows means ± SEM (n = 8) not sharing a common superscript letter are significantly different ($P < 0.05$) according to one-way ANOVA followed by Newman-Kauls post-hoc test.

basal levels could increase the tissue Se concentration and that there was a transfer of Se from the broodstock to the progeny. The bioavailability and transfer of Se was stronger in broodstock fed hydroxy-selenomethionine compared to selenite.

4.3. An altered antioxidant metabolism in deficient Se broodstock and offspring compensated by Se supplementation especially in organic form

In animals, selenoprotein P is a selenoprotein often found to be regulated by Se level (Yuan et al., 2013; Penglase et al., 2014a; Fontagné-Dicharry et al., 2015) and this was confirmed in the present study where the SelPa expression was significantly lower in the control group. In hepatic tissue of both males and females, the strongest effect was found in the group fed inorganic Se supplemented diets, while in the whole-body swim-up fry, the effect is detectable only in the organic Se supplemented groups. In humans, Steinbrenner et al. (2006) found that hepatocyte-derived selenoprotein P concentrations can stimulate the GPX1 expression and activity in astrocytes. Similar to selenoprotein P, GPX1 mRNA levels and activity are often described to decrease in mammals under Se deficiency (Chow and Tappel, 1974; Sunde et al., 2009) and have been found to be a good biomarker of Se status in fish

Table 7
Tocopherol content in offspring (oocyte and whole-body swim-up fry) from rainbow trout broodstock fed different Se sources over 6 months at a constant temperature of 8 ± 1 °C.

	Dietary group			Stage		*p*-value		
	NC	SS	SO	Oocyte	Swim-up fry	Diet	Stage	DxS
α-tocopherol	1815 ± 146[b]	2056 ± 176[ab]	2472 ± 168[a]	2157 ± 154	2071 ± 132	0.043	0.773	0.794
γ-tocopherol	254 ± 23	249 ± 26	254 ± 15	263 ± 17	241 ± 18	0.348	0.426	0.400
δ-tocopherol	5.15 ± 0.68	5.67 ± 0.69	4.68 ± 0.66	6.31 ± 0.36[a]	4.02 ± 0.60[b]	0.967	< 0.001	0.613

Values expressed in ng tocopherol per individual are means ± SEM calculated from samples of offspring (n = 8 per dietary group and stage). Within rows and within dietary groups ($n = 16$) and developmental stages ($n = 24$), means not sharing a common superscript letter are significantly different ($P < 0.05$) according to two-way ANOVA followed by a Newman-Keuls test.

(Bell et al., 1985; Penglase et al., 2014a; Fontagné-Dicharry et al., 2015; Pacitti et al., 2015). Likewise, the present results showed a lower GPX1a expression in male liver of the control group, compared to the inorganic Se treatment, but there was no effect in female liver or GPX activity in broodstock male or female liver. In whole-body swim-up fry, GPX1a1 and GPX1b2 expression, in correlation with GPX activity was increased in fry coming from broodstock fed the organic Se supplemented diet. In male liver, also GPX4a2 was influenced by dietary Se supplementation with significantly higher levels in the organic Se supplemented treatment, compared to the group fed inorganic Se, while in a study with rainbow trout fry GPX4a1 was found to be the most sensitive isoform in terms of low Se levels (Fontagné-Dicharry et al., 2015). Se deficiency was described to lead to decreased levels of MsrB protein activity and mRNA expression in various tissues (Moskovitz and Stadtman, 2003). In the present study, the selenoprotein MrsB1 was higher expressed only in male liver of the group fed organic Se-supplemented diets, while the non-selenoprotein MsrB2 was regulated by dietary Se not only in male liver of both supplemented groups, but also in the progeny of the group fed organic selenium. In comparison to mice, where the selenoprotein MsrB1 is the most dominant Msr in liver and affected by dietary Se levels (Novoselov et al., 2010), these results in rainbow trout could indicate a different MsrB hierarchy in fish as MsrB2 was more sensitive to low dietary Se levels than MsrB1.

These results showed that parental Se nutrition, especially supplementation with hydroxy-selenomethionine as an organic source of Se, can enhance not only the expression of proteins involved in antioxidant defense, such as SelP, GPX and MsrB2, but can also increase the activity of GPX in the progeny, while the expression of some hepatic proteins in the broodstock was more strongly affected even by a supplementation with selenite.

4.4. Contrasted oxidative status in progeny related to Se broodstock nutrition

As an integral part of several glutathione peroxidases, Se plays an important role in metabolizing hydrogen peroxides and lipid hydroperoxides into water and lipid alcohols (Brigelius-Flohé, 1999). This function is complementary to that of vitamin E as a lipid-soluble antioxidant and both are functioning in close link (Hamre, 2011). It has been shown that a combined deficiency of vitamin E and Se can lead to severe consequences, including suppressed growth, muscular dystrophy, exudative diathesis and death in fish (Poston et al., 1976; Gatlin III et al., 1986; Watanabe et al., 1997), while Se-deficiency symptoms appear less severe, if adequate levels of vitamin E are provided (Bell et al., 1985, 1986). In the present study, vitamin E requirements were assumed to be fulfilled as per the NRC, National Research Council (2011) with > 50 mg tocopherol per kg diet. As organic Se supplementation led to an increase in α-tocopherol levels in the progeny, this possibly indicates a Se deficiency or reduced Se availability in the control group and in the group receiving sodium selenite. Indeed, Se-deficiency has been reported to cause a reduction of vitamin E levels (Hamre, 2011). These results are in line with an earlier study in hens, describing significant increase in yolk vitamin E concentration by the supplementation of hydroxy-selenomethionine (Tufarelli et al., 2016). Elevated vitamin E and Se levels in the progeny of supplemented groups thus should provide them a better protection against oxidative damage. This was supported also by an elevated vitamin C level in organic Se-supplemented group in this study, as was also reported by Kumar et al. (2018) in striped catfish. However, protein carbonyls as an indicator of protein oxidation were not significantly affected by Se-supplementation in the present study, even though there was a tendency for lower protein carbonyl levels in the group with organic Se-supplementation. An effect might have been found at the single tissue level as in mice, where fewer protein carbonyls were found in isolated liver, kidney, cerebrum and cerebellum, when fed higher Se levels (Moskovitz and Stadtman, 2003). A closer look on 8-isoprostane levels showed a decrease only in the group with sodium selenite supplementation. This result is surprising as Küçükbay et al. (2009) found that, both inorganic and organic Se supplementation led to a linear decrease of both serum 8-isoprostane and malondialdehyde (MDA) levels. Similarly, in common carp, selenite supplementation did not even affect MDA levels, which were only decreased by feeding SeMet or nano-Se (Saffari et al., 2017). As 8-Isoprostanes are products of fatty acid (arachidonic acid) peroxidation, a more indirect effect of Se could be suspected in the present study as dietary Se has been shown to affect fatty acid composition in a previous study (Fontagné-Dicharry et al., 2015). Also, the GSH/GSSG ratio in the whole-body swim-up fry was found to be lower in supplemented groups compared to the control group, which is accompanied by an absence of effect on the mRNA levels of both glutamate-cysteine ligase catalytic subunit (Gclc), needed for the synthesis of glutathione, and glutathione reductase (GR), needed for the reduction of glutathione, while the GPX activity was higher in supplemented groups. In more developed fry fed with different Se level, not only the GPX activity, but also the GSH/GSSG ratio was higher in groups fed Se-supplemented diets (Fontagné-Dicharry et al., 2015). The different results might be connected to differences in the glutathione metabolism, which seems to be boosting at swim-up fry stage as levels of total glutathione increased about ten times compared to oocytes and oxidized glutathione levels only became detectable at swim-up fry stage in this study. The indirect effect of parental feeding on glutathione status in the progeny will need further investigation.

The absence of significant effects on some markers of oxidative status, especially linked to glutathione metabolism, may indicate that even with the clear difference in Se transfer from broodstock to the progeny, the Se deficiency in the non-supplemented group was not critical enough to provoke a strong oxidative stress. Otherwise, the Se deficiency in broodstock might induce other metabolic effects leading to changes in oxidative status such as modification of one-carbon metabolism as suggested by Speckmann et al. (2017) in mice.

5. Conclusion

The present results demonstrate that dietary Se-supplementation in plant-ingredient based diets with low basal selenium levels can enhance the total number of females spawning, while hydroxy-selenomethionine supplementation even led to an earlier spawning. It was demonstrated for the first time a parental transfer of Se in the progeny with the higher effect observed with the better bioavailability of organic Se. In the progeny before first feeding, the high Se levels led to an increase in the activity and mRNA expression of GPX and other proteins involved in antioxidant protection, along with elevated vitamin E and C levels. The impact of Se broodstock nutrition on glutathione metabolism in the progeny is intriguing and requires further investigation. Further work with juvenile fish at later developmental stages is warranted to show, if the parental effects persist after exogenous feeding of the progeny.

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5.2. Publication 2

Tissue localization of selenium of parental or dietary origin in rainbow trout fry using LA-ICP MS bioimaging

Objective:

The aim of this study was to examine the organs involved in the metabolism of selenium at different early developmental stages in rainbow trout and compare involvement of organs depending on selenium source given to the parents and during first feeding.

Approach:

Progeny of rainbow trout fed diets supplemented with different selenium sources were sampled at swim-up fry stage before the first feeding and after 11 weeks of cross-feeding diets with different selenium sources as whole fish. The frozen fish were embedded in a cryostat for consecutive cryosectioning. Then, the cryosections were analyzed by inductively coupled plasma mass spectrometry (LA ICP MS) to map the selenium abundance in different body tissues. In addition, pooled samples were used for total selenium analysis and selenium speciation.

Major findings:

- LA ICP MS allows co-localization of different microminerals in the body of rainbow trout fry
- Dietary sodium selenite showed lower retention compared to other dietary forms
- Both, the parental and fry feeding of hydroxy-selenomethionine increased body and muscle selenium levels in swim-up fry and 11-week fed fry relative to a negative control or sodium selenite feeding
- Sodium selenite supplementation in fry diets resulted in the highest liver selenium abundance in 11-week fed fry

Conclusion:

LA-ICP MS bioimaging demonstrated that tissue Se distribution could change pending on parental and dietary Se treatment in rainbow trout fry. While the Se levels before first feeding were influenced by parental Se nutrition, in the long-term the Se level in fish was dominated by its direct dietary intake of Se. This study suggests that muscle and liver Se concentration might be good indicators of the Se loading, pending on dietary form. Imaging analysis of the entire fry revealed that hydroxy-selenomethionine efficiently raised the muscle Se content in swim-up fry through parental nutrition and by direct feeding later in life.

Tissue localization of selenium of parental or dietary origin in rainbow trout (*Oncorhynchus mykiss*) fry using LA-ICP MS bioimaging

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Abstract

In relation to the decrease of Se content in aquafeeds, the impact of level and form of parental and dietary Se supplementation was investigated in rainbow trout fry using Laser Ablation-Inductively Coupled Plasma Mass Spectrometry (LA-ICP MS) bioimaging. The offspring of rainbow trout broodstock, fed either a control diet without any Se supplementation (0.3 mg Se/kg) or a diet supplemented with Se (0.6 mg Se/kg) either as sodium selenite or hydroxy-selenomethionine were sampled at swim-up fry stage or after 11 weeks of cross-feeding. Total body Se levels were influenced by parental Se nutrition in swim-up fry and by direct Se feeding in 11-week fry with higher levels in the Se-supplemented groups compared to the control and the highest levels in the hydroxy-selenomethionine treatment. The Se retention was lower for dietary sodium selenite. SeMet levels increased when Se was provided as hydroxy-selenomethionine. LA-ICP MS maps revealed yolk in swim-up fry and intestine, liver and kidney in 11-week fed fry as tissues with high Se abundance. In swim-up fry, muscle Se was the highest abundant when parents were fed hydroxy-selenomethionine. In 11-week fed fry, muscle Se abundance was higher in the head part of fry fed both Se-supplemented diets, but only in fry fed hydroxy-selenomethionine in the tail part. Liver Se abundance was higher in fry fed sodium selenite compared to the control diet. Therefore, the present results support the hypothesis that tissue Se distribution can be influenced by parental and dietary Se forms and levels.

Statement of significance

In aquaculture industry the on-going replacement of fishmeal with plant-ingredients in fish diets promotes sustainability while resulting in a steady decrease of dietary selenium levels. Selenium supplements could help to optimize the selenium status in farmed fish, which will be beneficial also for human consumption as sub-optimal selenium nutrition is widely spread in several countries. However, knowledge about metabolic differences related to dietary selenium forms is still incomplete. The present study provides insight in the tissue selenium distribution in rainbow trout at early developmental stages that are considered to be especially sensitive to nutritional stimuli.

1. Introduction

In pelleted aquafeeds, fishmeal, as the main natural selenium (Se) source, is increasingly replaced by plant ingredients with low Se content suggesting the need for application of dietary Se supplements.[1,2] On the market, Se products exist in both organic as well as inorganic forms and these differ fundamentally in their metabolism.[3] In general, the intestinal Se absorption was found to be high, independent from dietary level and form[4,5], but with a higher bioavailability and retention for selenomethionine (SeMet) compared to the widely used inorganic sodium selenite.[6]

In fish, Se exists mainly as SeMet and selenocysteine (SeCys)[7], which are also the predominant forms in proteins and selenoproteins, respectively. To be incorporated into selenoproteins, both, organic and inorganic dietary Se needs to be metabolized to hydrogen selenide, the universal Se donor for SeCys tRNA.[8] Selenite can either be reduced to selenide by thioredoxin reductase or by reacting with glutathione.[9] SeMet, on the other hand, can be absorbed by methionine transporters entering the methionine body pool. Then it is either metabolized via the methionine cycle and transselenation pathway to SeCys and further catabolized to selenide for the mediated incorporation into selenoproteins or directly and randomly incorporated into proteins at the methionine position.[9] The body Se pool is controlled in the liver from where Se can be distributed through the transport and storage protein selenoprotein P, which contains up to 17 SeCys in fish.[10,11]

The tissue distribution under limiting Se conditions is follows a hierarchical pattern including a possible redistribution.[12,13] In Atlantic salmon, total body Se concentrations were higher in fish fed a SeMet supplemented diet, but liver Se concentrations were highest when fed sodium selenite under non deficient conditions.[14,15] A similar effect was also observed in rats where Se accumulation in brain and muscle was more pronounced with SeMet compared to sodium selenite.[16]

In fish, Se provided by maternal nutrition during oogenesis represents the available pool during embryonic development. We previously reported that parental Se nutrition affects the amount of Se transferred to the progeny, which was higher in Se-supplemented treatments, especially when supplied as organic Se form.[17] Higher Se levels in the organic Se treatment were accompanied by a modified SeMet to SeCys ratio in swim-up fry before the first feeding. Therefore, it could be assumed that dietary Se form presented by both parental and direct feeding can influence the tissue distribution in the body of fry presenting a possible link to observed differences in metabolic response.

In this context, the understanding of biological processes involving trace elements can be improved through the study of their distribution in biological tissues. Considering imaging techniques, the spatial resolution (20-50 nm) offered by X-Ray Fluorescence is an advantage to localize metal at subcellular level as demonstrated in fish on otoliths of Sacramento Splittail.[18] However, its sensitivity (100-1000 $ng.g^{-1}$) can be about 2 orders of magnitudes higher than the one of mass spectrometry imaging techniques.[19] Among the latter, Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP MS) is an attractive technique thanks to its sensitivity (10 $ng.g^{-1}$) and its spatial resolution to localize trace elements at the micrometre level within thin sections of biological tissues or individuals.[20–22] Applications of LA-ICP MS bioimaging are numerous but none concerns Se mapping in whole rainbow trout sections as earlier studies focussed on specific tissues like the otoliths.[23]

The objective of this study was to investigate the effect of different dietary Se forms on the whole-body Se distribution by both parental and direct feeding in rainbow trout fry after cryosectioning and LA-ICP-MS imaging.

2. Material and Methods

2.1 Feeding trial with rainbow trout broodstock and fry

All procedures were performed in compliance with the European Directive 2010/63/EU for the protection of animals used for scientific purposes and the French Decree no. 2013-118 for animal experimentation.

The rainbow trout husbandry and diet preparation were previously described.[17,24] Briefly, rainbow trout broodstock were divided into three groups of 25 females and 15 males. The broodstock were fed one of the three experimental diets designed to differ only in Se content (Table 1). The diet Bnc was not supplemented with Se having a basal Se level of 0.3 mg/kg. The broodstock diet Bss was supplemented with 0.3 mg/kg sodium selenite to a target total Se concentration of 0.6 mg/kg, similar to Bso, which was, however, supplemented with 0.3 mg/kg hydroxy-selenomethionine (OH-SeMet, Selisseo®). The diets were given for six months prior to spawning. At spawning, oocytes from each spawning female were collected through stripping, pooled and fertilized with a pool of sperm retrieved from males of the same dietary treatment collected at the same day. The eggs were cultivated until swim-up fry stage. At swim-up fry stage, the pooled progeny of each parental treatment was subdivided into three fry feeding groups given one of the three fry diets designed at similar Se levels compared to the broodstock diets (Fnc, Fss, Fso) for 11 weeks (Table 1).

Table 1 Experimental treatments and dietary selenium levels in the rainbow trout feeding trial.

Broodstock diet	Target/analyzed Se (mg/kg diet)	Fry diet	Target/analyzed Se (mg/kg diet)	Fry treatment
Bnc; negative control	0.3/0.3	Fnc; negative control	0.3/0.3	BncFnc
		Fss: sodium selenite	0.6/0.5	BncFss
		Fso: OH-SeMet	0.6/0.6	BncFso
Bss: sodium selenite	0.6/0.8	Fnc; negative control	0.3/0.3	BssFnc
		Fss: sodium selenite	0.6/0.5	BssFss
		Fso: OH-SeMet	0.6/0.6	BssFso
Bso: OH-SeMet	0.6/0.7	Fnc; negative control	0.3/0.3	BsoFnc
		Fss: sodium selenite	0.6/0.5	BsoFss
		Fso: OH-SeMet	0.6/0.6	BsoFso

All diets were supplemented with 40 mg/kg $ZnSO_4$ and 30 mg/kg $CuSO_4$ $5H_2O$.

2.2 Sampling

Sampling took place at swim-up fry stage and at the end of the 11-week feeding period. Rainbow trouts were euthanized with an overdose of benzocaine, weighted and sampled as whole-body fry. Three fry from each treatment were individually spread, wrapped, while the rest of the fish were pooled. All samples were frozen in liquid nitrogen and stored at -80°C.

2.3 Total selenium and selenium speciation

Total Se and SeMet were measured in homogenized swim-up fry and 11-week fed fry. Total Se was determined using inductively coupled plasma mass spectrometry (ICP-MS, Agilent series 7500cx) by Ultra Trace Analysis Aquitaine (UT2A, Pau, France) according to Vacchina and Dumont.[25] SeMet was analyzed by liquid chromatography (HPLC, Agilent series 1200) coupled to ICP-MS by UT2A for 11-weeks fed fry[26] and, by the Institut des Sciences Analytiques et de Physico-Chimie pour l'Environnement et les Matériaux (IPREM, Pau, France) for swim-up fry as described[7].

2.4 Cryosectioning of fry bodies

The 11-week fed fish were cut with a scalpel at the beginning of the dorsal fin, leading to two parts: head and tail, in order to fit the cryosectioning block (2.5 cm diameter). Entire swim-up fry and head and tail parts of the 11-week fed fry were positioned and embedded in a resin (cryomatrix ThermoShadon, 6769006) on the specimen discs and placed in a cryostat (Microm HM 505 E) pre-cooled to -20°C. Then, serial sections of 40 µm were recovered on glass slides, alternating for either histological staining or LA-ICP MS imaging analysis.

2.5 Tissue staining

Tissue sections were fixed in 10% formalin solution for 10 min and then rinsed with distilled water for 5 min. They were stained in 1% alcian blue 8GX solution (Sigma-Aldrich, CAS 75881-23-1) for 20 min and rinsed in distilled water for 5 min. The periodic acid Schiff reaction was applied by incubating the slides in 0.5% periodic acid solution for 3 min, rinsing them with running tap water for 5 min and staining with Schiff's reagent (Sigma-Aldrich, 1.09033) for 5 min before rinsing in distilled water. Sections were then stained in Ehrlich' hematoxylin solution (Electron Microscopy Sciences, 26040-05) for 3 min and rinsed in distilled water for 5 min. Staining in Orange G solution was done for 5 min followed by 3 min rinsing in distilled water. Sections were then air-dried for 1h before pictures were taken[27] (Figure 1).

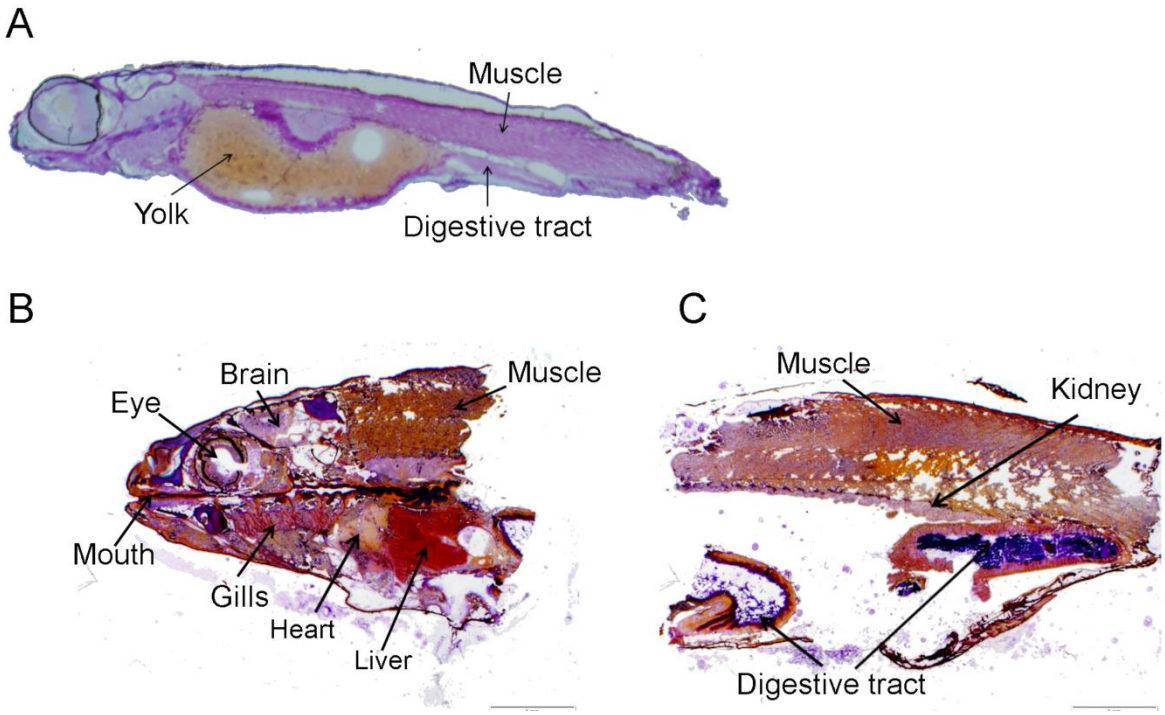

Figure 1 (A) Histologically stained rainbow trout fry at swim-up fry stage or (B) after 11 weeks of external feeding

2.6 Elemental imaging by Laser Ablation – Inductively Coupled Plasma Mass Spectrometry

A 7900x ICP MS (Agilent, Tokyo Japan) detector was used. The instrument was first tuned under liquid sample introduction (with 1 µg L^{-1} Y, Li, Tl, Ce, 2% HNO_3 tuning solution) and H_2

collision cell mode for optimal mass calibration, maximum sensitivity and stability as well as minimal Se background. For the LA-ICP MS analysis, a NWR213 Laser Ablation system (ESI, Freemont, CA, USA) equipped with a TV2 ablation cell was used. A 612 NIST glass reference material was scanned to verify operational conditions and slightly refine if necessary tuning of argon carrier gas, ion lenses voltages and H_2 collision cell flow rate. The ICP MS was operated in a dry plasma mode, using Ni cones and under H_2 cell gas mode (3.5 $mL.min^{-1}$). Sections of 11-week fed fry were sampled at 100 $\mu m.s^{-1}$ speed with a laser beam of 100 x100 µm, a 20 Hz laser shot frequency and a 4.6 $J.cm^{-1}$ fluency. A distance of 125 µm between each consecutive ablated line was set. In case of swim-up fry, scan speed, laser beam size and distance between 2 consecutives lines were modified to 50 $\mu m.s^{-1}$, 50 x 50 µm, and 75 µm, respectively. The laser-produced aerosol was transported toward the ICP source by He gas at 800 $mL.min^{-1}$. Integration time of 0.4 s was set to monitor both Se isotopes (^{77}Se, ^{78}Se) while copper (^{63}Cu) and zinc (^{64}Zn) isotopes were integrated for 0.7 s, leading to a total sampling time of 0.946 s. Every two days, laser tubing's, ablation cell and ICP MS cones were systematically cleaned to limit instrumental sensitivity decrease. A csv file was recorded for each scanned line and elemental spectrum and images were produced using a homemade program under Python and converted to a pdf file. Element intensity (cps) per pixel was mapped using a color code. The program allowed as well the integration of element intensity on a defined area (muscle for swim-up fry; muscle, liver and kidney for 11-week fed fry) of fish section, enabling to report a number of cps by μm^2.

2.7 Statistical analysis

Results are given as means ± standard error of mean. Data were analyzed using statistical software R (R Core Team). All data were tested for normality and homogeneity. Se and zinc (Zn) abundances were rank transformed before statistical analysis. In swim-up fry differences between groups were analyzed by one-way ANOVA, while in 11-week fed fry effect of parental and direct Se nutrition were analyzed by two-way ANOVA. The

significance level was set to $p < 0.05$ in all analysis and differences between groups were obtained using Tukey's HSD post hoc test. The correlation between and within thin-sections was performed using "Pearson method" (R, PerformanceAnalytics, version 2.0.4).

3. Results

3.1 Fry performance, body selenium and selenium speciation

The experimental diets were readily accepted by the fry from first feeding onwards. The parental nutritional history had no significant effect on the body weight or feed conversion ratio (FCR) in the 11-week fed fry (Table 2). The Se retention in 11-week fed fry was not affected by the parental nutritional history for Se. Similarly, final body weight and FCR were not significantly different according to fry Se nutrition. However, the Se retention was lower, when the fry were fed the Fss diet compared to feeding of Fnc or Fso.

Table 2 Performance and Se retention measured in whole-body fry originating from parents fed different selenium diets and then again fed different selenium diets for 11 weeks. Initial body weights at swim-up fry stage: 85 ± 3 mg in Bnc, 82 ± 3 mg in Bss and 85 ± 2 mg in Bso were not significantly different according to one-way ANOVA. (Broodstock diets: Bnc, control; Bss, + sodium selenite; Bso, +OH-SeMet and fry diets: Fnc, control; Fss, +sodium selenite; Bso, +OH-SeMet).

	Broodstock feeding (BF)			Fry feeding (FF)			p-values		
	Bnc	Bss	Bso	Fnc	Fss	Fso	BF	FF	BFxFF
Body 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
FCR[1]	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.44	0.26	0.71
Se retention[2]	54 ± 2	54 ± 2	54 ± 2	59 ± 1^{a}	46 ± 1^{b}	58 ± 1^{a}	0.73	<0.01	0.88

Values are means ± SEM (n=9 rearing tanks of three broodstock or three fry groups). Within rows and for each diet related effect (broodstock feeding, BF and fry feeding, FF), means not sharing a common superscript letter are significantly different according to two-way ANOVA followed by Tukey's HSD.

[1]FCR= dry feed intake [g] / wet weight gain [g]

[2]Se retention= [(Final body weight [g] x final whole body Se [µg/g])-(initial body weight [g] x initial whole body Se [µg/g])] / (total feed intake [g] x dietary Se content [µg/g])

Parental feeding with Se-supplemented diets increased the body Se content of swim-up fry with the highest Se levels in the Bso group (Figure 2A). The SeMet concentration was higher in Bso compared to the two other treatments. However, in the Bso as well as in the Bss group, the increase of total Se was also related to the increase of other seleno-compounds including SeCys.

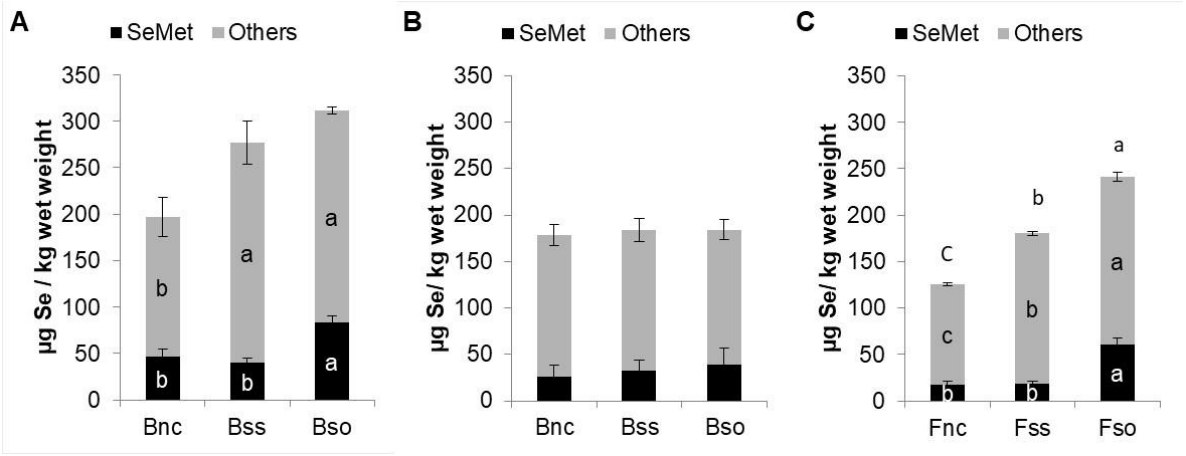

Figure 2 Body concentrations of SeMet and other seleno-compounds. (A) Parental selenium in swim-up fry, superscript letters show significant differences according to one-way ANOVA (n=3). (B) Parental effect on Se in 11-week fed fry analyzed by two-way ANOVA (n=9, three broodstock and three fry treatments). (C) Effect of Se feeding in 11-week fed fry analyzed by two-way ANOVA (n=9, three broodstock and three fry treatments). Superscript letters show significant differences in main effect. No significant interaction between selenium of parental and dietary origin was found. (Broodstock diets: Bnc, control; Bss, + sodium selenite; Bso, +OH-SeMet and fry diets: Fnc, control; Fss, +sodium selenite; Bso, +OH-SeMet).

Total Se analysis in 11-week fed fry revealed no differences in total body Se or SeMet content according to parental nutritional history (Figure 2B). Significantly higher Se concentrations were found in fry fed Fss compared to Fnc, but the highest Se content was found in fry receiving the Fso diet (Figure 2C). Similarly to the Bss treatment in swim-up fry, the Fss treatment showed no increase in SeMet concentration in 11-week fed fry that was only detected in the Fso treatment. In 11-week fed fry no significant interaction between parental and direct feeding on total body Se, SeMet or other seleno compound content was detected.

3.2 Localization of trace elements

As shown from example maps in Figure 3, the trace elements Se, Zn and copper (Cu) were detected in thin-sections of rainbow trout fry analyzed by LA-ICP MS. The Se abundance visually increased according to the dietary Se treatment, while Zn and Cu abundances were found to be comparable between the dietary treatments. From these images, it was possible to identify liver, kidney and digestive tract as organs with high Se abundance in all dietary treatments. However, the muscle Se abundances, although lower than in the previous organs, were especially high in fry subjected to organic Se treatment. Cu is mainly localized in liver and digestive tract in all dietary treatments. On the other hand, Zn appears more diffuse with higher abundance in the skin and periphery of the eyes in the body with co-localization to Se regardless of the dietary treatment.

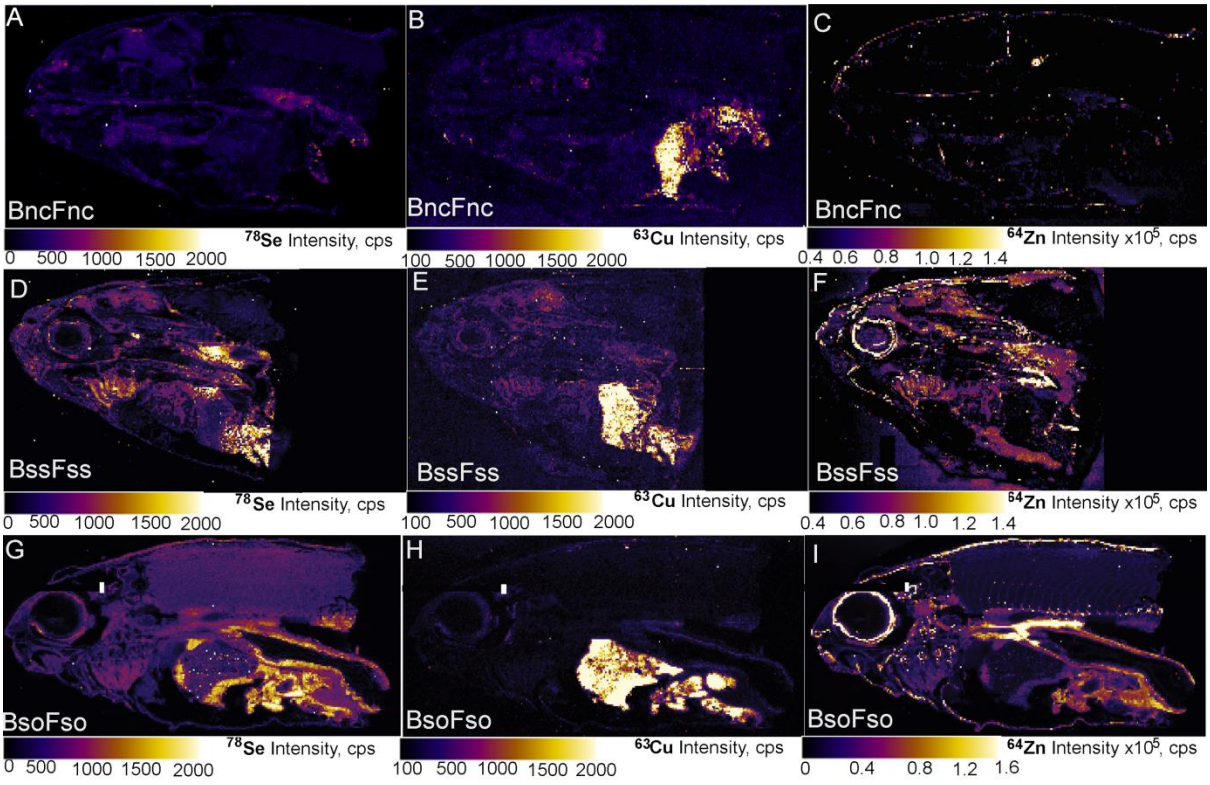

Figure 3 Thin-sections of the head part from three individual rainbow trout fed similar selenium regimes compared to the parents mapped for Se (A,D,G), Cu (B,E,H) or Zn (C,F,I). (Broodstock diets: Bnc, control; Bss, + sodium selenite; Bso, +OH-SeMet and fry diets: Fnc, control; Fss, +sodium selenite; Bso, +OH-SeMet).

3.3 Validation of LA-ICP MS methodology for inter section and tissue comparison

In order to quantify differences in Se abundance between dietary treatments the Se intensity was integrated over selected tissue areas to achieve an average ratio of intensity to analyzed area (cps/µm^2).

In swim-up fry, the Se abundance within two different thin-sections of the same individual fish (Figure 4A) and within different muscle areas of the same thin-section (Figure 4B) showed a high correlation. However, a drift in sensitivity was observed during analysis accompanied with high variability between two different thin-sections of the same individual.

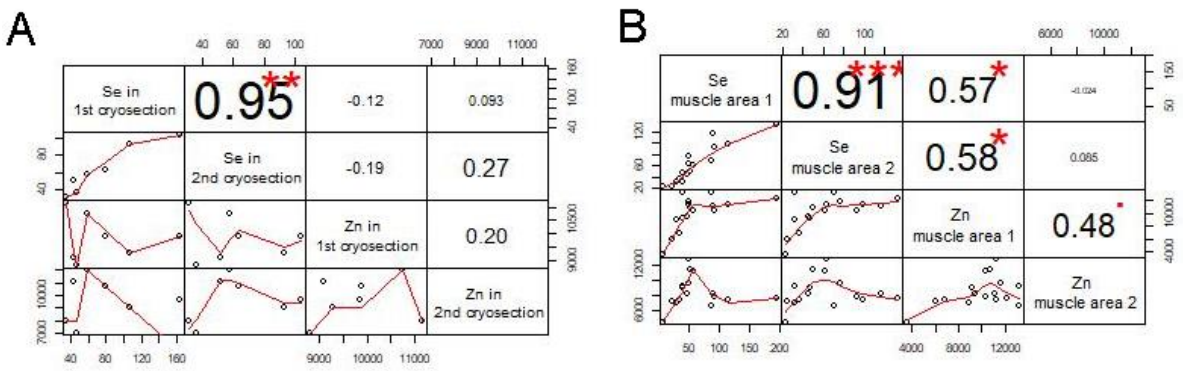

Figure 4 Correlation plots of Se and Zn abundance (cps/µm^2) integrated from LA-ICP MS in either two thin-sections of the same individual swim-up fry (A) or two muscle areas within the same thin-section (B).

Therefore, the possibility to use another endogenous element, monitored simultaneously, to standardize Se levels was assessed. In swim-up fry Zn distribution in thin-sections appeared homogenous between treatments by visual examination showing no effect of parental Se treatment. But, as shown in Figure 4 no correlation was detected for Zn between the different thin-sections of the same individual fish, and also the correlation between different areas of the same thin-section was weak.

In 11-week fed fry, the muscle Se abundance was strongly correlated in two areas within the same thin-section, but the muscle Se abundance in head and tail parts of the same fish showed only weak correlation (Figure 5). Kidney Se abundance analyzed in tail sections was correlated to muscle Se in the tail, but not in the head section. Zn muscle abundance was strongly correlated within the same thin-section, but not correlated in-between thin-sections (head and tail parts) of the same fish.

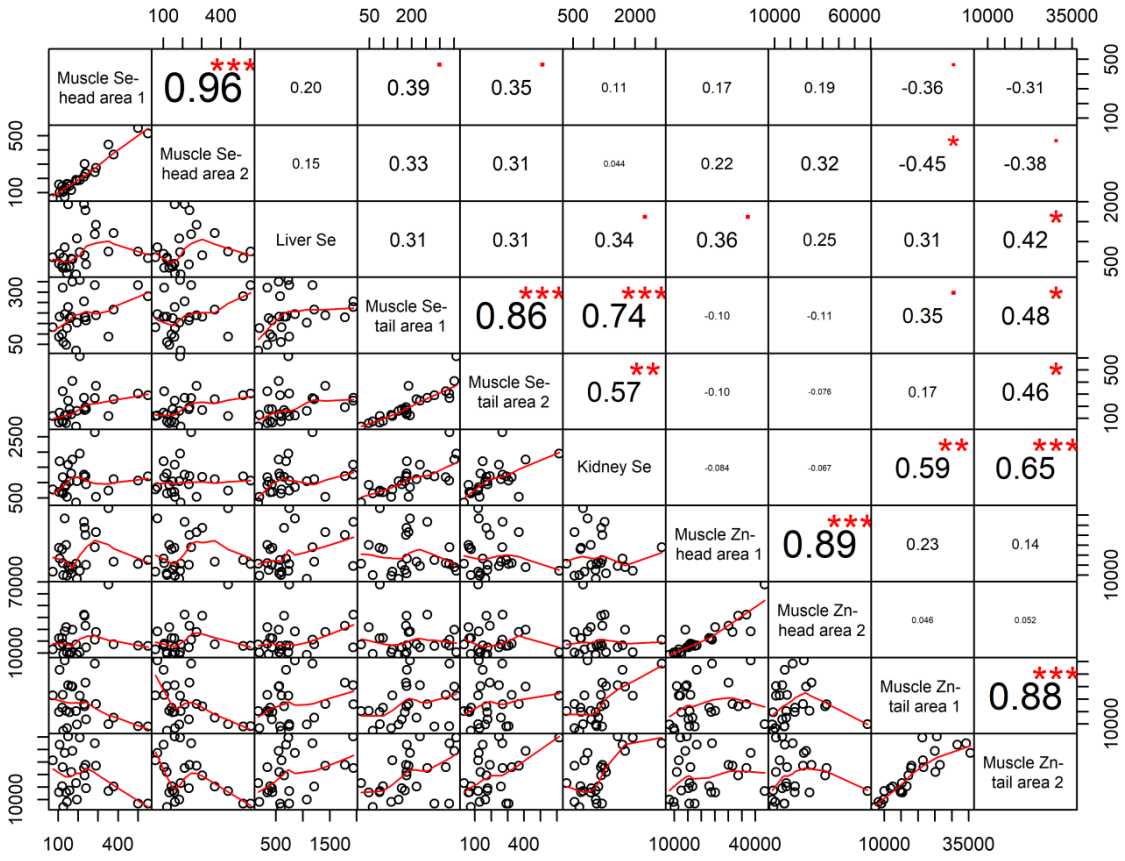

Figure 5 Correlation plot including integrated Se intensity (cps/µm²) from LA-ICP MS in two muscle areas in the head region, two muscle areas in the tail region, the liver and kidney in fry after the 11-week feeding trial. Zn abundance was only determined in corresponding muscle areas.

