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Dietary selenium required to achieve body homeostasis and attenuate pro-inflammatory responses in Atlantic salmon post-smolt exceeds the present EU legal limit

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ABSTRACT

Selenium (Se) supplementation either as inorganic or organic form was evaluated in Atlantic salmon post-smolt *in vivo* and *in vitro*. The basal diet was formulated to be low in fish meal and contain 0.24 mg Se kg^{-1}; six other diets with Se inclusion of 0.15, 0.4, 0.7 or 1.1 mg kg^{-1} as sodium selenite (SS) and 0.15 or 0.4 mg kg^{-1} as L-selenomethionine (SM) were formulated from the basal diet. The diets were fed to Atlantic salmon post-smolt (mean initial weight, 216 ± 27 g) in triplicate groups (35 fish $tank^{-1}$) and reared in flow-through seawater (33 ppt) at 10–12 °C for 9 weeks. At the end of the feeding trial, whole fish and tissues were sampled for *in vivo* assessment; whereas, liver cells and head kidney leukocytes (HKL) were isolated and their primary cultures used for *in vitro* assessment following exposure to hydrogen peroxide (H_2O_2), lipopolysaccharide (LPS) or poly I:C (PIC). Growth, feed intake, feed conversion ratio, specific growth rate, hepato-somatic index, proximate composition and mineral concentration of the whole fish (except for Se) were unaffected by dietary Se ($p > .05$). Hematocrit was significantly higher in fish fed the 0.4 mg Se supplemented feeds, irrespective of the Se source ($p = .02$). The Se concentration in whole body, liver, muscle, plasma, kidney and liver/kidney Se ratio increased with increasing dietary Se concentration ($p < .0001$). Level of oxidised glutathione (GSSG) in liver and head kidney followed a quadratic function ($p < .05$) indicating that the concentrations were lower at intermediate SS supplementation of 0.4 and 0.65 mg Se kg^{-1} (total Se, 0.65 and 0.87 mg Se kg^{-1}). Impact of Se sources on glutathione redox status was similar. Slope-ratio analysis revealed SM to be more efficient than SS in improving apparent availability, whole body or tissues Se status, Se retention and reducing Se loss to the environment. *In vitro*, the mRNA expression of *p38mapk* and *aif*, in liver cells were affected by the impact of dietary Se, but not by the treatment of H_2O_2 ($p < .05$). In the HKL, the LPS and PIC induced pro-inflamatory action of *il-1β*, *cox2*, *nfkβ* and *viperin* were attenuated by SM supplementation, but not by SS ($p < .001$). Dietary Se supplementation required to the basal diet containing 0.24 mg Se kg^{-1} was 0.41 mg Se as SS (total 0.65 mg kg^{-1}) or 0.17 mg Se as SM (total 0.41 mg kg^{-1}) based on body Se homeostasis or tissue Se status. SM inclusion at 0.4 mg kg^{-1} diet (total, 0.65 mg kg^{-1}) attenuated LPS or PIC induced pro-inflammatory responses *in vitro*. Overall, Se requirement of Atlantic salmon post smolt was 0.27 mg kg^{-1} diet, on availale basis. Dietary Se level required to maintain body Se homeostasis and improved health status of Atlantic salmon fed plant-based diets (0.65 mg kg^{-1} diet) exceed the existing EU maximum limit of 0.5 mg Se kg^{-1} diet. SM as the Se source in salmon feeds has the potential to improve salmon health and reduce Se emissions from Norwegan salmon farming by 60 to 70%.

Abbreviations: SS, sodium selenite.; SM, selenomethionine.; LPS, lipopolysaccharide.; PIC, polyinosinic:polycytidylic acid.; AAC, apparent availability coefficient.; il-1b, interleukin 1 beta.; p38mapk, p38 mitogen-activated protein kinases.; 5lox, Arachidonate 5-lipoxygenase.; cox2, cyclooxygenase2.; sod2, superoxide dismutase2.; casp3, caspase 3.; cat, catalase.; viperin, virus inhibitory protein, endoplasmic reticulum [ER]-associated, interferon [IN]-inducible.; bcl2, B-cell lymphoma 2.; tnfα, tumor necrosis factor alpha.; nfkβ, nuclear factor kappa-beta.; pparα, peroxisome proliferator activated receptor alpha.; gpx2, glutathione peroxidase 2.; gpx3, glutathione peroxidase 3.; aif, apoptosis-inducing factor.; β-actin, beta actin.; ef1α, elogation factor1 alpha.; rpl13, ribosomal protein L13.