3.4 Selenium abundance in fry tissues

Visual examination as shown in Figure 6 indicated that the intensity of Se was increased in swim-up fry by parental Se nutrition with the highest Se abundance found in the Bso treatment. Yolk was identified as a Se-rich tissue in all dietary treatments.

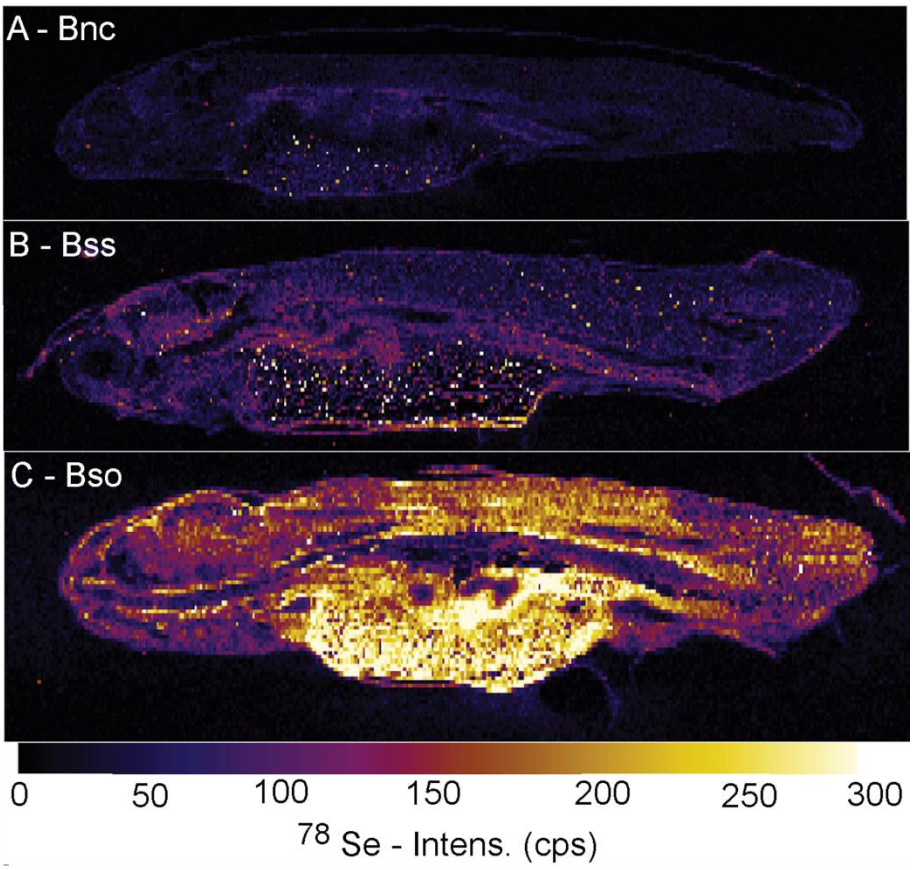

Figure 6 LA-ICP MS maps of Se in rainbow trout swim-up fry originating from parents fed either Bnc (A), Bss (B) or Bso (C) diet. (Broodstock diets: Bnc, control; Bss, + sodium selenite; Bso, +OH-SeMet)

Se appeared evenly distributed within muscle tissue. Muscle Se abundances were found to be higher in Se-supplemented groups, especially in the Bso treatment (Figure 5 and Table 3). The Se/Zn ratio increased with increasing Se abundance.

Table 3 Se and Zn abundance (cps/µm^2) in muscle areas of swim-up fry sections integrated from LA-ICP MS

	Bnc	Bss	Bso
Muscle Se	25 ± 5^c	49 ± 5^b	102 ± 18^a
Muscle Zn	6694 ± 1613	9789 ± 1007	9724 ± 327
Muscle Se/Zn	3.7 ± 0.1^c	5.1 ± 0.1^b	10.9 ± 1.9^a

Values are means ± SEM (n=3). Means not sharing a common superscript letter are significantly different according to one-way ANOVA on ranks followed by Tukey's HSD.

After 11 weeks of feeding, in comparison to swim-up fry, the visual examination of LA-ICP MS maps gave no indication for a long-term effect of parental Se nutrition on the body Se abundance or Se distribution in fry (Figure 7).

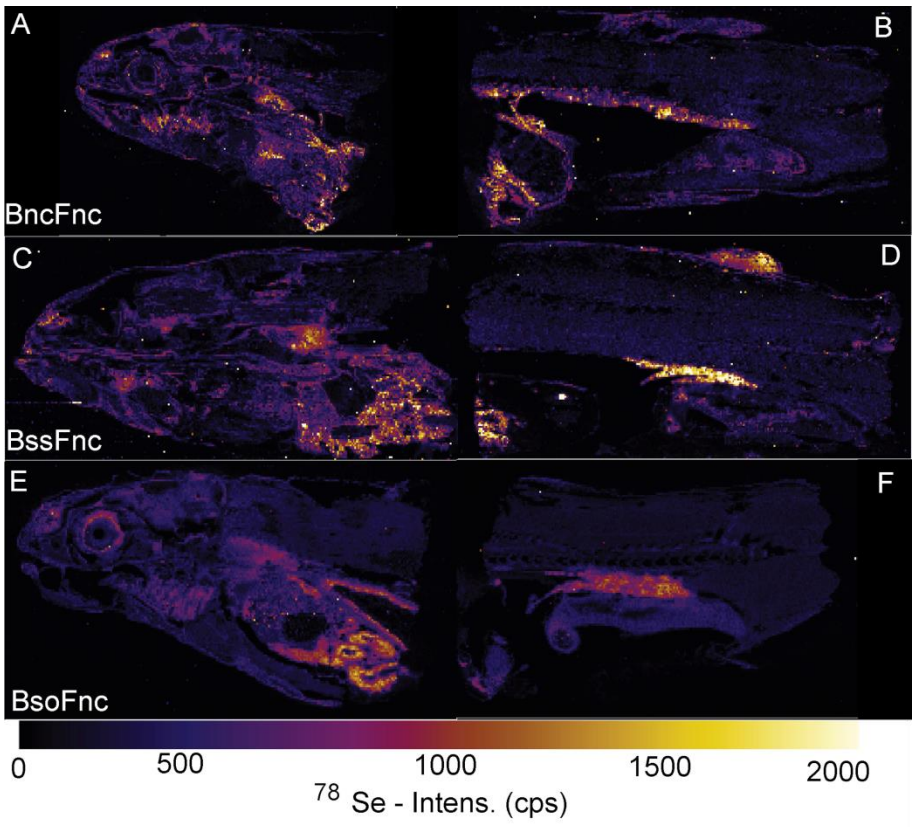

Figure 7 Thin-sections of rainbow trout fry fed the control diet Fnc for 11 weeks. LA-ICP MS maps of Se in head and tail of rainbow trout originating from parents fed either Bnc (A+B), Bss (C+D) or Bso (E+F) diet. (Broodstock diets: Bnc, control; Bss, + sodium selenite; Bso, +OH-SeMet and fry diets: Fnc, control; Fss, +sodium selenite; Bso, +OH-SeMet).

In 11-week fed fry the Se abundance in muscle, liver or kidney was not significantly affected by parental Se nutrition (Table 4). The parental effect on the Se/Zn ratio, which was decreased in the Bss compared to Bso treatment, is possibly related to the tendency of higher Zn abundance found in muscle in the head and tail parts of the Bss group compared to both other groups (2-way ANOVA, p=0.08).

Table 4 Se and Zn abundance [(cps/μm^2)/1000] in different tissues in head (H) and tail (T) parts of 11-week fed fry integrated from LA-ICP MS. (Broodstock diets: Bnc, control; Bss, + sodium selenite; Bso, +OH-SeMet and fry diets: Fnc, control; Fss, +sodium selenite; Bso, +OH-SeMet).

	Broodstock feeding			Feeding feeding			P-value		
	Bnc	Bss	Bso	Fnc	Fss	Fso	BF	FF	BFxFF
Selenium									
Muscle (H)	0.20 ± 0.03	0.19 ± 0.03	0.27 ± 0.06	0.14 ± 0.02^{b}	0.23 ± 0.03^{a}	0.27 ± 0.06^{a}	0.43	0.03	0.13
Muscle (T)	0.21 ± 0.05	0.16 ± 0.04	0.22 ± 0.03	0.12 ± 0.02^{b}	0.19 ± 0.03^{ab}	0.28 ± 0.04^{a}	0.24	0.01	0.59
Liver	0.95 ± 0.18	0.72 ± 0.16	0.69 ± 0.18	0.45 ± 0.07^{b}	1.22 ± 0.21^{a}	0.75 ± 0.11^{ab}	0.21	0.01	0.37
Kidney	1.12 ± 0.25	1.13 ± 0.14	1.03 ± 0.08	0.87 ± 0.13	1.30 ± 0.22	1.19 ± 0.15	0.88	0.14	0.87
Zinc									
Muscle (H)	20 ± 2	27 ± 5	16 ± 2	19 ± 3	29 ± 5	17 ± 2	0.08	0.13	0.12
Muscle (T)	23 ± 3	19 ± 3	14 ± 2	19 ± 3	20 ± 3	18 ± 3	0.08	0.86	0.86
Se/Zn*1000									
Muscle (H)	11 ± 2^{ab}	8 ± 1^{b}	18 ± 4^{a}	9 ± 2^{b}	10 ± 2^{ab}	17 ± 3^{a}	0.01	0.03	0.38
Muscle (T)	9 ± 2^{b}	8 ± 1^{b}	18 ± 4^{a}	7 ± 2^{c}	10 ± 1^{b}	18 ± 3^{a}	<0.01	0.01	0.03

Values are means ± SEM (n=9 rearing tanks of three broodstock or three fry groups). Within rows and for each diet related effect (broodstock feeding, BF and fry feeding, FF), means not sharing a common superscript letter are significantly different according to two-way ANOVA on ranks followed by Tukey's HSD.

The pictures obtained by LA-ICP MS clearly show higher Se abundances in fry fed the Se-supplemented diets Fss and Fso compared to the Fnc treatment (Figure 8). The Se abundance in muscle from the head part and liver was significantly increased in the Fss compared to the Fnc treatment in 11-week fed fry (Table 4). In the Fso treatment, liver Se abundance was not significantly different from Fnc or Fss, but Fso displayed higher Se abundance in muscle from both head and tail parts compared to the Fnc group. The muscle Se/Zn ratio in tail part was also significantly higher in Fss group compared to Fnc. The kidney Se abundance was not significantly different between dietary treatments. No interactive effects between Se from parental and dietary origin on Se or Zn tissue abundance were detected in 11-week fed fry.

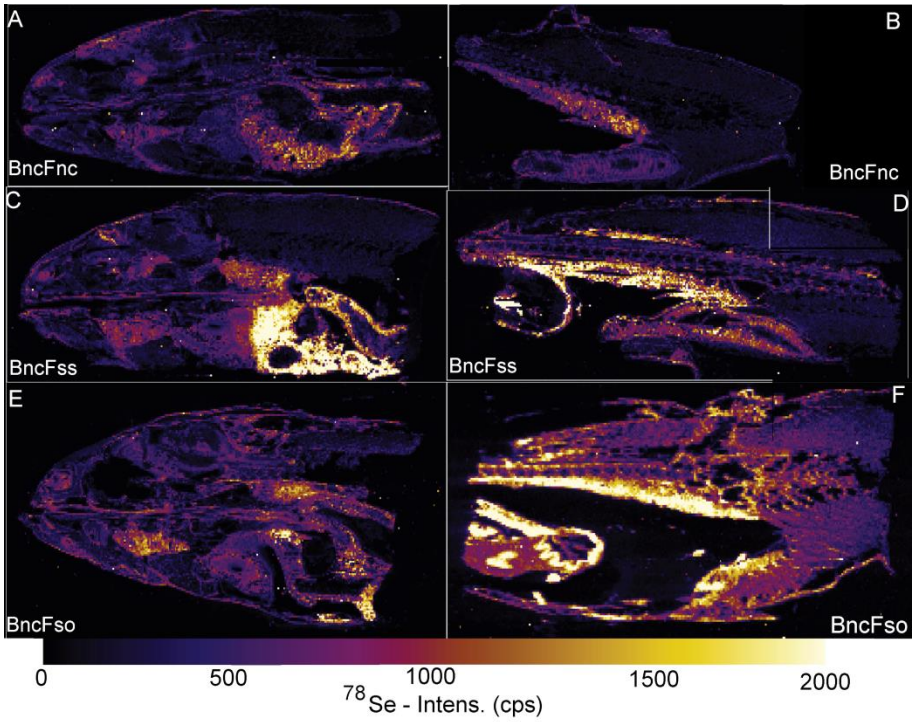

Figure 8 Thin-sections of rainbow trout fry originating from parents fed the control diet Bnc for 6 month prior to spawning. LA-ICP MS maps of Se in head and tail parts of rainbow trout fed either the Fnc diet (A+B), the Fss diet (C+D) or the Fso diet (E+F). (Broodstock diets: Bnc, control; Bss, + sodium selenite; Bso, +OH-SeMet and fry diets: Fnc, control; Fss, +sodium selenite; Bso, +OH-SeMet).

4. Discussion

4.1 The quantification of Se abundance: Methodological considerations

The results obtained by LA-ICP MS imaging support the hypothesis that tissue Se distribution in rainbow trout is not random with localization mainly in liver, kidney, muscle and digestive tract depending on the parental and dietary Se treatment. However, to determine differences between tissues or dietary treatments it seems inevitable to develop tools that allow the quantification of the abundance of the detected trace elements beside the visual examination. Therefore, we integrated the Se intensity of defined areas to obtain a representative Se abundance in selected tissues. For swim-up fry, the high correlation between different areas of the same tissue indicates that the analysis and data interpretation is valid for Se, while Zn abundance only weakly correlates, which might be related to a non-

homogenous Zn distribution in muscle. A high correlation of Se abundance between two sections of the same swim-up fry is promising for their comparison. However, in 11-week fed fry, the correlation of Se abundances between two thin-sections of the same fish was only weak, which might be either due to differences within the tissue by changing Se levels in head and tail area of the fish or due to instrumental sensitivity drift causing high variability between consecutively analyzed thin-sections. The possible sensitivity drift during one section imaging could be more noticeable for 11-week fed fry which are bigger in size than the swim-up fry, requiring thus longer analysis time and leading to a more pronounced clogging of the system at the end of imaging. The sensitivity drift can be compensated by the use of an internal standard homogeneously distributed in the sample for normalization purpose. The main difficulty in this study was the size of the 11-week fed fry samples (approx. 2 x 3 cm after cutting into 2 parts) requiring to cover a large surface with an internal standard, which requires further development. To reduce the impact of variability between sections our idea was to utilize Zn as an internal standard that should be more similar between dietary groups than Se. Even if in swim up fry, the Se/Zn ratios tended to increase with Se absolute abundance, this approach turned less promising due to the variability of Zn distribution particularly in 11-week fed fry body. In addition, strength of the methodology is its capability to map simultaneously other endogenous elements like the essential micronutrients Cu and Zn. In this study, the dietary Se treatment seems not to promote Cu and Zn redistribution in fry body letting us suppose that their biological functions are preserved. In contrast, another study highlights the correlation of Se and Zn accumulation in eyes and pigment containing tissue of zebrafish larvae exposed to toxic Se levels.[28] At the dietary Se levels utilized in the present study, Zn is also observed in pigments and eyes of 11-week fed fry, though with higher abundance compared to Se. The clear link between Zn and Se deposition in rainbow trout fry deserves further investigation in future studies, especially when considering that effects of dietary Se can be also driven by changes in other trace elements.

4.2 Short and long-term effect of parental Se nutrition on Se deposition in fry

With the decreasing Se levels in commercial fish diets, recent studies highlighted the beneficial effect of dietary Se supplementation on the antioxidant system in fish.[29] This should be especially important at sensitive stages like reproduction.[17] Our results of bioimaging and Se analysis show that feeding Se-supplemented diets to rainbow trout broodstock increased the body Se levels in the progeny in a dose and form dependent manner. The highest Se levels were found in swim-up fry fed diets supplemented with OH-SeMet, which possibly relates to the superior bioavailability of organic compared to inorganic Se compounds.[6] LA-ICP MS imaging of swim-up fry of the OH-SeMet group indicated also higher muscle Se abundance, meaning thus that the developing fry also transferred and deposited more available Se from yolk to muscle. Likewise, as shown in zebrafish broodstock fed high SeMet levels (30 mg Se/kg diet) by X-ray fluorescence imaging, Se can accumulate in the yolk but also in the eye and other tissues of zebrafish larvae.[30] This is similar to what is known in poultry where in fertilized eggs both embryonic and extra-embryonic Se levels were increased, especially when hens were fed organic Se supplied as Se-enriched yeast.[31] In chicken, higher Se levels in breeder diets increased Se concentrations in yolk, albumen, liver, and breast muscle of the developing embryo, but irrespective of the Se level presented to the breeders, embryonic liver and breast muscle concentrations were higher when fed SeMet compared to sodium selenite.[32] However, the importance of Se in the metabolism mainly relates to the activity of selenoproteins, where Se is incorporated as SeCys. Therefore, it would have been interesting to determine, if yolk and muscle Se in the progeny was protein bound considering that whole body SeMet levels were increased in the OH-SeMet treatment. A protein profiling in eggs and muscle tissue coupled to an imaging technique might be a promising approach in future studies to answer such questions. Nevertheless, in embryonic chicken the parental Se transfer was associated with higher glutathione peroxidase activity and an enhanced antioxidant system[33] and also we

have previously demonstrated that increased Se levels in the progeny of rainbow trout by parental OH-SeMet feeding resulted in increased glutathione peroxidase mRNA levels and activity[17]. Together, these results suggest that parental feeding with organic Se might be more beneficial for the developing progeny compared to inorganic Se forms. However, in the long term, the effect of parental Se nutrition on body Se levels was no more detectable probably due to the strong effect of fry Se nutrition. Indeed the quantity of parental Se represents potentially only 1 to 5% of total Se in 11-week fed fry.

4.3 Effect of direct Se feeding on the Se deposition in whole-body fry

Similar to previous studies, we detected that dietary Se supplementation increased the body Se levels in rainbow trout fry.[7] The Se retention (58%) in fry fed the basal diet or the diet supplemented with OH-SeMet was not found to differ. This might be related to the composition of basal diet as it has been previously shown that SeMet account for high amounts of the total Se in these plant-based diets.[7,34] Similarly, retention of 58-61% of dietary Se supplied as L-SeMet has been reported in Atlantic salmon post-smolts.[15] On the other hand, the Se retention was lowered to 46% when fry were fed diets supplemented with sodium selenite in accordance with other terrestrial animal and fish studies where inorganic Se sources have been described to be lower retained[35,36] with Se retention as low as 30-38% in Atlantic salmon[15]. In a previous study with rainbow trout fry fed plant-based diets supplemented with Se-enriched yeast, SeMet was found to represent 50% of total Se in whole-body fry[7] whereas in the present study, SeMet represented only 25% of total Se in whole-body fry fed OH-SeMet. This lower SeMet proportion is probably indicative of reduced Se storage. However, in the present study, whole-body total Se of 11-week fed fry ranged from 120 to 260 µg/kg wet weight similarly to previous study in rainbow trout fry fed plant-based diets supplemented with 0.3 mg Se/kg diet supplied either as sodium selenite or Se-enriched yeast.[29] Fish body Se homeostasis has been used in Atlantic salmon post-smolt to

define dietary Se requirement[15] and body Se levels lower than 200 µg/kg were indicative of dietary Se deficiency. So in the present study the control diet Fnc might be considered as Se-deficient but also the sodium-selenite supplemented diet Fss according to whole-body levels. However, glutathione peroxidase activity that has been used as a specific response criterion to estimate dietary Se requirements of fish[27] was higher in fry fed sodium selenite compared to the control treatment, the OH-SeMet treatment being intermediate[24]. In a previous study in rainbow trout fry with dietary Se levels ranging from 0.5-0.9 mg Se/kg diet, the non-supplemented diets containing 0.5 mg Se/kg diet were found to be Se deficient according to glutathione peroxidase activity.[29] So the question remains if the Se supplemented diets used in the present study containing 0.5-0.6 mg Se/kg diet, might be considered as sub-optimal for rainbow trout fry.

4.4 Effect of Se nutrition on the Se deposition in tissues of rainbow trout fry

Similar to earlier studies in rainbow trout juveniles of larger size[5], we detected high Se levels in liver and kidney of 11-week fed fry by means of bioimaging. In the present study, quite high levels were also noticed in the digestive tract. In juvenile rainbow trout fed high levels of SeMet, a marked increase of Se deposition was also observed in all major tissues including the brain[37], which was not noticed in the present study probably due to the use of lower dietary levels. In the same study[37], SeMet was found to be the predominant form of Se (up to 40%) in all of the tissues including liver and kidney involved in Se handling. The distribution of Se for the production of selenoproteins to different organs is mediated by the liver.[9] In this organ, integration of LA-ICP MS measurements gave the highest Se abundance in the sodium selenite treatment as described previously in rainbow trout juveniles[38]. It might suggest superior availability of selenite for inclusion in selenoproteins as described in pigs by serum glutathione peroxidase levels.[39] Hilton et al.[40] found that in rainbow trout liver Se levels rose disproportionally to dietary Se levels. While liver and

kidney Se levels readily increased, when rainbow trout were fed diets supplemented with sodium selenite up to a dietary concentration of 0.35 mg Se/kg dry feed, at higher dietary Se levels the liver Se concentrations disproportionally increased compared to kidney levels. Similar results for differentially deposition of Se in liver and kidney were observed in rainbow trout juveniles fed SeMet at levels of 10 and 40 mg/kg diet.[41] In the present study, the increase in kidney Se abundance as observed by LA-ICP MS imaging in Se-supplemented treatments was not significant. In accordance with these findings it could be suggested that at physiological basal Se levels (higher than 0.35 mg Se/kg diet), dietary Se supplementation with sodium selenite is beneficial for selenoprotein production in liver tissue, but at higher levels (above 1.25 mg Se/kg diet), liver Se accumulation might induce cytotoxicity that cannot be regulated through urinary excretion.

In the present study, bioimaging revealed that both OH-SeMet and sodium selenite nutrition increased muscle Se abundance in rainbow trout fry, but OH-SeMet was more effective. In Atlantic salmon juveniles, muscle Se levels were found to increase with feeding diets supplemented with SeMet and sodium selenite with a SeMet abundance in muscle tissue of over 90% in both treatments.[34] It cannot be assumed that sodium selenite is transformed to SeMet[9], but rather that the SeMet of the basal level is transferred to the muscle tissue in the sodium selenite treatment to larger amounts compared to the negative control, which is in accordance with the present results that only in the organic Se treatment body SeMet levels increased. However, on whole body level we also detected a significant increase of other seleno-compounds including SeCys in the organic Se treatment that could suggest a selenoprotein production or deposition in muscle tissue. Indeed, Wand et al.[42] found that a dietary supplementation with Se-yeast increased mRNA levels of several selenoproteins in muscle in comparison to liver where mainly SelP was affected. A combined study on tissue Se and selenoprotein levels might help to further elucidate the biological relevance of tissue Se abundance and how different Se forms are further metabolized.

Conclusion

LA-ICP MS bioimaging demonstrated that tissue Se distribution could change pending on parental and dietary Se treatment in rainbow trout fry. While the Se levels before first feeding were influenced by parental Se nutrition, in the long-term it was dominated by the feeding regime. This study supports that muscle and liver Se concentration might be good indicators of the Se loading, depending on dietary Se form. Imaging analysis of the entire fry revealed that OH-SeMet efficiently raised the muscle Se content in swim-up fry through parental nutrition and by direct feeding later in life.

Conflicts of interest

There are no conflicts to declare.

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5.3. Publication 3

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

Objective:

Aim of the study was to evaluate, if parental selenium nutrition in rainbow trout caused effects on the antioxidant metabolism of the progeny under normal and stressed conditions detectable after first feeding. In addition, a possible interaction between parental and direct selenium nutrition should be evaluated.

Approach:

The progeny of three groups of rainbow trout broodstock fed experimental diets with different form of selenium were cross-fed at first feeding different forms of selenium for 11 weeks. The fry were sampled either before or after subjected to a hypoxic stress and analyzed for oxidative stress and antioxidant parameters.

Major findings:

- Programming effects by parental selenium nutrition were detectable in long-term
- Parental and direct inorganic selenium feeding had opposite effects on GPx
- Hypoxic stress induced an antioxidant response in rainbow trout fry
- Direct selenium feeding improved the stress resistence towards hypoxia
- Parental selenium decreased the stress resistence towards hypoxia

Conclusion:

Parental selenium causes long-term modifications in the antioxidant system of the progeny in rainbow trout possibly related to modifications in the glutathione metabolism. The nutritional programming effects by parental selenium oppose the effects of selenium nutrition and require a more detailed investigation of underlying mechanisms. Higher selenium levels in the diets might be required to obtain clearer results and raises the question, if both basal and supplemented diets might have been within the deficiency range of rainbow trout fry.

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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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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*, *nfκb*, *keap1X2*) and decreased mRNA expression of antioxidant enzymes (*trxr*, *sod*, *gstπ*) indicated a transcriptional regulation of the antioxidant response. In stressed fry, the mRNA expression of several antioxidant genes including *gr*, *msr* and *gstπ* 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 were 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, Clextral, 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, 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).

Table 1
Formulation and composition of experimental diets.

Diet	Broodstock diets			Fry diets		
	Bnc	Bss	Bso	Fnc	Fss	Fso
Ingredients (%)						
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	–
Selisseo® (μg/g diet)[g]	–	–	15	–	–	15
Analytical composition (DM, %)						
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% astaxantin

[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; $CaHPO_4{\cdot}2H_2O$, 33 g; $CaCo_3$, 2.15 g; $Mg(OH)_2$, 1.24 g; KCl, 0.9 g; NaCl, 0.4 g; $FeSO_4{\cdot}7H_2O$, 0.2 g; $ZnSO_4{\cdot}7H_2O$, 40 mg; $MnSO_4{\cdot}H_2O$, 30 mg; $CuSO_4{\cdot}5H_2O$, 30 mg; NaF, 10 mg; KI, 0.4 mg; $CoCl_2{\cdot}6H_2O$, 0.2 mg. All ingredients were diluted with α-cellulose.

[g] Sodium selenite contained 42% Se (Sigma-Aldrich) and Selisseo contained 2% Se (Adisseo).

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 N_2. 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
selpa1	EE605178	gcccaaacaggaagatgtgt	gggcagggagatatggtagg	100
selpa2	HF969249.1	cagccacctggttggagtat	cctggagtagggccacca	82
selpb	HF969250.2	gtgtcgttcctcatcgtgaa	ggaacgtcagtctcccacat	187
gpx1a	HE687021	aatgtggcgtcactctgagg	caattctcctgatggccaaa	131
gpx1b1	CA357669.1	cgagctccatgaacggtacg	tgcttcccgttcacatccac	183
gpx1b2	HE687023	tcggacatcaggagaactgc	tccttcccattcacatccac	121
gpx4a1	HE687024	gaaaggcttcctgggaaatg	ctccaccacactgggatcat	112
gpx4a2	HE687025	agaaatacaggggcgacgtt	gcatctccgcaaactgagag	90
gpx4b	CA344428.1	ttggaggtcaggagccaggt	acccttttcccttgggctgtt	152
trxr3a	HF969246.1	gagcctccctcaagtgtgac	agtgaactccagaggccaga	158
trxr3b	HF969247.1	acaaaatcaaggcgaccaac	ggcagagagaacaggtcgtc	148
msrb1a	BX313019.3	ctggcctgccttcactgaga	ttcccacatcggaccttgaa	87
msrb2	BX311214.3	aggggacagagatgcccttc	cccatgagcctctttgaacg	149
msrb3	CX041708.1	tcaggttggccttccttcta	cgaggtgagaaccacactga	118
msra1	BT073399.1	gatggctatgtttgggatgg	acctgtgcaggtctcttcgt	136
sod1	AF469663.1	tggtcctgtgaagctgattg	ttgtcagctcctgcagtcac	201
sod2	CA352127.1	tccctgacctgacctacgac	ggcctcctccattaaacctc	201
cat	BX087110.3	tgatgtcacacaggtgcgta	gtgggctcagtgttgttgag	195
gr	HF969248.1	ctaagcgcagcgtcatagtg	acacccctgtctgacgacat	108
gclc1	GSONMT00071788001	aggccagagtatggcagcta	cagcctaaccttgggaatga	176
gclc2	GSONMT00065033001	aggccagagtatggcagcta	ttgggaatgatgtgatggtg	166
gstπ	BX302932.3	tcgctgactggacgaaagga	cgaaggtcctcaacgccatc	196
nrf2	CA360709.1	tgagctgcagcaatgtctga	gttgggcaatgggtagaagc	124
keap1X1	GSONMT00034445001	gctacgtgatgtctgcccct	ggtacctcatagcggccagt	116
keap1X2	GSONMT00019673001	actgggaggttatgacggga	cctccgtcctctcatgcttc	179
nfκb	BX880658.3	cagcgtcctaccaggctaaa-gagat	gctgttcgatccatccgcactat	181
Iκb	BT074199.1	agagacagactgcgctccac	cggccttcagtagcctctct	72
Iκb2	CDQ64494.1	agacacgcttctccaccttg	gacgctccactatctcagcc	164
tnfα3	HE798146.1	ccccaagcaccctaacaacc	gtcttcggtgccgtagcttg	82
hsp70	AB062281.1	gcaggacgctgacaaataca	tgttctccaaccaggaaatg	194
hif1ab1	XM_021584966	cctcacccttagctacactgat	aactttctcttgcgctgtgag	183
hif1ab2	NM_001124288.1	cccaaccctagagtgctc	tggtgagtaaggaagcaggg	183
ahrα	AF065137.3	ctctcgcctggacaaactct	gcgttcatcccattcatgtt	138
ahrβ	AF065138.4	ccaggctctgaatggctttg	tgactgatggaagcccaggt	97
pparα	AY494835.1	ctggagctggatgacagtga	ggcaagtttttgcagcagat	192
pparγ	CA345564.1	cccacggaaactcaccgttt	ggatctggatacggcggaag	168
EF1α	AF498320.1	tcctcttggtcgtttcgctg	acccgagggacatcctgtg	159
β-actin	AJ438158.1	gatgggccgaaagacagcta	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; nfκb, nuclear factor kappa-light chain-enhancer of activated B cells; Iκb, nfκb inhibitor; ahr2, aryl hydrocarbon receptor 2; hsp70, 70 kDa heat shock protein; pparα, peroxisome proliferator activated receptor α; pparγ, peroxisome proliferator activated receptor γ; hif1α, hypoxia inducible factor 1 subunit α; tnfα3, 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 phytoprostanes (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*-16B_{1t}-PhytoP (PhytoP5), *ent*-9L_{1t}-PhytoP (PhytoP6), *ent*-9L_{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 docosahexaenoic 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	PExDF
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, selenoindependent 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-troughput qRT-PCR was used on the RNA extracts to quantify gene expression at the GenotoulPlatform (http://genomique.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.

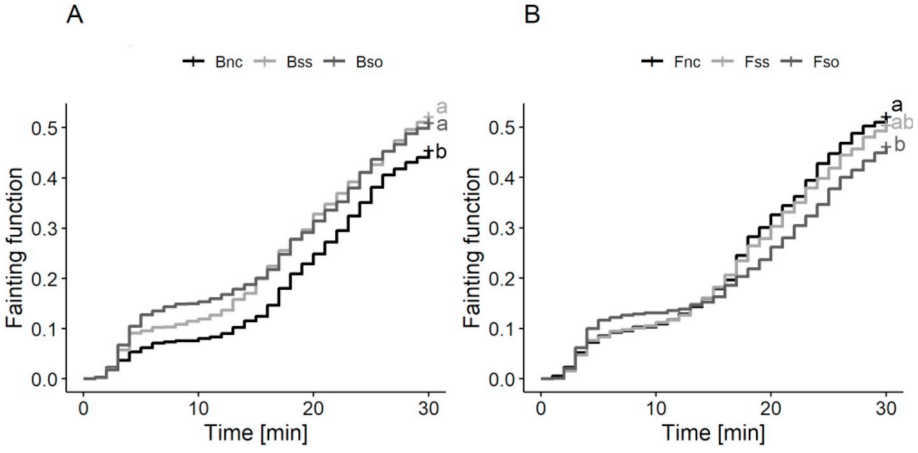

Fig. 1. Empirical fainting function based on Kaplan-Meier [1-(number of surviving fish/total number of fish)] by parental nutritional history (A) or direct Se feeding group (B). Superscript letters represent significant differences between survival curves based on a log rank test.

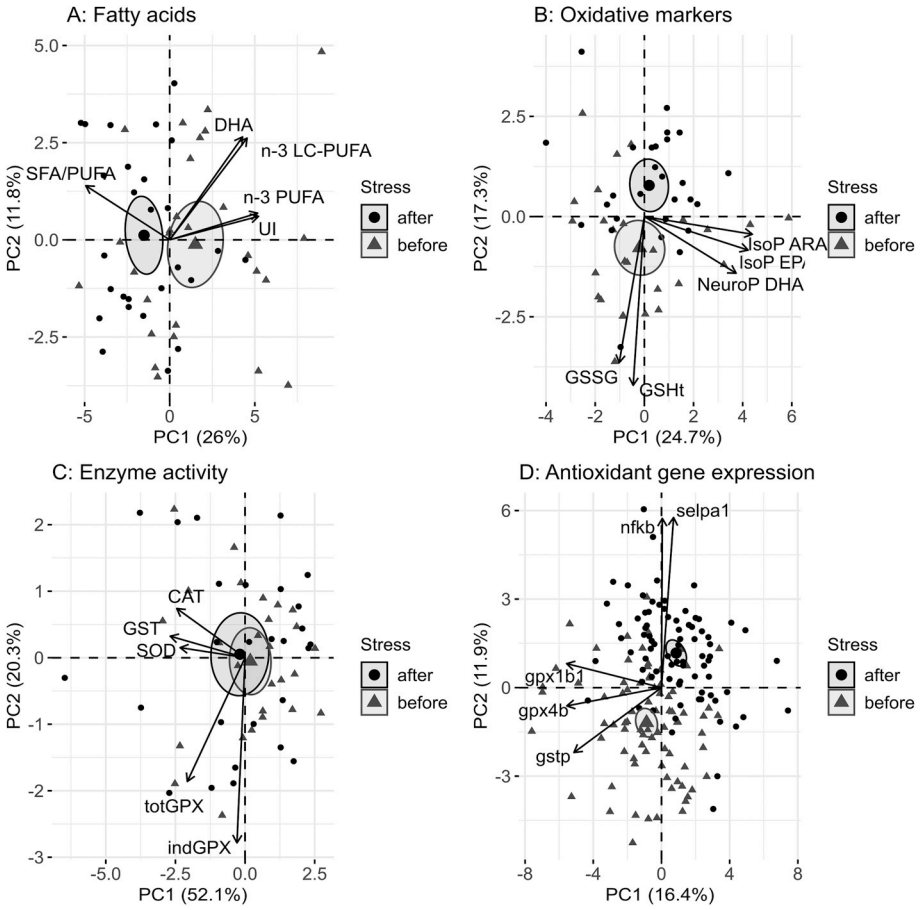

Fig. 2. PCA biplots on fatty acids (A), oxidative markers (B), specific antioxidant enzyme activity (C) and gene expression of antioxidant proteins (D) measured in whole fry before and after the hypoxic stress challenge. Arrows represent the 5 most contributing variables to the model, respectively A: 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; *gstπ*, glutathione-S-transferase π; *selpa1*, selenoprotein P; *nfκb*, 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_{2t}-IsoP[c]	**0.33 ± 0.02**[a]	**0.25 ± 0.02**[b]
F_{3t}-IsoP1[c]	0.16 ± 0.01	0.15 ± 0.01
F_{3t}-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 ± 1% in BssFss vs BncFss and 68 ± 1 vs 59 ± 2% in BsoFso vs BncFso).

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

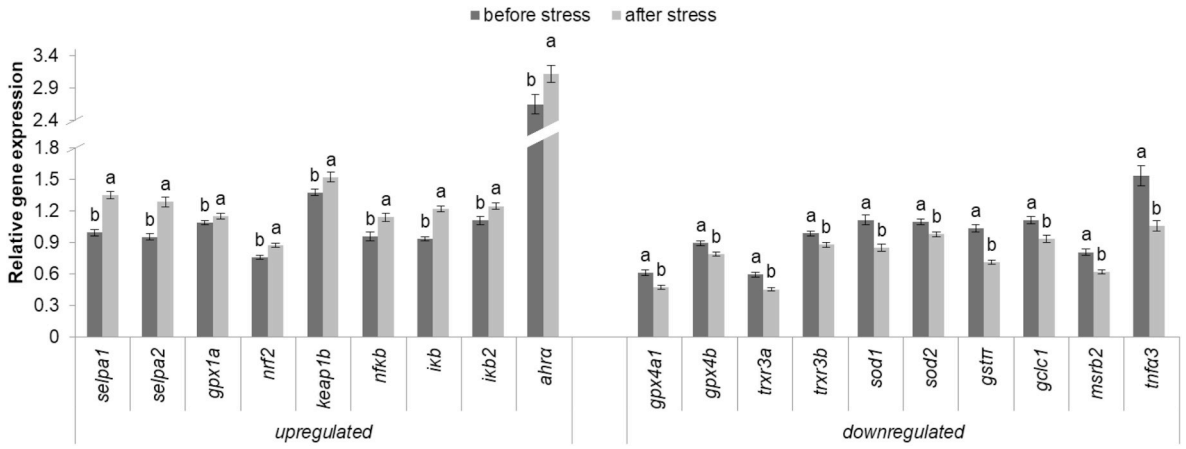

Fig. 3. Relative gene expression of significantly different expressed genes in whole fry before or after they were exposed to an acute hypoxic stress challenge for maximum 30 min. Data were normalized to a geometric mean of *ef1α* and *β-actin* and expressed as fold-changes of mRNA abundance compared to a sample from before stress in the BncFnc group. Bars represent means ± 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.

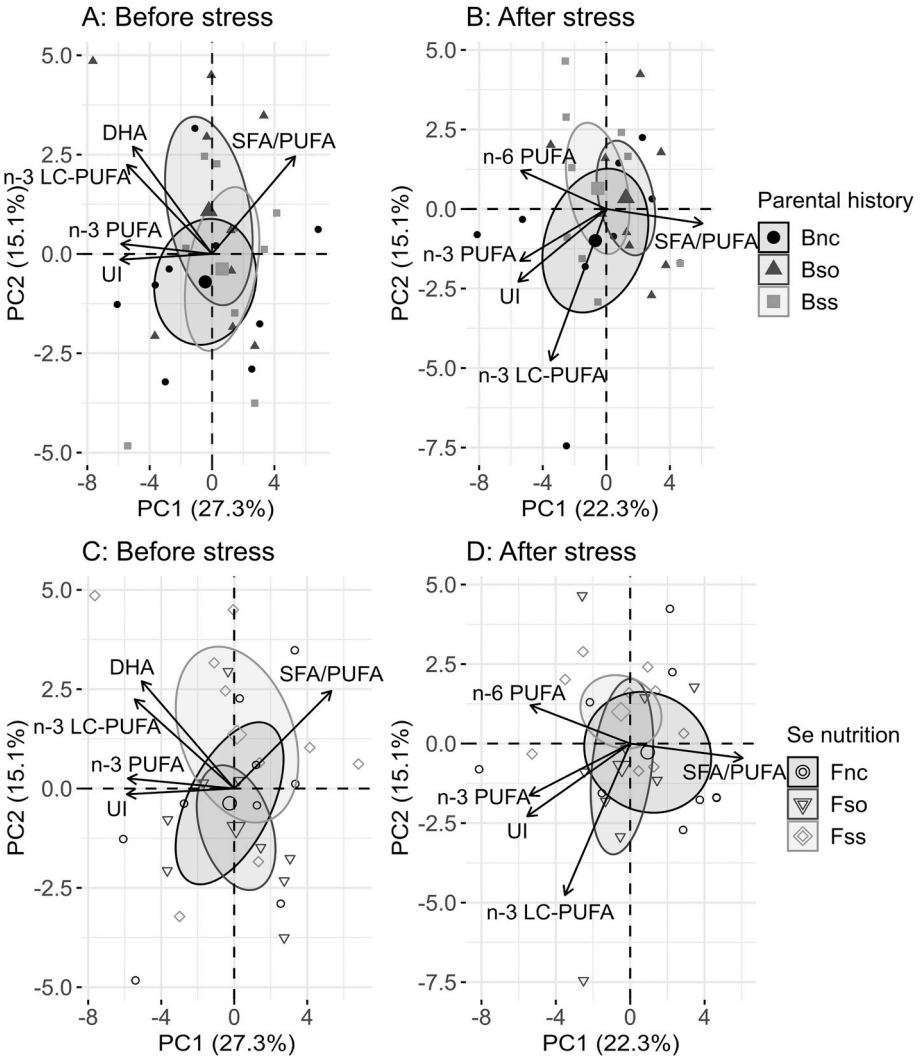

Fig. 4. PCA biplots on fatty acids grouped by either their parental nutritional history (A and B) or direct Se feeding (C and D) measured in whole fry before (A and C) and after (B and D) hypoxic stress. Arrows represent the 5 most contributing variables to the model, respectively (UI, unsaturation index; 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).