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1. Introduction

Selenium (Se) is an essential nutrient exerting its role as selenoproteins (SeCys) and selenium containing proteins (SeMet) in numerous biological processes involving free radical and antioxidant metabolism, immune and inflammatory responses (Regina, 2015). Diet is the major route of Se supply to fish (Janz, 2012) and the dietary Se level essential to satisfy the requirement to fish ranges between 0.15 and 1.85 mg kg^{-1} diet, across different fish species (Antony Jesu Prabhu et al., 2016). Among salmonids, rainbow trout has a minimal requirement in the range of 0.15–0.38 mg Se kg^{-1} diet (Hodson and Hilton, 1983); whereas, the Se requirement is yet to be established in Atlantic salmon (NRC, 2011). An indication of a dietary Se range (0.6 to 0.9 mg kg^{-1} diet) to satisfy requirement in post-smolt Atlantic salmon was obtained recently from a multi-nutrient regression study (Antony Jesu Prabhu et al., 2019). Over the years, salmonid feeds included considerable levels of fish meal (Aas et al., 2019), a good source of Se (Julshamn et al., 1978). The fish meal based salmonid feeds often contain close to 1 mg Se kg^{-1} diet and therefore Se supplementation was not considered essential to meet the minimal Se requirements (Bell and Cowey, 1989; Lorentzen et al., 1994). Reduction in fish meal with concomitant increase in plant protein sources has reduced Se supply (Betancor et al., 2016; Fontagne-Dicharry et al., 2015; Sissener et al., 2013), altered Se speciation (Godin et al., 2015; Sele et al., 2018) and reduced dietary availability (Antony Jesu Prabhu et al., 2014; Silva et al., 2019). Nevertheless, the maximal permitted Se concentrations in animal feeds, including fish feeds in the EU is 0.5 mg Se kg^{-1} in complete feed (European Commission, 2014). Therefore, the importance of Se supplementation in plant ingredient based fish feeds gained significance ever more and with it, the bioavailability of different Se forms.

The chemical form of Se differentially affects the bioavailability and toxicity in fish feeds (Berntssen et al., 2017). Different inorganic (sodium selenite and sodium selenate) or organic Se forms (selenomethionine, selenocysteine, selenised yeast) have been studied in salmonids and found to vary in their bioavailability and toxicity thresholds (Bell and Cowey, 1989; Berntssen et al., 2018; Berntssen et al., 2017; Fontagne-Dicharry et al., 2015; Hamilton et al., 1990; Hardy et al., 2010; Hilton et al., 1980, 1982; Hodson and Hilton, 1983; Pacitti et al., 2016; Pacitti et al., 2015; Rider et al., 2010; Silva et al., 2019). A common consensus from the above listed studies is that, compared to inorganic Se, the organic Se forms show greater availability and higher ability to increase the Se status of the fish (Antony Jesu Prabhu et al., 2016).

Selenium, through selenoproteins influence innate and acquired immune responses, especially under conditions of stress (Arthur et al., 2003; Turner and Finch, 1991). During stress, Se stored as SeMet in Se-conatining proteins is liberated by transselenation *via* the transsulfuration pathway for the synthesis of selenoproteins (Burk and Hill, 2015). In accordance, organic Se is suggested to have a better ability to protect fish against physical, chemical or pathogen-induced stressors (Khan et al., 2017). In mammals, Se mediates inflammation by modifying cellular redox tone, which in turn regulates the *nfkb, mapk* and *tnfα* signalling pathways, affecting *cox* and *lox* enzymes (Bonizzi and Karin, 2004; Mattmiller et al., 2013). These pathways also regulate inflammation in fish and are responsive to dietary Se levels and sources, *in vivo* (Pacitti et al., 2016; Zheng et al., 2018a; Zheng et al., 2018b). Further, the stress mitigating effect of organic Se was observed often at supra-nutritional levels exceeding the minimal dietary requirement (Küçükbay et al., 2009; Pacitti et al., 2016; Rider et al., 2009; Wang et al., 1997; Zheng et al., 2018a; Zheng et al., 2018b). On the other hand, excess supply of dietary Se will increase load to the environment (Tornero and Hanke, 2016). The key to minimize Se load to environment is by improving dietary Se availability and retention. Therefore, it is important to improve the availability of dietary Se for better fish health, minimize environmental impact and to comply with the legislation.