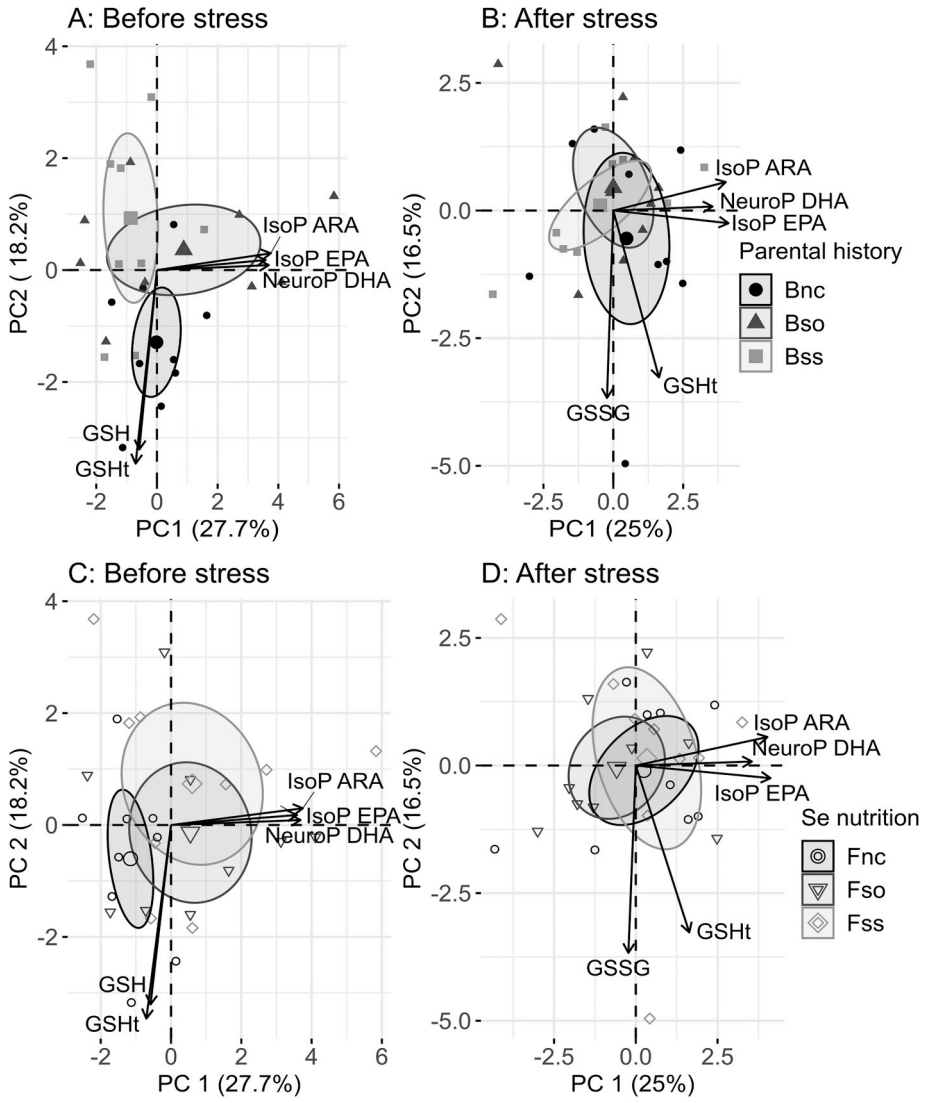

Fig. 5. PCA biplots on marker for oxidative stress grouped by either their parental nutritional history (A and B) or direct Se feeding (C and D) measured in whole fry before (A and C) and after (B and D) hypoxic stress. Arrows represent the 5 most contributing variables to the model, respectively: IsoP 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 ± 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 F_2-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 *selpa1* and the transcription factor *nfκb*. 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	PExDF
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*, *gstπ*, *gclc1* and *msrb2* was reduced after the hypoxic stress challenge. On the other hand, transcription factors including *keap1b*, *nfκb* with its inhibitors *iκb* and *iκb2* 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 *ahrα*.

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

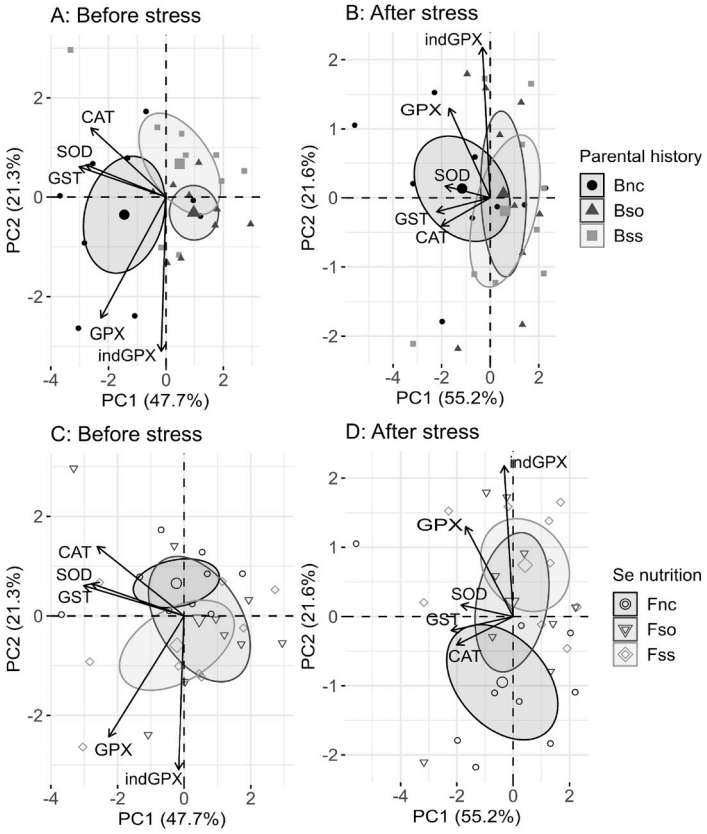

Fig. 6. PCA biplots on specific antioxidant enzyme activity grouped by either their parental nutritional history (A and B) or direct Se feeding (C and D) measured in whole fry before (A and C) and after (B and D) hypoxic stress. Arrows represent the 5 most contributing variables to the model, respectively (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	PExDF
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

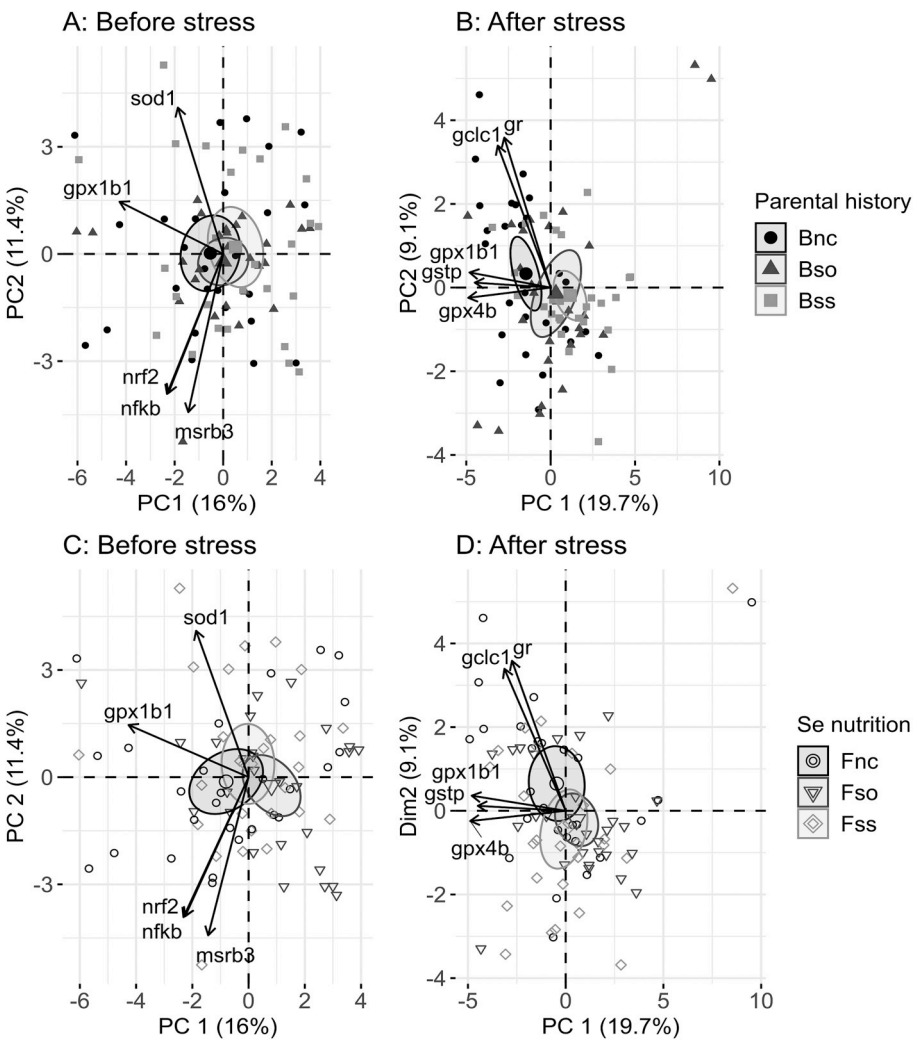

Fig. 7. PCA biplots on gene expression of antioxidant proteins grouped by either their parental nutritional history (A and B) or direct Se feeding (C and D) measured in whole fry before (A and C) and after (B and D) subjected to hypoxic stress. Arrows represent the 5 most contributing variables to the model, respectively (gpx, glutathione peroxidase with gpx1b1 and gpx4b; sod1, superoxide dismutase; gr, glutathione reductase; gclc, glutathione-cysteine ligase catalytic subunit; gstπ, glutathione-s-transferase π; nrf2, nuclear factor erythroid 2-related factor 2; nfκb, 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 *pparα* 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*, *hifαb2*, *ahrα* and *pparα* 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 *gstπ*, *gclc1* and *iκb* mRNA levels were reduced compared to Bnc. The gene *tnfα3* 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 *msrb1α* was lower in both Se-supplemented groups compared to the control group

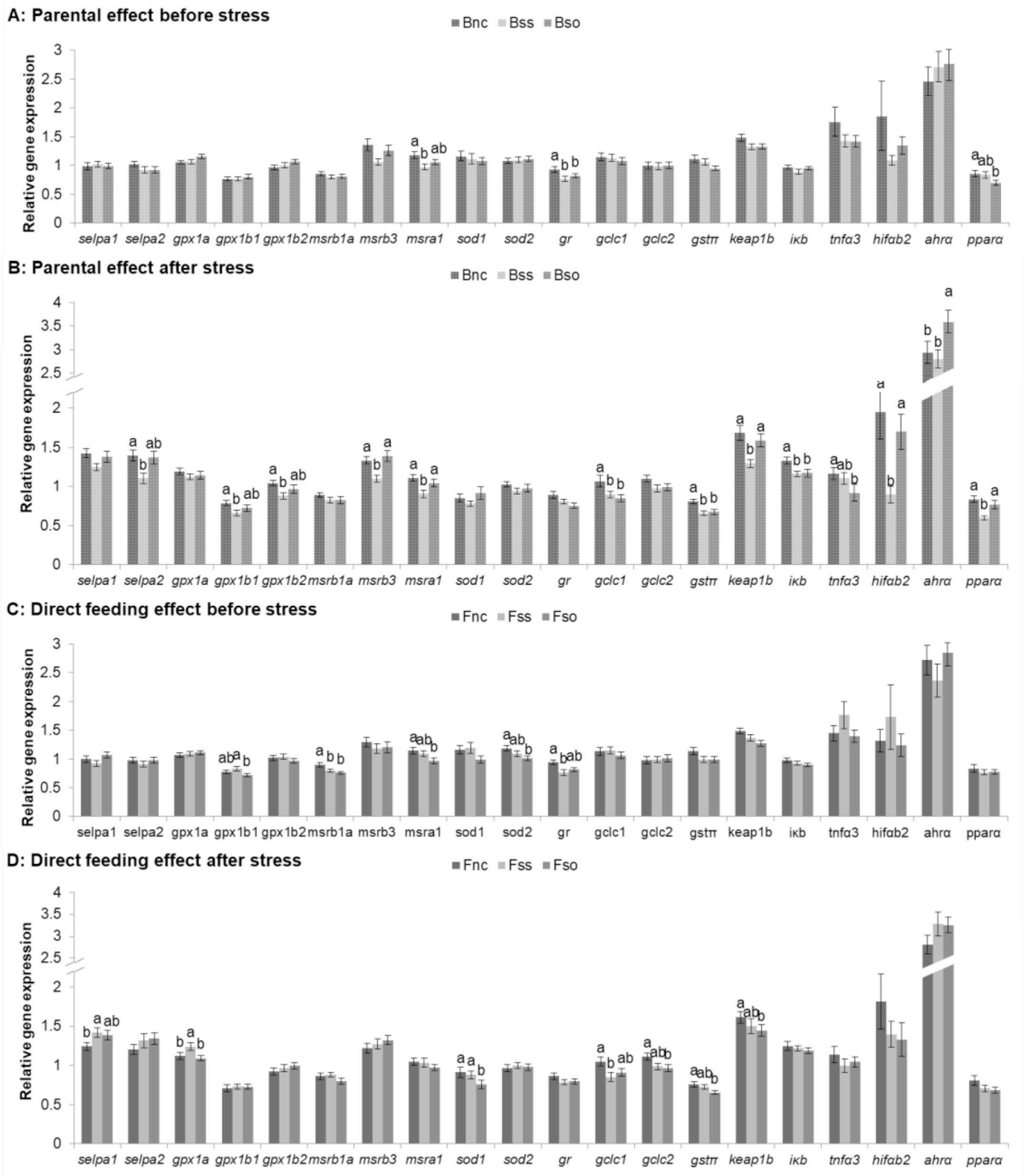

Fig. 8. Genes showing a significant main effect for parental (A + B) or direct feeding (C + D) analyzed in fry before (A + C) and after (B + D) the hypoxic stress challenge. Relative gene expression normalized to a geometric mean of EF1α and β-actin and expressed as fold-changes of mRNA abundance compared to a sample from before stress in the BncFnc control group. Bars represent means ± 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*, *gstπ*, *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 F_2-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 isoprostanoids, 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 isoprostanoid 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 were 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 were 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-kB 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

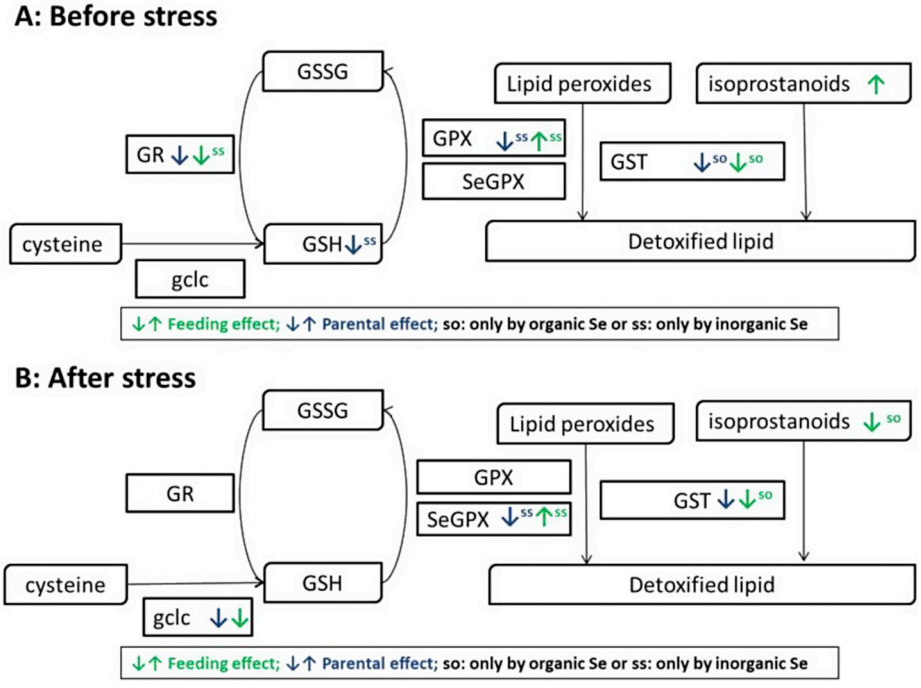

Fig. 9. Effect of parental and direct Se nutrition on the glutathione metabolism in rainbow trout fry before (A) and after (B) subjected to a hypoxic stress challenge.

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 malonodialdehyde and F_2-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 were 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 HIF1α levels *in vitro* in neuroblastoma cells. In the present study two results can be noted, first, the mRNA expression of *hif1α* was not significantly different before and after stress and second, the *hif1αb2* 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 *hifα* 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.

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5.4. Publication 4

Parental selenium nutrition affects the one carbon metabolism and hepatic DNA methylation pattern in the progeny of rainbow trout (*Oncorhynchus mykiss*)

Objective:

Aim of the study was to investigate the influence of parental selenium on the one carbon metabolism that might relate to epigenetic modifications as underlying mechanisms of the nutritional programming effects by selenium observed in rainbow trout fry.

Approach:

Rainbow trout broodstock were divided into three groups and either fed a control diet or a diet supplemented with selenium in organic or inorganic form until spawning. At spawning broodstock liver and oocytes were sampled for analysis of 1C metabolites. The rest of the oocytes were fertilized and cultivated until the swim-up fry stage. Swim-up fry were either sampled in a pool as whole fish or dissected for liver tissue analyzed by RRBS to obtain the whole genome methylation pattern.

Major findings:

- Parental selenium decreased transsulfuration pathway in the progeny
- Selenium decreased SAM/SAH ratio in broodstock liver and whole fry
- Parental selenium induced strong changes in hepatic DNA methylation pattern of progeny
- Differentially methylated genes were associated to various metabolic pathways

Conclusion:

Rainbow trout broodstock selenium nutrition was found to modify the 1C metabolism in parental fish as well as their progeny. The progeny showed elevated selenium levels that might underlay the observed changes in the 1C metabolism, while on the other hand strong modifications in hepatic DNA methylation pattern suggest a nutritional programming by selenium in rainbow trout fry. The affected genes were related to different metabolic pathways, but further investigations are required to identify physiological changes related to parental selenium nutrition. High methylation differences were found for genes related in (neuronal) cell signal transduction, inflammation and immunology.

life

MDPI

Article

Parental Selenium Nutrition Affects the One-Carbon Metabolism and the Hepatic DNA Methylation Pattern of Rainbow Trout (*Oncorhynchus mykiss*) in the Progeny

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Abstract: Selenium is an essential micronutrient and its metabolism is closely linked to the methionine cycle and transsulfuration pathway. The present study evaluated the effect of two different selenium supplements in the diet of rainbow trout (*Onchorhynchus mykiss*) broodstock on the one-carbon metabolism and the hepatic DNA methylation pattern in the progeny. Offspring of three parental groups of rainbow trout, fed either a control diet (NC, basal Se level: 0.3 mg/kg) or a diet supplemented with sodium selenite (SS, 0.8 mg Se/kg) or hydroxy-selenomethionine (SO, 0.7 mg Se/kg), were collected at swim-up fry stage. Our findings suggest that parental selenium nutrition impacted the methionine cycle with lower free methionine and S-adenosylmethionine (SAM) and higher methionine synthase (*mtr*) mRNA levels in both selenium-supplemented treatments. DNA methylation profiling by reduced representation bisulfite sequencing (RRBS) identified differentially methylated cytosines (DMCs) in offspring livers. These DMCs were related to 6535 differentially methylated genes in SS:NC, 6890 in SO:NC and 7428 in SO:SS, respectively. Genes with the highest methylation difference relate, among others, to the neuronal or signal transmitting and immune system which represent potential targets for future studies.

Keywords: selenium; methionine cycle; transsulfuration; nutritional programming; DNA methylation; rainbow trout

1. Introduction

Selenium (Se) is an essential micronutrient in humans and animals, with selenoproteins exerting various metabolic functions [1]. Among vertebrates, fishes have been described to have a well-developed selenoproteome [2], but there is concern within the aquaculture sector with present feed formulations. The ongoing replacement of Se-rich fishmeal with plant protein sources [3,4] is associated with a decrease in dietary Se level provided to farmed fish reared over a long period [5,6]. Many of the characterized selenoproteins are known to influence antioxidant metabolism, but knowledge on the effects of dietary Se on other metabolic pathways is not well characterized [7].

As shown *in vivo*, in the case of a Se deficiency, an increase in glutathione levels possibly relates to a feedback mechanism by changes in redox state [8,9]. The major source for glutathione is cysteine,

which is synthesized from homocysteine via the transsulfuration pathway [10]. On the other hand, Se deficiency can impair the transsulfuration pathway with decreased levels of cysteine, cystathionine and homocysteine [11,12]. Homocysteine is a key metabolite in the methionine cycle, which links the antioxidant system to one-carbon (1C) metabolism [13]. Studies in mice have confirmed that Se affects methionine metabolism with decreased betaine homocysteine methyltransferase activity and S-adenosylhomocysteine (SAH) levels [14–16]. Comparable studies in fish are lacking.

In the methionine cycle, methionine is activated by S-adenosylmethionine synthetase to form S-adenosylmethionine (SAM). In the cell, SAM is the universal donor for methylation reactions forming SAH. DNA methylation is a major regulatory mechanism for epigenetic modifications [17]. DNA methylation at repeated cytosine phosphate guanine (CpG) residues, especially when localized at the promoter region, is considered to influence gene expression [18]. Dietary supplementation of Se has been associated with both hyper- and hypomethylation in mice, but the relationship between Se and epigenetic mechanisms is still not fully understood [19]. The present work therefore aims to study the effect of parental Se nutrition in rainbow trout (*Oncorhynchus mykiss*) on the 1C metabolism and the hepatic DNA methylation pattern of the progeny.

The period of embryonic development is extremely sensitive to environmental-induced epigenetic modifications. For example, the allocation of maternal gene products and nutrients to the yolk has been associated with regulation of key embryonic developmental processes and persisting changes in the phenotype of the progeny [20,21]. In zebrafish (*Danio rerio*), dietary inclusion of methyl group donors did not lead to changes in hatching rate or survival, but mRNA sequencing of the embryos revealed "hidden" effects of parental nutrition [22] which led to phenotypic changes at later life stages [23]. In rainbow trout, the maternal Se nutrition during oogenesis increased not only the number of spawning females, but also the Se levels in the oocytes, especially when provided in the form of organic Se [6]. Changing Se levels in the progeny during embryonic development were also associated with modifications in the oxidative status.

In fish diets, Se supplementation becomes increasingly important to make up for the low Se levels detected in diets based on plant protein sources [24]. With regard to Se supplements, in addition to the widespread use of sodium selenite in terrestrial livestock nutrition, selenomethionine is the naturally dominant dietary seleno-compound, known to be a highly bioavailable form of Se also in mineral premixes [25]. These seleno-compounds, however, might exert different impacts on the 1C metabolism as they are metabolized through different routes [26]. Seleno amino acids are metabolized interchangeably with their sulfur analogues making selenomethionine to follow the methionine cycle, while inorganic Se compounds such as sodium selenite can be directly reduced to selenide to be incorporated into selenoproteins as selenocysteine [24].

In this context, the present study aims to make a comparison between the use of sodium selenite and hydroxy-selenomethionine (OH-SeMet), a pure form of the hydroxy-analogue of selenomethionine, as dietary supplements in plant protein-rich feeds for rainbow trout broodstock on the 1C metabolism and the hepatic DNA methylation pattern of the progeny.

2. Results

2.1. Parental Selenium Affects Transsulfuration Metabolites in Swim-Up Fry

A decrease in cysteine and cysteinyl-glycine was detected in liver of female broodstock only when fed sodium selenite (SS) compared to the non-supplemented control (NC). In addition, homocysteine levels were higher in fish fed OH-SeMet (SO) compared to the two other groups. No effect of the dietary Se on hepatic aminothiol concentrations was detected in males, which had generally lower hepatic aminothiol levels compared to females (Table 1).

Table 1. Aminothiol concentrations (μg/g sample) measured in liver and oocytes of rainbow trout (*Oncorhynchus mykiss*) broodstock fed diets containing different levels and source of Se.

		Homocysteine	Cysteine	Cysteinyl-Glycine	Glutathione	γ-Glutamyl-Cysteine
Oocyte	NC	0.3 ± 0.0	6.7 ± 1.0	3.7 ± 0.4	16 ± 1	1.0 ± 0.1
	SS	0.3 ± 0.0	6.7 ± 0.6	4.1 ± 0.5	17 ± 1	1.0 ± 0.1
	SO	0.2 ± 0.0	8.1 ± 1.1	3.1 ± 0.3	13 ± 1	1.0 ± 0.1
p-value		*0.21*	*0.48*	*0.35*	*0.07*	*0.48*
Female liver	NC	1.4 ± 0.1 ^{b}	33 ± 4 ^{a}	53 ± 4 ^{a}	551 ± 32	30 ± 4
	SS	1.1 ± 0.1 ^{b}	17 ± 2 ^{b}	37 ± 3 ^{b}	530 ± 32	23 ± 2
	SO	3.2 ± 0.3 ^{a}	38 ± 3 ^{a}	48 ± 3 ^{ab}	526 ± 47	28 ± 3
p-value		***<0.01***	***<0.01***	***0.01***	*0.88*	*0.29*
Male liver	NC	0.7 ± 0.1	21 ± 3	26 ± 3	511 ± 64	21 ± 2
	SS	1.0 ± 0.2	24 ± 5	23 ± 3	300 ± 66	10 ± 2
	SO	1.1 ± 0.3	33 ± 8	27 ± 4	465 ± 30	16 ± 5
p-value		*0.49*	*0.33*	*0.80*	*0.06*	*0.48*
Average	Female	1.9 ± 0.2 ^{a}	29 ± 2	46 ± 2 ^{a}	536 ± 22 ^{a}	27 ± 2 ^{a}
	Male	0.9 ± 0.1 ^{b}	26 ± 3	25 ± 2 ^{b}	434 ± 39 ^{b}	13 ± 2 ^{b}
p-value		***<0.01***	*0.43*	***<0.01***	***0.02***	***<0.01***

Values are the mean ± SEM (n = 8 in female tissue and n = 5 in males). ^{a,b} Within-rows values not sharing a common superscript letter are significantly different ($p < 0.05$) according to one-way ANOVA followed by Tukey's HSD.

A parental effect of Se in swim-up fry could be detected for cysteine as well as cysteinyl-glycine, which were both significantly lower in fry originating from parents fed Se-supplemented diets compared to the NC group (Table 2). This was accompanied by a decrease in pyridoxamine levels, but other B vitamins (folate and vitamin B12) including the pyridoxamine derivate pyridoxal were not significantly affected. Cystathionine, glutathione and γ-glutamyl-cysteine levels were not significantly different between the Se treatments. Similarly, parental Se treatment had no significant effect on the homocysteine level detected in swim-up fry.

Table 2. Free amino acid, aminothiol, and B vitamin composition of swim-up fry from broodstock fed the different diets.

Dietary Group	NC	SS	SO	*p*-Value
Essential amino acids [1]	1972 ± 79 ^{a}	1737 ± 54 ^{b}	1400 ± 65 ^{c}	**<0.01**
Non-essential amino acids [1]	2415 ± 50 ^{a}	2351 ± 41 ^{a}	2109 ± 60 ^{b}	**<0.01**
Methionine [1]	99 ± 5 ^{a}	83 ± 3 ^{b}	56 ± 4 ^{c}	**<0.01**
Homocysteine [1]	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	0.86
Cystathionine [1]	9 ± 1	6 ± 1	7 ± 1	0.21
Cysteine [1]	21 ± 1 ^{a}	17 ± 1 ^{b}	17 ± 0 ^{b}	**0.01**
Cysteinyl-glycine [1]	28 ± 1 ^{a}	24 ± 1 ^{b}	24 ± 1 ^{b}	**0.02**
Glutathione [1]	179 ± 7	159 ± 7	169 ± 13	0.35
γ-Glutamyl-cysteine [1]	18 ± 1	17 ± 1	16 ± 1	0.25
Taurine [1]	688 ± 17	751 ± 18	724 ± 16	0.05
Pyridoxamine [2]	0.24 ± 0.01 ^{a}	0.21 ± 0.02 ^{b}	0.18 ± 0.01 ^{b}	**0.01**
Pyridoxal [2]	1.82 ± 0.08	1.65 ± 0.06	1.85 ± 0.10	0.17
Folate [2]	0.36 ± 0.03	0.37 ± 0.02	0.28 ± 0.02	0.05
Cobalamine [2]	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.51

[1] (μg/g sample); [2] (μg/mg sample). Values are the mean ± SEM (n = 8). ^{a,b,c} Within-rows values not sharing a common superscript letter are significantly different ($p < 0.05$) according to one-way ANOVA followed by Tukey's HSD.

2.2. Parental Selenium Nutrition Affects the Methionine Metabolism in Swim-Up Fry

In the whole body of swim-up fry, the methionine concentration was significantly decreased, when parents received Se-supplemented diets compared to fry from the NC group, with the lowest concentration observed in fries from SO treatment (Table 1). The decreased methionine levels were accompanied by a general decrease in both essential and non-essential amino acids. PCA analysis of free amino acids and N-metabolites in fry revealed a strong clustering of the data according to the three parental groups dominated by essential amino acids, with the main contributing variables being lysine, isoleucine, valine, leucine, methionine, threonine and histidine besides glutamine, ammonium chloride

and glycine (Figure 1). The only amino acid that was significantly higher in Se-supplemented treatments compared to the control was asparagine, with a 18 ± 4 µg/mg sample in NC vs. a 34 ± 3 µg/mg sample in SS and a 44 ± 5 µg/mg sample in SO.

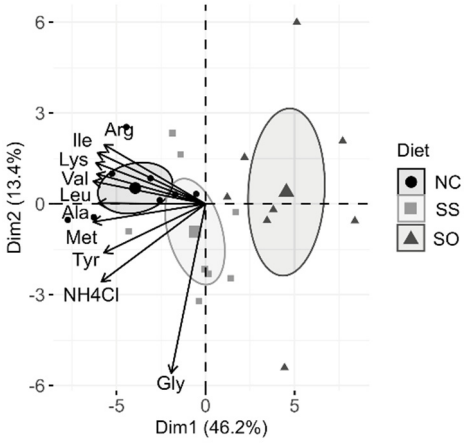
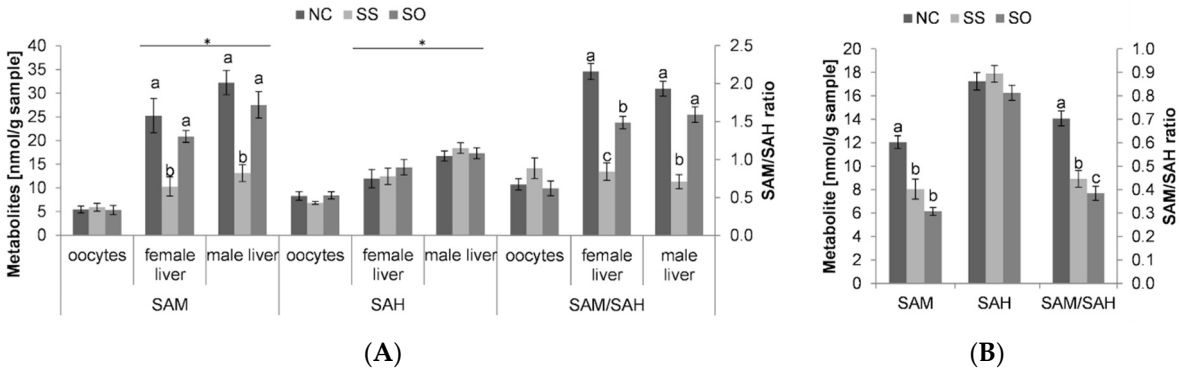


Figure 1. PCA biplot of free amino acids and related compounds measured in whole-body swim-up fry. Arrows represent the 10 most contributing variables to the model. Ellipses represent the 95% confidence intervals around a center of eight pooled samples per dietary treatment.

In broodstock liver tissue, a reduction in the SAM/SAH ratio was detected for the Se-supplemented groups (Figure 2A). SAM levels in males and females were, however, strongly affected by inorganic Se, without a significant difference between NC and SO in males that showed higher SAM as well as SAH levels compared to females. In oocytes, no significant difference in SAM or SAH levels and the SAM/SAH ratio was detected between treatments.



(**A**) (**B**)

Figure 2. (**A**) SAM, SAH and the SAM/SAH ratio in whole-body swim-up fry; (**B**) SAM, SAH and the SAM/SAH ratio in broodstock tissue. Bars are the mean ± SEM (n = 8 in swim-up fry and female tissues and n = 5 in males). Means not sharing a common superscript letter are significantly different ($p < 0.05$) according to one-way ANOVA followed by Tukey's HSD.

In the whole body of swim-up fry, the SAM/SAH ratio was low in both Se-supplemented treatments compared to the control with the lowest SAM/SAH ratio detected in the SO group (Figure 2B). The decrease in the SAM/SAH ratio can be related to the comparatively lower SAM levels observed in this group.

2.3. Parental Selenium Affects mRNA Levels of Genes Related to the One-Carbon Metabolism in Swim-Up Fry

Gene expression levels in female liver tissue were not significantly different between groups. Methionine synthase (*mtr*) expression was higher in male liver tissue when the fish were fed Se-supplemented diets, with the highest expression in the SS treatment (Figure 3A). In addition,

in male liver tissue, the expression of adenosylmethionine decarboxylase 1a (*amd1a*) was higher in SO compared to NC and that of glycine N-methyltransferase (*gnmt*) in SS compared to the other two dietary treatments. Except for adenosylmethionine decarboxylase 1b (*amd1b*) and *gnmt*, the expression of the 1C metabolism-related genes analyzed was higher in the females than in the males.

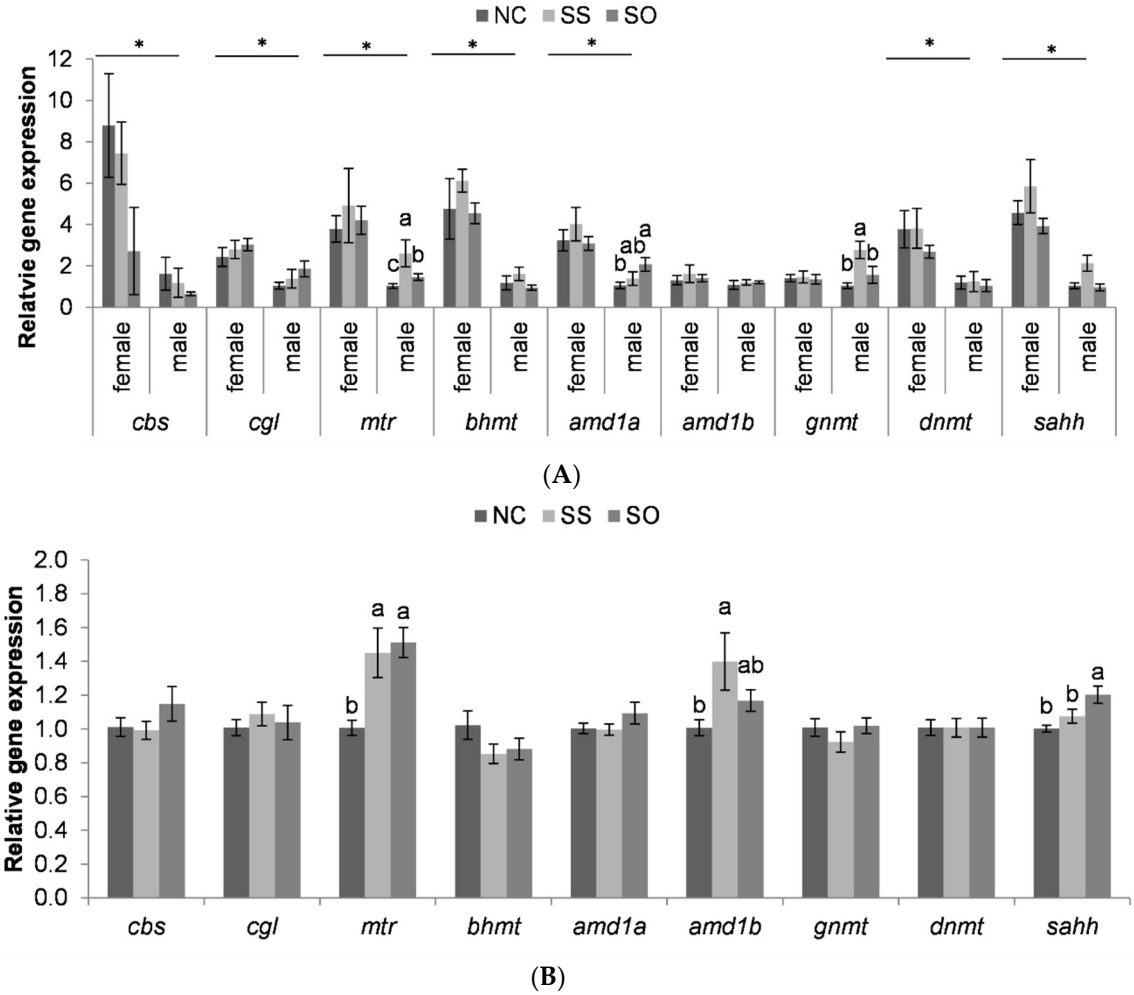


Figure 3. (**A**) Relative mRNA levels in whole-body swim-up fry from rainbow trout subjected to different Se treatments; (**B**) relative mRNA levels in parental liver tissue from rainbow trout subjected to different Se treatments. Data are normalized to β-actin and expressed as fold changes compared with the control group NC. In A, values are expressed relative to NC males. Bars are the mean ± SEM (A: n = 8; B n = 8 in female liver and n = 5 in male liver). Means not sharing a common superscript letter are significantly different ($p < 0.05$) according to one-way ANOVA followed by Tukey's HSD.

Parental feeding of both SS and SO increased *mtr* gene expression in the swim-up fry compared to NC feeding (Figure 3B). In addition, the expression of *amd1b* was higher in SS compared to NC and that of adenosylhomocysteinase (*sahh*) was higher in SO compared to the two other groups.

2.4. Parental Selenium Resulted in a Weak Group-Wise DNA Methylation Clustering

Reduced representation bisulfite sequencing (RRBS) data were first processed and aligned to the rainbow trout genome (Table A1). For downstream analysis, only uniquely mapped reads (47.6 ± 1.4%) were used. Of the 12 samples sequenced (4 per dietary group), none appeared as an outlier. In the search for the global methylation pattern with all the mapped CpG sites, t-SNE (t-distribution stochastic neighbor embedding) was used. The individual methylation pattern was stronger compared to group-wise global patterns, with only a weak group-wise clustering identified when using the 95th

percentile of the CpC variance (Figure 4). The stronger individual variation compared to group-wise clustering was confirmed using other methods including PCA, hierarchical clustering and correlation analysis (Figure A1).

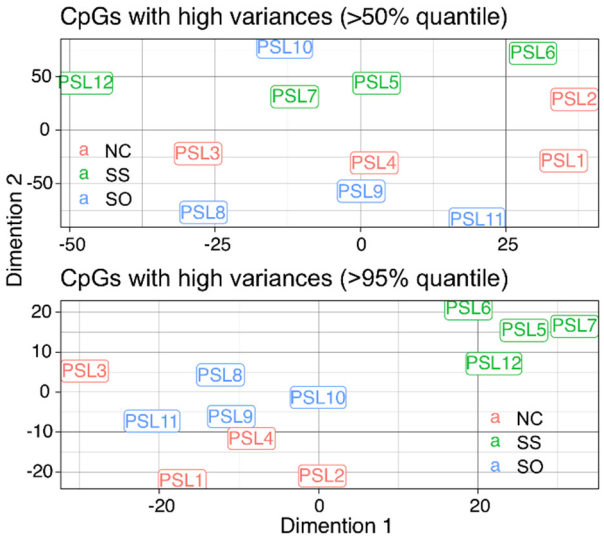
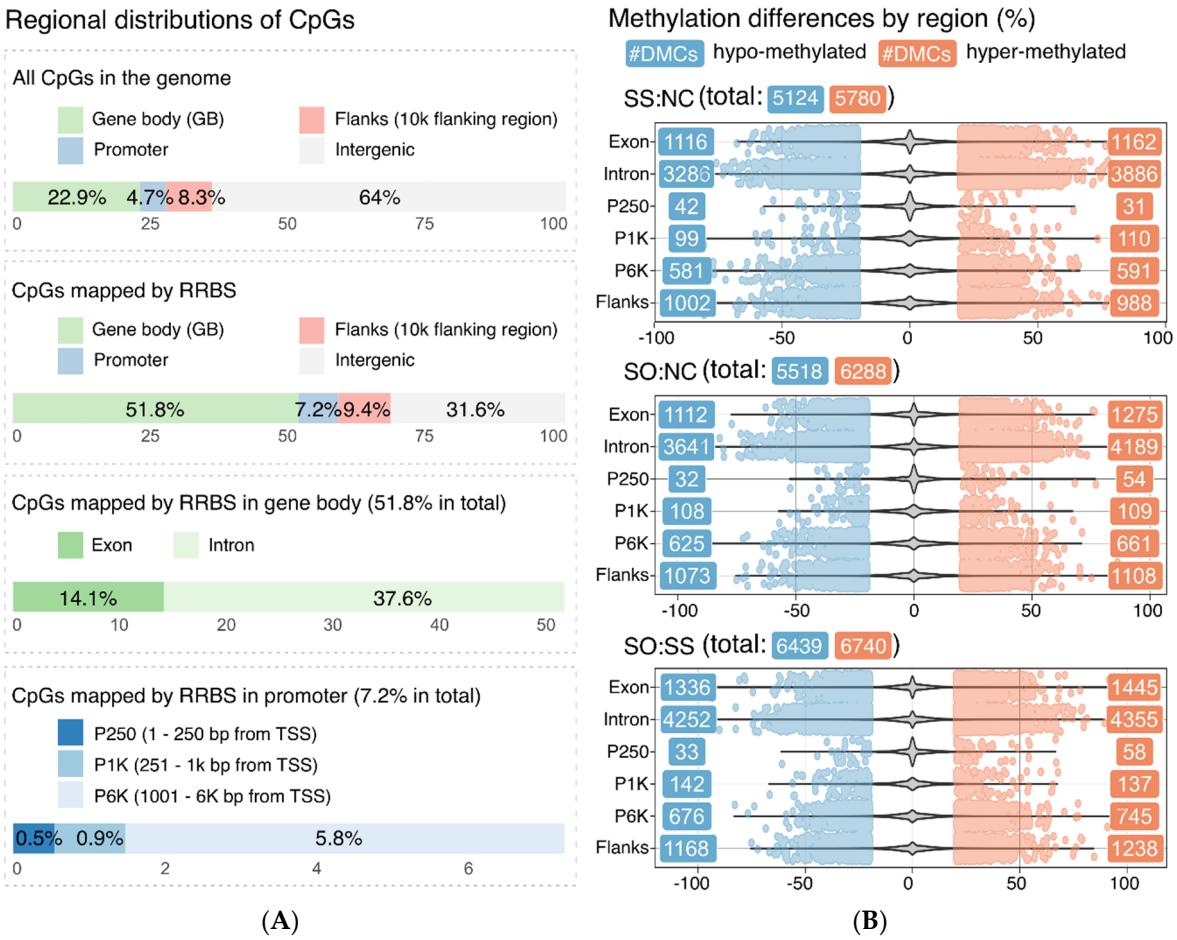


Figure 4. t-SNE analysis with the CpG sites using either the > 50% or > 95% quantile.