Building on the information on the dietary Se level to satisfy the requirement with a multi-nutrient design (Antony Jesu Prabhu et al., 2019), this study was a targeted approach designed to determine dietary Se requirement and relative efficiency of two Se sources in Atlantic salmon post-smolt. Classical *in vivo* responses were complemented with a pathogen-mimic challenge *in vitro* to assess Se bioavailability and requirement in Atlantic salmon post-smolt fed diets low in fish meal.

2. Materials and methods

2.1. In vivo study

2.1.1. Experimental design

The study followed a regression design with 5 graded levels of dietary Se supplemented as sodium selenite (SS) to determine the dietary Se level in plant-based diets at which Se requirement is satisfied in Atlantic salmon post-smolt. Additionally, to assess the relative bioavailability of an organic Se source namely selenomethionine (SM), two diets with graded inclusion of SM were tested. The former was assessed through linear and non-linear regression models, while the latter using slope-ratio method.

2.1.2. Diet formulation and selenium levels

The basal mix was formulated to be low in Se concentration and thus the fish meal inclusion level was considerably reduced compared to the formulation used in commercial Atlantic salmon post-smolt feeds (Table 1). The selenium concentration of the major ingredients used in the basal mix was analysed from three different batches prior to feed production to ensure low basal Se levels (Table 2). The basal mix was supplemented with Se either as (i) sodium selenite (SS; $Na2SeO_3$, 4.5% Se, DSM nutritional products, Basel, Switzerland) or (ii) as L-selenomethionine (SM; $C_5H_{11}NO_2Se$, 0.16% Se, Orffa Additives, Werkendam, The Netherlands) to formulate seven experimental diets. The analysed Se concentrations in the experimental feeds were 0.24 (basal diet), 0.34, 0.65, 0.87 and 1.4 mg kg^{-1} in SS diets, 0.38 and 0.63 mg kg^{-1} in SM diets (Table 3). The Se levels were selected based on the range of dietary Se level at which tissue saturation occured in Atlantic salmon post-smolt, 0.6 to 0.9 mg kg^{-1} diet following the inclusion of a multi-nutrient package, including Se (Antony Jesu Prabhu et al., 2019). The actual Se concentration in the basal diet (0.24 mg kg^{-1}) was higher

Table 1
Formulation and composition of the basal experimental diet.

Ingredients (%)	
Whole wheat	10.0
Corn gluten	10.0
Wheat gluten	20.0
Soy protein concentrate	30.0
Faba beans, whole	1.0
Fish meal *	1.0
Fish oil *	10.5
Rapeseed oil †	13.0
Micro-ingredients and Se-free premixes ‡	4.4
Yttrium premix ¥	0.1
Proximate composition (analysed, n = 7)	
Dry weight (%)	93.1 ± 0.4
Energy (kJ/g)	23.9 ± 0.2
Lipid (%)	25.5 ± 0.5
Protein, analysed as N * 6.25 (%)	44.0 ± 0.1
Ash (%)	3.8 ± 0.1
Phosphorus (%)	0.82 ± 0.02

* North-Atlantic. † European, non-GM. ‡ Contains monoamonium phosphate, histidine HCl, L-lysine and DL-methionine and astaxanthin; standard vitamin and mineral mix, excluding selenium (Se). ¥ contains 10% yttrium oxide as the inter marker for apparent availability estimation.

Table 2
Selenium concentration of feed ingredients used in Atlantic salmon feeds.