2.5. Data Alignment Gives a Balanced Hepatic Methylation Pattern between Groups

The regional annotation showed that most of the mapped CpG originated with 51.8% from the gene bodies compared to the whole rainbow trout genome, where it accounts for 22.9% (Figure 5A). With 37.6%, most of the mapped CpG in the gene body were coming from the intron region. Further, promoters were more targeted by RRBS compared to the whole rainbow trout genome, with an increase from 4.7% to 7.2%.

The total number of differentially methylated cytosine (DMC) was comparable for the groups SS:NC (10904), SO:NC (11806) and SO:SS (13179) and, within each group, the number of hyper- and hypomethylated CpG sites was balanced even when divided into different sub-regions, exon and intron for the body and P250 for the proximal promoter, P1K for the promoter and P6K for the distal promoter region as well as flanks (Figure 5B).



(**A**) (**B**)

Figure 5. (**A**) Regional distributions of mapped/original CpG. (**B**) Regional distributions of methylation differences of the differentially methylated cytosines (DMC) with a 20% threshold. Three violin plots show the density of overall methylation differences for SS:NC, SO:NC, and SO:SS, with scattered dots indicating DMC.

2.6. Parental Selenium Affects the DNA Methylation Pattern in Several Metabolic Pathways

Comparing NC with the SS treatment showed a total of 6535 differentially methylated genes (DMGs) from which 1142 DMGs had DMCs located in the promoter region (Figure 6). Similarly, in total, 6890 DMGs were detected between NC and the treatment receiving SO with 1250 DMGs showing DMCs located in the promoter region. The highest number of DMGs was detected between the two different Se-supplemented treatments with a total of 7428 genes of which 1340 DMGs had DMCs located in the promoter region. A synergetic effect of Se in NC vs. SS and NC vs. SO was detected on 3663 genes, whereas SS vs. SO displays a specific effect of the Se source on 2387 genes.

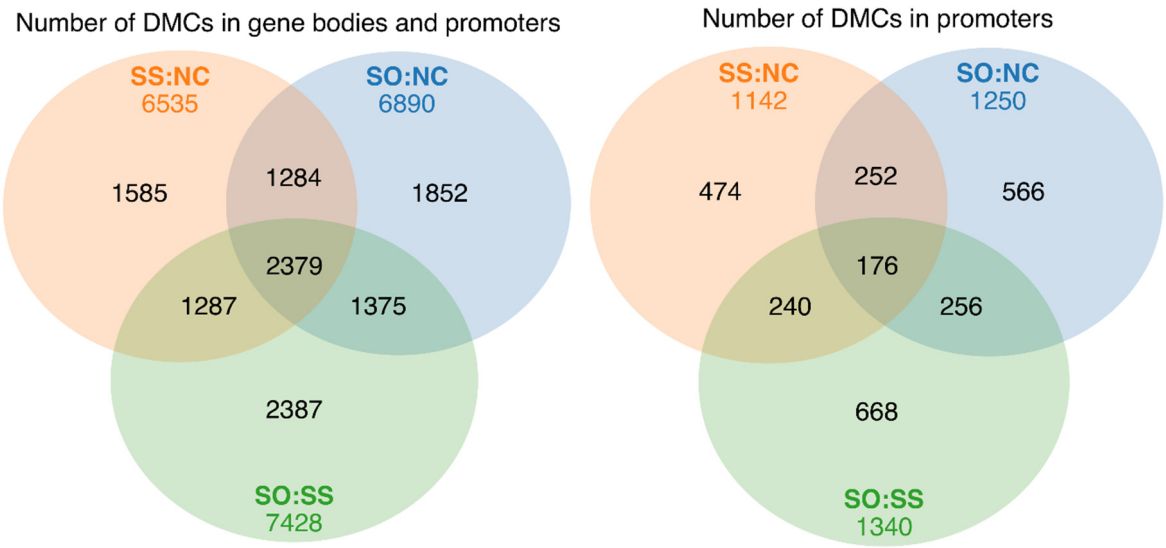


Figure 6. Venn diagrams summarizing the analysis of genes with different methylation patterns in NC vs. SS, NC vs. SO and SS vs. SO. (**A**) Genes are included with at least one DMC in gene body or promoter region; (**B**) Genes are included with at least one DMC in the promoter region.

In all datasets, multiple KEGG pathways were significantly enriched—22 in SS:NC, 18 in SO:NC and 20 in SO:SS (Figure A2). These KEGG pathways relate to diverse biological mechanisms, mainly cellular metabolism and environmental information processing, but also the organismal system and cellular processing.

Among the five genes with the highest number of DMCs listed for each of the sub-regions (exon, intron, proximal promoter, promoter and distal promoter) in Tables A2–A4, three genes were common. The limbic system-associated membrane, transcript variant X5 protein (*Isamp*) belongs to the immunoglobulin super-family, known to be expressed and excreted in the developing forebrain showed 15 DMCs in SS:NC, 24 in SO:NC and 22 in SO:SS, all located in the intron region. The methylation pattern revealed both hyper- and hypomethylated CpG sites. The DMCs of the other two genes were located in the promoter region. The radical S-adenosyl methionine domain containing protein 2-like (*viperin*) is a cytoplasmic antiviral protein that is induced by interferons. *Viperin* had five DMCs in SS:NC and three DMCs in SO:NC and SO:SS, respectively. In the inorganic Se treatment, the CpG sites were hypomethylated, but they were hypermethylated in the organic Se treatment. The third gene was gamma-aminobutyric acid receptor subunit rho-2 (*gabrr2*), which is an inhibitory neurotransmitter in the vertebrate brain. The DMCs of *gabrr2* were located in the distal promoter region and similar to *viperin* the gene was hypomethylated in SS:NC and hypermethylated in SO:NC.

2.7. Parental Selenium also Affects Methylation in Genes Related to the 1C Metabolism

Several genes related to the methionine cycle and transsulfuration pathway were identified, but mostly they contained only single DMC sites (Table 3). An effect on the genes that provide selenocysteine for the selenoprotein synthesis was only detected in the organic Se treatment. Nevertheless, selenoprotein I and selenoprotein U had DMCs in both Se-supplemented treatments.

Table 3. Differentially methylated genes (DMGs) related to sulfur and selenium metabolism.

DMGs		Hyper-/Hypomethylated DMC		
Gene ID	Gene name	SS:NC	SO:NC	SO:SS
Methionine Cycle				
110530927	S-adenosylmethionine synthase	0/1		
110537066	S-adenosylmethionine synthase-like		0/1	0/2
110502651	S-adenosylmethionine synthase-like	1/0 [P]	0/2	1/0
110529528	S-adenosylmethionine decarboxylase proenzyme-like		0/1	0/1
110538418	DNA (cytosine-5)-methyltransferase 1-like, transcript variant X1		0/1	
110505844	DNA (cytosine-5)-methyltransferase 3A-like		1/0	
110532515	DNA (cytosine-5)-methyltransferase 3A-like, transcript variant X2			1/0
110497603	DNA (cytosine-5)-methyltransferase 3A-like, transcript variant X6	1/0		
110492301	DNA (cytosine-5)-methyltransferase 3B-like, transcript variant X1			1/0
110500231	Putative adenosylhomocysteinase 3		0/1 [P]	
110494352	S-adenosylhomocysteine hydrolase-like protein 1 transcript variant X1		1/0	
110490243	Adenosylhomocysteinase 3-like	2/1	4/2	0/1
110522167	Putative adenosylhomocysteinase 3, transcript variant X2			0/1
Glutathione Metabolism				
110521555	Glutamate—cysteine ligase regulatory subunit-like	0/1		
110502703	Glutamate—cysteine ligase catalytic subunit-like, transcript variant X2	0/1		
110532297	Glutathione synthetase		0/1 [P]	
110522620	Glutathione-specific gamma-glutamylcyclotransferase 1-like		2/0	
110537206	Gamma-glutamyltransferase 5-like,transcript variant X1	0/1 [P]	1/0 [P]	2/0 [P]
100305229	Glutathione S-transferase kappa 1, transcript variant X1			1/0 [P]
110492369	Glucose-6-phosphate 1-dehydrogenase-like, transcript variant X2		2/0	0/1
100305228	Peroxiredoxin 6, transcript variant X2		1/0	
110532317	Spermidine synthase		0/1 [P]	
110535309	5-oxoprolinase (ATP-hydrolyzing)		0/1	1/2
110501851	Isocitrate dehydrogenase [NADP] cytoplasmic-like	1/0 [P]		0/1 [P]
110520228	Isocitrate dehydrogenase [NADP] cytoplasmic-like		1/1	
Selenoprotein Synthesis and Selenoproteins				
110494778	Methionyl-tRna synthetase 1		0/1	0/1
110488988	Methionyl-tRNA synthetase 2, mitochondrial	1/0		
110500323	Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase			1/0 [P]
110512109	tRNA selenocysteine 1-associated protein 1-like		0/1	
110528137	Eukaryotic elongation factor, selenocysteine-tRNA specific		1/0	
110529243	Selenoprotein I	0/3	5/0	7/0
100499413	Selenoprotein U	0/1 [P]	1/0 [P]	1/0 [P]
110532070	Selenoprotein K, transcript variant X1		0/1	
110497881	Selenoprotein O-like			1/0
110525853	Thioredoxin reductase 2			0/1

[P] located at the promoter region.

3. Discussion

3.1. Parental Selenium Nutrition and the Transsulfuration Pathway in the Progeny

Decreased levels of cysteine in rainbow trout fry originating from the parental group fed Se-supplemented diets are in contrast to reports in adult rats, mice and chicken, where rather an impaired transsulfuration with decreased cystathionine and cysteine levels has been described under

dietary Se deficiency [12,14,15]. Decreased levels of cysteine were also detected in liver tissue of female broodstock of the sodium selenite treatment, indicating an effect independent of life stage in rainbow trout (Figure 7). In the present study, swim-up fry of the Se-supplemented treatments had lower pyridoxamine levels compared to the control group, which indicates an increased demand for vitamin B6 by parental Se nutrition. The combined effect of maternal Se and pyridoxal nutrition has been studied in porcine embryos by Dalto et al. [27,28], who described that the co-supplementation increased plasma seleno-dependent glutathione peroxidase levels in the progeny in the long term. In addition, the supply of organic Se and vitamin B6 stimulated the expression of elongation factors, biological processes related to translation and the mitotic cell cycle in five-day-old embryos [27]. Although the sulfur and seleno amino acids follow a similar pathway, the most important reaction for selenocysteine is its reduction to selenide via selenocysteine lyase and further by selenophosphate synthetase that donates Se to the Sec-tRNA for the selenoprotein synthesis [26]. Both these enzymes are vitamin B6 dependent, highlighting the importance of this vitamin in Se metabolism. This reaction is independent of dietary Se form as both inorganic and organic forms undergo the reduction to selenide [29]. The impact of dietary Se form as observed in the present study could be questioned as in an earlier study it was shown that even in the parental sodium selenite treatment more than 94% of the Se in oocytes was either selenocysteine or selenomethionine [6]. Nevertheless, the higher selenomethionine levels corresponding to the higher total Se levels in the organic Se treatment might contribute to changes in the methionine cycle, providing methyl groups and homocysteine/Se-homocysteine for the transsulfuration pathway. Thus, it can be inferred that the higher redox status of Se-homocysteine compared to homocysteine might favor transsulfuration, as its enzymes are readily regulated through the redox status [30].

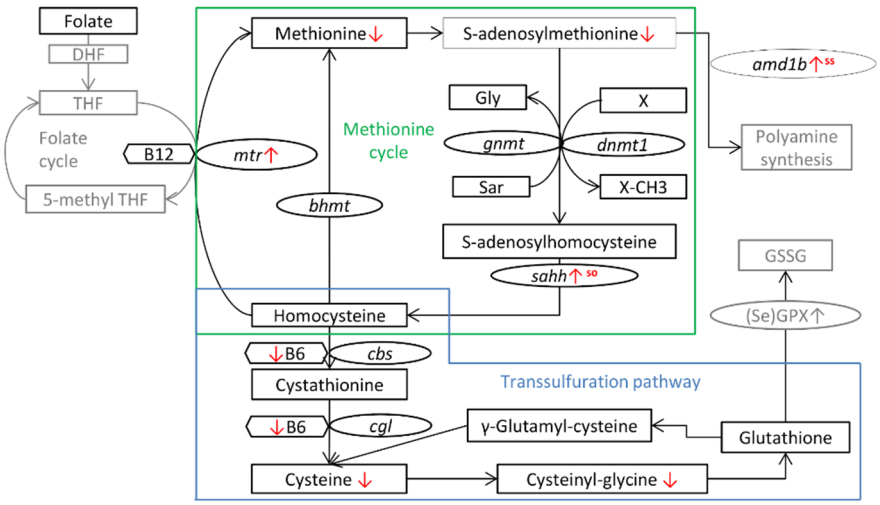


Figure 7. Effect of parental Se nutrition on the methionine cycle and transsulfuration pathway in the progeny of rainbow trout. Superscript indicates that the effect was only detected in the respective treatment.

3.2. Parental Selenium Nutrition and the Methionine Cycle in the Progeny

In the present study, parental Se had no effect on homocysteine levels in the swim-up fry, while studies in mice indicate an inverse correlation between liver Se and homocysteine levels [31,32]. An increased mRNA level of *mtr* in both Se-supplemented treatments could indicate that in rainbow trout, parental Se favors the re-methylation of homocysteine to methionine in the offspring. An increase in homocysteine can result in the accumulation of SAH, which is a competitive inhibitor of methyl-transferases and therefore associated to global hypomethylation [19]. In mammals, selenomethionine supplementation resulted in decreased hepatic SAH, but selenite on the contrary increased hepatic SAH [16,33]. In the present study, the difference was not significant. Nevertheless,

increased mRNA levels of *sahh* indicate that SAH might be increasingly metabolized to homocysteine in the organic Se treatment (Figure 7). The decrease in the SAM/SAH ratio in swim-up fry of Se-supplemented treatments due to a decrease in SAM levels is possibly due to the lower methionine levels. In the OH-SeMet treatment, it cannot be excluded that a competition of selenomethionine on active transporters reduced the methionine uptake in the gut [34]. However, considering the small fraction that selenomethionine represents compared to dietary methionine levels in this study, it might be rather indicative of a higher methionine flux in the organic Se treatment. The sequence of Se-compounds in the metabolism creates an additional drain on methyl groups, as selenide and other highly reactive seleno-compounds can be spontaneously methylated [35]. This process is of importance for inorganic Se sources which do not follow the methionine cycle and are directly reduced to selenide [36]. This hypothesis is supported by our data on broodstock liver, where a significant decrease in SAM was only observed in the selenite treatment. This might be an explanation why Speckmann et al. [16] reported a high SAM/SAH ratio in response to selenomethionine supplementation using Se-deficient conditions in mice where no methylation of seleno-compounds for removal could be expected. Several other studies in mice using higher Se levels detected no effect of Se on the SAM/SAH ratio [14,33,37]. A depletion of the methyl donor SAM can result in decreased DNA methyltransferases (DNMT) activity [38]. If in human colon carcinoma cells, administration of selenite inhibits DNMT activity [39], *dnmt1* expression in the present study was not affected by Se, similar to what was reported on hepatic mRNA levels in mice [16].

3.3. Parental Selenium Nutrition Affects the DNA Methylation Pattern of Genes Related to Several Metabolic Pathways

Analyzed liver tissue of rainbow trout fry revealed that the DNA methylation patterns of several genes are sensitive to parental Se nutrition. Alterations in DNA methylation by Se have been reported in several murine studies, although with somewhat contradictory results, as Se could be associated with both hyper- and hypomethylation [16,33,37,40]. The methylation of DNA can possibly regulate the spatial-temporal expression pattern of genes driving towards the development of a specific phenotype [18]. Genes directly related to the sulfur and Se metabolism presented methylation differences according to parental Se nutrition. Therefore, epigenetic marks might relate to metabolic differences observed in the present as well as in an earlier study on the expression of genes involved in the glutathione and antioxidant metabolism in rainbow trout fry [6]. Although an expected enrichment of the glutathione pathway could not be detected, genes of the glutathione metabolism including glutathione synthetase and glutathione-s-transferase kappa 1 were detected as DMGs. It has been reported that in cancer cells, selenite supplementation reactivates the transcription of glutathione-s-transferase π, another member of the glutathione-s-transferase family by a hypermethylation of the promoter region [41]. Most studies with cancer cells generally report the methylation of selenoproteins like glutathione peroxidase 1 and 3, methionine sulfoxide reductase B1 and selenium binding protein 1 in the promoter region [42]. In the present study with rainbow trout, parental Se nutrition did not result in changing methylation pattern for these genes, contrary to selenoprotein I, a potential target of parental Se nutrition on the progeny. Selenoprotein I is a protein involved in the formation of the glycerophospholipid phosphatidylethanolamine [43], belonging to the glycophospholipid metabolism KEGG pathway that was enriched in the sodium selenite treatment. A silencing of the gene has been associated with impaired neural development as it is essential in the myelination process [44]. In general, several genes with high changes in DNA methylation like *gabrr2* were related to brain signaling pathways and neurotransmission. Under physiologically relevant conditions, Se nutrition has been associated with a neuroprotective role on γ-aminobutyric acidergic neurons [45]. It remains unclear whether the changes in DNA methylation of neuronal signaling genes as observed here in the hepatic tissue would be similarly detected in other organs such as the brain. DNA methylation works on a time and spatial dimension as genes gain tissue-dependent importance and are also activated and deactivated at different developmental stages [46,47]. High methylation

differences were also identified for genes with a role in immune protection, including the antiviral protein *viperin* that showed several DMC sites in the promoter region. *Viperin* was up-regulated by supra-nutritional Se feeding in rainbow trout as well as Atlantic salmon (*Salmo salar*) in earlier studies [48,49]. This indicates that the impact of Se on the inflammatory response in fish might not be limited to direct feeding effects, but also be exerted through an epigenetic process. In this context, similar to other natural feed additives [50], Se might act as an immunostimulant, improving fish immunity in the long term.

4. Materials and Methods

4.1. Experimental Set up

The experiment was conducted at the INRAE experimental fish farm in Lées-Athas, France. Fish maintenance and experimental procedures were conducted by trained personnel in compliance with the European Directive 2010/63/EU for the protection of animals used for scientific purposes and the French Decree no. 2013–118 for animal experimentation.

Three-year-old rainbow trout (*Oncorhynchus mykiss*) broodstock (initial mean weight: 1.1 ± 0.2 kg in females and 0.9 ± 0.3 kg in males) from the same genetic group produced at the INRAE facilities of Lées-Athas (permit no. A64-104-1) were individually tagged and divided into three groups consisting of 25 females and 15 males. The fish were reared under natural photoperiod, as previously described [6], over six months and fed the respective diets once daily to apparent satiation. At spawning, oocytes from eight females per group were fertilized with pooled sperm received from males of the same dietary treatment collected on the same day. Fertilized eggs from each female were reared separately in small trays until swim-up fry stage supplied with flow-through spring water at 8 ± 1 °C.

4.2. Experimental Diets

The diets were based on plant ingredients with an 8% fish oil inclusion and designed to differ only in their Se content (Table 4), as previously described [6]. The NC diet at a basal Se level of 0.3 mg/kg was not supplemented with Se. The SS diet was supplemented with sodium selenite to a target level of 0.6 mg/kg (analyzed concentration, 0.8 mg/kg) and the SO diet was supplemented to the same target level of 0.6 mg/kg with OH-SeMet (Selisseo®, Adisseo SAS, Antony, France), resulting in a final Se concentration of 0.7 mg/kg.

Table 4. Dietary composition.

Diet	NC	SS	SO
Ingredients			
Plant meals [1]	74	74	74
Crystalline amino acids and attractant mixture [2]	3.14	3.14	3.14
Soybean lecithin[3]	2	2	2
Fish oil [3]	8	8	8
Vegetable oils [4]	8	8	8
Astaxanthin (μg/g diet) [5]	40	40	40
Vitamin and mineral mixture without Se [6]	4.82	4.82	4.82
Sodium selenite (μg/g diet) [7]	–	**0.71**	–
Hydroxy-selenomethione (μg/g diet) [7]	–	–	**0.75**
Analytical composition			
Dry matter (DM, %)	96	98	97
Crude protein (% DM)	49	50	50
Total lipid (% DM)	23	22	23
Gross energy (kJ/g DM)	25	25	25
Ash (% DM)	6	6	6
Phosphorus (% DM)	1.2	1.1	1.2
Selenium (mg/kg dry feed) [8]	**0.3**	**0.8**	**0.7**

[1] 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). [2] 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. [3] Soybean lecithin from Louis François and fish oil from Sopropêche. [4] Vegetable oils (% diet): 4% rapeseed oil, 2.4% linseed oil, 1.6% palm oil (Daudry). [5] Provided as Carophyll®pink (DSM). [6] Vitamin and mineral mixture without Se (per kg diet): retinol acetate, 55,000 IU; cholecalciferol, 2,500 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; $CaHPO_4 \cdot 2H_2O$, 33 g; $CaCo_3$, 2.15 g; $Mg(OH)_2$, 1.24 g; KCl, 0.9 g; NaCl, 0.4 g; $FeSO_4 \cdot 7H_2O$, 0.2 g; $ZnSO_4 \cdot 7H_2O$, 40 mg; $MnSO_4 \cdot H_2O$, 30 mg; $CuSO_4 \cdot 5H_2O$, 30 mg; NaF, 10 mg; KI, 0.4 mg; $CoCl_2 \cdot 6H_2O$, 0.2 mg. All ingredients were diluted with α-cellulose. [7] Sodium selenite contained 42% Se (Sigma-Aldrich) and hydroxy-selenomethionine contained 40% Se provided as Selisseo®(Adisseo). [8] Total Se was determined using inductively coupled plasma mass spectrometry (ICP MS, Agilent series 7500cx) by Ultra-Trace Analysis Aquitaine (UT2A, Pau, France) according to Vacchina and Dumont [51], with a calculated uncertainty of 15 μg/kg and a limit of quantification of 3 μg/kg.

4.3. Sampling

The broodstock fish were anaesthetized with benzocaine for stripping and afterwards euthanized by a sharp blow to the head for liver dissection in both males and females. For each individual female, samples of pooled oocytes after stripping (1 g sized samples) and progeny at swim-up fry stage (whole-body fry) killed by an overdose of benzocaine were withdrawn. Moreover, a total of 36 individual swim-up fry livers were randomly dissected on the same day at the Ecology and Fish Population Biology facility in Saint-Pée-sur-Nivelle, France [52], originating from 12 females (n = 4 females per dietary treatment). The three individual livers per female were pooled into a single sample tube for DNA extraction. All collected samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

4.4. Metabolite Analysis

In 0.1 g of pooled whole-body swim-up fry, free amino acids and other N-metabolites were analyzed using the Biochrome Analyzer and post column ninhydrin reaction following deproteinization, as previously described [53]. The aminothiols of the transsulfuration and glutathione pathway including homocysteine, cysteine, γ-glutamyl-cysteine, reduced glutathione and cysteinyl-glycine as well as SAM and SAH were measured by HPLC using one sample extract. First, 0.3 g of broodstock liver tissue, 1 g of pooled oocytes or 1 g whole-body swim-up fry were homogenized with an ultra-turrax in a 20 mM phosphate, 1 mM EDTA (pH = 6.4) buffer. After centrifugation (10,000 g, 15 min, 4 °C), deproteinization of the supernatant was performed using a 10% metaphosphoric acid solution. The protocol for aminothiol analysis was adapted from Toyooka and Imai [54]. Derivatization was performed by adding

62.5 µL AccQ·Fluor™ borate buffer (Waters, Guyancourt, France), 5 µL 1.55N NaOH and 4.5 mM ABD-F buffer to 25 µL sample aliquot for 20 min at 60 °C. After, the reaction was stopped by addition of 12.5 µL of 1N HCL and cooling at 4 °C for 15 min. Separation was performed using a AccQTaq^{TM} column at 40 °C using gradient elution: 0–2 min 97% A, 3% C; 20 min: 96% A, 4% C; 25 min: 20% B, 80% C; 30–35 min: 97% A, 3% C with (A) aqueous solution of AccQTag^{TM} Eluent A; (B) ultra-pure water and (C) methanol. Aminothiols were detected with fluorescence (excitation 385 nm, emission 515 nm). SAM/SAH measurement was adapted from She et al. [55] with separation on a Revolve C18 at 40 °C with the following gradient: 0–10 min 95% A, 5% B; 20 min 30% A, 70% B; 35–45 min 95% A, 5% B with (A) 20mM phosphate buffer with 8mM OSA (pH 2.7, TFA adjusted) and (B) methanol. In 0.1 g of pooled whole-body swim-up fry, pyridoxine, pyridoxal and pyridoxamine were measured by ultra-performance liquid chromatography (UPLC) [56] and vitamin B12 and total folate were analyzed microbiologically using *Lactobacillus delrueckii ssp. lactis* and *Lactobacillus rhamnosus*, respectively, as previously described [57].

4.5. RNA Extraction and RT-qPCR

The RNA was extracted and analyzed by quantitative RT-qPCR on 0.1 g samples of broodstock liver and a pool of three whole-body swim-up fry, as previously described [6]. The primer sequences are given in Table 5.

Table 5. Oligonucleotide primers used to assay mRNA levels by Fluidigm PCR.

Gene	Accession No.	Forward Primer	Reverse Primer	Amplification Size
amd1a	XM_021611778.1	ccgtaccatcccaaggtttga	tcctgcttgtcggtctttgt	87
amd1b	XM_021600287.1	cagccagattttcccaaacgg	gcatgctcgttctcccagaa	108
bhmt	FR908041.1	cagagaagcacggtaactgg	ttctttgtgctgcatcaggt	188
cbs	NM_001124686.1	ccacctcaggcaatacaggt	aacatccaccttctccatgc	107
cgl	EU315111.1	caccaaccccaccatgaaag	gcgctggaagtaggctgaca	118
dnmt1	XM_021557911.1	ttgccagaagaggagatgcc	cccaggtcagcttgccatta	152
gnmt	XM_021585680.1	ctcaagtacgcgctgaagga	cactctggtccccttgaagt	187
mtr	XM_021576690.1	aatgcaggtctgcccaatac	ctgatgtgtgcaggagtcgt	137
sahh	XM_021609053.1	atcaaacgggccacagatgt	tcgtaccttccatggcagc	167
β-actin	AJ438158.1	gatgggccgaaagacagcta	tcgtcccgtggtgacgat	105

amd1, adenosylmethionine decarboxylase 1; *bhmt*, betaine-homocysteine S-methyltransferase 1; *cbs*, cystathionine beta-synthase; *cgl*, cystathionine gamma-lyase; *dnmt1*, DNA methyltransferase 1; *gnmt*, glycine N-methyltransferase; *mtr*, methionine synthase; *sahh*, adenosylhomocysteinase.

4.6. Statistical Analysis on Metabolic Analysis and Gene Expression Data

Results are given as the mean ± SEM. Statistical analysis was performed using statistical software R (R Core Team). All data were tested for normality and homogeneity. Gene expression data were rank transformed before further analysis. Principle component analysis (PCA) was performed on the free amino acid dataset in search for biological clusters and outliers (R: factoextra [58]). One-way ANOVA was used to identify differences between Se treatments or sex. Tukey's HSD was used as a post hoc test in case a significant difference ($p < 0.05$) was detected.

4.7. DNA Extraction, RRBS Library Preparation and Sequencing

DNA extraction on swim-up fry livers was performed using a QIAGEN DNeasy Blood and Tissue Kit (cat. no. 69504), following the manufacturer's instruction. DNA quantity was measured using Qubit fluorometric quantitation (Life Technologies, Carlsbad, California, USA), ensuring that the sample contained a minimum of 200 ng of DNA. The DNA extract was stored at −20 °C before DNA methylation was measured by reduced representation bisulfate sequencing (RRBS) performed at the Biomedical Sequencing Facility BSF in Vienna, Austria.

The RRBS library preparation was performed, as previously described [23], on 100 mg genomic DNA including DNA digestion (Msp1 20 U, 16h at 37 °C), enzymatic adapter ligation (T4 DNA

Ligase rapid), quantification and pooling. Bisulfite conversion was performed using EZ DNA Methylation-Direct Kit D5020, Zymo Research, but conversion reagent was used at 0.9× concentration with incubation for 20 cycles of 1 min at 95 °C, 10 min at 60 °C and a desulphonation time of 30 min to increase the number of CpG nucleotides covered. Enrichment PCR was performed after AMPure XP clean up and library concentrations were quantified with the Qubit Fluorometric Quantitation system (Life Technologies) and size distribution by a Bioanalyzer High Sensitive DNA Kit (Agilent). Sequencing was performed on Illumina HiSeq 3000/4000 instruments. The data have been stored in SRA [59] under the accession number PRJNA629594.

4.8. Rainbow Trout Genome and Genomic Annotation

The reference genome data of rainbow trout (Omyk_1.0) were downloaded from the NCBI assembly site (https://www.ncbi.nlm.nih.gov/assembly/ GCF_002163495.1).

For genes with multiple RefSeq sequences, only the longest sequence was kept after eliminating overlapped isoforms. All the CpG sites in the genome were identified and split into four regions—gene body (GB), promoter (P), flanking regions around mRNA (flanks), and intergenic. Gene body was further divided into two sub-regions, intron and exon, whereas promoter was also divided based on the distance from the transcriptional start site (TSS) as P250 (1 bp–250 bp), P1K (251 bp–1000 bp) and P6K (1001 bp–6000 bp). Flanks were defined as a combination of 4K upstream from the 5′ end of P6Ks (equivalently 6001–10,000 bp from TSS) and 10K downstream of the 3′ end of mRNA. All the regions outside of gene bodies, promoters and flanks were annotated as intergenic. Each CpG site was defined as a unique and non-redundant region or sub-region according to the precedence of exon > intron > P250 > P1K > P6K > flanks > intergenic.

4.9. RRBS Data Processing

Illumina2bam tools (1.17.3; https://github.com/wtsi-npg/illumina2bam) were used to de-multiplex pooled samples. SAMtools [60] was used to convert BAM files into FASTQ, before quality check by FastQC (Babraham Institute; https://www.babraham.ac.uk) and MultiQC [61]. Adapters and low-quality reads in the RRBS mode based on Cutadapt [62] were removed with Trim Galore! (Babraham Institute). Long reads were trimmed to 50 bp, and reads were selected by in-house python scripts to keep only those digested by MspI and TaqI.

Reads were aligned to the rainbow trout genome by Bismark [63] with Bowtie 1 [64]. Two Bismark tools, bismark_methylation_extractor and coverage2Cystosine, were used to retrieve methylation calls at CpG sites. Reads were filtered by methylKit tool [65] when either the number of reads was above 99.9th percentile or less than or equal to 10.

Cluster analysis was performed by Rtsne [66] for t-SNE [67], with perplexity = 2 and factoextra [58] for PCA, scree plot and hierarchical clustering with Ward's method.

Prior to differential methylation calculation, the unite function of methylKit was used to form SS:NC and SO:NC with NC as control and SO:SS with SS as control. Methylation differences were calculated by methylKit for all the CpG sites with methylation calls as a percentage and *p*-values by logistic regression. The SLIM method [68] was used to calculate q-values. CpG sites with a q-value of < 0.01 and $\geq 20\%$ methylation difference were defined as differentially methylated cytosines (DMCs). Genes with at least one DMC in the gene body or promoter region are considered to be differentially methylated genes (DMGs).

In-house R and Python scripts were coordinated in a pipeline by using Snakemake [69].

4.10. Functional Annotation and Statistical Analysis of DMGs

To find Kyoto Encyclopedia of Genes and Genomes (KEGG) [70] orthologues that correspond to rainbow trout genes, the results of BLASTKoala, GhostKoala [71] and KEGG Automatic Annotation Server (KAAS) [72] were merged. The precedence of BLASTKoala > GhostKoala > KASS was applied when conflicting annotation occurred. A total of 22501 orthologues along with 168 KEGG pathways

were identified. Over representation analysis (ORA) on KEGG pathways and Gene Ontology (GO) terms [73] was performed on DMGs by the R package clusterProfiler [74,75].

The Wilcoxon signed-rank test (Wilcox) was used to test the differences of methylation rates between two groups in a pair-wise manner for KEGG pathways.

A bootstrap version of the Kolmogorov–Smirnov test (KS-boot; the number of iterations: 1000) was used to test the methylation differences that are associated with a KEGG pathway against the methylation differences of the whole CpG sites in a region. All three methods of enrichment analysis were performed for all the defined regions, and the *p*-values were adjusted by the Benjamini–Hochberg procedure.

5. Conclusions

Our results demonstrate that in rainbow trout, parental Se nutrition decreased transsulfuration and modified the methionine cycle, as summarized in Figure 7. A decrease in the methyl donor SAM was noticed in parental fish and their offspring by Se supplementation. In the offspring, significant changes in the DNA methylation pattern were identified, especially for genes related to signal transmission and immune function, by parental Se supplementation with organic and inorganic Se forms. It could be suspected that such epigenetic changes might persist during subsequent growth and development of the fish, leading to long-term molecular and metabolic alterations in the progeny, which deserves further investigation.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

amd	Adenosylmethionine decarboxylase
bhmt	Betaine-homocysteine S-methyltransferase
cbs	Cystathionine beta-synthase
cgl	Cystathionine gamma-lyase
CpG	Cytosine phosphate guanine
DMCs	Differentially methylated cytosines
DMGs	Differentially methylated genes
DNMT	DNA methyltransferase
gnmt	Glycine N-methyltransferase
mtr	Methionine synthase
NC	Non-supplemented control treatment
OH-SeMet	Hydroxy-selenomethionine
RRBS	Representation bisulfite sequencing
SAH	S-adenosylhomocysteine
sahh	Adenosylhomocystenase
SAM	S-adenosylmethionine
SO	OH-SeMet treatment
SS	Sodium selenite treatment

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Appendix A

Table A1. Alignment statistics of 12 RRBS samples.

No	Name	Diet	Total Reads	Uniquely Mapped	(%)	Multi-Mapped	(%)	Non-Mapped	(%)
1	PSL1	NC	53 987 762	26 310 305	48.7	19 820 505	36.7	7 856 952	14.6
2	PSL2	NC	39 364 790	19 543 117	49.6	14 534 198	36.9	5 287 475	13.4
3	PSL3	NC	54 833 812	27 173 708	49.6	20 233 149	36.9	7 426 955	13.5
4	PSL4	NC	63 481 758	30 305 331	47.7	24 622 757	38.8	8 553 670	13.5
5	PSL5	SS	59 570 698	27 527 855	46.2	23 489 555	39.4	8 553 288	14.4
6	PSL6	SS	45 143 094	21 814 078	48.3	17 312 386	38.4	6 016 630	13.3
7	PSL7	SS	67 495 806	31 151 354	46.2	27 389 024	40.6	8 955 428	13.3
8	PSL8	SO	54 472 443	25 214 607	46.3	22 345 586	41.0	6 912 250	12.7
9	PSL9	SO	69 558 057	33 562 782	48.3	26 840 717	38.6	9 154 558	13.2
10	PSL10	SO	79 827 373	38 293 256	48.0	31 322 516	39.2	10 211 601	12.8
11	PSL11	SO	55 391 941	25 516 081	46.1	21 796 848	39.4	8 079 012	14.6
12	PSL12	SS	51 950 394	23 789 256	45.8	20 152 309	38.8	8 008 829	15.4

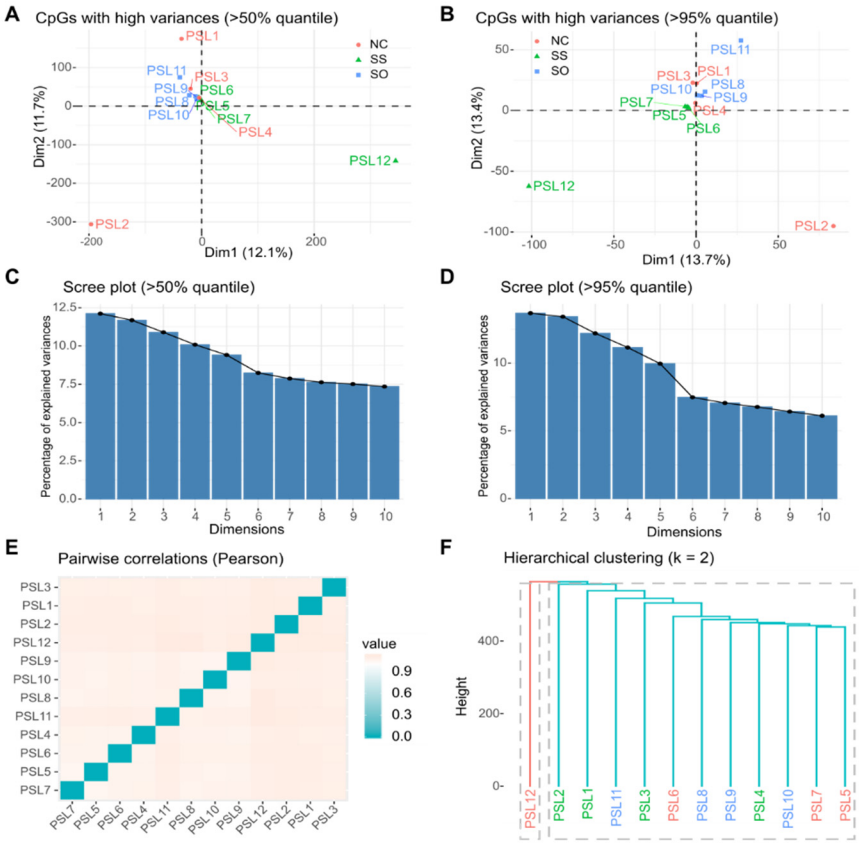

Figure A1. Clustering of the 12 RRBS samples by different algorithms. (**A + B**) PCA biplot by dietary treatment. (**C + D**) Scree plot with percentage of explained variance within the top 10 dimensions of the PCA. (**E**) Heatmap with sample–sample distance calculated by normalized Pearson's correlation coefficient in a range between 0 and 1, with $d = 0$ as $r = 1$ and d=1 as $r = -1$. (**F**) Dendrogram with hierarchical clustering.

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Appendix B

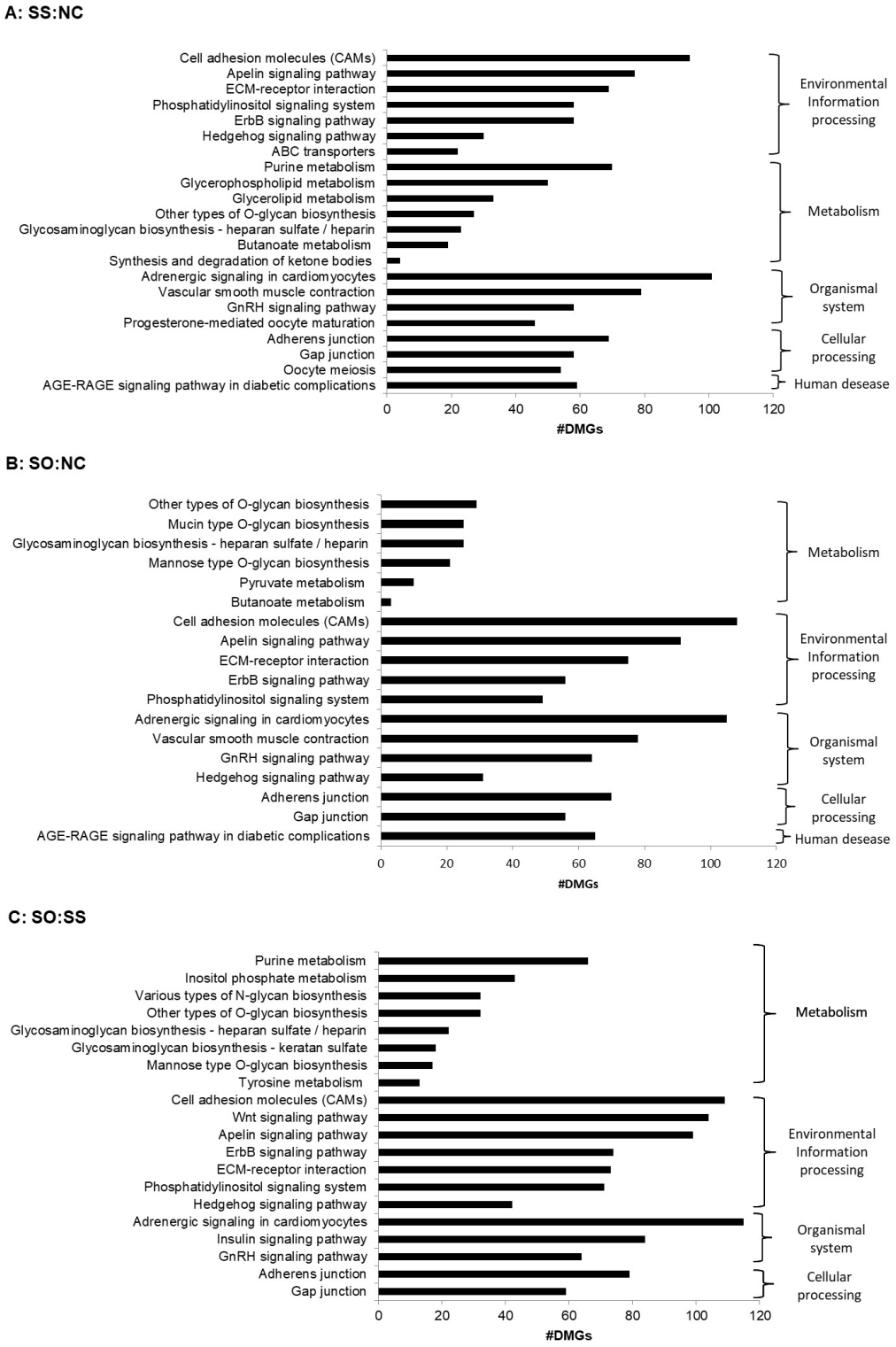

Figure A2. Enriched KEGG pathways in SS:NC (**A**), SO:NC (**B**) and SO:SS (**C**), with the number of differentially methylated genes (DMGs).

Appendix C

Table A2. Top five DMGs in the dataset SS:NC, with the highest number of CpG in each sub-region.

	Gene Symbol	Gene ID	Gene Name	Total DMC	DMC in the Region	Hyper-/Hypomethylated CpG
Exon	LOC110496419	110496419	Glycoprotein endo-alpha-1,2-mannosidase-like protein	10	10	0/10
	LOC110503414	110503414	TCDD-inducible poly [ADP-ribose] polymerase-like	10	8	8/0
	LOC110497587	110497587	SAM and SH3 domain-containing protein 1-like, transcript variant X2	10	7	0/7
	LOC110520294	110520294	Von Willebrand factor C domain-containing protein 2-like, transcript variant X1	7	7	5/2
	LOC110529233	110529233	MAM domain—containing glycosylphosphatidylinositol anchor protein 2-like	6	5	5/0
Intron	CTNNA2	110534326	Catenin alpha-2	18	18	11/7
	alk	110506268	ALK receptor tyrosine kinase	17	17	13/4
	lsamp	**110507545**	**Limbic system-associated membrane protein, transcript variant X5**	**15**	**15**	**9/6**
	NRXN2-like	110531840	Neurexin-2-like	16	12	5/7
	CDH4-like	110532012	Cadherin-4-like	13	12	5/7
P250	**LOC110498119**	**110498119**	**Radical S-adenosyl methionine domain-containing protein 2-like**	**5**	**5**	**0/5**
	LOC110493563	110493563	Septin-9-like	4	2	0/2
	COX6A2	100335037	Cytochrome c oxidase subunit VIa	3	2	0/2
	TIMP2-like	110487076	Metalloproteinase inhibitor 2-like	2	2	0/2
	LOC110490026	110490026	Mitogen-activated protein kinase kinase kinase 8-like	2	2	2/0
P1K	LOC110534101	110534101	Matrin-3-like	4	4	4/0
	LOC110489756	110489756	Transmembrane protein 14C-like	4	3	0/3
	LOC110486159	110486159	Proline-rich protein 15-like protein A	3	3	2/1
	TNFR11B-like	110506163	Tumor necrosis factor receptor superfamily member 11B-like	3	3	0/3
	TPPP3X2-like	110518569	Tubulin polymerization-promoting protein family Member 3-like, transcript variant X2	3	3	0/3
P6K	COX4I2-like	110492636	Cytochrome c oxidase subunit 4 isoform 2, mitochondrial-like	8	8	8/0
	LOC110523211	110523211	Oocyte zinc finger protein XlCOF6-like, transcript Variant X2	8	8	0/8
	LOC110498688	110498688	Fatty acid-binding protein, liver-type-like	7	7	7/0
	LOC110505815	**110505815**	**Gamma-aminobutyric acid receptor subunit rho-2-like**	**6**	**5**	**0/5**
	LOC110503024	110503024	Ras-related C3 botulinum toxin substrate 3-like	5	4	0/4

Genes in bold are commonly highly affected also in SO:NC and SO:SS.

Table A3. Top five DMGs in the dataset SO:NC, with the highest number of CpG in each sub-region.

	Gene Symbol	Gene ID	Gene Name	Total DMC	DMC in the Region	Hyper-/Hypomethylated CpG
Exon	LOC110490066	110490066	E3 ubiquitin-protein ligase rififylin-like, transcript variant X2	6	6	5/1
	LOC110520294	110520294	von Willebrand factor C domain-containing protein 2-like, transcript variant X1	6	6	3/3
	LOC110531157	110531157	Serine/threonine-protein kinase WNK2-like	7	5	0/5
	LOC110497587	110497587	SAM and SH3 domain-containing protein 1-like, transcript variant X2	6	5	1/4
	LOC110506316	110506316	Muscarinic acetylcholine receptor M4-like, transcript variant X1	6	5	0/5

Table A3. *Cont.*

	Gene Symbol	Gene ID	Gene Name	Total DMC	DMC in the Region	Hyper-/Hypomethylated CpG
Intron	**lsamp**	**110507545**	**Limbic system-associated membrane protein, transcript variant X5**	**24**	**24**	**9/15**
	LOC110505581	110505581	Placenta growth factor-like	15	15	9/6
	LOC110501635	110501635	Serine/threonine-protein kinase BRSK2-like	14	14	10/4
	LOC110506270	110506270	Protein kinase C-binding protein NELL1-like, transcript variant X1	13	13	5/8
	Catenin alpha-2	110534326	Catenin alpha-2	13	13	6/7
P250	LOC110501919	110501919	Alkyldihydroxyacetonephosphatesynthase,peroxisomal-like, transcript variant X2	5	3	2/1
	LOC110494831	110494831	Complement C1q-like protein 2	4	3	0/3
	LOC110508922	110508922	MARVEL domain-containing protein 2-like, transcript variant X2	4	3	3/0
	CRIP2-like	110505831	Cysteine-rich protein 2-like	3	3	3/0
	LOC110497531	110497531	Uncharacterized LOC110497531, transcript variant X2	4	2	2/0
P1K	LOC110493345	110493345	Gastrula zinc finger protein XlCGF17.1-like, transcript variant X1	4	4	4/0
	LOC110521247	110521247	Lactadherin-like, transcript variant X1	4	4	4/0
	LOC110533598	110533598	Ras-related protein Rab-24-like, transcript variant X1	4	4	0/4
	LOC110498119	**110498119**	**Radical S-adenosyl methionine domain-containing protein 2-like**	**3**	**3**	**3/0**
	NFATC3-like	110506757	Nuclear factor of activated T-cells, cytoplasmic 3-like	3	2	0/2
P6K	**LOC110505815**	**110505815**	**Gamma-aminobutyric acid receptor subunit rho-2-like**	**8**	**5**	**4/1**
	LOC110519930	110519930	Uncharacterized LOC110519930, transcript variant X2	6	5	0/5
	LOC110520086	110520086	Collagen alpha-1(XXVIII) chain-like	5	5	3/2
	taf6l	110531860	TATA-box binding protein associated Factor 6 like, transcript variant X1	5	5	5/0
	LOC110537362	110537362	Glutamate receptor 3, transcript variant X3	5	5	5/0

Genes in bold commonly highly affected also in SS:NC and SO:SS.

Table A4. Top five DMGs in the dataset SO:SS, with the highest number of CpG in each sub-region.

	Gene Symbol	Gene ID	Gene Name	Total DMC	DMC in the Region	Hyper-/Hypomethylated CpG
Exon	CDH2-like	110506386	Neural-cadherin-like	10	10	7/3
	PLEKHG7-like	110497700	Pleckstrin homology domain-Containing family G member 7-like	9	9	0/9
	NRXN2-like	110531840	Neurexin-2-like	21	8	2/6
	LOC110496419	110496419	Glycoprotein endo-alpha-1,2-mannosidase-like protein	8	8	8/0
	LOC110496815	110496815	Glutamate receptor ionotropic, kainate5-like	8	7	7/0
Intron	**lsamp**	**110507545**	**Limbic system-associated membrane protein, transcript variant X5**	**22**	**22**	**9/13**
	LOC110500600	110500600	Adhesion G protein-coupled receptor L3-like, transcript variant X5	23	20	17/3
	FBXL17X1	110525966	F-box and leucine rich repeat protein 17, transcript variant X2	18	16	13/3
	LOC110535694	110535694	Glutamate receptor ionotropic, delta-2, transcript variant X3	17	15	5/10
	ZNF407-like	110501552	Zinc finger protein 407-like	16	15	3/12

Table A4. *Cont.*

	Gene Symbol	Gene ID	Gene Name	Total DMC	DMC in the Region	Hyper-/Hypomethylated CpG
P250	LOC110488021	110488021	Calcitonin gene-related peptide type 1 receptor-like	8	7	7/5
	GATA 2-like	110494514	GATA-binding factor 2-like	7	6	6/0
	LOC110498119	**110498119**	**Radical S-adenosyl methionine domain-containing protein 2-like**	**4**	**3**	**3/0**
	LOC110508922	110508922	MARVEL domain-containing protein 2-like, transcript variant X2	3	3	3/0
	CRIP2-like	110505831	Cysteine-rich protein 2-like	4	2	2/0
P1K	LOC110527134	110527134	Methyltransferase-like protein 7A	5	5	0/5
	SEMA5A-like	110489566	Semaphorin-5A-like	6	4	0/4
	LOC110493345	110493345	Gastrula zinc finger protein XlCGF17.1-like, transcript variant X1	4	4	4/0
	LOC100135939	100135939	Proteoglycan 4, transcript variant X2	4	4	1/3
	GRTP1a-like	110527156	Growth hormone-regulated TBC Protein 1-A-like	4	4	0/4
P6K	LOC110523211	110523211	Oocyte zinc finger protein XlCOF6-like,transcript variant X2	8	8	8/0
	MARVELD2-like	110523471	MARVEL domain-containing protein 2-like	8	8	8/0
	MED12-like	110488993	Mediator of RNA polymerase II transcription subunit 12-like	9	7	7/0
	LOC110522593	110522593	F-box only protein 31-like, transcript variant X1	7	7	0/7
	LOC110505815	**110505815**	**Gamma-aminobutyric acid receptor subunit rho-2-like**	**6**	**6**	**6/0**

Genes in bold are commonly highly affected also in SS:NC and SO:NC.

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5.5. Publication 5

Long-term effect of parental selenium on the one-carbon metabolism in rainbow trout (Oncorhynchus mykiss) fry exposed to hypoxic stress

Objective:

Aim of the study was to investigate the long-term effect of parental selenium in combination with selenium nutrition on the one-carbon metabolism in rainbow trout fry.

Approach:

The progeny of three groups of rainbow trout broodstock fed different experimental diets with different form of selenium were cross-fed different forms of selenium for 11 weeks. The fry were sampled either before or after subjected to a hypoxic stress and analyzed for 1C metabolites.

Major findings:

- In normoxic fry glutathione levels were higher, when originating from parents fed Se-supplemented diets
- Expression of *bhmt* was reduced by parental and Se feeding in normoxic fry
- Dietary selenium decreased *cgl* mRNA levels in normoxic fry
- Hypoxic stress reduced levels of transsulfuration metabolites homocysteine, cysteine and cysteinyl-glycine, but increased mRNA levels of transsulfuration enzyme *cbs*
- Hypoxic stress reduced SAM, but not SAH levels
- In stressed fry originating from parents fed Se-supplemented diets *bhmt* and *amd1a* mRNA levels were decreased

Conclusion:

In rainbow trout fry, increased metabolite levels in the transsulfuration pathway, from parental selenium nutrition, suggest a degree of nutritional programming by Se in the glutathione metabolism. Under hypoxia, glutathione synthesis through transsulfuration might have been impaired by the depletion of a glutathione precursor. The combination of both parental Se nutrition and hypoxic stress on 1C gene expression induced also long-term impact on the methionine cycling and polyamine synthesis.

Running head: Parental selenium and 1C metabolism in trout

Long-term effect of parental selenium on the 1C metabolism in rainbow trout (*Oncorhynchus mykiss*) fry exposed to hypoxic stress

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Keywords: selenium; nutritional programming; rainbow trout; one-carbon metabolism; hypoxia

Abstract

This study evaluates how different forms of Se supplementation into rainbow trout broodstock diets modify the one-carbon metabolism of the progeny after the beginning of exogenous feeding further challenged with changing dissolved oxygen levels in the rearing water. The progeny of three groups of rainbow trout broodstock fed either a control diet (Se level: 0.3 µg/g) or a diet supplemented with inorganic sodium selenite (Se level: 0.6 µg/g) or organic hydroxy-selenomethionine (Se level: 0.6 µg/g) were cross-fed with diets of similar Se composition for 11 weeks. Offspring were sampled either before or after being subjected to an acute hypoxic stress (1.7 mg/L dissolved oxygen) for 30 minutes. In normoxic fry, parental Se supplementation allowed higher glutathione levels compared to fry originating from parents fed the control diet. Parental hydroxy-selenomethionine treatment also increased cysteine and cysteinyl-glycine concentrations in fry. Direct Se feeding decreased *cgl* mRNA levels. Hydroxy-selenomethionine feeding also lowered the levels of some essential amino acids. Both, organic Se feeding to parents and fry reduced *bhmt* expression in fry. The hypoxic stress decreased whole-body homocysteine, cysteine, cysteinyl-glycine and glutathione levels. Together with the higher mRNA levels of *cbs*, a transsulfuration enzyme, this suggests that under hypoxia, glutathione synthesis through transsulfuration might have been impaired by the depletion of a glutathione precursor. In stressed fry, S-adenosylmethionine levels were significantly decreased, but S-adenosylhomocysteine remained stable. Decreased *bhmt* and *amd1a* mRNA levels in stressed fry suggest a nutritional programming by parental Se also on methionine metabolism of rainbow trout.

1. Introduction

Selenium (Se) content in commercial feeds for salmonids has been reported to decrease in recent years[1–4]. This is a result of the ongoing reduction of fishmeal as the major Se source in such diets possibly needing Se supplements in order to meet the requirement of fish[5,6]. We have recently demonstrated that in a fishmeal-free rainbow trout broodstock diet, Se supplementation can improve reproductive performance[7], while causing long-term modifications in the antioxidant system of the progeny[8] that might relate to epigenetic modifications at early stages possibly reflecting a phenomenon known as nutritional programming[9].

The methyl-groups for methylation reactions are provided by the one carbon (1C) metabolism, a term used to describe three closely linked metabolic pathways[10]. The folate metabolism is a cyclic pathway, where one-carbon units from serine or glycine are transferred to tetrahydrofolate to form methyl-tetrahydrofolate, which is used in methionine metabolism to methylate homocysteine (Hcys) to methionine (Met) for the production of S-adenosylmethionine (SAM), a general donor of methyl groups. Demethylation results in S-adenosylhomocysteine (SAH), which is further metabolized to Hcys. Hcys can either remain in the methionine cycle by re-methylation to Met or is metabolized via the transsulfuration pathway. In the transsulfuration pathway Hcys is formed to cysteine that accounts for the majority of the glutathione (GSH) production[11].

The metabolic pathway of dietary Se components is in close link to the 1C metabolism[12]. Similar to its sulfur counterpart, selenomethionine (SeMet) can be demethylated to seleno-homocysteine and metabolized through the transsulfuration pathway to form selenocysteine for further reduction to selenide and final translation into selenoproteins. Inorganic Se can be directly reduced to selenide[13]. Dietary Se supplementation has been reported to modify the activity of enzymes of the Hcys metabolism such as betaine-homocysteine S-methyltransferase (BHMT), cystathionine beta synthase (CBS) and glutamate-cysteine ligase resulting in an increased Hcys flux through the transsulfuration pathway and reduced

re-methylation[14–16]. The Hcys metabolism was shown to be redox sensitive *in vitro* in human hepatic cells lines as well as *in vivo* in mouse liver where BHMT activity was reduced by oxidants, however with an increase of transsulfuration enzyme activity[11,17]. The redox sensitivity of the transsulfuration pathway therefore presented a feedback mechanism for the production of GSH, the major cellular redox molecule.

On this basis, we wanted to evaluate in rainbow trout, if the inorganic and organic forms of Se supplementation, in combination with cross-feeding of the progeny might cause long-term modifications influencing the activity of the 1C metabolism. Furthermore, the progenies were subjected to a hypoxic stress challenge in order to evaluate the interaction between Se supplementation and the active regulation of 1C metabolism.

2. Material and methods

2.1. Experimental diets

Diets were manufactured using a twin-screw extruder at the INRAE experimental facilities[7,8]. Diets of similar composition based on plant-derived protein sources and with similar levels of crude protein (50%) and total lipid (22%) that differed only in Se content were prepared for broodstock and progeny from first feeding onwards (Table 1). The negative control diet without any additional Se supplementation had a basal Se level of 0.3 mg/kg in broodstock (Bnc) and fry (Fnc). The diets supplemented with 0.3 mg/kg inorganic sodium selenite had a final dietary Se content of 0.8 mg/kg in broodstock (Bss) and 0.5 mg/kg in fry (Fss). Similarly, diets were supplemented organic hydroxy-selenomethionine (OH-SeMet) resulting in a final Se concentration of 0.7 mg/kg in the broodstock diet (Bso) and 0.6 mg/kg in the corresponding fry diet (Fso).

Table 1. Formulation and composition of experimental diets (g/100g dry weight).

Diet	Broodstock diets			Fry diets		
	Bnc	Bss	Bso	Fnc	Fss	Fso
Ingredients						
Plant ingredients[1]	84	84	84	84	84	84
Fish oil[2]	8	8	8	8	8	8
Premixes[3,4]	8	8	8	8	8	8
Sodium selenite (µg/g diet)[5]	-	0.7	-	-	0.7	-
Selisseo® (µg/g diet)[5]	-	-	15	-	-	15
Analytical composition						
Dry matter (DM, %)	96	98	97	95	94	95
Crude protein (% DM)	49	50	50	51	50	50
Total lipid (% DM)	23	22	23	20	21	21
Gross energy (kJ/g DM)	25	25	25	25	25	25
Ash (% DM)	6	6	6	6	6	6
Phosphorus (% DM)	1.2	1.1	1.2	1.1	1.1	1.1
Se (mg/kg)	0.3	0.8	0.7	0.3	0.5	0.6

[1]Plant ingredients (% 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), 2% soybean lecithin (Louis François, 4% rapeseed oil (Daudry), 2.4% linseed oil (Daudry), 1.6% palm oil (Daudry)

[2]Fish oil from Sopropêche.

[3]Premixes (per kg diet): L-lysine, 13.4 g; DL-methionine, 3 g; glucosamine, 5 g; taurine, 3 g; betaine, 3 g; glycine, 2 g; alanine, 2 g; retinol acetate, 55,000 IU; cholecalciferol, 2,500 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; $CaHPO_4 \cdot 2H_2O$, 33 g; $CaCo_3$, 2.15 g; $Mg(OH)_2$, 1.24 g; KCl, 0.9 g; NaCl, 0.4 g; $FeSO_4 \cdot 7H_2O$, 0.2 g; $ZnSO_4 \cdot 7H_2O$, 40 mg; $MnSO_4 \cdot H_2O$, 30 mg; $CuSO_4 \cdot 5H_2O$, 30 mg; NaF, 10 mg; KI, 0.4 mg; $CoCl_2 \cdot 6H_2O$, 0.2 mg. All ingredients were diluted with α-cellulose.

[4]Broodstock dietary premixes contained also 40 mg astaxanthin/kg diet supplied as Carophyll pink® (DSM).

[5]Sodium selenite contained 46% Se (Sigma-Aldrich) and Selisseo contained 2% Se (Adisseo).

2.2. Experimental set up

All procedures were performed in compliance with the European Directive 2010/63/EU for the protection of animals used for scientific purposes and the French Decree no. 2013-118 for animal experimentation by trained personal at the INRAE experimental fish farm in Donzacq.

The progenies of three broodstock groups (Bnc, Bss, Bso) fed the experimental diets for six months prior to spawning were used in this feeding trial[(7)]. At swim-up fry stage, the pooled offspring from each of the three parental groups were randomly sub-divided into three fry feeding groups (Fnc, Fss and Fso) resulting in nine dietary treatments as previously described[(8)]. Each group of fry was allocated to triplicate 50L fiberglass tanks with 250 fish per tank (n= 27 tanks) supplied with flow-through spring water at a dissolved oxygen

concentration of 9.2 ± 0.1 mg/L and at a temperature of 17.5 ± 0.5 °C. The fry were hand fed six times daily during the first three weeks of the trial and four times daily afterwards to apparent satiation.

At the end of the 11-week feeding period (average final body weight: 4.6 ± 0.1 g), the fish were either sampled or subjected to a hypoxic stress challenge as previously described[8]. Shortly, one hundred fish from each group were transferred to a tank containing water with low dissolved oxygen of 1.7 ± 0.2 mg/L. When the fish started to show signs of discomfort they were transferred back in tanks with normally oxygenated water for recovery until the termination of the stress challenge after 30 min. Fish sampled before and after the stress were killed with an overdose of benzocaine and frozen as whole fish in liquid nitrogen for storage at -80°C until further analysis.

2.3. Gene expression

The mRNA was extracted from whole fry three individuals per tank (=nine individuals per dietary treatment) and analyzed by high-throughput qRT-PCR at the GenotoulPlatform (http://genomique.genotoul.fr/) using the BioMark® 96:96 Dynamic Array (Fluidigm Corporation, San Francisco, CA, USA) as previously described[8]. The primer sequences are given in Table 2. Relative expression of the target genes was calculated at a geometric mean of the housekeeping genes *ef1α* and *β-actin* and a control originating from parents fed Bnc that also received the fry control diet Fnc (BncFnc) sampled before the stress challenge by the ΔΔCT method[18].

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

Gene	Accession no.	Forward primer	Reverse primer	Amplification size
amd1α	XM_021611778.1	ccgtaccatcccaaggtttga	tcctgcttgtcggtctttgt	87
amd1b	XM_021600287.1	cagccagattttcccaaacgg	gcatgctcgttctcccagaa	108
cbs	NM_001124686.1	ccacctcaggcaatacaggt	aacatccaccttctccatgc	107
mtr	XM_021576690.1	aatgcaggtctgcccaatac	ctgatgtgtgcaggagtcgt	137
dnmt1	XM_021557911.1	ttgccagaagaggagatgcc	cccaggtcagcttgccatta	152
gnmt	XM_021585680.1	ctcaagtacgcgctgaagga	cactctggtccccttgaagt	187
cgl	EU315111.1	caccaaccccaccatgaaag	gcgctggaagtaggctgaca	118
sahh	XM_021609053.1	atcaaacgggccacagatgt	tcgtaccttccatggcagc	167
bhmt	FR908041.1	cagagaagcacggtaactgg	ttctttgtgctgcatcaggt	188
ef1α	AF498320.1	tcctcttggtcgtttcgctg	acccgagggacatcctgtg	159
β-actin	AJ438158.1	gatgggccgaaagacagcta	tcgtcccgtggtgacgat	105

amd1, adenosylmethionine decarboxylase 1; *cbs*, cystathionine beta-synthase; *mtr*, methionine synthase; *dnmt1*, DNA methyltransferase 1; gnmt, glycine N-methyltransferase; *cgl*, cystathionine gamma-lyase; *sahh*, adenosylhomocysteinase; *bhmt*, betaine-homocysteine S-methyltransferase; *ef1α*, eukaryotic translation elongation factor 1α

2.4. Metabolite analysis

Three individual frozen fish per tank were dissected for a pool of 100 mg fillet after removal of skin at the left side above the lateral line to be analyzed for free amino acids and other N-metabolites using the Biochrome Analyzer and post column ninhydrin reaction following deproteinization as previously described[19]. Extraction and analysis of the aminothiols Hcys, cysteine, γ-glutamyl-cysteine, GSH and cysteinyl-glycine as well as SAM and SAH was done by HPLC on 300 mg homogenized whole-body fry as previously described[9]. Pyridoxine, pyridoxal and pyridoxamine were measured by ultra-performance liquid chromatography (UPLC)[20]. Vitamin B12 and folate were analyzed microbiologically[21] using *Lactobacillus delruceckii spp. Lactis* and *Lactobcillus rhamnosus*, respectively.

2.5. Statistical analysis

Results are given as mean ± standard error (SEM). Statistical analysis was performed using statistical software R (R Development Core Team, 2008). Data were tested for homogeneity and normal distribution resulting in rank transformation of gene expression data before further analysis. In the free amino acid data, one sample of the BssFss treatment collected

before stress was identified as a systematic outlier using boxplots and principle component analysis and was subsequently removed from the statistical analysis. Parental and direct feeding effects in fed and stressed fry were analyzed using two-way ANOVA and differences between fed and stressed fry using one-way ANOVA followed by Tukey's HSD post hoc test. The significance level was set to $p < 0.05$ in all analyses.

3. Results

3.1. Aminothiol concentration

In fry sampled before the hypoxic stress, the concentrations of cysteine, cysteinyl-glycine, γ-glutamyl-cysteine and GSH were significantly higher, when originating from parental groups fed Bso compared to fry originating from the Bnc treatment (Table 3). Cysteinyl-glycine and GSH were additionally found to be higher in the group originating from parents fed the Bss compared to Bnc. No parental effect was found for Hcys. None of the aminothiols showed any significant difference according to the direct feeding of Se-supplemented diets. All analyzed aminothiol concentrations decreased during the hypoxic stress challenge. In stressed fry, no significant differences according to parental Se or Se nutrition were detectable.

Table 3. Aminothiol concentrations [µg/g sample] measured in whole fry originating from parents subjected to different Se treatments (Bnc, Bss, Bso) and then fed diets containing different levels and sources of Se (Fnc, Fss, Fso) before and after exposed to hypoxic stress

		Hcys	Cys	CysGly	GSH	GluCys
Before stress	Bnc	0.29 ± 0.01	1.04 ± 0.08^{b}	0.22 ± 0.01^{b}	12 ± 1^{b}	0.56 ± 0.10^{b}
	Bss	0.34 ± 0.03	1.27 ± 0.09^{ab}	0.29 ± 0.01^{a}	15 ± 1^{a}	0.90 ± 0.15^{ab}
	Bso	0.33 ± 0.02	1.36 ± 0.07^{a}	0.27 ± 0.02^{a}	15 ± 1^{a}	1.04 ± 0.12^{a}
	p-value	*0.20*	***0.03***	***<0.01***	***<0.01***	***0.05***
	Fnc	0.31 ± 0.02	1.14 ± 0.09	0.25 ± 0.02	13 ± 1	0.74 ± 0.11
	Fss	0.34 ± 0.02	1.29 ± 0.09	0.28 ± 0.02	14 ± 1	0.99 ± 0.17
	Fso	0.29 ± 0.02	1.24 ± 0.09	0.26 ± 0.02	14 ± 1	0.77 ± 0.11
	P-value	*0.19*	*0.41*	*0.18*	*0.43*	*0.37*
	Bdiet x Fdiet	*0.40*	*0.22*	*0.31*	*0.11*	*0.62*
After stress	Bnc	0.15 ± 0.01	0.97 ± 0.09	0.18 ± 0.02	11 ± 1	0.39 ± 0.04
	Bss	0.13 ± 0.01	1.10 ± 0.12	0.19 ± 0.02	12 ± 1	0.46 ± 0.07
	Bso	0.14 ± 0.01	0.96 ± 0.05	0.16 ± 0.01	11 ± 1	0.43 ± 0.04
	p-value	*0.53*	*0.38*	*0.31*	*0.69*	*0.68*
	Fnc	0.15 ± 0.01	1.05 ± 0.11	0.18 ± 0.02	11 ± 1	0.46 ±0.07
	Fss	0.14 ± 0.01	1.09 ± 0.08	0.18 ± 0.01	12 ± 1	0.44 ± 0.05
	Fso	0.13 ± 0.01	0.89 ± 0.11	0.16 ± 0.02	11 ± 1	0.38 ± 0.07
	P-value	*0.38*	*0.19*	*0.53*	*0.71*	*0.46*
	Bdiet x Fdiet	*0.92*	*0.08*	***<0.01***	*0.07*	*0.24*
Before vs after stress	before	0.32 ± 0.01^{a}	1.22 ± 0.05^{a}	0.26 ± 0.01^{a}	14 ± 0^{a}	0.83 ± 0.08^{a}
	after	0.14 ± 0.01^{b}	1.01 ± 0.05^{b}	0.17 ± 0.01^{b}	11 ± 0^{b}	0.43 ± 0.03^{b}
	P-value	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***

Values are means ± SEM (n=9 for fed and stressed fry and n=27 for fed vs stressed fry). Within columns and groups values not sharing a common superscript letter are significantly different according to one- or two-way ANOVA followed by TukeyHSD.
Homocysteine (Hcys); cysteine (Cys); cysteinyl-glycine (CysGly); Glutathoine (GSH); γ-glutamyl-cysteine (GluCys)

3.2. B vitamin levels

Vitamin B6, folate or vitamin B12 were not significantly affected by parental or direct Se nutrition, whether before or after stress (Table 4). Pyridoxine and pyridoxamine as well as folate decreased significantly in stressed fish, while vitamin B12 concentrations remained stable.

Table 4. Concentration of B vitamins [µg/kg fresh weight] measured in whole fry originating from parents subjected to different Se treatments (Bnc, Bss, Bso) and then fed diets containing different levels and sources of Se (Fnc, Fss, Fso) before and after exposed to hypoxic stress

		Pyridoxamine	Pyridoxal	Folate	Cobalamin
Before stress	Bnc	0.37 ± 0.01	1.00 ± 0.02	0.45 ± 0.03	0.01 ± 0.00
	Bss	0.38 ± 0.02	1.01 ± 0.02	0.38 ± 0.03	0.01 ± 0.00
	Bso	0.38 ± 0.02	1.07 ± 0.03	0.37 ± 0.03	0.01 ± 0.00
	p-value	0.87	0.17	0.10	0.57
	Fnc	0.38 ± 0.01	1.03 ± 0.02	0.41 ± 0.03	0.01 ± 0.00
	Fss	0.39 ± 0.01	1.03 ± 0.03	0.41 ± 0.04	0.01 ± 0.00
	Fso	0.37 ± 0.02	1.02 ± 0.03	0.39 ± 0.02	0.01 ± 0.00
	p-value	0.61	0.94	0.88	0.23
	Bdiet x Fdiet	0.52	0.68	0.35	0.86
After stress	Bnc	0.34 ± 0.01	0.91 ± 0.02	0.24 ± 0.02	0.01 ± 0.00
	Bss	0.36 ± 0.01	0.88 ± 0.03	0.28 ± 0.02	0.01 ± 0.00
	Bso	0.36 ± 0.01	0.88 ± 0.03	0.24 ± 0.03	0.01 ± 0.00
	p-value	0.32	0.17	0.43	0.84
	Fnc	0.35 ± 0.01	0.91 ± 0.02	0.22 ± 0.02	0.01 ± 0.00
	Fss	0.35 ± 0.01	0.87 ± 0.02	0.26 ± 0.03	0.01 ± 0.00
	Fso	0.35 ± 0.01	0.89 ± 0.03	0.28 ± 0.03	0.01 ± 0.00
	p-value	0.97	0.94	0.19	0.54
	Bdiet x Fdiet	0.70	0.68	0.25	0.98
Before vs after stress	before	0.38 ± 0.01[a]	1.03 ± 0.01[a]	0.40 ± 0.02[a]	0.01 ± 0.00
	after	0.35 ± 0.01[b]	0.89 ± 0.01[b]	0.25 ± 0.01[b]	0.01 ± 0.00
	p-value	**<0.01**	**<0.01**	**<0.01**	0.06

Values are means ± SEM (n=9 for fed and stressed fry and n=27 for fed vs stressed fry). Within columns and groups values not sharing a common superscript letter are significantly different according to one- or two-way ANOVA followed by TukeyHSD.

3.3. SAM and SAH contents

SAM and SAH concentrations were not significantly affected by parental or direct Se nutrition, whether before or after stress (Table 5). The SAM/SAH ratio was decreased in fry by the hypoxic stress challenge related to a significant decrease of SAM.

Table 5. SAM and SAH [nmol/g sample] measured in whole fry originating from parents subjected to different Se treatments (Bnc, Bss, Bso) and then fed diets containing different levels and sources of Se (Fnc, Fss, Fso) before and after exposed to hypoxic stress

		SAM	SAH	SAM/SAH
Before stress	Bnc	18 ± 1	3.6 ± 0.3	5.1 ± 0.2
	Bss	16 ± 1	3.7 ± 0.3	4.5 ± 0.2
	Bso	16 ± 1	3.6 ± 0.4	4.5 ± 0.3
	p-value	*0.42*	*0.97*	*0.09*
	Fnc	15 ± 1	3.1 ± 0.2	5.1 ± 0.3
	Fss	16 ± 1	3.8 ± 0.3	4.4 ± 0.3
	Fso	18 ± 2	4.0 ± 0.3	4.6 ± 0.1
	P-value	*0.32*	*0.10*	*0.08*
	Bdiet x Fdiet	*0.91*	*0.29*	*0.09*
After stress	Bnc	13 ± 1	4.0 ± 0.5	3.6 ± 0.4
	Bss	14 ± 1	4.1 ± 0.3	3.4 ± 0.3
	Bso	15 ± 1	4.0 ± 0.4	3.9 ± 0.5
	p-value	*0.74*	*0.98*	*0.73*
	Fnc	14 ± 1	4.2 ± 0.5	3.8 ± 0.5
	Fss	15 ± 1	4.2 ± 0.5	3.6 ± 0.4
	Fso	13 ± 1	3.7 ± 0.4	3.6 ± 0.3
	P-value	*0.52*	*0.57*	*0.96*
	Bdiet x Fdiet	*0.68*	*0.40*	*0.65*
Before vs after stress	before	17 ± 1[a]	3.6 ± 0.2	4.7 ± 0.1[a]
	after	14 ± 1[b]	4.1 ± 0.2	3.7 ± 0.2[b]
	P-value	***0.01***	*0.12*	***<0.01***

Values are means ± SEM (n=9 for fed and stressed fry and n=27 for fed vs stressed fry). Within columns and groups values not sharing a common superscript letter are significantly different according to one- or two-way ANOVA followed by TukeyHSD.

3.4. Gene expression

Pre-stress, the expression of a majority of the analyzed genes were not significantly affected by parental or direct Se nutrition (Table 6). However, *cgl* mRNA levels were significantly higher in fry fed one of the Se-supplemented diets compared to fry fed Fnc. In addition, an interactive effect was found for *bhmt*, which was especially reduced in fry fed OH-SeMet (Fso), when originating from parents fed Se-supplemented diets (Bss or Bso) (Figure 1A).

Table 6. Relative gene expression of 1C genes in whole fry originating from parents subjected to different Se treatments (Bnc, Bss, Bso) and then fed diets containing different levels and sources of Se (Fnc, Fss, Fso) before and after exposed to hypoxic stress

	Parental effect (PE)			Direct feeding effect (FE)			p-values		
	Bnc	Bss	Bso	Fnc	Fss	Fso	PE	FE	PExFE
Before stress									
cbs	1.7 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	0.12	0.39	0.13
cgl	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.1^{a}	0.9 ± 0.0^{b}	1.0 ± 0.1^{b}	0.82	**0.01**	0.40
mtr	1.5 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	0.26	0.23	0.05
bhmt	1.2 ± 0.1^{a}	1.1 ± 0.1^{b}	1.0 ± 0.0^{b}	1.2 ± 0.1^{a}	1.1 ± 0.1^{ab}	0.9 ± 0.0^{b}	**<0.01**	**<0.01**	**<0.01**
amd1a	1.3 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	0.10	0.69	0.59
amd1b	1.7 ± 0.2	1.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.6 ± 0.2	1.5 ± 0.1	0.51	0.89	0.48
gnmt	1.7 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	0.52	0.22	0.78
dnmt1	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.2	0.46	0.72	0.22
sahh	1.1 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	0.52	0.33	0.89
After stress									
cbs	2.2 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.0 ± 0.1	0.71	0.08	0.48
cgl	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.0	0.89	0.64	0.97
mtr	1.6 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	0.39	0.45	0.18
bhmt	0.9 ± 0.0^{a}	0.8 ± 0.0^{b}	0.8 ± 0.0^{ab}	0.8 ± 0.0	0.9 ± 0.0	0.8 ± 0.0	**0.04**	0.38	0.78
amd1a	1.7 ± 0.1^{a}	1.4 ± 0.1^{b}	1.4 ± 0.1^{b}	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	**<0.01**	0.33	**0.04**
amd1b	1.8 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	1.5 ± 0.1	0.08	0.07	0.31
gnmt	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.0	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	0.14	0.81	**0.05**
dnmt1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.92	0.86	0.84
sahh	0.9 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.9 ± 0.0	0.44	0.71	0.19

Values are means ± SEM (n=27). Within columns and groups values not sharing a common superscript letter are significantly different according to two-way ANOVA followed by TukeyHSD.

The genes *gnmt*, *bhmt* and *sahh* were significantly lower expressed in fry post-exposure compared to pre-exposure to the hypoxic stress challenge (Figure 2). In contrast, *amd1a*, *mtr* and *cbs* were significantly higher expressed in stressed compared to non-stressed fry. The genes *dnmt1*, *amd1b* and *cgl* showed no significant difference according to the hypoxic stress challenge.

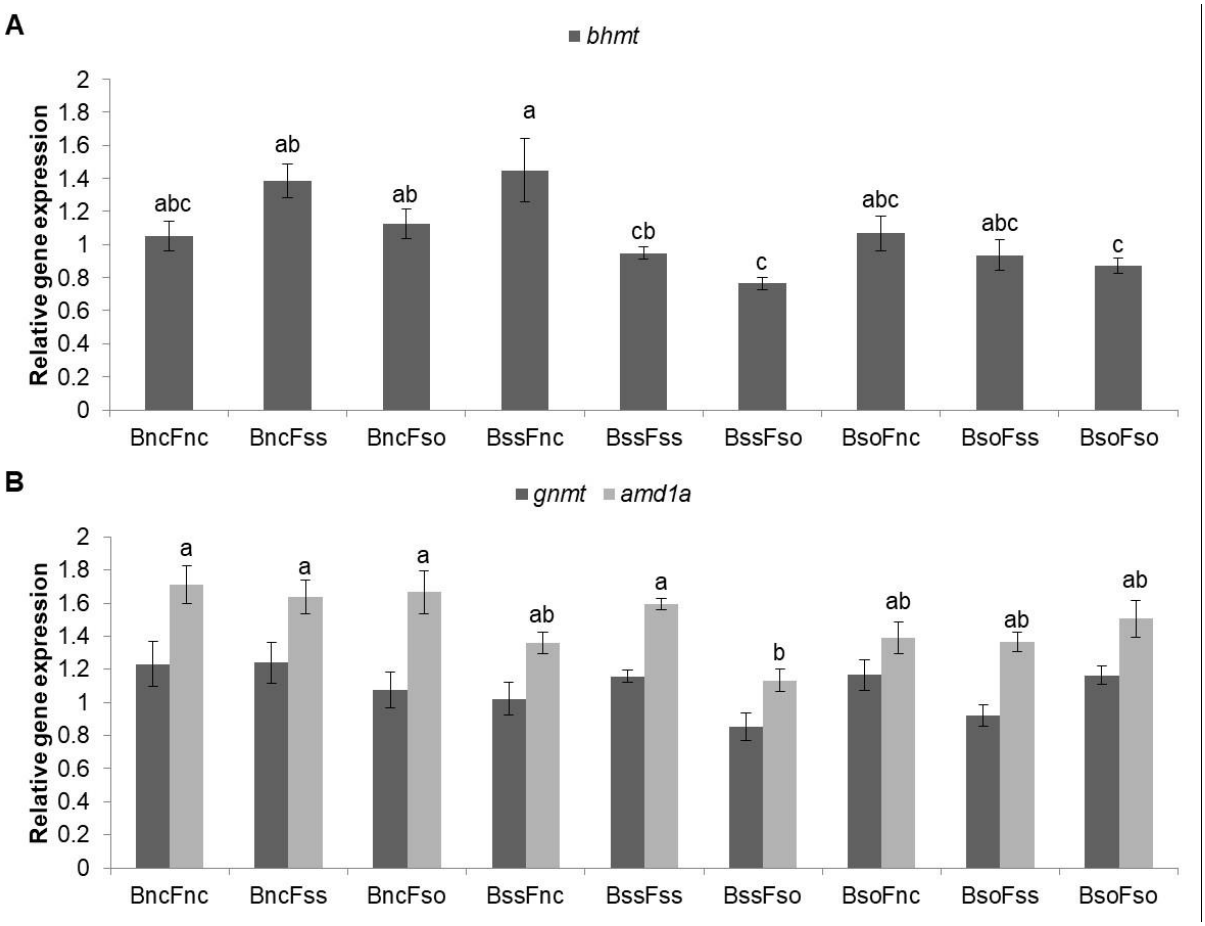

Figure 1. Interactive effect of parental and direct Se nutrition on the relative expression of *bhmt* in fry before stress (A) and *gnmt* and *amd1a* in stressed fry (B). Gene expression was normalized to β-actin and ef1α and expressed as fold-changes of mRNA abundance compared to a sample of fry from the BncFnc treatment before stress. Bars represent mean ± SEM (n=9). Means not sharing a common superscript letter are significant different according to two-way ANOVA followed by Tukey's HSD post hoc test.