Ingredient	Analysed Se concentration (mg kg^{-1})			Mean ± SD
	Batch 1[a]	Batch 2[b]	Batch 3[c]	
Fish meal, North Atlantic	2.72	2.48	3.12	2.77 ± 0.31
Whole wheat	0.02	0.16	0.11	0.10 ± 0.07
Wheat gluten	0.19	0.08	0.12	0.13 ± 0.06
Corn gluten	0.12	0.15	0.16	0.14 ± 0.02
Faba bean, dehulled	0.08	0.03	0.04	0.05 ± 0.03
Hi-Pro Soya	0.04	0.01	0.09	0.05 ± 0.04
Soy protein concentrate	0.03	0.04	0.18	0.08 ± 0.08

In every batch, two samples were analysed (n = 2).
[a] Analysed June 2017.
[b] Analysed Dec. 2017.
[c] Analysed April 2018.

than the desired level of 0.1 mg kg^{-1} possibly due to high variations in Se concentration of the ingredients between batches (Table 2). Consequently, the Se concentrations in the experimental feeds were also higher than desired (Table 3). The mean analysed protein, lipid, ash and energy content of the diets were respectively 44%, 26%, 3.8% and 24 kJ g^{-1}. All the diets contained yttrium oxide at 0.01% as inert digestibility marker. The diets were extruded and the pellet size was 4.0 mm (Skretting ARC).

2.1.3. Fish, feeding and rearing

Seven hundred and thirty-five Atlantic salmon post-smolt (mean weight, 216 ± 27 g), were distributed among 21 tanks with 35 fish in each (mean biomass, 7.56 ± 0.04 kg). The seven experimental diets were randomly assigned to the 21 tanks in triplicates. Prior to experimental feeding, the fish were maintained on commercial feeds (Spirit V 75-50A, 3.0 mm, Skretting). The fish were reared in circular tanks (volume, 1 m^3) supplied with flow-through seawater (salinity, 33 ppt) at 10–12 °C. The fish were fed the experimental diets twice a day to apparent satiation for 9 weeks. The uneaten feed pellets were collected to estimate the actual feed intake.

2.1.4. Fish sampling and analytical methods

The experiment was approved by the Norwegian Food Safety Authority (Mattilsynet) and conducted in accordance with the Norwegian laws and regulations concerning experiments with live animals. The feeding trial lasted 9 weeks and the all fish were individually weighed and measured for total length at the start and the end of the trial. Twenty fish were sampled at the start of the trial and homogenized for whole body proximate and mineral composition analysis. At the end of 9-week experimental feeding, all the fish were euthanized with an overdose (6 ml L^{-1}) of tricaine methanesulphonate (MS-222; PharmaQ, Bergen, Norway). All the fish were stripped and the collected faeces sample was pooled per tank, freeze dried for 72 h at −80 °C and stored at room temperature until further analysis (Silva et al., 2019). Ten fish per tank were then evacuated for any residual gut content and homogenized for whole body analysis. Further, blood samples were collected from six fish per tank using heparinized vacutainers and the plasma separated after centrifugation for 5 min at 3000*g* and the recovered plasma was collected as individual samples. Hematocrit was measured from a subsample of the blood (Antony Jesu Prabhu et al., 2017). After sampling for blood, the fish were dissected and sampled for liver, kidney and muscle, each tissue was pooled and homogenized per tank. All the tissue samples were stored at −20 °C until analysis. Individual samples of liver and head kidney from three fish per tank were flash frozen in liquid nitrogen and subsequently stored at −80 °C for the analysis of GSH, GSSG and calculation of the associated redox potential (Hamre et al., 2016). The diets were homogenized and analysed for determination of dry matter, ash, lipid, protein following standard procedures. Briefly, dry matter was measured after drying at 103 °C for 24 h; ash content determined by combustion in a muffle furnace at 550 °C for 16–18 h; lipid was determined following ethyl-acetate and acid-extraction in fish tissue and feeds, respectively (Lie, 1991); protein (6.25 x nitrogen) was measured with a nitrogen analyser (Vario Macro Cube, Elementar Analysensysteme GmbH, Germany) according to AOAC official methods of analysis (Sweeney and Rexroad, 1987). Selenium analysis in diets, faeces and tissue samples; and yttrium in feed and faeces samples were performed by inductively coupled plasma mass spectrometry (ICP-MS) (Silva et al., 2019).

2.1.5. Calculations

Apparent availability coefficient, % = 100 - [100*(yttrium in feed/yttrium in faeces)*(selenium in faeces/selenium in feed)].

Selenium retention, % = [(final biomass * final Se content) - (initial biomass * initial Se content)] * 100/ (Total feed intake * Se content in feed).please correct as required.