Post-stress, a significant main effect was found on *bhmt* for parental feeding with higher expression in Bnc compared to Bss (Table 6). A significant interaction between parental and direct feeding effects was found for *amd1a* and *gnmt* in stressed fry that was illustrated in Figure 1B. If these two genes exhibited the same pattern of expression, in the case of *gnmt*, the Tukey's HSD post hoc test did not allow discriminating the groups contrary to *amd1a.* The genes were lower expressed in BssFso compared to fry originating from rainbow trout fed Bnc, irrespective of their fry diet.

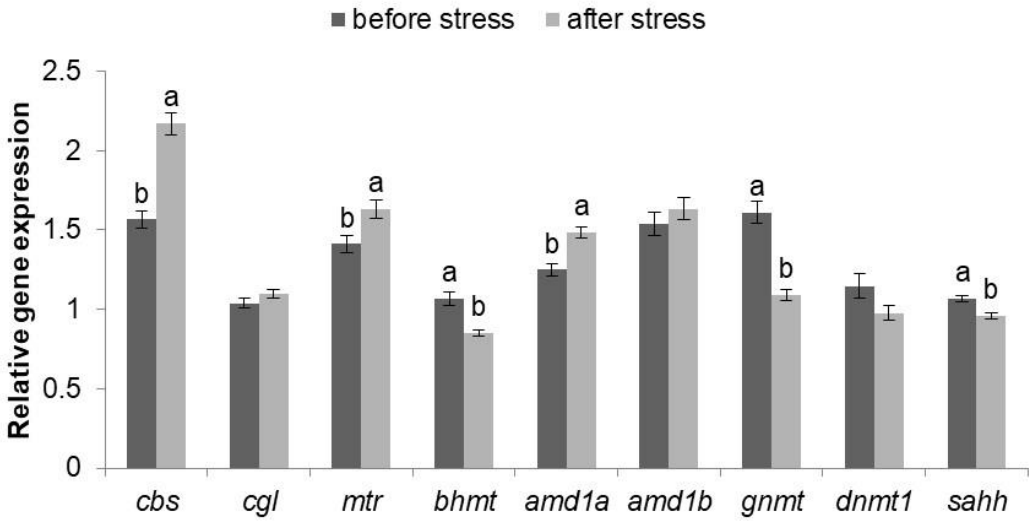

Figure 2. Effect of hypoxic stress on the relative gene expression of C1 genes. Gene expression was normalized to β-actin and ef1α and expressed as fold-changes of mRNA abundance compared to a sample of fry from the BncFnc treatment before stress. Bars represent mean ± SEM (n=81). Means not sharing a common superscript letter are significant different according to one-way ANOVA followed by Tukey's HSD post hoc test.

3.5. Muscle free amino acids

Fry sampled before stress displayed lower alanine and sarcosine concentrations when originating from parents fed Bso compared to Bnc (Table 7). For alanine, decreasing concentrations were also detected in the Bss treatment. Glycine and alpha-amino-n-butyric acid were higher in Bso compared to Bss. A direct feeding effect was detected for arginine, isoleucine, leucine, threonine, valine and DL-beta-aminoisobutyric acid with lower concentrations in Fso group compared to Fnc and Fss treatments.

Table 7. Essential and non-essential amino acids [µg/g sample] in muscle of fry originating from parents subjected to different Se treatments (Bnc, Bss, Bso) and then fed diets containing different levels and sources of Se (Fnc, Fss, Fnc)

	Parental effect (PE)			Direct feeding effect (FE)			p-values		
	Bnc	Bss	Bso	Fnc	Fss	Fso	PE	FE	PExFE
EAA	*779 + 16*	*777 + 34*	*798 + 20*	*809 + 23*	*793 + 19*	*755 + 20*	*0.78*	*0.26*	*0.95*
Arg	30 + 2	30 + 2	29 + 2	31 + 2^{a}	32 + 1^{a}	26 + 1^{b}	0.79	**0.02**	0.59
His	579 + 12	559 + 25	590 + 11	577 + 15	574 + 16	579 + 17	0.47	0.96	0.84
Iso	11 + 1	12 + 1	12 + 1	12 + 1^{a}	12 + 0^{a}	10 + 0^{b}	0.80	**0.03**	0.56
Leu	24 + 1	25 + 1	26 +1	26 + 2^{ab}	27 + 1^{a}	21 + 1^{b}	0.83	**0.02**	0.61
Lys	19 + 3	31 + 7	19 + 4	29 + 7	20 + 4	18 + 3	0.19	0.31	0.70
Met	15 + 1	14 +1	15 + 1	15 + 1	15 + 1	14 + 1	0.41	0.46	0.36
Phe	11 + 0	11 + 0	12 + 0	12 + 0	12 + 0	10 + 0	0.60	0.07	0.69
Thr	70 + 4	75 + 7	76 + 7	84 + 6^{a}	78 + 2^{a}	60 + 3^{b}	0.62	**<0.01**	0.46
Val	20 + 1	20 + 2	21 + 2	22 + 2^{a}	22 + 1^{a}	17 + 1^{b}	0.78	**0.04**	0.60
NEAA	*2983 + 81*	*2786 + 58*	*2875 + 105*	*2900 + 110*	*2871 + 48*	*2881 + 89*	*0.36*	*0.96*	*0.83*
Ala	268 + 8^{a}	236 + 17^{b}	240 + 10^{b}	248 + 7	260 + 11	238 + 15	**0.01**	0.17	**<0.01**
Asn	71 + 30	63 + 17	92 + 27	76 + 34	69 + 24	82 + 15	0.75	0.94	0.52
Asp	85 + 5	82 + 8	89 + 8	89 + 8	92 + 5	77 + 7	0.80	0.37	0.62
bAla	99 + 7	82 + 13	84 + 5	93 + 6	93 + 9	81 + 10	0.18	0.42	0.05
Cit	6 + 0	5 + 0	6 + 0	6 + 0	6 + 0	6 + 0	0.11	0.34	0.13
Gln	68 + 7	65 + 8	69 + 6	64 + 6	66 + 7	72 + 7	0.94	0.76	0.83
Glu	123 + 7	101 + 9	104 + 5	104 + 5	115 + 9	111 + 8	0.09	0.57	0.70
Gly	390 + 19^{ab}	367 + 18^{b}	439 + 13^{a}	394 + 19	402 + 21	404 + 17	**0.03**	0.92	0.98
Hyp	62 + 3	52 + 3	62 + 3	58 + 4	62 + 3	56 + 3	0.10	0.64	0.96
Orn	5 + 0	4 + 0	5 + 0	5 + 1	5 + 0	4 + 0	0.85	0.34	0.76
Pro	154 + 21	111 + 18	133 + 23	130 + 22	163 + 24	111 +13	0.41	0.31	0.99
Ser	141 + 9	141 + 9	148 + 11	140 + 12	152 + 4	139 + 11	0.84	0.67	0.91
Tau	1501 + 54	1467 + 37	1393 + 59	1483 + 53	1375 + 28	1492 + 62	0.35	0.28	0.79
Tyr	10 + 0	10 + 1	11 + 0	11 + 0	11 + 0	10 + 0	0.30	0.14	0.66
Others	*2598 + 85*	*2774 + 115*	*2631 +68*	*2660 + 65*	*2783 + 120*	*2562 + 58*	*0.23*	*0.11*	*0.12*
AABA	4 + 0^{ab}	4 + 0^{b}	5 + 0^{a}	5 + 0	5 +1	4 + 0	**0.02**	0.26	0.77
NH4Cl	104 + 3	100 + 2	103 + 2	101 + 2	106 + 2	101 + 2	0.46	0.21	0.56
BAA	7 + 0	7 + 0	7 + 0	7 + 0^{a}	7 + 0^{ab}	6 + 0^{b}	0.08	**0.01**	0.09
Ans	1021 + 26	1015 + 31	1077 + 30	1028 + 26	1026 + 36	1060 + 26	0.31	0.67	0.91
Car	148 + 5	133 + 10	154 + 10	144 + 11	152 + 8	141 + 8	0.30	0.78	0.50
Cysta	16 + 2	16 + 2	21 + 3	18 + 3	20 + 2	15 + 2	0.32	0.32	0.98
1MHis	19 + 3	17 + 3	11 + 3	15 + 3	17 + 4	16 + 3	0.10	0.81	0.19
PEA	14 + 1	15 + 1	14 + 1	14 + 1	14 + 1	14 + 1	0.34	0.86	0.13
Sar	15 + 1^{a}	14 + 1^{ab}	11 + 1^{b}	13 + 1	13 + 1	14 +1	**0.04**	0.69	0.93
Urea	1250 + 81	1453 + 109	1228 + 70	1315 + 67	1422 + 121	1190 + 56	0.07	0.07	0.11

Values are means ± SEM (n=9). Within columns and groups values not sharing a common superscript letter are significantly different according to two-way ANOVA followed by TukeyHSD.

Essential amino acids (EAA); Non-essential amino acids (NEAA); Arginine (Arg); Histidine (His); Isoleucine (Iso); Leucine (Leu); Lysine (Lys); Phenylalanine (Phe); Threonine (Thr); Valine (Val); Alanine (Ala); Aspartic acid (Asp); bAla (b-Alanine); Cit (Citrulline); Glutamine (Gln); Glutamic acid (Glu);Glycine (Gly); Hydroxy-L-Proline (Hyp); Ornithine (Orn); Proline (Pro); Serine (Ser); Taurin (Tau); Tyrosine (Tyr); L-alfa-amino-n-butyric acid (AABA); DL-beta-aminoisobutyric acid (BAA); Anserine (Ans); Carnosine (Car); Cystathionine (Cysta); 1-Methyl-L-Histidine (1MHis); O-Phosphoethanolamine (PEA); Sarcosine (Sar)

During the hypoxic stress challenge, the sum of essential amino acids significantly decreased in the muscle of fry (Figure 3A) including arginine, lysine, methionine, threonine and valine, besides asparagine and aspartic acid (Figure 3B). An opposing effect with a significant increase during the stress was detected for isoleucine and leucine (Figure 3B). On the other hand, the sums of non-essential amino acids and N-metabolites in muscle were not detected to significantly change during stress (Figure 3A). Nevertheless, beta-alanine, hydroxy-proline, ornithine, serine, alpha-amino-n-butyric acid, carnosine, cystathionine, sarcosine and urea contents were significantly lower in stressed fry, while anserine concentration was higher in stressed compared to non-stressed fry (Figure 3B).

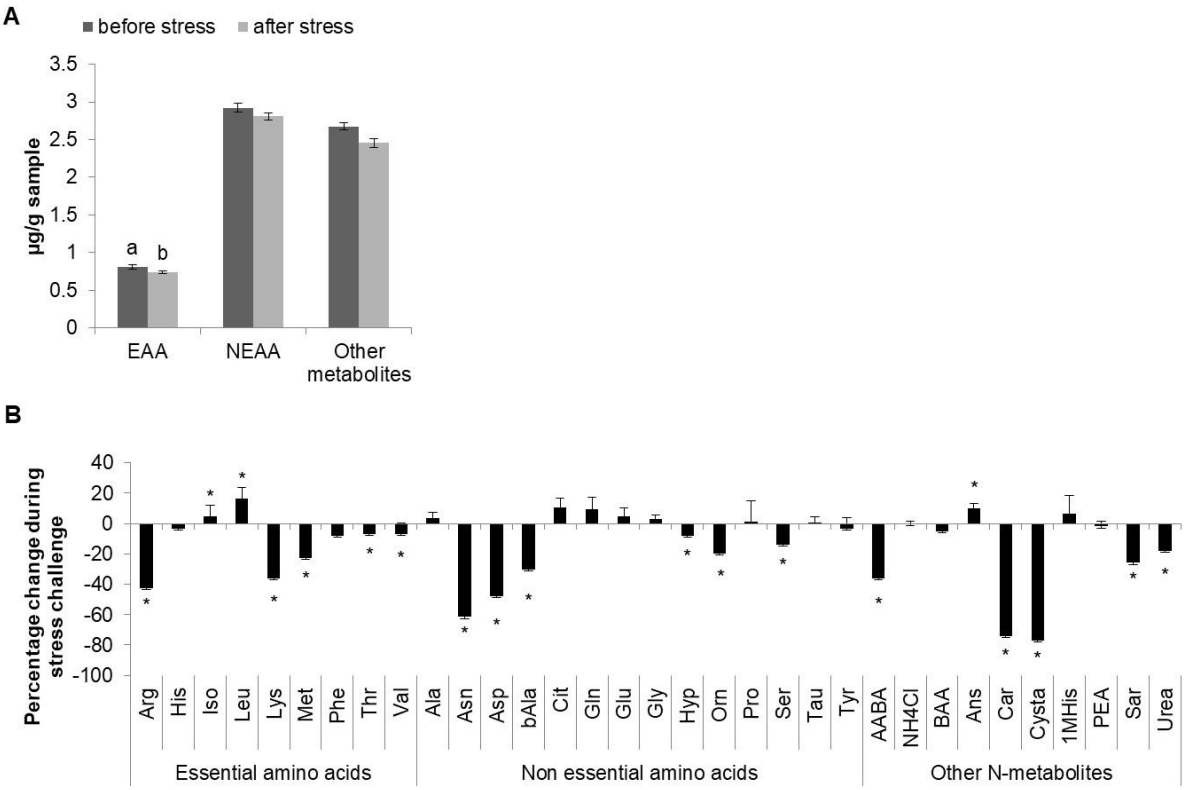

Figure 3. Concentrations of essential free amino acids (EAA), non-essential amino acids (NEAA) and other N-metabolites in muscle filet of fry before and after exposed to hypoxic stress (A). Percentage change in fillet concentration free amino acids during the hypoxic stress challenge (B). Stars represent a significant increase or decrease in the metabolite assessed by one-way ANOVA.
Arginine (Arg); Histidine (His); Isoleucine (Iso); Leucine (Leu); Lysine (Lys); Phenylalanine (Phe); Threonine (Thr); Valine (Val); Alanine (Ala); Aspartic acid (Asp); bAla (b-Alanine); Cit (Citrulline); Glutamine (Gln); Glutamic acid (Glu);Glycine (Gly); Hydroxy-L-Proline (Hyp); Ornithine (Orn); Proline (Pro); Serine (Ser); Taurin (Tau); Tyrosine (Tyr); L-alfa-amino-n-butyric acid (AABA); DL-beta-aminoisobutyric acid (BAA); Anserine (Ans); Carnosine (Car); Cystathionine (Cysta); 1-Methyl-L-Histidine (1MHis); O-Phosphoethanolamine (PEA); Sarcosine (Sar)

In stressed fry, parental effects were found for aspartic acid and sarcosine with the highest concentrations in the Bss treatment and DL-beta-amino-isobutyric acid with the highest concentration in the Bso treatment, while B-alanine content was the highest in Bnc (Table 8). Fry sampled after stress showed lower leucine and threonine levels when fed Fso compared to Fnc, similarly to pre-stressed fry. Likewise in stressed fry, also aspartic acid, ornithine and proline were significantly lower in Fso compared to Fnc, while valine was higher in Fnc compared to Fss (Table 8).

Table 8. Essential and non-essential amino acids [µg/g sample] in muscle of fry originating from parents subjected to different Se treatments (Bnc, Bss, Bso) and then fed diets containing different levels and sources of Se (Fnc, Fss, Fso)

	Parental effect (PE)			Direct feeding effect (FE)			p-values		
	Bnc	Bss	Bso	Fnc	Fss	Fso	PE	FE	PExFE
EAA	*779 + 16*	*777 + 34*	*798 + 20*	*809 + 23*	*793 + 19*	*755 + 20*	*0.78*	*0.26*	*0.95*
Arg	30 + 2	30 + 2	29 + 2	31 + 2^{a}	32 + 1^{a}	26 + 1^{b}	0.79	**0.02**	0.59
His	579 + 12	559 + 25	590 + 11	577 + 15	574 + 16	579 + 17	0.47	0.96	0.84
Iso	11 + 1	12 + 1	12 + 1	12 + 1^{a}	12 + 0^{a}	10 + 0^{b}	0.80	**0.03**	0.56
Leu	24 + 1	25 + 1	26 +1	26 + 2^{ab}	27 + 1^{a}	21 + 1^{b}	0.83	**0.02**	0.61
Lys	19 + 3	31 + 7	19 + 4	29 + 7	20 + 4	18 + 3	0.19	0.31	0.70
Met	15 + 1	14 +1	15 + 1	15 + 1	15 + 1	14 + 1	0.41	0.46	0.36
Phe	11 + 0	11 + 0	12 + 0	12 + 0	12 + 0	10 + 0	0.60	0.07	0.69
Thr	70 + 4	75 + 7	76 + 7	84 + 6^{a}	78 + 2^{a}	60 + 3^{b}	0.62	**<0.01**	0.46
Val	20 + 1	20 + 2	21 + 2	22 + 2^{a}	22 + 1^{a}	17 + 1^{b}	0.78	**0.04**	0.60
NEAA	*2983 + 81*	*2786 + 58*	*2875 + 105*	*2900 + 110*	*2871 + 48*	*2881 + 89*	*0.36*	*0.96*	*0.83*
Ala	268 + 8^{a}	236 + 17^{b}	240 + 10^{b}	248 + 7	260 + 11	238 + 15	**0.01**	0.17	**<0.01**
Asn	71 + 30	63 + 17	92 + 27	76 + 34	69 + 24	82 + 15	0.75	0.94	0.52
Asp	85 + 5	82 + 8	89 + 8	89 + 8	92 + 5	77 + 7	0.80	0.37	0.62
bAla	99 + 7	82 + 13	84 + 5	93 + 6	93 + 9	81 + 10	0.18	0.42	0.05
Cit	6 + 0	5 + 0	6 + 0	6 + 0	6 + 0	6 + 0	0.11	0.34	0.13
Gln	68 + 7	65 + 8	69 + 6	64 + 6	66 + 7	72 + 7	0.94	0.76	0.83
Glu	123 + 7	101 + 9	104 + 5	104 + 5	115 + 9	111 + 8	0.09	0.57	0.70
Gly	390 + 19^{ab}	367 + 18^{b}	439 + 13^{a}	394 + 19	402 + 21	404 + 17	**0.03**	0.92	0.98
Hyp	62 + 3	52 + 3	62 + 3	58 + 4	62 + 3	56 + 3	0.10	0.64	0.96
Orn	5 + 0	4 + 0	5 + 0	5 + 1	5 + 0	4 + 0	0.85	0.34	0.76
Pro	154 + 21	111 + 18	133 + 23	130 + 22	163 + 24	111 +13	0.41	0.31	0.99
Ser	141 + 9	141 + 9	148 + 11	140 + 12	152 + 4	139 + 11	0.84	0.67	0.91
Tau	1501 + 54	1467 + 37	1393 + 59	1483 + 53	1375 + 28	1492 + 62	0.35	0.28	0.79
Tyr	10 + 0	10 + 1	11 + 0	11 + 0	11 + 0	10 + 0	0.30	0.14	0.66
Others	*2505 + 60*	*2487 + 171*	*2369 + 81*	*2426 + 143*	*2463 + 88*	*2470 + 70*	*0.61*	*0.95*	*0.37*
AABA	4 + 0^{ab}	4 + 0^{b}	5 + 0^{a}	5 + 0	5 +1	4 + 0	**0.02**	0.26	0.77
NH4Cl	104 + 3	100 + 2	103 + 2	101 + 2	106 + 2	101 + 2	0.46	0.21	0.56
BAA	7 + 0	7 + 0	7 + 0	7 + 0^{a}	7 + 0^{ab}	6 + 0^{b}	0.08	**0.01**	0.09
Ans	1021 + 26	1015 + 31	1077 + 30	1028 + 26	1026 + 36	1060 + 26	0.31	0.67	0.91
Car	148 + 5	133 + 10	154 + 10	144 + 11	152 + 8	141 + 8	0.30	0.78	0.50
Cysta	16 + 2	16 + 2	21 + 3	18 + 3	20 + 2	15 + 2	0.32	0.32	0.98
1MHis	19 + 3	17 + 3	11 + 3	15 + 3	17 + 4	16 + 3	0.10	0.81	0.19
PEA	14 + 1	15 + 1	14 + 1	14 + 1	14 + 1	14 + 1	0.34	0.86	0.13
Sar	15 + 1^{a}	14 + 1^{ab}	11 + 1^{b}	13 + 1	13 + 1	14 +1	**0.04**	0.69	0.93
Urea	1250 + 81	1453 + 109	1228 + 70	1315 + 67	1422 + 121	1190 + 56	0.07	0.07	0.11

Values are means ± SEM (n=9). Within columns and groups values not sharing a common superscript letter are significantly different according to two-way ANOVA followed by TukeyHSD.

Essential amino acids (EAA); Non-essential amino acids (NEAA); Arginine (Arg); Histidine (His); Isoleucine (Iso); Leucine (Leu); Lysine (Lys); Phenylalanine (Phe); Threonine (Thr); Valine (Val); Alanine (Ala); Aspartic acid (Asp); bAla (b-Alanine); Cit (Citrulline); Glutamine (Gln); Glutamic acid (Glu);Glycine (Gly); Hydroxy-L-Proline (Hyp); Ornithine (Orn); Proline (Pro); Serine (Ser); Taurin (Tau); Tyrosine (Tyr); L-alfa-amino-n-butyric acid (AABA); DL-beta-aminoisobutyric acid (BAA); Anserine (Ans); Carnosine (Car); Cystathionine (Cysta); 1-Methyl-L-Histidine (1MHis); O-Phosphoethanolamine (PEA); Sarcosine (Sar)

4. Discussion

4.1. Impact of parental selenium nutrition on the 1C metabolism in rainbow trout fry

In the present study, we found that parental Se nutrition increased the levels of metabolites of the transsulfuration pathway in fry. This includes cysteine, cysteinyl-glycine and GSH, but also γ-glutamyl-cysteine an intermediate for the recovery of cysteine from GSH without impacting mRNA levels of transsulfuration enzymes. This effect is opposite to the parental Se effect that we previously described at swim-up fry stage where cysteine and cysteinyl-glycine levels were decreased by parental OH-SeMet nutrition[9]. It has been shown in mammalian and avian studies that Se supplementation favors the transsulfuration of Hcys with higher cystathionine and cysteine levels[22,23], while in suboptimal Se conditions the transsulfuration can be severely impaired[15]. In the present study, betaine-homocysteine S-methyltransferase (BHMT*)* expression was reduced in fry originating from parents that received Se-supplemented diets, but only when similarly fed Se-supplemented diets. This interactive effect indicates that Se nutrition can have long-term consequences on *bhmt* mRNA levels and therefore possibly on the re-methylation of Hcys to Met. A parental effect on *bhmt* expression or DNA methylation pattern was not detected at swim-up fry stage suggesting that the parental effect relates to the further inclusion of dietary Se at first feeding suggesting that epigenetic modifications work in a time and spatial dimension that might underlay nutritional programming effects[9].

4.2. Impact of direct selenium feeding on the 1C metabolism in rainbow trout fry

In contrast to the parental Se effect, we found that direct Se nutrition decreased mRNA levels of the second step transsufuration enzyme *cgl* without impacting the metabolite levels. The enzyme cystathionine gamma-lyase (CGL) catalyzes the reaction from cystathionine to form cysteine and is similarly active on selenocystathionine to form selenocysteine in the

SeMet metabolism[24]. Therefore, it is surprising that Se supplementation reduced mRNA expression of *cgl* in the present study. Previously, in male and female liver tissue of rainbow trout broodstock, no effect of Se feeding on *cgl* mRNA expression was detected[9]. However, CGL has been described to be regulated post-transcriptionally by androgens and estrogen sex hormones that result in tissue specific CGL activity, a mechanism that has been suggested to account for sexual dimorphism in selenoprotein production[25], but might also regulate the CGL activity throughout the lifecycle. A post transcriptional regulation of CGL is also known during early embryonic development in mammals, where the enzyme is expressed but inactive[26], which has been suggested to favor the coincidental incorporation of SeMet into proteins rather than the active selenoprotein production from SeMet during early embryonic development[27]. Nevertheless, in the present study Se feeding had no impact on Hcys levels in whole fry, which has been previously described to decrease in blood plasma in accordance with increased transsulfuration by Se treatment[16,28]. However, Geillinger (13) described higher hepatic Hcys levels in Se deficient mice as a tissue specific effect under limiting Se conditions. Hcys has been shown to be produced, but translocated from the liver to the plasma possibly avoiding re-methylation of Hcys through the 1C metabolism due to limiting Se conditions[29]. Such effects might have been masked in the present study, where Hcys levels were determined in whole-body fry or because the dietary Se level was not enough low in the control treatment. Also, we found reduced *bhmt* mRNA levels in the OH-SeMet supplemented treatment compared to the control thus inhibition of BHMT can be associated with hyperhomocysteinemia[30]. However, the increase of transsfulfuration enzymes by Se possibly pulled Hcys towards the transsfulfuration pathway resulting in the unchanged Hcys levels as similarly suggested in rats[16,28,31].

4.3. Impact of acute hypoxic stress on the 1C metabolism in rainbow trout fry

In response to the acute hypoxic stress we found decreased aminothiol concentrations in rainbow trout fry. GSH is a major contributor in the cellular redox state that detoxifies reactive oxygen species by oxidation. Therefore, oxidative stress typically involves an

alteration of the intra and extra-cellular thiol concentrations[32,33]. The observed decrease of GSH under acute hypoxia is in line with the study of Pérez-Jiménez[34], who found lower liver GSH in gilthead sea bream after exposure to hypoxic conditions for 6h. On the other hand, in common carp, 6h exposure to hypoxia was not associated with changes in GSH[35]. Such differences might be species specific[36,37] or relate to differences in study design. In the present study, the similar decrease of Hcys, cysteine, cysteinyl-glycine and free muscle cystathionine as well as increased mRNA levels of the cystathionine beta-synthase (CBS) gene might suggest that an elevated GSH production via transsulfuration was impaired by the depletion of GSH precursors. Also in human glioblastoma and rat phaechromocytoma cells, hypoxia stimulated CBS gene expression and activity mediated through the hypoxia inducible factor[38] and in human brain the CBS-mediated cysteine limitation resulted in neurological deficits under hypobaric hypoxia[39]. On the contrary, in the liver of developing and neonatal rats, CBS activity was decreased by hypoxic stress[40,41], however, without any changes in mRNA levels. This was possibly related to the observed decrease in SAM levels as SAM is a well-known regulator of CBS[42]. A decrease of SAM during hypoxia was also found in other mammalian studies[43–45], similar to our present findings in rainbow trout fry. In hepatocytes of male rats, ATP-dependent SAM formation was identified as a rate-limiting step in GSH synthesis under limiting oxygen conditions, while GSH formation from cysteine was not impaired[46]. Together with other genes in the amino acid metabolism, transcriptomic analysis found an upregulation of S-adenosylmethionine synthase under hypoxic conditions in liver and muscle of fish[47,48]. Similar to the present results, decreasing Met levels under hypoxia have been described in various organisms[49–51]. The decrease of many free essential amino acids in muscle together with the decrease of asparagine and aspartic acid levels revealed an active stimulation of gluconeogenesis from amino acids in muscle. The branched chain amino acids are, however, preserved in stressed fry demonstrating that the ketogenic pathway is not involved in neoglucogenesis. Furthermore, the large decrease in sarcosine and beta-alanine and the increase in anserine in stressed fish suggest alterations in buffering capacity of muscle tissue. This could be related to the general decrease of pH

observed in stressed fish[52], but the increase in anserine content suggests that other mechanisms could be involved. However, the transcriptomic response of liver aminotransferases in fish to hypoxia was also found to be dynamic during exposure time[47].

4.4. Impact of selenium on the 1C metabolism in stressed rainbow trout fry

The detected modifications of the transsulfuration pathway by parental Se in fry were not detected anymore after application of the acute hypoxic stress. It could be speculated that the impact was masked by the strong decrease of these metabolites during hypoxia. However, similar to normoxic fry, in stressed fry *bhmt* mRNA levels were lower when originating from broodstock fed the inorganic Se-supplemented diet Bss. This persistent effect by parental Se suggests a strong nutritional programming effect on the methionine cycling. Although, in our previous study, we found no differences in whole-body expression or hepatic DNA methylation pattern of the *bhmt* gene, other genes of the 1C metabolism including *mtr* and *amd1b* were upregulated in swim-up fry in response to parental Se nutrition[9]. In the present study, stressed fry of the BssFso treatment had lower S-adenosylmethionine decarboxylase (AMD) expression compared to fry originating from the broodstock control group Bnc. This might indicate that parental inorganic Se feeding could inhibit polyamine synthesis under stressful conditions. Polyamine synthesis has been formerly described to be increased by both acute hypoxia[53] as well as Se treatment[54]. Similarly, also glycine N-methyltransferase (GNMT) expression showed interactive effects between direct and parental Se nutrition in stressed fry. GNMT converts SAM to SAH, while generating sarcosine from glycine[55]. Even though the post hoc test could not discriminate between treatments for *gnmt* mRNA levels, the highest expression was related to fry originating from the broodstock control group and the lowest for fry fed differing Se forms compared to their parents. The fact that two dietary Se forms exert different effects on the sarcosine metabolism was also suggested by sarcosine levels that were higher in fry

originating from parents fed sodium selenite compared to fry originating from the hydroxy-selenomethionine treatment. GNMT activity and sarcosine levels can relate to changes in DNA methylation pattern, which are underlying epigenetic mechanisms in nutritional programming effects[56,57]. Therefore, the long-term impact of parental Se on polyamine synthesis and the impact of inorganic and organic Se forms on the sarcosine metabolism in fish might deserve further investigation.

5. Conclusion

In rainbow trout fry, the increased metabolite levels by parental Se nutrition in the transsulfuration pathway suggest a nutritional programming by Se in the glutathione metabolism. Under hypoxia, glutathione synthesis through transsulfuration might have been impaired by the depletion of a glutathione precursor. The combination of both parental Se nutrition and hypoxic stress on 1C gene expression also induced also long-term impacts on the methionine cycling and polyamine synthesis.

Conflict of interest

None.

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Author statement:

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6. GENERAL DISCUSSION

Selenium is an essential micronutrient in humans and animals, including fish, which is required to maintain health, especially the antioxidant system [319]. During the past years Se levels in fish diets have been noticed to decrease as a result of the ongoing replacement of fishmeal with more sustainable ingredients [320]. A previous study in our laboratory found that a complete replacement of fishmeal in rainbow trout fry diets might result in sub-optimal Se levels [18], raising the question, if a dietary supplementation might be necessary to maintain antioxidant status and health. However, the Se requirements in fish remain largely unknown, while in terrestrial livestock production Se supplementation is widely used to improve broodstock performance and maintain progeny health [20]. Therefore, this PhD investigated if the complete exchange of fishmeal with plant ingredients in rainbow trout diets might result in sub-optimal levels in broodstock fish and their progeny, that could be avoided by using an inorganic (sodium selenite) or organic (hydroxy-selenomethionine) dietary Se supplementation to improve performance, body Se levels and antioxidant status.

6.1. Dietary selenium supplementation in non-supplemented fishmeal-free diet reveals sub-optimal levels at different developmental stages

Using the negative control diet without any Se supplementation (Se level: 0.3 mg/kg diet) did not cause any impaired growth or survival in broodstock fish or first feeding fry (publication 1 and 3). There exist no comparable studies in broodstock fish, but Poston et al. [313] found increased mortality using diets with 0.1 mg Se/kg diet in first feeding Atlantic salmon. We did not observe this effect in fry probably because we used higher dietary Se levels (0.3 vs 0.1 mg/kg). In addition, it has been speculated that these severe signs of Se deficiency might be limited to the phase of rapid growth during first feeding as even at such low levels of 0.06-0.07 mg Se/kg dry feed did not result in impaired growth or survival in 1.3-10g sized rainbow trout [300,311]. Also, at later stages, especially at reproduction, growth is usually low due to the high energy demand for reproduction that could mask the impact of dietary Se at this stage. In

addition, clinical deficiency signs of Se have been found to be mitigated, when other dietary antioxidants, especially vitamin E are provided in adequate levels. This is in accordance with our findings that in stressed fish, vitamin C levels were preserved, when the fish were fed Se-supplemented diets contrary to stressed fish fed non Se-supplemented diets (publication 3). These results show that growth might be a poor indicator in fish to determine the Se status.

Seleno-dependent GPx (SeGPx) has been used to determine Se requirements in mammals and also in fish. Hilton et al. [300] found increased liver and plasma GPx activity in rainbow trout, when a basal diet of 0.06 mg Se/kg dry feed was supplemented to 0.87 mg/kg. It was surprising that in the present work in fry fed the sodium selenite supplemented diet, we detected a significant increase in total, but not SeGPx activity, while hydroxy-selenomethionine feeding had no significant impact. Also, the expression of gpx1b1 was significantly higher in fry of the selenite treatment than in fry fed the hydroxy-selenomethionine supplemented diet, but it was not significantly different in comparison to fry fed the control diet (publication 3). However, as the Se range was narrower (0.5-0.6 vs. 0.3 mg Se per kg diet) and as analyses were performed on whole body levels, tissue specific effects might have been masked. We found that sodium selenite feeding elevated liver Se abundance compared to hydroxy-selenomethionine (publication 2). As the liver is considered as a key organ in selenoprotein production [321] this might favor the sodium selenite treatment for GPx production. Nevertheless, in an earlier study, using higher Se levels (0.9 vs. 0.5 mg/kg diet for the Se-supplemented and not supplemented diets, respectively), sodium selenite and Se-enriched yeast commonly increased whole-body *gpx1b2* and *gpx4a1* mRNA levels and an increase in SeGPx activity was found only in the organic Se treatment [18]. We hypothesized that our diets might have been deficient in fry even in the supplemented groups resulting in the observed differences. Indeed, the level of 0.5 mg Se/kg corresponds in the present study to the Se-supplemented groups whereas considered to be deficient as non Se-supplemented group in the previous study. This is also in agreement with Atlantic salmon post-smolt, where Se requirements were calculated at a total dietary Se level of 0.65 mg/kg in plant-based diets [322]. This could either show that within the deficiency range

higher Se levels are not associated to increased SeGPx levels as similarly suspected in a study in broilers [323] or that the increase in SeGPx levels is too low and not detected due to the impact of individual variability. Higher supplementation levels might help to provoke stronger responses in this case and the use of graded Se levels to determine if the levels used in this study were within a deficiency range.

In fed fry, the beneficial effect of Se supplementation became obvious only after the fish were subjected to an acute hypoxic stress (publication 3). Fish fed Se-supplemented diets showed better resistance towards this stress compared to fry of the control group. The effect of Se supplementation in fish to support stress resistance has been previously studied at supra-nutritional levels, which resulted in the assumption that Se requirements would increase under stressful conditions [310,324,325]. We observed in stressed fry that *gpx1a* mRNA levels were increased by sodium selenite supplementation. Such effects are similar to our observations in broodstock males, where increased hepatic *gpx1a* expression was detected in the selenite treatment (publication 1). In general, Se-supplementation affected mRNA levels mainly in males, which suggest a sex specific effect on gene expression in broodstock fish. It was not surprising to find a sexual dimorphism in gene expression as the selenoproteome is known to show sex specific expression patterns [81]. This might be especially important in pre-reproductive period, where Se is needed for spermatogenesis [222,326] and also directed to oogenesis as shown in publication 1. The effect of Se in physiological relevant levels on mature fish has only been poorly studied. In zebrafish, Se supplementation increased male testes weight and reproductive success [327] as well as spermatozoa concentration in common carp [326]. In the present work, the impact of Se on male reproduction has not been further investigated, but we detected a tendency for higher Se levels in sperm that might have resulted in beneficial effects as described above and would have deserved a more detailed investigation. But, our study could, to our knowledge, demonstrate for the first time that also female reproduction in rainbow trout can benefit from Se supplementation as we found an increasing number of spawning females in Se-supplemented treatments and earlier spawning, when fed diets

supplemented with hydroxy-selenomethionine. Similar to the mentioned zebrafish trial, also in our experiment no significant difference in fecundity was detected. However, chronic exposure to dietary SeMet at 4.54 mg Se/kg wet weight has been described to increase steroidogenesis in female rainbow trout [317] indicating that might interfere with the balance of sex hormones possibly related to the observed changes. These results clearly show that sub-optimal Se level can impair reproductive success in fish. Further investigation might be necessary to determine the role of dietary Se in fish reproduction and describe sex specific effects.

6.2. Selenium analysis revealed differences in tissue Se localization and Se composition pending on dietary selenium form

As expected, tissue Se levels were increased in broodstock fish as well as in fry when fed Se-supplemented diets (publication 1 and 2). In 11-week fed fry, the provision of sodium selenite in supplemented diets resulted in an increase of total body Se levels of 44%, while hydroxy-selenomethionine increased body Se levels even by 93%. It corresponds to reduced Se retention of dietary sodium selenite compared to hydroxy-selenomethionine, which is in accordance to other studies on terrestrial animals and in fish. The body Se concentrations of 120-260 µg/kg wet weight are in similar range to previous studies in rainbow trout fed a plant-based diet supplemented with 0.3 mg Se/kg diet supplied as sodium selenite or Se-enriched yeast [308]. However, according to Se homeostasis in Atlantic salmon post-smolt, body Se levels lower than 200 µg/kg wet weight were indicative for dietary Se deficiency [322], which supports the suggestion that the Se-supplemented diets in the present study might have been within the deficiency range of rainbow trout fry. In the following, we investigated the effect of Se supplementation on the tissue Se distribution, where effects might be different as Se tissue supply has been previously described to follow a hierarchical principle. We found that tissue distribution, but also Se composition within tissues varied according to dietary Se source. SeMet is known to increase muscle Se content more effectively than other Se forms [328], which is in

accordance to our findings in both 11-week fed fry, and broodstock fish. The increase of SeMet levels in relation to hydroxy-selenomethionine nutrition should be related to the suggestion that SeMet remains largely intact after ingestion and might occur in free form also in rainbow trout tissues [329]. The metabolism for SeMet incorporation into proteins might be slower compared to inorganic Se sources [329] as SeMet is then further metabolized pending on dietary methionine concentration with which it builds a common pool [56,308]. Sodium selenite, on the other hand, can be reduced and incorporated into selenoproteins in the liver [321]. In 11-week fed fry, liver Se abundance was the highest when fry were fed diets supplemented with sodium selenite, but hepatic Se levels in broodstock females were not found to differ between groups. The liver is the main organ responsible for the body distribution of Se through the transport protein SelP, where Se is incorporated as SeCys [321]. Nevertheless, in broodstock males and females we found elevated hepatic SelP mRNA levels in the Se-supplemented treatments with the highest expression in the sodium selenite treatment (publication 1). It could be indicative that while SeMet elevates the body Se levels, sodium selenite might be more available for the production of selenoproteins as suggested in various mammalian cell lines [330]. In Se-deficient rats, plasma Se injection increased SelP levels similarly in males and females [331], but we found a sex specific peculiarity between the dietary Se forms as SeMet increased SelP mRNA levels only in males and not in females. A possible explanation is that in females, SeMet, which is the main product of hydroxy-selenomethionine, was directed towards the oocytes and might have been utilized for selenoprotein production to a lesser extent, which can help to explain the missing effect of Se on hepatic selenoprotein mRNA levels mentioned above. This theory is supported by the significant increase in SeMet levels in oocytes and probably not in milt for males fed hydroxy-selenomethionine as total Se was not different between dietary groups and Se levels were ten-fold lower compared to oocytes. Se speciation revealed that more than ninety percent of the Se in oocytes was either SeCys or SeMet, but SeMet proportion was increased by hydroxy-selenomethionine supplementation. It remains the question, if the maternally transferred Se was protein bound,

which would suggest that SeCys might have been incorporated in SelP, especially because protein bound SeCys is more stable compared to the free form [329]. However, care should be taken to not exceed Se requirement levels in broodstock diets as a linear relationship between maternal body Se and egg Se concentrations have been detected in rainbow trout at tissue levels between 3 and 23 µg Se/g and were associated to toxicity effects and deformities in the progeny [332]. Even at the much lower levels, from 0.3 to 0.8 mg Se/kg diet in our study, the Se gradient in offspring by parental Se nutrition remained detectable through embryonic development until swim-up fry stage. High proportions of the Se were still localized in the yolk, but muscle Se levels were the highest in the organic Se treatment indicating that the parental transferred SeMet might have been well deposited in the developing fry (publication 2). A long-term effect of parental Se nutrition on body Se levels was not detected and might have been dominated by the nutritionally ingested Se that then represented the large majority of 95-99% from the total body Se concentrations.

6.3. Parental selenium improved the antioxidant status of the offspring in short-term but long-term studies gave unexpected changes in the metabolism of glutathione

We hypothesized that the maternally transferred Se to the progeny at physiological relevant levels might support their antioxidant system. Indeed, we found that in swim-up fry originating from the Se-supplemented treatments, the (Se-)GPx activity was increased following the detected gradient of body Se levels with the highest effect in the hydroxy-selenomethionine treatment (publication 1). This is similar to what is known from studies in poultry, where hatchlings of female chicken fed Se-supplemented diets had higher liver SeGPx activity that even remained detectable during two weeks post-hatching period [333]. We found that fry originating from parents fed Se-supplemented diets also showed higher mRNA levels of antioxidant proteins and body levels of vitamin C and α-tocopherol, but only with hydroxy-

selenomethionine supplementation. This might relate to the better bioavailability of this Se source. Together, these results indicate a clear beneficial effect of parental Se on the antioxidant metabolism in the progeny before first feeding. However, opposed was the decrease in GSH/GSSG ratio in swim-up fry that was commonly detected for Se-supplemented groups and that was contrary to known effects of dietary Se in rainbow trout fry [18]. A decreased GSH/GSSG ratio is associated with oxidative stress, but other measures including protein carbonyls and 8-isoprostanes were not affected. The level of 8-isoprostanes, the product of arachidonic acid peroxidation was even lower in the sodium selenite treatment, suggesting an antioxidant effect of parental sodium selenite supplementation. The decrease in GSH probably relates to an impaired transsulfuration in swim-up fry by parental Se (publication 4), which is surprising as dietary Se in other animal studies has been associated with increased transsulfuration [334–336]. In a subsequent study, we confirmed that parental Se had a long-lasting effect on the glutathione metabolism (publication 3 and 5). The metabolite levels in the transsulfuration pathway were increased by parental Se nutrition in 11-week fed fry; while on the other hand, especially under stress, mRNA levels of enzymes of the glutathione metabolism including *gr*, *gpx1b*, *gclc* and *gstπ* were decreased by parental Se nutrition. This suggests a nutritional programming effect by parental Se on the glutathione metabolism. Nutritional programming describes a phenomenon where prenatal or postnatal nutritional stimuli can have long-term consequences on physiological functions in later life [251,337]. Epigenetic modifications are considered as one of the underlying mechanisms for nutritional programming resulting in modified gene expression patterns. Here we described that the parental Se caused adverse effects compared to direct Se feeding on the glutathione metabolism, which would suggest that the progeny of the control group is either showing compensatory effects or that the progeny of Se-supplemented treatments might be less tolerant towards low dietary Se levels and require higher dietary Se levels later in life.

6.4. Selenium interferes in the one-carbon metabolism and causes changes in DNA methylation pattern of genes that reveal new potential target pathways of parental selenium

Homocysteine in the 1C metabolism presents an important link between the glutathione and methionine metabolism. As seleno amino acids are co-metabolized with their sulfur counterparts, we hypothesized that the nutritional programming related to changes in the 1C metabolism causing epigenetic modifications [338]. Parental selenium nutrition caused no changes in progeny Hcys levels in short or long-term, but hepatic Hcys levels were elevated in broodstock females of the hydroxy-selenomethionine treatment (publication 4 and 5). Murine studies found an inverse correlation between liver Se and Hcys levels [335,336], but especially under limiting Se conditions also a translocation of Se from liver to the plasma has been found, possibly to avoid re-methylation [339,340]. Such effects could have been masked by analysis of progeny metabolites on whole-body level. In broodstock males, the mRNA levels of the re-methylation enzyme *mtr* were higher in Se-supplemented groups compared to the control group. On the other hand, in 11-week fed fry, mRNA levels of *bhmt,* the other enzyme involved in re-methylation of methionine, were lower, when the fry were fed hydroxy-selenomethionine. However, as the effect was only detectable in fry originating from one of the Se-supplemented treatments, this suggests that parental Se nutrition can cause long-term modifications in the re-methylation pathway on *bhmt*, when the progeny is presented with hydroxy-selenomethionine later in life. Indeed, no differences were found earlier at swim-up fry stage where parental Se had no impact on whole-body *bhmt* mRNA levels.

The methionine cycle provides S-adenosylmethionine (SAM), the universal donor in cellular methylation reactions. We found that sodium selenite supplementation decreased liver SAM levels in broodstock males and females, while Se nutrition had no impact on whole-body SAM levels in 11-week fed fry (publication 4 and 5). The procession of Se-compounds in the metabolism can create a drain on methyl groups as selenide and other highly reactive seleno-

compounds can be spontaneously methylated [341]. This process is especially important for inorganic Se sources that do not follow the methionine cycle and might be underlying observed differences in other studies [256,259,335,342]. We also found that parental Se in swim-up fry resulted in lower SAM content in swim-up fry, while in the long-term parental effects were no more detected. The ratio between SAM and S-adenosylhomocysteine is also known as methylation potential, and decreasing methylation potential is usually associated to DNA hypomethylation [343]. DNA methylation is an important epigenetic mechanism that can modify the gene expression pattern of an organism in long-term and thus considered as one of the underlying mechanisms of nutritional programming effects.

We used reduced representation bisulfite sequencing to identify genes showing differences in liver DNA methylation pattern at swim-up fry stage between the three parental treatments (publication 4). The results show that the DNA methylation pattern in the progeny is sensitive to parental Se nutrition. We not only identified genes related to the sulfur, Se and glutathione metabolism, but also genes related to several other metabolic pathways. This is a useful dataset that points towards interesting targets in future studies. We presented genes that show the highest differences in methylation pattern, which included genes involved in neuron or signal transmission as well as the immune system. These genes were found to differ according to Se level, but also according to Se form. However, pending on research question also other filtering options would be possible. Research questions targeting the Se level might specifically check for genes that have common differentially methylated cytosines in the genes. It would have been interesting to match the present information on methylation differences with transcriptomic data as differences in methylation pattern are not necessarily resulting in different gene expression pattern.

6.5. Complex interaction between parental nutritional history, direct selenium nutrition, selenium form and other environmental stimuli

Determining the epigenetic contributions towards a specific phenotype is complex and requires a detailed understanding on the metabolism that cannot be explained yet. We have shown that fry originating from parents fed Se-supplemented diets had higher weight gain, when fed the respective Se-supplemented diet during first feeding (publication 3). This might relate to changes in the energy metabolism as also in sheep, parental Se feeding induced nutritional programing resulting in higher growth rates of the progeny [344]. However, in the mentioned study, Se-enriched yeast was the sole utilized Se form and the impact of dietary Se form remains to be investigated. In contrast to the positive impact of parental Se nutrition on weight gain, fry originating from parents fed Se-supplemented diets showed reduced stress resistance towards hypoxia. This effect might partly relate to the adverse effect of parental Se on the glutathione metabolism and especially GPx that has a key role in the antioxidant response to oxidative stress. However, we also detected that also several other genes were transcriptionally regulated according to parental Se treatment under oxidative stress. The enzyme S-adenosylmethionine decarboxylase (AMD) was lower expressed in fry originating from parents fed sodium selenite compared to fry from the control group, when fed hydroxy-selenomethionine, which might indicate that parental selenite feeding could inhibit polyamine synthesis under stressful conditions (publication 5). Polyamine synthesis has been formerly described to be increased by both acute hypoxia [345] and Se treatment [346]. Similarly, also glycine N-methyltransferase (GNMT) expression showed interactive effects between direct and parental Se nutrition in stressed fry. GNMT converts SAM to SAH, while generating sarcosine from glycine [347]. Even though the post hoc test could not discriminate between treatments for *gnmt* mRNA levels, the highest expression was related to fry originating from the broodstock control group and the lowest for fry fed differing Se forms compared to their parents. The fact that two dietary Se forms exert different effects on the sarcosine metabolism was also suggested by sarcosine levels

that were higher in fry originating from parents fed sodium selenite compared to fry originating from the hydroxy-selenomethionine treatment.

7. CONCLUSION AND PERSPECTIVES

7.1. Conclusion

A complete replacement of fishmeal in aquafeeds can result in sub-optimal Se levels in rainbow trout. Although classical parameters including growth, survival and GPx expression or activity were not or only weakly affected by direct Se feeding, dietary Se supplementation could improve stress resistance in fry and reproductive success in broodstock.

Hydroxy-selenomethionine elevated total body Se levels more effectively compared to sodium selenite possibly related to Se storage in muscle tissue. Sodium selenite feeding resulted in higher liver Se abundance that could be well available for selenoprotein incorporation within the dietary range levels used in our study. In broodstock fish the Se tissue distribution might not only be dependent on dietary Se form, but be also sex specific. The maternal transfer of Se to the offspring could be proven at physiologically relevant concentrations.

In swim-up fry, parental Se feeding resulted in higher tissue Se concentrations and supported the antioxidant system. However, parental Se also modified the glutathione metabolism of the progeny in the long-term, causing adverse effects that even resulted in poorer stress resistance of progeny of Se-supplemented treatments. In order to compensate such contrary effects higher dietary Se levels in the offspring diets might be required, when parental diets are supplemented with dietary Se. Transgenerational studies could help to further elucidate the long-term effect of Se on the glutathione metabolism.

Parental and direct selenium nutrition were shown to affect the methionine cycle. The methylation potential was decreased in broodstock fish and swim-up fry, where the DNA methylation pattern was strongly affected by parental sodium selenite as well as hydroxy-selenomethionine nutrition. Further research might be required to link such epigenetic changes to physiological modifications.

In the long-term parental Se nutrition showed significant interactions with direct Se nutrition and between dietary Se forms. The hypoxic stress challenge also showed that other environmental factors can interact with effects of dietary and parental Se supplementation that

complicate their detection and interpretation. The understanding of such interactions requires a detailed knowledge of metabolic relations, which requires further investigation.

7.2. Perspectives

Comparable data on the effect of dietary Se in rainbow trout broodstock are missing and also the studies in other fish species are rare. However, our finding that dietary Se supplementation can improve the number of spawning females and that females spawn earlier, when presented hydroxy-selenomethionine deserve further investigation. Related to the previous work of Wisemann et al. [317] which reported that dietary SeMet influences steroidogenesis in rainbow trout and numerous studies in mammals that found a relation between selenium and estrogen [232,233,237], future studies might focus on the relation between Se and female sex hormones in fish. For females, the deposition of large amounts of Se in the oocytes also raises the question, if Se requirements might be different in broodstock male and female fish and therefore the studies on the nutritional requirements, especially in broodstock fish should include a sex specific design. In addition, it remains unanswered how Se is included in the oocytes by females. As the proportion of SeMet inclusion in oocytes was elevated by parental hydroxy-selenomethionine nutrition and SeMet is suspected to be partly present in free form in the muscle tissue of rainbow trout [329], it could be speculated, if the Se included in the oocytes was protein-bound or not. This is particularly true also for SeCys that represented together with SeMet the major Se forms in oocytes. SeCys is less stable in free form, but on the other hand, SeCys is the key-component in selenoproteins. The Se form-dependent tissue deposition of Se raises the question, how form-specific effects work on a tissue dimension. In view of this, a limitation of the present work is that we analyzed the progeny almost exclusively on whole-body level. Generally, the liver is considered as a key organ in selenium and the antioxidant system [321]. We found that mRNA levels of GPx and other selenoproteins was often higher, when fed sodium selenite, in broodstock liver tissue and in whole fry, however at swim-up fry

stage the antioxidant system better responded to the parental hydroxy-selenomethionine treatment, where the yolk contained high levels of Se in all treatments, but hydroxy-selenomethionine showed the highest muscle Se abundance. Nevertheless, it was not possible to quantify Se in yolk and to identify the liver in swim-up fry for body Se mapping by LA-ICP MS bioimaging. Therefore, an improvement in sample preparation technique possibly by separation of yolk or different body positioning with focus on liver might be required, when targeting the role of liver in the metabolism of parental Se. We dissected liver at swim-up fry stage and found that the liver methylation pattern was sensitive to the parental Se treatment in relation to both level and form. In view of the fact that parental Se nutrition caused persistent changes on the methionine cycle, transsulfuration pathway, glutathione system and antioxidant system in the F1 generation further studies on the long-term impact of parental Se nutrition throughout generations should be conducted. Also, at swim-up fry stage, the decrease of vitamin B6 levels in combination with impaired transsulfuration suggests that the requirements of other one carbon nutrients might be increased by Se treatment. The positive impact of a dietary co-supplementation of vitamin B6 and Se on the glutathione metabolism has been described in pigs [348]. An overlap between the DNA methylation data at swim-up fry stage and the long-term parental effect in fed fry was identified in the nrf2-keap1 redox signaling pathway that deserves further investigation also due to its link on the cellular inflammatory response. The strong changes in DNA methylation pattern of genes related to inflammation and immunology at swim-up fry stage point towards epigenetic modifications on these pathways.

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ANNEX

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Effect of selenium sources in plant-based diets on antioxidant status and oxidative stress-related parameters in rainbow trout juveniles under chronic stress exposure

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ABSTRACT

Selenium (Se) is an essential micronutrient required for normal development and antioxidant protection. Oxidative stress induced by stressful conditions has been shown to be reduced by dietary Se supplementation. However, replacing fishmeal, an important source of Se, with ingredients of plant origin usually results in decreased Se content in aquafeeds. The objective of the study was hence to assess the impact of dietary Se supplementation by an inorganic or organic source on the antioxidant defense system of rainbow trout (*Oncorhynchus mykiss*) juveniles fed practical plant-based feeds under normal or chronic stress conditions. Fish (initial mean weight: 42 ± 2 g) were maintained under normal (25 fish/tank, dissolved oxygen: 7.9 mg/l) or stressful conditions combining decreased water quality with hypoxia and increased stocking density (50 fish/tank, dissolved oxygen: 5.9 mg/l) for 12 weeks at 17 °C. Rainbow trout juveniles were fed three plant-derived protein-based diets containing 0.5 mg Se/kg diet supplemented or not with 0.3 mg Se/kg diet supplied as sodium selenite or Se-enriched yeast, Selsaf®. The apparent digestibility coefficients (ADC) of dry matter, protein, ash and Se were improved by Se-enriched yeast supplementation while the ADC of Se was reduced by sodium selenite supplementation and the ADC of dry matter, starch, energy, ash and phosphorus were reduced by chronic stress exposure. Dietary Se supplementation had no significant impact on growth performance contrary to chronic stress exposure which led to decreased final body weight and feed intake. Total Se content of fish was increased by both dietary Se supplementations but to a larger extent with Se-enriched yeast. Fish fed Se-enriched yeast displayed increased hepatic selenomethionine content and decreased muscle anisidine value. Blood glutathione content was reduced by chronic stress exposure. While hepatic catalase activity and transcript expression were increased by chronic stress exposure, both hepatic and renal glutathione peroxidase (GPX) activity and hepatic transcript expression of *gpx1b2* and *gpx4a2* were decreased in non-Se supplemented fish. These results suggest the necessity to supplement plant-based diets with Se for rainbow trout juveniles, independently of chronic stress exposure and highlight the superiority of organic form of Se to fulfil the dietary Se requirement and sustain the antioxidant status of fish.

1. Introduction

Selenium (Se) is an essential trace element for animals, including fish, required for normal development and antioxidant protection (Watanabe et al., 1997; Surai, 2006; NRC, 2011; Khan et al., 2017). This role is mainly attributed to the presence of Se within proteins and enzymes, named selenoproteins, in the form of selenocysteine, considered as the 21st amino acid. The selenoproteins involved in the protection of cell from deleterious damages of oxidized compounds include glutathione peroxidases (GPX) used as a specific response criterion to es-

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timate dietary Se requirements of fish (Antony Jesu Prabhu et al., 2016), thioredoxine reductases (TR) and methionine sulfoxide reductase B (MSRB).

Se can be added as a supplement to aquafeeds in either inorganic or organic forms. There is evidence in rainbow trout as in other animals that inorganic Se (sodium selenite or selenate) is less bioavailable and bioactive than organic Se, such as Se-yeast or selenomethionine (Küçükbay et al., 2009; Rider et al., 2009; Rider et al., 2010; Fontagné-Dicharry et al., 2015; Godin et al., 2015; Antony Jesu Prabhu et al., 2016). If levels of 0.15 to 0.38 mg Se/kg diet as sodium selenite were required to provide maximum growth and GPX activity in rainbow trout according to Hilton et al. (1980), other recent studies recommend much higher doses of organic Se supplementation, up to 2–4 mg Se/kg diet, to promote growth and flesh quality in rainbow trout (Wang et al., 2018a, 2018b). Fish meal-based diets generally provide sufficient Se to satisfy the nutritional requirements of fish (Watanabe et al., 1997), but can contain levels of Se well above the maximum dietary levels of 0.5 mg/kg permitted by European Food Safety Authority (European Commission, 2003), even though this upper limit only applies when Se is added to the feed, not when it appears naturally from the feed ingredients (Sissener et al., 2013). However, currently, there is significant reduction in fishmeal levels in aquafeeds, particularly in salmonid feeds, replaced to a large extent by plant protein sources (Tacon and Metian, 2008) that vary widely in their Se content (Watanabe et al., 1997; Sauvant et al., 2004; Rayman et al., 2008). Accordingly, recent reports suggest that dietary Se required in plant-based feeds for Atlantic salmon and gilthead seabream may exceed the present EU legal limit of 0.5 mg/kg (Antony Jesu Prabhu et al., 2020; Domínguez et al., 2020).

Some studies in fish have shown that dietary Se supplementation can reduce oxidative stress induced by stressful conditions such as handling (Rider et al., 2009), crowding (Küçükbay et al., 2009), heavy metal contamination (Lin and Shiau, 2007; Talas et al., 2008; Penglase et al., 2014a) or oxidized oil ingestion (Chen et al., 2013). Hypoxia, another stressful condition, is recognized as a major concern in aquaculture as this naturally occurring phenomenon in most aquatic environments, especially in intensive fish farming with high stoking density, has been reported to have many adverse effects (Van Raaij et al., 1996; Wu et al., 2016; Li et al., 2018; Del Rio et al., 2019). Hypoxia induces metabolic changes that may elicit oxidative stress due to the generation of reactive oxygen species (Lushchak and Bagnyukova, 2006; Bickler and Buck, 2007; Pérez-Jiménez et al., 2012). The antioxidant status of fish exposed to hypoxia can nonetheless be restored by proper supplementation of dietary antioxidants (Varghese et al., 2017).

However, with the fishmeal replacement by plant ingredients, the dietary Se level tends to decline in aquafeeds (Sissener et al., 2013). The objective of the study was hence to assess the impact of dietary Se supplementation by an organic or inorganic source on the antioxidant defense system of rainbow trout juveniles fed practical plant-based feeds under normal or stressful conditions combining decreased water quality with hypoxia and increased stocking density.

2. Materials and methods

2.1. Experimental diets

Three practical diets based on plant-derived proteins were formulated to contain 51% crude protein and 23% crude lipid with different levels and forms of selenium (Table 1). Diets were supplemented with mineral mixture to meet all the essential mineral requirements of rainbow trout (NRC, 2011), except for Se. The Se0 diet containing 0.5 mg Se/kg feed was not supplemented with Se and served as control. The other two diets were supplemented with either sodium selenite (SeS diet) or the Se-enriched yeast Selsaf from Phileo by Lesaffre (France) containing 2.2 g Se/kg with 99% of organic Se (SeY diet) at a concentration of 0.3 mg Se/kg feed. All the diets were prepared with incorporation of an inert marker, (yttrium oxide at 0.01%) for apparent digestibility measurements, manufactured using a twin-screw extruder (BC 45, Clextral), vacuum-coated with oils and stored at 4 °C at the INRAE experimental facilities in Donzacq (Landes, France).

Table 1
Formulation and composition of the experimental diets.

Diet	Se0	SeS	SeY
Ingredients (%)			
Plant meals[a]	75.1	75.1	75.1
Vegetable and fish oils[b]	15.6	15.6	15.6
Soybean lecithin[c]	2.0	2.0	2.0
Crystalline amino acids[d]	1.6	1.6	1.6
Attractant premix[e]	1.5	1.5	1.5
Vitamin premix[f]	1	1	1
Mineral premix without Se[g]	3.2	3.2	3.2
Yttrium oxide (g/kg diet)	0.1	0.1	0.1
Sodium selenite (mg/kg diet)	–	0.7	–
Se-enriched yeast, Selsaf (mg/kg diet)	–	–	150
Proximate composition			
Dry matter (DM, %)	97	97	97
Crude protein (% DM)	51	51	52
Total lipid (% DM)	24	23	23
Gross energy (kJ/g DM)	25	25	25
Ash (% DM)	5	5	5
P (% DM)	1.1	1.1	1.1
Se (mg/kg)	0.5	0.9	0.9

[a] Plant meals (% diet): wheat gluten (Roquette, France), 20; corn gluten meal (Inzo, France), 18; soybean protein concentrate (Estril, Sopropêche, France), 17; soybean meal (Sud-Ouest Aliment, France), 5; white lupin meal (Farilup 500, Terrena, France), 5; rapeseed meal (Primor 00, Sud-Ouest Aliment, France), 4; dehulled pea meal (Primatex, Sotexpro, France), 3; whole wheat (Sud-Ouest Aliment, France), 3.1.

[b] Vegetable and fish oils (% diet): linseed oil (Daudruy, France), 7.8; fish oil (Sopropêche, France), 7.8.

[c] Supplied by Louis François (France).

[d] Crystalline amino acids (% diet): L-lysine (Ajinomoto-Eurolysine, France), 1.34; DL-methionine (Evonik, Germany), 0.3.

[e] Attractant premix (g/kg diet): glucosamine, 5; taurine, 3; betaine, 3; glycine, 2; alanine, 2.

[f] Vitamin premix (IU or g/kg diet): retinyl acetate, 5000 IU; cholecalciferol, 2500 IU; DL-α-tocopheryl 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; *myo*-inositol, 0.3 g; D-biotin, 0.2 mg; folic acid, 1 mg; cyanocobalamin, 10 μg; L-ascorbyl-2-polyphosphate, 50 mg; choline, 1 g. All ingredients were diluted with α-cellulose.

[g] Mineral mixture (g/kg diet): $CaHPO_4{\cdot}2H_2O$, 27; $CaCO_3$, 2.15; NaCl, 0.4; $FeSO_4{\cdot}7H_2O$, 0.2; $MnSO_4{\cdot}H_2O$, 0.03; $ZnSO_4{\cdot}7H_2O$, 0.04; $CuSO_4{\cdot}5H_2O$, 0.03; $CoCl_2{\cdot}6H_2O$, 0.0002; KI, 0.0004; NaF, 0.01; MgO, 1.24; KCl, 0.9.

2.2. Apparent digestibility trial

Rainbow trout juveniles (initial body weight: 84 g) from the same parental stock were held (12 for normal conditions or 24 fish per unit for stressful conditions) in 18 cylindro-conical fiberglass tanks (each 60 l) connected to a re-circulation system (one per condition) with a flow rate of 2 l/min at Saint-Pée-sur-Nivelle, INRAE, France. The fish were fed one of the three diets (Se0, SeS or SeY) in triplicate groups. The water was well aerated and thermostatically maintained at 17.0 ± 0.5 °C with dissolved oxygen levels of 7.5 ± 0.4 mg/l for normal condition and 5.8 ± 0.2 mg/l for stressful conditions representing 78 and 60% saturation, respectively. This low dissolved oxygen level was selected to be within the minimum range of dissolved oxygen level for farmed trout (5–6 mg/l) to avoid sub-lethal effects (Ellis et al., 2002). Other water quality parameters were monitored every 3 days to ensure animal welfare (pH: 7.4 ± 0.2, ammonia: non detectable, nitrite: < 0.015 mg/l). The fish were allowed to adapt to the rearing conditions for 2 weeks, after which collection of feces was performed by the method of Choubert et al. (1982). The feces were

collected on a daily basis for a period of 2 weeks and stored at −20 °C. The feces collected over 2 weeks from each tank were pooled, freeze-dried and used for further chemical analysis. The apparent digestibility coefficients (ADC, %) of Se and other dietary components were calculated using yttrium oxide as digestibility marker as follows ADC (%) = 100 − [100 ∗ (% X in feces / % X in diet) ∗ (% Y_2O_3 in diet / % Y_2O_3 in feces)] where X is the dietary component or energy.

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.

2.3. Experimental fish and dietary trial conditions

The feeding trial was carried out in the INRAE experimental fish farm at Donzacq supplied with flow through spring water at 17.5 ± 0.5 °C. Rainbow trout (*Oncorhynchus mykiss*) juveniles from the same parental stock with a mean body weight of 42 ± 2 g were randomly allocated to 150-l fiberglass tanks receiving a natural photoperiod. Fish were maintained under normal (N, 25 fish/tank, 20 kg/m^3) and stressful (S, 50 fish/tank, 42 kg/m^3) conditions for 12 weeks after acclimatization to the rearing conditions two weeks prior to the start of the experiment. These two stocking densities were selected to be within the normal density range of 15–40 kg/m^3 for rainbow trout reared in tanks (Ellis et al., 2002). For stressful conditions, the flow rate was reduced from 24 l/min to below 12 l/min to decrease dissolved oxygen levels from 7.9 ± 0.4 mg/l to 5.9 ± 1.0 mg/l, representing 83 and 62% saturation, respectively. Fish were hand fed twice a day to visual satiation. Each diet was distributed to three replicate groups of fish over a 12-week growth trial.

2.4. Sampling

At the beginning of the feeding trial, 6 fish were sampled for analysis of whole-body composition. During the study, fish from each tank were bulk weighed every three weeks. Feed was withheld for 16 h before every weighing and sampling. At the end of the 12-week growth trial, a pooled sample of 3 fish from each experimental unit was taken for final body composition analysis. Further, 3 more fish from each replicate were withdrawn, anaesthetized (benzocaine, 30 mg/l) and blood samples were collected from the caudal vein into heparinized syringes. Fish were euthanized subsequently by a sharp blow to the head and liver, kidney and muscle were dissected. The liver was weighed for calculating hepato-somatic indice (HSI). Tissues were immediately frozen in liquid nitrogen and stored at −80 °C before analysis.

2.5. Proximate, mineral and fatty acid analyses

Proximate composition of diets was determined according to following procedures: dry matter after drying at 105 °C for 24 h, protein (N × 6.25) by the Kjeldahl method after acid digestion, ash by incineration at 550 °C for 10 h and gross energy in an adiabatic bomb calorimeter. Starch content was determined as glucose by a kit, using the amyloglucosidase/hexokinase/glucose-6-phosphate dehydrogenase method (Invivo Labs, France). Total lipid was extracted and measured gravimetrically according to Folch et al. (1957) using dichloromethane instead of chloroform. Total P was determined by the molybdate blue/ascorbic acid method at 802 nm after mineralization and acid digestion (AFNOR, 1992). Total Se and yttrium concentrations were determined after acid digestion using concentrated HNO_3 and H_2O_2, using inductively coupled plasma-mass spectrometry (ICP-MS) as previously described (Fontagné-Dicharry et al., 2015). Typical analytical precision was lower than 5% (relative standard deviation) and detection limit of 3 μg/kg. Selenomethionine (SeMet) and selenocysteine (SeCys) concentrations were determined in rainbow trout tissues after enzymatic digestion using HPLC-ICP QMS as previously described (Godin et al., 2015). Fatty acid methyl esters were prepared and analyzed according to Fontagné et al. (2008).

2.6. Oxidative status

Conjugated dienes (E232) and trienes (E268) were measured as specific extinctions at the wavelengths of 232 and 268 nm, respectively (CEN, 2002). Anisidine value (AV) was determined according to standard procedures (CEN, 2000). Lipid-soluble fluorescent products (LSFP) and thiobarbituric acid-reactive substances (TBARS) were analyzed as previously described (Fontagné-Dicharry et al., 2014). Oxidized glutathione (GSSG) and reduced glutathione (GSH) were measured in blood and whole-body using Cayman glutathione assay kit (Bertin Pharma, Montigny-le-Bretonneux, France) according to the manufacturer's instructions with protein concentration assessed by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.7. Antioxidant enzyme activity

Total and Se-dependent GPX (EC 1.11.1.9), glutathione reductase (GR, EC1.6.4.2), glutathione-S-transferase (GST, EC2.5.1.18), superoxide dismutase (SOD, EC1.15.1.1) and catalase (CAT, EC1.11.1.6) activities were assayed in liver as previously described (Fontagné et al., 2008; Fontagné-Dicharry et al., 2014). Total and Se-dependent GPX activities were also assayed in kidney. Specific enzymatic activities were assessed with protein concentration determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.8. Antioxidant transcript level

Total RNA was isolated from liver using Trizol reagent (Invitrogen, Cergy-Pontoise, France). For quantitative RT-PCR, complementary DNA was generated from 1 mg total RNA using SuperScript III RT (Invitrogen) and a mix of oligo(dT)15 and random primers (Promega, Charbonnières, France). For each sample, RT was performed in duplicate and quantitative PCR analyses were performed in LightCycler® 480 Instrument II (Roche Diagnostics, Meylan, France) using LightCycler® 480 SYBR Green I Master mix (Roche Diagnostics). Total reaction volume was 6 μl, with 2 μl of the diluted RT reaction mixture (dilution 40) and 4 μl of master mix added with 0.4 mM of each primer (Table 2). The PCR protocol was initiated at 95 °C for 10 min for initial denaturation of the cDNA and hot-start Taq-polymerase activation, followed by 45 cycles of a three-step amplification program (15 s at 95 °C, 10 s at the melting temperature of 60 °C, 4.8 s at 72 °C). Melting curves were systematically monitored (5 s at 95 °C, 1 min at 65 °C, temperature slope at 0.11 °C/s from 65 to 97 °C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Relative quantification of target gene transcripts were performed using Elongation Factor 1α (EF1α) as the reference gene and Se0-N as the reference group using the ΔΔCt method (Pfaffl, 2001).

2.9. Statistical analyses

Data are expressed as mean ± standard deviation (SD). Differences between dietary groups were analyzed using two-way ANOVA to test the effect of dietary Se supplementation, rearing conditions and their interaction. The Newman-Keuls multiple range test was used to compare means when a significant difference was found. Gene transcript data were rank-transformed before analysis. Statistical analyses were performed with the computing program SigmaStat 3 (SPSS, Chicago, IL, USA) and differences were considered significant when P values were < 0.05.

Table 2
Oligonucleotide primers used to assay gene transcript levels by real-time quantitative RT-PCR.

Gene	Accession no.	Forward primer sequence (5′ → 3′)	Reverse primer sequence (5′ → 3′)	Amplicon size (bp)
gpx1a[a]	HE687021	AATGTGGCGTCACTCTGAGG	CAATTCTCCTGATGGCCAAA	131
gpx1b1[a]	CA357669.1	CGAGCTCCATGAACGGTACG	TGCTTCCCGTTCACATCCAC	183
gpx1b2[a]	HE687023	TCGGACATCAGGAGAACTGC	TCCTTCCCATTCACATCCAC	121
gpx4a1[a]	HE687024	GAAAGGCTTCCTGGGAAATG	CTCCACCACACTGGGATCAT	112
gpx4a2[a]	HE687025	AGAAATACAGGGGCGACGTT	GCATCTCCGCAAACTGAGAG	90
gpx4b[a]	CA344428.1	TTGGAGGTCAGGAGCCAGGT	ACCCTTTCCCTTGGGCTGTT	152
selpa2	MH085054.1	ACCTTGCTGAGCCAGAAACT	CAGACGACCACACCTGTCAT	129
tr3a	HF969246.1	GAGCCTCCCTCAAGTGTGAC	AGTGAACTCCAGAGGCCAGA	158
tr3b	HF969247.1	ACAAAATCAAGGCGACCAAC	GGCAGAGAGAACAGGTCGTC	148
msrb2	CA354807.1	AGGGGACAGAGATGCCCTTC	CCCATGAGCCTCTTTGAACG	149
sod1	AF469663.1	TGGTCCTGTGAAGCTGATTG	TTGTCAGCTCCTGCAGTCAC	201
sod2	CA352127.1	TCCCTGACCTGACCTACGAC	GGCCTCCTCCATTAAACCTC	201
cat	BX087110.3	TGATGTCACACAGGTGCGTA	GTGGGCTCAGTGTTGTTGAG	195
gr	HF969248.1	CTAAGCGCAGCGTCATAGTG	ACACCCCTGTCTGACGACAT	108
gstπ	BX302932.3	TCGCTGACTGGACGAAAGGA	CGAAGGTCCTCAACGCCATC	196
gclc	FR904377.1	AGGCCAGAGTATGGCAGCTA	TTGGGAATGATGTGATGGTG	166
nrf2	CA360709.1	TGAGCTGCAGCAATGTCTGA	GTTGGGCAATGGGTAGAAGC	124
iκbα	BT074199.1	AGAGACAGACTGCGCTCCAC	CGGCCTTCAGTAGCCTCTCT	72
ahrα	AF065137.3	CTCTCGCCTGGACAAACTCT	GCGTTCATCCCATTCATGTT	138
ef1α	AF498320.1	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	159

gpx1, cytosolic glutathione peroxidase; gpx4, phospholipid hydroperoxide glutathione peroxidase; selp, selenoprotein P; tr, thioredoxin reductase; msrb, methionine-R-sulfoxide reductase B; sod1, cytosolic Cu/Zn superoxide dismutase; sod2, mitochondrial Mn superoxide dismutase; cat, catalase; gr, glutathione reductase; gst, glutathione S-transferase; gclc, glutamate-cysteine ligase catalytic subunit; nrf2, nuclear factor erythroid-2-related factor 2; iκbα, nuclear factor kappa-light chain-enhancer of activated B cells inhibitor *α*; ahr*α*, aryl hydrocarbon receptor α; ef1*α*, elongation factor 1*α*.

[a] As described by Pacitti et al. (2013).

Table 3
Apparent digestibility coefficients (ADC) of Se and other nutrients in rainbow trout juveniles fed the experimental diets not supplemented with Se (Se0), supplemented with sodium selenite (SeS) or Se-enriched yeast, Selsaf (SeY) and kept under normal (N) or stressful (S) conditions for 2 weeks at 17 ± 1 °C.

ADC (%)	Dietary Se supplementation			Rearing conditions		Interaction
	Se0	SeS	SeY	N	S	
Dry matter	77 ± 2[b]	77 ± 3[b]	79 ± 1[a]	79 ± 1[z]	76 ± 3[y]	0.091
Protein	91.7 ± 0.4[b]	91.9 ± 0.4[b]	92.5 ± 0.3[a]	92.2 ± 0.4	91.9 ± 0.4	0.588
Starch	88 ± 3	88 ± 5	89 ± 4	92 ± 2[z]	85 ± 2[y]	**0.041**
Energy	84 ± 1	83 ± 1	84 ± 1	85 ± 1[z]	83 ± 1[y]	0.240
Ash	21 ± 5[b]	22 ± 6[b]	28 ± 4[a]	28 ± 3[z]	20 ± 4[y]	0.236
Phosphorus	37 ± 5	38 ± 6	39 ± 4	42 ± 2[z]	34 ± 2[y]	0.161
Selenium	89 ± 1[b]	87 ± 1[c]	91 ± 1[a]	89 ± 2	89 ± 2	0.743

Values are means ± SD of six or nine rearing tanks (two conditions per Se level or three diets per condition). Within rows and for each effect: dietary Se supplementation or rearing condition, means not sharing a common superscript letter are significantly different according to two-way ANOVA. Significant interactions are highlighted in bold type.

3. Results

3.1. Diet digestibility

The ADC of dry matter, protein, ash and Se were significantly affected by diet composition, being improved by Se-enriched yeast (Selsaf) supplementation (Table 3). The ADC of Se was significantly reduced by sodium selenite supplementation. A significant interaction of dietary Se supplementation with rearing conditions was observed for ADC of starch as a significant decrease was noticed in Se0 group compared to SeS group but only in N conditions. The ADC of dry matter, starch, energy, ash and phosphorus were significantly reduced by chronic stressful conditions.

3.2. Growth performance

Rainbow trout juveniles readily accepted the plant-based diets from the beginning of the study and maintained normal behavior throughout the growth trial. The survival was not significantly affected by dietary treatments and conditions (Table 4). Rainbow trout from all dietary groups almost tripled their body weight at the end of the 12-week feeding trial and attained an individual final body weight ranging from 109 g in S groups to 124 g in N conditions. Daily growth index, feed intake and hepato-somatic index were also significantly reduced by stressful conditions and not affected by dietary Se supplementation. On the other hand, feed conversion ratio was not significantly affected by stressful conditions and no significant interaction between dietary Se supplementation and rearing conditions was noticed on the different growth parameters.

3.3. Se contents in rainbow trout

Final whole-body dry matter and ash contents were not significantly affected by Se supplementation and rearing conditions (Table 5). Total Se content of whole-body rainbow trout was significantly increased by both dietary Se supplementations but to a larger extent with Se-enriched yeast compared to sodium selenite (Fig. 1). Both dietary Se supplementations increased also total Se content in liver and rainbow trout fed Se-enriched yeast displayed increased SeMet content in liver whereas hepatic SeCys content was not significantly affected. No significant effect of stress exposure or interaction between dietary Se supplementation and rearing conditions was noticed on the different rainbow trout Se contents (whether total Se content in whole-body and liver, or SeMet and SeCys content in liver).

Table 4
Growth performance of rainbow trout juveniles fed the experimental diets not supplemented with Se (Se0), supplemented with sodium selenite (SeS) or Se-enriched yeast, Selsaf (SeY) and kept under normal (N) or stressful (S) conditions for 12 weeks at 17 ± 1 °C.

	Dietary Se supplementation			Rearing conditions		Interaction
	Se0	SeS	SeY	N	S	
Survival (%)	78 ± 9	93 ± 9	86 ± 16	80 ± 14	91 ± 9	0.977
Mean body weight (g)	112 ± 13	118 ± 12	118 ± 10	124 ± 11[a]	109 ± 6[b]	0.837
Daily growth index[1]	1.6 ± 0.3	1.7 ± 0.2	1.7 ± 0.2	1.8 ± 0.2[a]	1.5 ± 0.1[b]	0.848
Absolute feed intake[2]	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1[a]	1.0 ± 0.1[b]	0.952
Feed intake[3]	8.3 ± 0.6	9.4 ± 0.7	9.0 ± 1.2	9.3 ± 0.9[a]	8.5 ± 0.8[b]	0.794
Feed conversion ratio[4]	1.5 ± 0.3	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.3	1.4 ± 0.1	0.803
Hepatosomatic index[5]	1.3 ± 0.2	1.4 ± 0.2	1.4 ± 0.1	1.5 ± 0.2[a]	1.3 ± 0.1[b]	0.475

Values are means ± SD of six or nine rearing tanks (two conditions per Se level or three diets per condition). Within rows and for each effect: dietary Se supplementation or rearing condition, means not sharing a common superscript letter are significantly different according to two-way ANOVA.

[1] Daily growth index was calculated as 100 × [(final mean body weight)$^{1/3}$ − (initial mean body weight)$^{1/3}$] / duration of experimental period (84 days).

[2] Absolute feed intake, expressed in g DM per fish per day, was calculated on dry matter (DM) basis as FI_{tot} / (n × t) where FI_{tot} is the total feed intake per tank (in g DM) over experimental period, n is the number of fish in tank and t is the duration of experimental period.

[3] Feed intake per metabolic body weight, expressed in g DM per kg$^{0.8}$ per day was calculated as absolute feed intake / [√(final mean body weight × initial mean body weight) / 1000]$^{0.8}$.

[4] Feed conversion ratio was calculated as dry feed intake (g) / wet weight gain (g).

[5] Hepato-somatic index was calculated as wet liver weight (g) / fish weight (g).

Table 5
Whole-body composition and oxidative status of rainbow trout juveniles fed the experimental diets not supplemented with Se (Se0), supplemented with sodium selenite (SeS) or Se-enriched yeast, Selsaf (SeY) and kept under normal (N) or stressful (S) conditions for 12 weeks.

	Dietary Se supplementation			Rearing conditions		Interaction
	Se0	SeS	SeY	N	S	
Dry matter (% wet weight)	30.9 ± 0.8	31.2 ± 0.6	30.9 ± 1.3	31.2 ± 1.1	30.7 ± 0.8	0.081
Ash (% wet weight)	2.3 ± 0.2	2.3 ± 0.3	2.5 ± 0.2	2.3 ± 0.3	2.4 ± 0.2	0.386
TBARS (µg/g wet weight)	5.8 ± 3.9	4.9 ± 1.7	3.9 ± 1.5	6.0 ± 3.1	3.7 ± 1.4	0.934
GSH (µmol/mg protein)	1.5 ± 0.5	1.7 ± 0.7	1.9 ± 0.8	1.7 ± 0.7	1.7 ± 0.8	0.059
GSSG (µmol/mg protein)	0.7 ± 0.5	0.8 ± 0.5	0.8 ± 0.4	0.8 ± 0.4	0.8 ± 0.6	0.709
GSSG/GSH ratio	0.7 ± 0.5	0.6 ± 0.3	0.5 ± 0.4	0.5 ± 0.2	0.7 ± 0.5	0.611

Values are means ± SD of six or nine rearing tanks (two conditions per Se level or three diets per condition). Within rows and for each effect: dietary Se supplementation or rearing condition, means not sharing a common superscript letter are significantly different according to two-way ANOVA.

3.4. Oxidative status in rainbow trout

The oxidative status in whole-body rainbow trout assessed by TBARS and glutathione contents was not significantly affected by dietary Se supplementation and rearing conditions (Table 5). Total glutathione contents were lower in whole-body than in blood (2.4 vs. 5.2 µmol/mg protein), with GSH and GSSG mean values of 1.7 and 0.8 for whole-body and 5.0 and 0.2 for blood (Fig. 2), respectively. The GSSG/GSH ratio was thus higher in whole-body than in blood (0.61 vs. 0.04, respectively). Unlike whole-body glutathione levels, chronic stress exposure significantly reduced blood glutathione levels, both reduced and oxidized forms, leading to a reduced GSSG/GSH ratio (Fig. 2).