Faecal selenium loss, % = 100 – apparent availability coefficient.

Non-faecal selenium loss, % = 100*[(dietary Se intake - body Se gain – faecal Se loss)/dietary Se intake].

Body Se homeostasis = Final Se concentration – initial Se concentration.

Environmental load calculations: The retention and loss were extrapolated to estimate the Se load from Norwegian salmon farming assuming the annual Atlantic salmon production and feed consumption data (Aas et al., 2019). In addition, the median concentration of Se in Norwegian salmon feeds from the Norwegian fish feed surveillance program (Sanden et al., 2017) were used to estimate the intake, retention and loss considering a 'business-as-usual' scenario. The quantity of Se retained or lost to the environment was then compared with the three different scenarios of dietary Se found to satisfy requirement in this study, SS (0.4 ppm supplementation), SM (0.15 ppm supplementation) and SM (0.4 ppm supplementation).

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

$E_h = E^{0\prime} - RT/nF \ln([GSH]^2/[GSSG])$.where the GSH and GSSG

Table 3
Study design, selenium (Se) supplementation and analysed concentration of Se in the experimental diets.

Se source	Diet code	Se inclusion (mg kg^{-1} diet)	Desired Se levels (mg kg^{-1} diet)	Rationale and reference to Se levels tested	Analysed Se levels (mg kg^{-1} diet)
Basal diet	A	0	0.1	Low[1]	0.24
SS (Na_2SeO_3)	B	0.15	0.25	Sub-optimal [1]	0.34
	C	0.4	0.5	Requirement range[1]; Max limit[2]	0.65
	D	0.7	0.8	Requirement range[1]	0.87
	E	1.1	1.2	Above requirement[1]	1.4
SM (Se-Met)	F	0.15	0.25	Low[1]	0.38
	G	0.4	0.5	Requirement range[1]; Max limit[2]	0.63

[1] Dietary total Se level needed for tissue saturation of Atlantic salmon post-smolt in seawater, 0.6 to 0.9 mg kg^{-1} diet (Antony Jesu Prabhu et al., 2019). [2]Maximum permitted concentration for total Se in complete fish feeds with 12% moisture is 0.5 mg kg^{-1} diet (European Commission, 2014). SS, sodium selenite, Na2SeO_3, 4.5% Se, DSM nutritional products, Basel, Switzerland : SM, L-selenomethionine, $C_5H_{11}NO_2Se$, 0.16% Se, Orffa Additives, Werkendam, The Netherlands.

Table 4
Nucleotide Sequence of primers used in qPCR for mRNA expression analysis of target and house-keeping genes.

Gene	Forward (5′- > 3′)	Reverse (5′- > 3′)	Ascension No.	Reference
il1β	GTATCCCATCACCCCATCAC	GCAAGAAGTTGAGCAGGTCC	NM_001123582	Stenberg et al. (2019)
p38mapk	GGCACACAGACGATGAGATG	ACAGCGTTCTGCCAGTGAG	EF123661	
5lox	ACTAAGTTTGCTGCTTCGG	CTGACTCCAGACCTCGTG	CA387866	
cox2	GGAGGCCTACTCCAACCTATT	CGAACATGAGATTGGAACC	AY848944	
sod2	CCACGTCCATGCCTTTGG	TCAGCTGCTGCAGTCACGTT	DY718412	
casp3	ACAGCAAAGAGCTAGAGGTCCAACAC	AAAGCCAGGAGACTTTGACGCAG	DQ008070	Martins et al. (2019)
cat	CCAGATGTGGGCCGCTAACAA	TCTGGCGCTCCTCCTCATTC	Est04a09	
viperin	TCCTTGATGTTGGCGTGGAA	GCATGTCAGCTTTGCTCCACA	NM_001140939	
bcl2	TGACAGATTTCATCTACGAGCGG	GCCATCCAGCTCATCTCCAATC	NM_001141086	
tnfα	GGCGAGCATACCACTCCTCT	TCGGACTCAGCATCACCGTA	AY848945	
nfkβ	CAGCGTCCTACCAGGCTAAAGAGAT	GCTGTTCGATCCATCCGCACTAT	CA341859	Holen et al. (2015)
pparα	TCCTGGTGGCCTACGGATC	CGTTGAATTTCATGGCGAACT	DQ294237	Holen et al. (2014)
gpx2	TGTACCTCAAGGAGAAGCTGCCGT	ATTAAGGCCATGGGATCGTCGC	Est04e05	Todorčević et al. (2010)
gpx3	CCTTCCAGTACCTGGAGTTGAATGC	CTCATGATTGTCTCCTGGCTCCTGT	CA345853	
aif	AGGTGGAGTCCCAAGGAATCTGC	CCCCAAGAAACCTCCTCCAATG	TC37490	Kjær et al. (2016)
β-actin	CCAAAGCCAACAGGGAGAA	AGGGACAACACTGCCTGGAT	BG933897	Stenberg et al. (2019)
ef1α	TGCCCCTCCAGGATGTCTAC	CAGCGTGATAGACTCGTTGC	AF321836	
rpl13	CCAATGTACAGCGCCTGAAA	CGTGGCCATCTTGAGTTCCT	NM_001141291	Hevrøy et al. (2015)