Lipid peroxidation and fatty acid profile were also assessed specifically in the flesh (Table 6). Levels of conjugated and lipid-soluble fluorescent products in rainbow trout muscle were not significantly affected by dietary Se supplementation or rearing conditions (Table 6). On the contrary, anisidine value was decreased by Se-enriched yeast supplementation compared to control group Se0 (Fig. 3). Levels of eicosapentaenoic acid (EPA) were increased by both sodium selenite supplementation and chronic stress exposure whereas other fatty acid

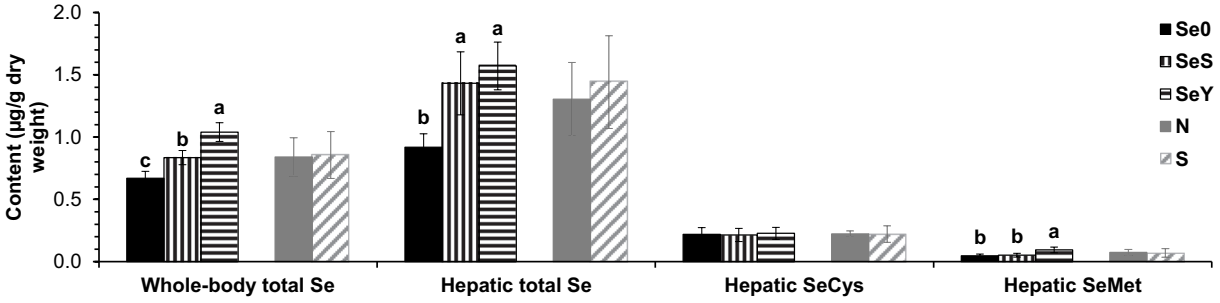

Fig. 1. Se contents of rainbow trout juveniles fed the experimental diets not supplemented with Se (Se0), supplemented with sodium selenite (SeS) or Se-enriched yeast, Selsaf (SeY) and kept under normal (N) or stressful (S) conditions for 12 weeks. Bars represent means ± SD of six or nine rearing tanks (two conditions per Se level or three diets per condition). Means not sharing a common superscript letter are significantly different ($P < 0.05$) according to two-way ANOVA followed by a Newman-Keuls test. The interaction between dietary Se supplementation and rearing conditions was not significant.

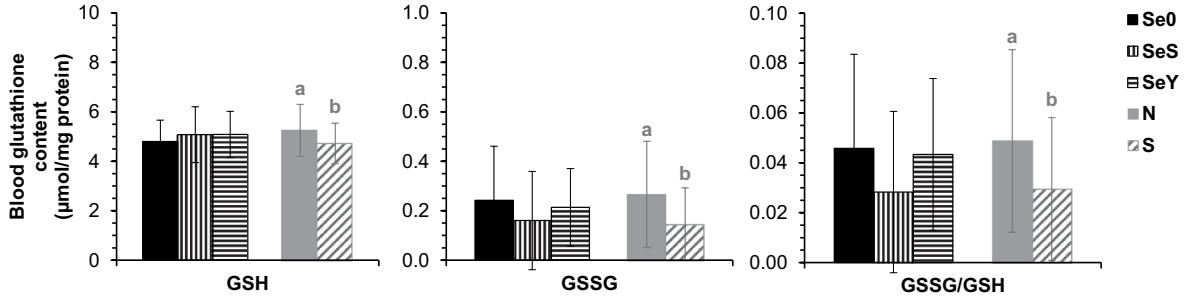

Fig. 2. Glutathione contents in blood of rainbow trout juveniles fed the experimental diets not supplemented with Se (Se0), supplemented with sodium selenite (SeS) or Se-enriched yeast, Selsaf (SeY) and kept under normal (N) or stressful (S) conditions for 12 weeks. Bars represent means ± SD of 18 or 27 fish (two conditions per Se level or three diets per condition). Means not sharing a common superscript letter are significantly different ($P < 0.05$) according to two-way ANOVA followed by a Newman-Keuls test. The interaction between dietary Se supplementation and rearing conditions was not significant.

levels were not significantly affected (Table 6).

No significant interaction between dietary Se supplementation and rearing conditions was noticed on the different oxidative status parameters (Tables 5 and 6, Fig. 2).

3.5. Antioxidant enzyme activity and transcript levels in rainbow trout

In both liver and kidney, total and Se-dependent GPX activities were increased by dietary Se supplementation whether as sodium selenite or Se-enriched yeast (Fig. 4) while hepatic CAT activity was increased by chronic stress exposure (Table 7). Other antioxidant enzyme activities were not significantly affected and no significant interaction between dietary Se supplementation and rearing conditions was recorded (Table 7).

The transcript levels of *gpx1b2* and *gpx4a2* were increased in both Se-supplemented groups similar to GPX activity (Table 8). Chronic stress exposure modulated antioxidant transcript expression rather differently to antioxidant enzyme activity: transcript levels of *gpx1a*, *gpx4a2* and *gpx4b* were reduced by chronic stress exposure whereas *tr3a* and *msrb2* were increased by chronic stress exposure. Only *cat* mRNA content was increased by chronic stress exposure similar to CAT activity. No significant interaction between dietary Se supplementation and rearing conditions was noticed on the transcript level according to two-way ANOVA.

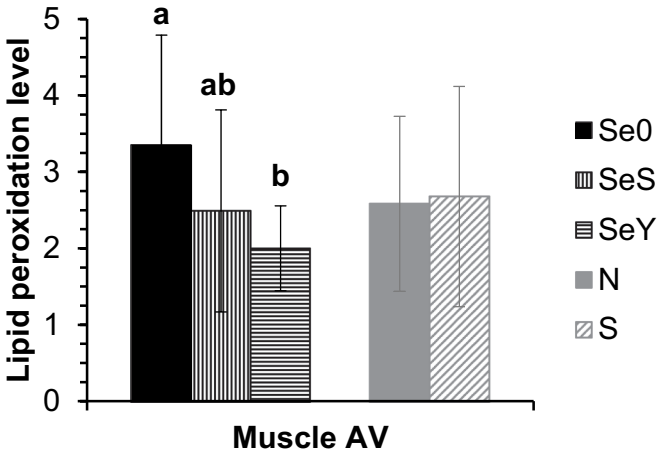

Fig. 3. Anisidine value (AV) in muscle of rainbow trout juveniles fed the experimental diets not supplemented with Se (Se0), supplemented with sodium selenite (SeS) or Se-enriched yeast, Selsaf (SeY) and kept under normal (N) or stressful (S) conditions for 12 weeks. Bars represent means ± SD of 18 or 27 fish (two conditions per Se level or three diets per condition). Means not sharing a common superscript letter are significantly different ($P < 0.05$) according to two-way ANOVA followed by a Newman-Keuls test. The interaction between dietary Se supplementation and rearing conditions was not significant.

Table 6
Lipid peroxidation and fatty acid composition in flesh of rainbow trout juveniles fed the experimental diets not supplemented with Se (Se0), supplemented with sodium selenite (SeS) or Se-enriched yeast, Selsaf (SeY) and kept under normal (N) or stressful (S) conditions for 12 weeks.

	Dietary Se supplementation			Rearing conditions		Interaction
	Se0	SeS	SeY	N	S	
Total lipid (% wet weight)	3.8 ± 0.7	3.5 ± 0.5	3.7 ± 0.4	3.7 ± 0.5	3.6 ± 0.6	0.288
Lipid peroxidation level						
E_{232}	12 ± 2	13 ± 2	12 ± 2	13 ± 2	12 ± 2	0.594
E_{268}	4.3 ± 1.7	4.7 ± 1.4	3.9 ± 1.2	4.4 ± 1.3	4.2 ± 1.6	0.615
LSFP	3.9 ± 0.4	3.8 ± 0.2	3.8 ± 0.2	3.8 ± 0.3	3.9 ± 0.3	0.051
FA composition (% total FA)						
Saturated FA	23.0 ± 1.0	22.8 ± 1.1	22.6 ± 0.7	23.0 ± 1.0	22.7 ± 0.8	0.572
Monounsaturated FA	23.0 ± 1.7	22.2 ± 1.2	22.3 ± 1.4	22.4 ± 1.2	22.6 ± 1.7	0.294
n-6 PUFA	18.0 ± 0.7	18.0 ± 0.6	18.4 ± 0.4	18.2 ± 0.6	18.0 ± 0.6	0.516
n-3 PUFA	32.4 ± 1.6	33.3 ± 1.0	33.3 ± 1.3	32.8 ± 1.1	33.2 ± 1.6	0.812
Long-chain n-3 PUFA	15.6 ± 1.7	16.3 ± 1.3	15.9 ± 1.1	15.8 ± 1.3	16.1 ± 1.4	0.540
EPA	4.8 ± 0.4^{b}	5.1 ± 0.3^{a}	4.9 ± 0.4^{ab}	4.8 ± 0.4^{y}	5.1 ± 0.5^{z}	0.978
DHA	8.3 ± 1.2	8.7 ± 1.1	8.4 ± 0.7	8.4 ± 1.0	8.5 ± 1.1	0.230
Unsaturation index	203 ± 7	207 ± 5	206 ± 5	204 ± 5	206 ± 6	0.852

E_{232}, conjugated dienes; E_{268}, conjugated trienes; LSFP, lipid-soluble fluorescent products; FA, fatty acid; PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Unsaturation index = sum (fatty acid percent) × (number of double bonds).
Values are means ± SD of 18 or 27 fish (two conditions per Se level or three diets per condition). Within rows and for each effect: dietary Se supplementation or rearing condition, means not sharing a common superscript letter are significantly different according to two-way ANOVA.

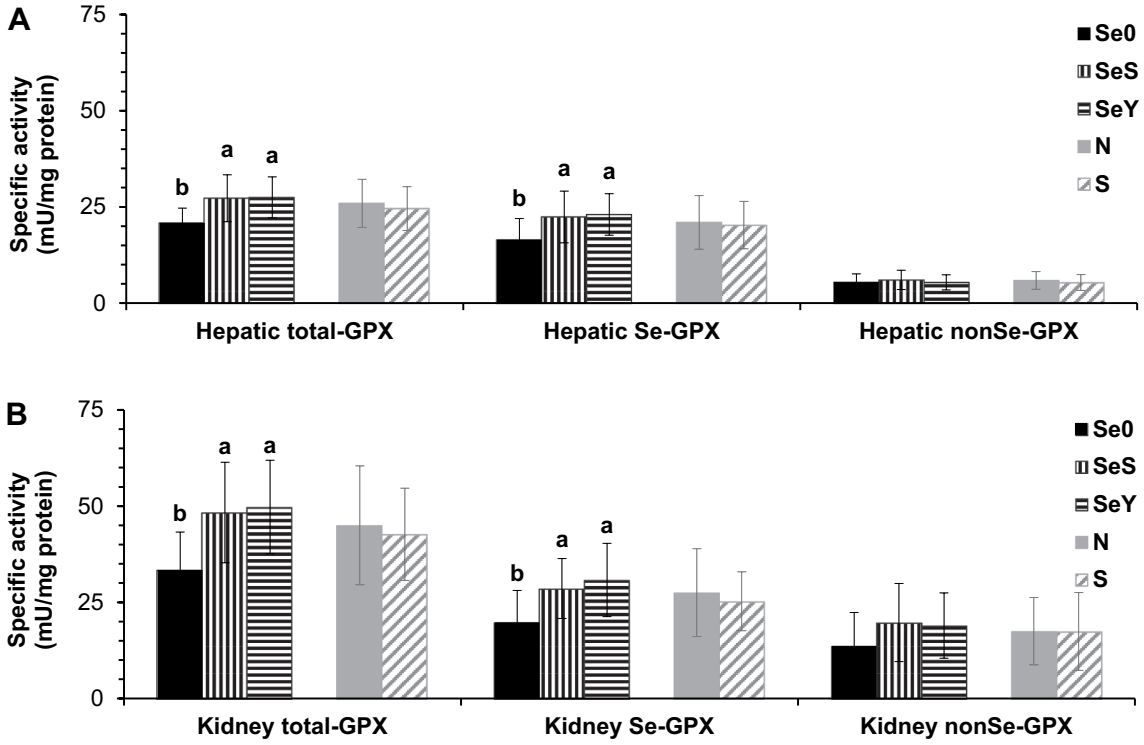

Fig. 4. Total, Se-dependent and non-Se dependent glutathione peroxidase activity in liver (A) and kidney (B) of rainbow trout juveniles fed the experimental diets not supplemented with Se (Se0), supplemented with sodium selenite (SeS) or Se-enriched yeast, Selsaf (SeY) and kept under normal (N) or stressful (S) conditions for 12 weeks. Bars represent means ± SD of 18 or 27 fish (two conditions per Se level or three diets per condition). Means not sharing a common superscript letter are significantly different ($P < 0.05$) according to two-way ANOVA followed by a Newman-Keuls test. The interaction between dietary Se supplementation and rearing conditions was not significant.

4. Discussion

4.1. Impact of Se supplementation in plant-based diets on antioxidant metabolism in rainbow trout juveniles

Similar to our previous study in rainbow trout fry (Fontagné-Dicharry et al., 2015), growth performance of juveniles was not affected by dietary Se sources and levels ranging from 0.47 to 0.93 mg/kg diet. These dietary Se levels correspond to available Se levels of 0.42 mg/kg diet for the non-Se supplemented diet Se0, 0.81 mg/kg diet for the selenite- supplemented diet, SeS and 0.83 mg/kg diet for the SeMet-supplemented diet, SeY. This finding is in accordance with the earlier results of Hilton et al. (1980), who reported a Se requirement of 0.15 mg/kg diet based on weight gain as response criterion or 0.38 mg/kg diet based on maximal plasma GPX activity. According to the meta-analysis of Antony Jesu Prabhu et al. (2016), hepatic GPX activity is a more stringent and robust criterion than weight gain to define Se deficiency in fish. In the present study, both hepatic and kidney GPX activities were improved by dietary Se supplementation (from 31 to 40% in liver to 45–56% in kidney), suggesting that the basal dietary Se level of 0.47 mg/kg diet meaning 0.42 mg/kg diet of available Se in the non-Se supplemented diet Se0 was not sufficient to sustain proper GPX activity in rainbow trout juveniles fed plant-based diets. Besides, this suggests that kidney GPX activity can be a reliable marker to define Se deficiency. These results are consistent with our previous observations of reduced whole-body GPX activity in rainbow trout fry fed a similar plant-based diet containing 0.50 mg Se/kg diet (Fontagné-Dicharry et al., 2015). These plant-based diets contained predominantly SeMet, with some selenite and selenate (Godin et al., 2015). This is coherent with the present ADC of Se that is intermediate for the non-Se supplemented diet Se0 compared to the selenite- and SeMet-supplemented diets, SeS and SeY, respectively. So, the higher dietary need for Se in rainbow trout from the present study compared to the study of Hilton et al. (1980) using semi-purified diets supplemented with sodium selenite might not be attributed to a reduced Se availability in the basal diet Se0. On the other hand, an interacting effect of dietary vitamin E

Table 7
Antioxidant enzyme activity in liver of rainbow trout juveniles fed the experimental diets not supplemented with Se (Se0), supplemented with sodium selenite (SeS) or Se-enriched yeast, Selsaf (SeY) and kept under normal (N) or stressful (S) conditions for 12 weeks.

	Dietary Se supplementation			Rearing conditions		Interaction
	Se0	SeS	SeY	N	S	
SOD (U/mg protein)	31 ± 9	25 ± 5	28 ± 4	27 ± 6	31 ± 7	0.686
CAT (U/mg protein)	542 ± 94	511 ± 111	531 ± 97	489 ± 86[b]	568 ± 98[a]	0.545
GR (mU/mg protein)	24 ± 15	26 ± 9	25 ± 13	28 ± 12	23 ± 13	0.540
GST (mU/mg protein)	334 ± 66	319 ± 46	310 ± 54	318 ± 54	324 ± 59	0.292

Values are means ± SD of 18 or 27 fish (two conditions per Se level or three diets per condition). Within rows and for each effect: dietary Se supplementation or rearing condition, means not sharing a common superscript letter are significantly different according to two-way ANOVA.

Table 8
Antioxidant transcript levels in liver of rainbow trout juveniles fed the experimental diets not supplemented with Se (Se0), supplemented with sodium selenite (SeS) or Se-enriched yeast, Selsaf (SeY) and kept under normal (N) or stressful (S) conditions for 12 weeks.

	Dietary Se supplementation			Rearing conditions		Interaction
	Se0	SeS	SeY	N	S	
gpx1a	0.9 ± 0.4	0.9 ± 0.3	1.0 ± 0.2	1.0 ± 0.3^{z}	0.8 ± 0.2^{y}	0.959
gpx1b1	1.0 ± 0.3	0.9 ± 0.3	1.1 ± 0.3	1.0 ± 0.3	1.0 ± 0.3	0.795
gpx1b2	1.1 ± 0.3^{b}	1.2 ± 0.3^{a}	1.5 ± 0.3^{a}	1.2 ± 0.4	1.3 ± 0.3	0.876
gpx4a1	0.9 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.965
gpx4a2	1.0 ± 0.3^{b}	1.4 ± 0.3^{a}	1.4 ± 0.3^{a}	1.4 ± 0.4^{z}	1.1 ± 0.3^{y}	0.123
gpx4b	0.8 ± 0.2	0.9 ± 0.3	0.8 ± 0.2	0.9 ± 0.2^{z}	0.7 ± 0.2^{y}	0.089
selpa2	1.1 ± 0.3	1.0 ± 0.4	1.1 ± 0.2	1.0 ± 0.3	1.1 ± 0.3	0.605
tr3a	1.3 ± 0.6	1.1 ± 0.4	1.2 ± 0.5	1.0 ± 0.4^{y}	1.3 ± 0.5^{z}	0.837
tr3b	1.0 ± 0.5	0.9 ± 0.4	1.0 ± 0.4	1.0 ± 0.3	0.9 ± 0.5	0.928
msrb2	1.1 ± 0.3	1.1 ± 0.4	1.2 ± 0.3	1.0 ± 0.3^{y}	1.3 ± 0.3^{z}	0.169
sod1	1.1 ± 0.4	1.1 ± 0.5	1.4 ± 0.7	1.2 ± 0.6	1.2 ± 0.5	0.631
sod2	0.9 ± 0.2	0.8 ± 0.2	0.9 ± 0.3	0.9 ± 0.2	0.9 ± 0.3	0.213
cat	1.0 ± 0.3	0.8 ± 0.3	1.0 ± 0.3	0.9 ± 0.3^{y}	1.1 ± 0.3^{z}	0.506
gr	1.1 ± 0.3	1.0 ± 0.3	1.2 ± 0.5	1.1 ± 0.4	1.1 ± 0.4	0.356
gst	1.2 ± 0.4	1.1 ± 0.4	1.1 ± 0.4	1.1 ± 0.3	1.2 ± 0.4	0.736
gclc	1.2 ± 0.3	1.3 ± 0.5	1.2 ± 0.4	1.1 ± 0.3	1.3 ± 0.5	0.762
nrf2	1.0 ± 0.5	0.9 ± 0.2	0.9 ± 0.3	0.9 ± 0.2	1.0 ± 0.4	0.520

Values are means ± SD of 18 or 27 fish (two conditions per Se level or three diets per condition), normalized to EF1α RNA and expressed as fold-changes of mRNA abundance compared with the control group Se0-N. Within rows and for each effect: dietary Se supplementation or rearing condition, means not sharing a common superscript letter are significantly different according to two-way ANOVA.

level could not be excluded. The dietary vitamin E level used in the present study, even meeting the vitamin E requirement of rainbow trout according to NRC (2011), was eight times lower than the one used by Hilton et al. (1980) and could have increased the dietary need for Se in this study as was suggested in the meta-analysis by Antony Jesu Prabhu et al. (2016).

The reduction of hepatic and kidney Se-GPX activities in fish fed the non-Se supplemented diet was associated with a reduction of total-GPX activity. There was no compensatory increase of non-Se-dependent GPX activity in agreement with Bell et al. (1985, 1986) in the liver of rainbow trout but contrary to our previous findings in whole-body of rainbow trout fry (Fontagné-Dicharry et al., 2015). Besides, the other antioxidant enzymes were not either affected in agreement with Fontagné-Dicharry et al. (2015) and Bell et al. (1985) but contrary to Bell et al. (1986) and Gatlin et al. (1986) who reported an increased hepatic GST activity in Se-deficient fish. The reduced activity of Se-GPX in fish fed Se0 was associated with concomitant decreased expression of the Se-GPX genes *gpx1b2* and *gpx4a2* confirming the observations on GPX transcript expression in rainbow trout hepatocytes (Pacitti et al., 2013), fry (Fontagné-Dicharry et al., 2015) and progeny (Wischhusen et al., 2019). On the other hand, *gpx1a* and *gpx4a* were not affected by increased Se intake in the present study whereas these transcripts were reported to be the most sensitive ones among cytosolic and phospholipid hydroperoxide GPX (Pacitti et al., 2013). Except for *gpx1b2* and *gpx4a2*, antioxidant or selenoprotein transcript expressions were not affected by Se intake, in conjunction with antioxidant enzyme activities. The selenoprotein P, considered as a major selenoprotein involved in the transport and delivery of Se to remote tissues (Papp et al., 2007) and a good marker of Se status in rainbow trout fry and broodstock (Fontagné-Dicharry et al., 2015; Wischhusen et al., 2019), was not affected by dietary Se level in the present study with rainbow trout juveniles whereas diet compositions, Se levels and forms were comparable. In zebrafish, the expression of the selenoprotein P paralogues has been shown to differ, with only *sepp1a* responding to Se status (Penglase et al., 2014b). Similar results were reported in rainbow trout broodstock with *selpa* and *selpa2* responding to Se status contrary to *selpb* (Wischhusen et al., 2019). In the present study, we assessed the expression of *selpa2*, a putative Se-sensitive paralogue and this difference of regulation might be attributed to a difference of sensitivity between life stages, with juvenile stages being less sensitive or able to adapt more quickly to Se deficiency than fry or broodstock as suggested for oxidative stress (Fontagné-Dicharry et al., 2018). If selenoprotein P level has been proved to be a good biomarker of Se status in higher vertebrates, its mRNA level is not always decreased by Se deficiency contrary to GPX1 (Sunde and Raines, 2011). Such difference between protein and mRNA levels might exist in fish and it would have been interesting to also analyze tissue selenoprotein P levels in the present study to obtain more information about the hierarchy of Se regulation of the various selenoproteins and their mRNA.

The oxidative status was mildly altered by dietary Se levels and forms with only a decrease of anisidine value in muscle of rainbow trout juveniles fed Se-enriched yeast compared to the control group. This result supports the superiority of organic Se form to sustain the antioxidant status of fish reported by Küçükbay et al. (2009) and Fontagné-Dicharry et al. (2015) in rainbow trout and by Saffari et al. (2016) and Mechlaoui et al. (2019) in other fish species. This finding is probably related to the superior bioavailability of the organic form, resulting in improved body Se contents compared to the inorganic form (171 vs. 156% compared to the non-Se supplemented diet Se0). Nevertheless, this could not be due only to the higher digestibility of the organic form (91 vs 87%) that results in a small difference of available Se between yeast and selenite (201 vs. 194% compared to the non-Se supplemented diet Se0) but probably also to a different metabolism between Se forms. In Atlantic salmon, organic Se intake has been shown to result in improved body Se especially in muscle (Lorentzen et al., 1994; Antony Jesu Prabhu et al., 2020) as SeMet (Sele et al., 2018) compared to inorganic Se intake. However, if organic Se supplementation has been shown to increase SeMet levels in liver of rainbow trout, hepatic SeCys levels were not significantly affected similar to observations in the whole-body of rainbow trout fry (Godin et al., 2015). These results would suggest the non-specific incorporation of dietary Se as SeMet into hepatic protein rather than its incorporation as SeCys into functional selenoenzymes such as GPX, even if this assumption is not sustained by the hepatic GPX activity and transcript expression data. Nevertheless, different metabolism of selenite and SeMet between liver and muscle probably occur, resulting in differential Se deposition as suggested in salmon (Lorentzen et al., 1994; Sele et al., 2018; Antony Jesu Prabhu et al., 2020). It would have been interesting to also analyze muscle Se

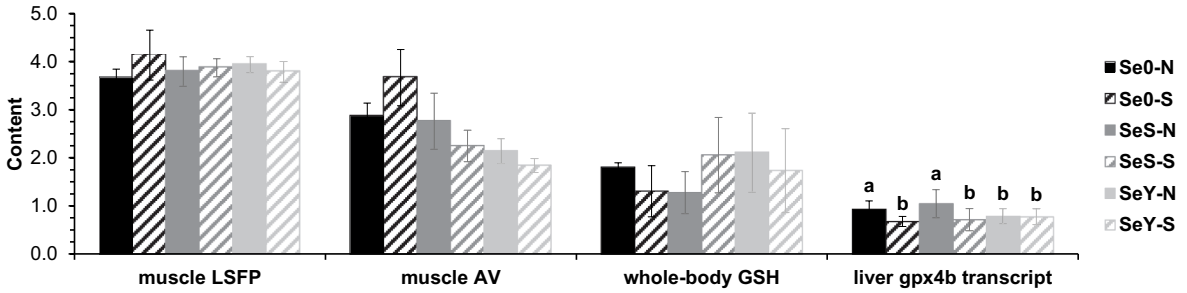

Fig. 5. Oxidative stress parameters displaying a tendency towards an interaction between dietary Se supplementation and rearing conditions in rainbow trout juveniles fed the experimental diets not supplemented with Se (Se0), supplemented with sodium selenite (SeS) or Se-enriched yeast, Selsaf (SeY) and kept under normal (N) or stressful (S) conditions for 12 weeks. Means not sharing a common superscript letter are significantly different ($P < 0.05$) according to one-way ANOVA followed by a Newman-Keuls test.

levels in the present study, coupled with the muscle selenoenzymes to figure out the role of non-specific SeMet incorporation into proteins on the antioxidant protection in rainbow trout muscle.

4.2. Impact of chronic stress exposure on oxidative stress in rainbow trout juveniles

Similar to our results on the impact of chronic stress exposure combining stocking density and hypoxia, reduced growth and feed intake were reported in rainbow trout subjected to crowding conditions but with reduced feed efficiency (Trenzado et al., 2006; Küçükbay et al., 2009; Naderi et al., 2017). Morever, contrary to the present study with chronic stress exposure, even in different tissues, Küçükbay et al. (2009) also reported a reduction of serum and muscle Se levels and serum GPX activity, with an increase of serum and muscle TBARS content with crowding conditions and Naderi et al. (2017) observed a decrease of the serum total antioxidant capacity and hepatic vitamin E content. In the present study, we only observed a reduction of some hepatic GPX mRNA contents that could relate to the changes of oxidative stress markers reported in the former studies. These differences could be linked to the intensity of the stress that was lower in the present study with a density of 42 kg/m^3 compared to the three previous ones with a density of 100 kg/m^3. Moreover, according to Ellis et al. (2002), the magnitude of the density effect depends upon study-specific conditions, including behavioral interactions and water quality deterioration with a special attention to dissolved oxygen level.

The reduction of growth, feed intake and nutrient digestibility by chronic stress exposure combining both decreased water quality with hypoxia and increased stocking density noticed in the present study is in accordance with results reported in a rainbow trout isogenic line exposed to hypoxia by Magnoni et al. (2018). However, the effects of chronic stress exposure on oxidative stress induction were less clear as blood GSH level was reduced and hepatic CAT activity and mRNA content were increased as well as *msrb2* and *tr3a* transcript levels but along with some GPX mRNA contents that were reduced as well as blood GSSG level and GSSG/GSH ratio. Such discrepancy between antioxidant enzymes has been highlighted in different fish species by Lushchak and Bagnyukova (2006) who distinguished a conventional response to hypoxic stress with antioxidant enzymes remaining unchanged or being lowered over hypoxia in fish and the preparation to oxidative stress response with an increase of antioxidant enzymes only in cyprinids exposed to hypoxia. Likewise, Magnoni et al. (2019) reported no increase of oxidative stress in liver of a rainbow trout isogenic line contrary to Varghese et al. (2017) who noticed an oxidative stress induction in the mrigal carp, *Cirrhinus mrigala*. On the other hand, Pérez-Jiménez et al. (2012) recorded dual response of antioxidant enzymes similar to our results, but with decreased SOD and increased GPX activity in gilthead sea bream exposed to an acute hypoxic challenge and CAT activity was increased by hypoxia only if fish were fed a methionine-supplemented diet.

4.3. Interaction between dietary Se supplementation and chronic stress exposure on oxidative stress in rainbow trout juveniles

In the present study, no significant interaction on oxidative stress induction between chronic stress exposure combining hypoxia and stocking density and dietary Se supplementation was detected. The beneficial effect of dietary Se supplementation was observed only for starch digestibility under normal conditions but not under stressful conditions. A tendency for an increased lipid peroxidation with reduced antioxidant transcript level in stressful conditions could be noticed only in non-supplemented (for muscle LSFP, AV and hepatic *gpx4b* data) or selenite-supplemented fish (for whole-body GSH and hepatic *gpx4b* data) but it was significant only for hepatic gpx4b mRNA levels according to one-way ANOVA (Fig. 5). If significant, it could have indicated that Se requirements may not be met by the non-supplemented or selenite-supplemented diet in stressed fish similar to the results reported by Rider et al. (2009) in juvenile rainbow trout fed a non-supplemented diet containing 0.73 mg Se/kg diet subjected to chronic physical stress. However, this interaction was not significant for almost all parameters. So according to the present results, dietary Se supplementation did not appear to alleviate the detrimental effects of chronic stress exposure that were, however, quite limited on oxidative stress induction. Similarly, no effect of dietary Se supplementation was noticed on rainbow trout under high stocking density conditions with 80 kg/m^3 by Naderi et al. (2017) contrary to dietary vitamin E supplementation. On the other hand, Rider et al. (2009) and Küçükbay et al. (2009) indicated that dietary Se supplementation could prevent the negative effects of chronic physical stress (daily handling and confinement stressors) and crowding conditions with 100 kg/m^3 in rainbow trout juveniles. In the present study, even if the stocking density was doubled in stressful conditions to reach 42 kg/m^3, this could not be considered as crowding conditions for rainbow trout according to the review of Ellis et al. (2002). So stress conditions were different and could explain the observed differences between studies. In order to ascertain this, the impact of different stress conditions on oxidative stress induction and antioxidant metabolism in rainbow trout deserves further studies to gain a better understanding of the mechanisms involved.

5. Conclusion

In summary, our results suggest the necessity to supplement plant-based diets with Se for rainbow trout juveniles, independently of chronic stress exposure and highlight the superiority of organic form to fulfil the dietary Se requirement probably due in part to a better

apparent availability and to sustain the antioxidant status of fish. However, dietary Se supplementation did not allow to alleviate the detrimental effects on growth performance and antioxidant metabolism induced by chronic stress exposure.

CRediT authorship contribution statement

Stéphanie Fontagné-Dicharry: Conceptualization, Formal analysis, Writing - original draft, Visualization, Supervision, Funding acquisition.**Vincent Véron:**Investigation.**Laurence Larroquet:** Investigation.**Simon Godin:**Investigation.**Pauline Wischhusen:** Investigation.**Pierre Aguirre:**Investigation.**Frédéric Terrier:** Investigation.**Nadège Richard:**Visualization, Project administration. **Maïté Bueno:**Conceptualization, Methodology, Validation, Supervision.**Brice Bouyssière:**Conceptualization, Methodology, Supervision.**Philip Antony Jesu Prabhu:**Conceptualization, Writing - review & editing.**Philippe Tacon:**Resources, Project administration, Funding acquisition.**Sadasivam J. Kaushik:**Conceptualization, Writing - review & editing, Funding acquisition.

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.

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[1] **P. Wischhusen***, M. Parailloux, P.-A. Geraert, M. Briens, M. Bueno, S. Mounicou, B. Bouyssiere, P. Antony Jesu Prabhu, S.J. Kaushik, B. Fauconneau, S. Fontagné-Dicharry, Effect of dietary selenium in rainbow trout (*Oncorhynchus mykiss*) broodstock on antioxidant status, its parental transfer and oxidative status in the progeny, Aquaculture. 507 (2019) 126–138. https://doi.org/10.1016/j.aquaculture.2019.04.006

[2] **P. Wischhusen***, C. Arnaudguilhem, M. Bueno, G. Vallverdu, B. Bouyssiere, M. Briens, P. Antony Jesu Prabhu, P.-A. Geraert, S.J. Kaushik, B. Fauconneau, S. Fontagné-Dicharry, S. Mounicou, Tissue localization of selenium of parental or dietary origin in rainbow trout (*Oncorhynchus mykiss*) fry using LA-ICP MS bioimaging. (submitted to Metallomics)

[2] **P. Wischhusen***, L. Larroquet, T. Durand, C. Oger, J.-M. Galano, A. Rocher, C. Vigor, P. Antony Jesu Prabhu, V. Véron, M. Briens, J. Roy, S.J. Kaushik, B. Fauconneau, S. Fontagné-Dicharry, 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. 155 (2020) 99–113. https://doi.org/10.1016/j.freeradbiomed.2020.05.006

[3] **P. Wischhusen***, T. Saito*, C. Heraud, S. Kaushik, B. Fauconneau, P. Antony Jesu Prabhu, S. Fontagné-Dicharry, K.H. Skjærven, Parental selenium affects the one carbon metabolism and hepatic DNA methylation pattern in rainbow trout (*Oncorhynchus mykiss*) fry. Life, 121 (2020) 10(8). https://doi.org/10.3390/life10080121

[5] **P. Wischhusen***, C. Heraud, K.H. Skjærven, S. Kaushik, B. Fauconneau, P. Antony Jesu Prabhu, S. Fontagné-Dicharry, Long-term effect of parental selenium on the 1C metabolism in rainbow trout (*Oncorhynchus mykiss*) fry exposed to hypoxic stress. (submitted to British journal of Nutrition)

[6] S. Fontagné-Dicharry*, V. Veron, L. Larroquet, S. Godin, **P. Wischhusen**, P. Aguirre, F. Terrier, N. Richard, M. Bueno, B. Bouyssiere, P. Antony Jesu Prabhu, P. Tacon, S. Kaushik, Effect of selenium sources in plant-based diets on antioxidant status and oxidative stress-related parameters in rainbow trout juveniles under chronic stress exposure, Aquaculture. 529 (2020) 735684, https://doi.org/10.1016/j.aquaculture.2020.735684

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LIST OF COMMUNICATIONS

[1] **P. Wischhusen***, M. Briens, P.-A. Geraert, P. Antony Jesu Prabhu, L. Larroquet, V. Veron, S.J. Kaushik, B. Fauconneau, S. Fontagne-Dicharry, Parental and direct feeding effects of dietary selenium in rainbow trout (*Oncorhynchus mykiss*) fry, (2019). Aquaculture Europe 2019, Berlin, Germany.

[2] **P. Wischhusen***, P. Antony Jesu Prabhu, B. Fauconneau, S. Fontagne-Dicharry, S. Panserat, Nutritional programming in rainbow trout, (2019). Current perspectives in Aquaculture: Fish epigenetics, Tromso, Norway.

[3] **P. Wischhusen**, M. Parailloux, P.-A. Geraert, M. Briens, M. Bueno, S. Mounicou, B. Bouyssiere, P. Antony Jesu Prabhu, S.J. Kaushik, B. Fauconneau, S. Fontagné-Dicharry*, Influence du selenium de l'aliment des geniteurs de truite arc-en-ciel sur le statut anti-oxydant de la descendance, (2019). Journées de la recherche filière piscicole, Paris, France.

[4] **P. Wischhusen***, M. Parailloux, M. Briens, P.-A. Geraert, P. Antony Jesu Prabhu, S.J. Kaushik, B. Fauconneau, S. Fontagne-Dicharry, Effect of organic and inorganic dietary selenium supplementation in rainbow trout Oncorhynchus mykiss broodstock on the oxidative status in progeny, (2018). AQUA2018, Montpellier, France.

[5] **P. Wischhusen***, M. Briens, P.-A. Geraert, M. Parailloux, B. Bouyssière, M. Bueno, S. Mounicou, P. Antony Jesu Prabhu, K. Skjaerven, B. Fauconneau, S. Fontgné-Dicharry, Effect of dietary inorganic and organic selenium supplementation on reproduction and egg quality in rainbow trout (*Oncorhynchus mykiss*), (2018). ISFNF2018, Las Palmas de Gran Canaria, Spain.

[6] S. Fontagne-Dicharry*, V. Véron, S. Godin, L. Larroquet, P. Aguirre, F. Terrier, **P. Wischhusen**, M. Bueno, B. Bouyssière, N. Richard, Effects of different dietary selenium sources on antioxidant status and oxidative stress-related parameters in rainbow trout juveniles fed plant ingredients, (2018). ISFNF2018, Las Palmas de Gran Canaria, Spain.

[7] **P. Wischhusen***, S. Mounicou, C. Arnauldguilhem, M. Bueno, B. Bouyssiere, M. Briens, P.-A. Geraert, P. Antony Jesu Prabhu, B. Fauconneau, S. Fontagne-Dicharry, Parental selenium and antioxidant status in fish, (2018). MIRA, Anglet, France.

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Abstract: Selenium (Se) nutrition in rainbow trout broodstock and their F1 progeny fed plant-based diets was studied. The diets were unsupplemented or added with 0.3 ppm Se as inorganic (sodium selenite) or organic (hydroxy-selenomethionine, OH-SeMet). Parental Se, especially as organic form, improved reproduction performance and increased body Se levels in the progeny before first feeding. In the F1 progeny, tissue Se distribution was modified pending on parental and dietary Se level and form. OH-SeMet efficiently raised muscle Se content, but the direct sodium selenite feeding resulted in higher liver Se levels. In short-term the antioxidant system including glutathione peroxidase (GPx) was supported by parental OH-SeMet, even if a decreased ratio of reduced to oxidized glutathione was also noticed. We found that parental Se can cause long-term modifications in the glutathione metabolism that even persist after the beginning of exogenous feeding. Fingerling showed lower stress tolerance towards hypoxia when originating from parents fed Se-supplemented diets, but direct Se feeding increased stress resistance. The contrary effect observed between direct and parental Se nutrition on the GPx and antioxidant system might relate to a nutritional programming effect, where either the progeny of the control group compensated the low dietary Se levels or the fry originating from Se-supplemented groups was less tolerant towards low dietary Se levels. Epigenetic modifications can underlay nutritional programming effects and indeed we found that the liver DNA methylation pattern at swim-up fry stage was sensitive towards the parental Se regime in terms of both Se level and form. Genes identified in the study point towards several metabolic pathways that might be affected by parental Se nutrition. Overall, this work highlighted modifications in short and long term of different metabolic pathways including antioxidant system by parental and dietary Se in rainbow trout fry in interaction with dissolved oxygen levels, which could allow further optimization of new feed formulations for broodstock and fry stages.

Résumé : L'objectif de cette thèse a été d'étudier le rôle du sélénium (Se) dans l'alimentation des géniteurs de truites arc-en-ciel et leur progéniture (génération F1). Les aliments à base d'ingrédients végétaux ont été supplémentés avec 0,3 ppm de Se apporté sous forme inorganique (sélénite de sodium) ou organique (hydroxy-sélénomethionine, OH-SeMet). Le Se, plus particulièrement la forme organique, dans l'alimentation des géniteurs a amélioré les performances de reproduction et augmenté les teneurs corporelles en Se de la progéniture avant le premier repas. Chez la progéniture F1, la distribution tissulaire du Se a été affectée à la fois par le Se présent dans l'aliment des géniteurs et celui des alevins. L'OH-SeMet a amélioré nettement la teneur en Se du muscle alors que le sélénite de sodium de l'aliment des alevins a augmenté le teneur en Se du foie. A court terme, les défenses anti-oxydantes avec notamment la glutathion peroxydase (GPx) ont été stimulées par la supplémentation en OH-SeMet des géniteurs, même si une diminution du rapport glutathion réduit/glutathion oxydé a également été notée. Nous avons pu montrer que la supplémentation en Se des géniteurs pouvait entraîner des modifications à long terme du métabolisme du glutathion qui ont persisté après le début de l'alimentation exogène. Une moins bonne résistance au stress hypoxique a été observée chez les alevins issus des géniteurs nourris avec des régimes supplémentés en Se, alors que la supplémentation en Se dans l'alimentation des alevins a amélioré la résistance au stress. L'effet antagoniste entre l'apport en Se par l'aliment ou les parents sur la GPx et les défenses anti-oxydantes pourrait être lié à une programmation nutritionnelle où soit la progéniture issue du lot contrôle compenserait les faibles niveaux alimentaires de Se, soit la progéniture issue des groupes supplémentés en Se serait moins tolérante aux faibles niveaux alimentaires en Se. Des modifications épigénétiques pourraient être à l'origine de cette programmation nutritionnelle et, en effet, nous avons montré que le niveau de méthylation de l'ADN du foie des alevins émergents était sensible à la composition en Se du régime des géniteurs, à la fois en terme de teneur et de forme. Les gènes identifiés dans cette étude indiquent que plusieurs voies métaboliques pourraient être affectées par l'alimentation en Se des géniteurs. L'ensemble de ce travail a mis en évidence des modifications à court et long terme de différentes voies métaboliques incluant les défenses anti-oxydantes par le Se d'origine parentale ou alimentaire chez les alevins de truite en interaction avec la teneur en oxygène dissous de l'eau ce qui devrait permettre à terme d'améliorer la formulation des nouveaux aliments piscicoles pour les stades géniteurs et alevins.

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