il-1b, interleukin 1 beta; p38mapk, p38 mitogen-activated protein kinases; 5lox, Arachidonate 5-lipoxygenase; cox2, cyclooxygenase2; sod2, superoxide dismutase2; casp3, caspase 3; cat, catalase; viperin, virus inhibitory protein, endoplasmic reticulum [ER]-associated, interferon [IN]-inducible; bcl2, B-cell lymphoma 2; tnfα, tumor necrosis factor alpha; nfkβ, nuclear factor kappa-beta; pparα, peroxisome proliferator activated receptor alpha; gpx2, glutathione peroxidase 2; gpx3, glutathione peroxidase 3; aif, apoptosis-inducing factor; β-actin, beta actin; ef1α, elogation factor1 alpha; rpl13, ribosomal protein L13.

concentrations are in M and E_h is given in volts. $E^{0\prime}$ is the standard reduction potential at pH 7 and 25 °C and was assumed to be −0.240 V (Kemp et al., 2008; Schafer and Buettner, 2001).

2.2. In vitro experiments

One week prior to the final sampling, fish from five treatment groups namely basal diet (A), two SS groups (B and C) and two SM groups (F and G) were sampled for isolation of liver cells (n = 5) and head kidney leucocytes *in vitro* (n = 3). Tissue samples were also collected from these fish to study gene expression changes *in vivo*. The cells were isolated as described originally in Espe and Holen (2013) following modifications as per Martins et al. (2019).

2.2.1. Isolation and preparation of liver cells

Briefly, the fish were anesthetized in MS-222 (0.5 ml L^{-1}), liver perfused clean with a 0.09 M HEPES buffer (1.4 M NaCl, 0.067 M KCl and 0.03 M EDTA, pH 7.4), at a flow rate of 4 ml min^{-1}. The liver was digested in 0.1% type IV collagenase (prepared in 0.9 M HEPES perfusion buffer), the cells harvested in 10 ml of 10% PBS buffer (0.002 M KH_2PO_4, 0.02 M Na_2HPO_4, 0.03 M KCl and 0.14 M NaCl, pH 7·4), centrifuged at 50*g* for 5 min, filtered (sterile, 100 mm filter) and washed twice in the perfusion buffer, resuspended in Leibovits-15 medium supplemented with 10% fetal bovine serum (FBS, BioWhittaker), 1% glutamax (Gibco), 1% antibiotic Antimycotic (Penicillin-Streptomycin 50 U ml^{-1}, BioWhittaker) and assessed for viability with the Trypan Blue exclusion test (Lonzo; Medprobe).

2.2.2. Isolation and preparation of head kidney leucocytes

In brief, the head kidneys from each fish was aspirated, added to sterile isolation buffer (150 mM NaCl and 24 mM EDTA, pH 7.2) and then squeezed through a 40 μM Falcon cell strainer to 50 mL tubes. The cell filtrate was washed by centrifugation (400*g* for 5 min, at 4 °C). The resulting cell pellet was re-suspended in the isolation buffer and layered carefully on top of equal amounts of diluted Percoll at a density of 1.08 g ml^{-1}. The tubes were then centrifuged at 800 *g* for 5 min, at room temperature. The cell layer in the interface was collected and the cells were pelleted by centrifugation (400 *g* for 5 min, at 4 °C). The cell pellet was then re-suspended in Leibovits-15 medium for culture and use in challenge experiments.

2.2.3. Primary cell culture and challenge

The isolated liver cells and head kidney leucocytes were cultured *in vitro* (Martins et al., 2019). The cells were cultured on 6 well tissue culture plates in 2 ml of L15 medium for 24 h, incubated at 9 °C. Subsequently, on day two, the liver cells were challenged with a pro-oxidant, 200 μM H_2O_2 (Espe et al., 2015) and the head kidney leucocytes with 100 μg ml^{-1} lipopolysaccharide (LPS, Sigma-Aldrich) or 50 μg ml^{-1} polyinosinic:polycytidylic acid (Poly I:C or PIC, Sigma-Aldrich) (Martins et al., 2019). The LPS is a gram-negative bacterial cell wall component and PIC is a viral mimic. Untreated cells from each fish served as parallel controls in the challenge study. The cells were further incubated for 24 h, harvested, treated with 600 μl RTL-Plus buffer (RNeasy®Plus kit Qiagen), centrifuged and frozen at −80 °C.

2.3. RNA extraction and qPCR

The impact of dietary Se levels and sources on the transcriptional changes in oxidative, immune and inflammatory response markers *in vivo* (liver and head kidney tissue) and *in vitro* (primary cultures of liver cells and head kidney leucocytes) were studied. The procedure for RNA extraction, reverse transcription and qPCR followed were as described in (Stenberg et al., 2019), except for the reference genes used to normalise the expression of target genes using the program geNorm version 3.5. The reference genes used for liver tissue were EF1a and β-actin; while RPL and β-actin were used in liver cells. In the head kidney, it was β-actin and EF1a for both tissue and isolated cells. The details of the qPCR primers used for amplification of the reference and target genes are provided in Table 4.

2.4. Data analysis and statistics

The data from the *in vivo* and *in vitro* studies were treated using tanks (n = 3) and individual fish (n = 3 to 5) as experimental units, respectively. Differences between groups were analysed using one-way or two-way ANOVA, following Tukey's multiple comparison test, for normally distributed datasets (Anderson-Darling normality test, p > .05). Non-normally distributed datasets (Anderson-Darling normality test, p < .05) were analysed using Kruskal-Wallis non-parametric test following Dunn's multiple comparison. Regression analysis was used to compare the efficacy of Se sources by slope-ratio method and dose response analysis to determine requirement estimates. The

Table 5
Growth and zoo-technical indices of Atlantic salmon post-smolt fed different dietary levels and sources of selenium (Se) for 8 weeks.

Se source	Se inclusion level (mg kg^{-1} diet)	Analysed Se level in diets (mg kg^{-1} diet)	Final body weight (g)	Weight gain (g)	Feed intake (% day^{-1})	Feed conversion ratio (FCR)	Specific growth rate (SGR)	Hepato-somatic index (HSI)	Hematocrit (Hct)
Basal diet	0	0.24	526.6	310.8	1.15	0.75	1.42	1.16	41.9^{ab}
SS (Na_2SeO_3)	0.15	0.34	533.0	315.6	1.15	0.76	1.42	1.15	39.9^{a}
	0.4	0.65	530.2	313.8	1.16	0.75	1.43	1.16	44.6^{b}
	0.7	0.87	516.3	300.7	1.14	0.76	1.40	1.16	42.0^{ab}
	1.1	1.4	523.9	308.9	1.16	0.76	1.41	1.17	42.4^{ab}
SM (Se-Met)	0.15	0.38	524.5	309.4	1.14	0.75	1.41	1.17	42.3^{ab}
	0.4	0.63	534.2	318.9	1.14	0.73	1.44	1.17	44.5^{b}
pSD			14.9	14.3	0.03	0.01	0.04	0.03	1.1
p-value			ns	ns	ns	ns	ns	ns	0.02

Data presented as mean and pooled standard deviation (n = 3). Treatments effects were considered significant when $p < .05$ upon ANOVA followed by Tukey's post-hoc multiple comparison analysis. Different superscripts within a column indicate statistically significant difference between the groups; ns, treatment effects not significant, $p > .05$.

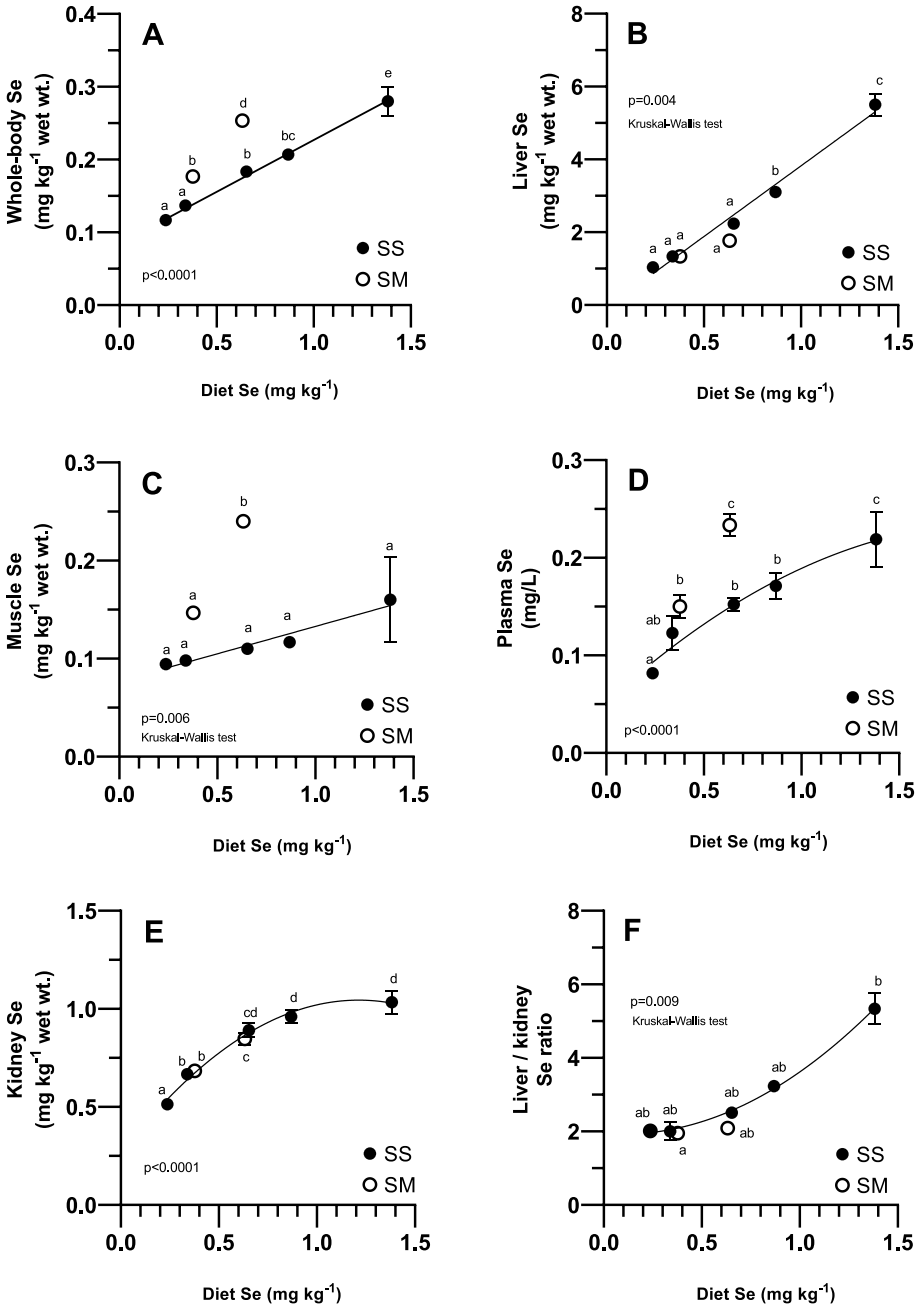


Fig. 1. Selenium (Se) concentration in Atlantic salmon post-smolt whole body (Fig.1A), liver (Fig. 1B), muscle (Fig. 1C), plasma (Fig. 1D), kidney (Fig. 1E) and liver/kidney ratio (Fig. 1F). Dark circles and regression line represent sodium selenite (SS) fed groups, including the basal diet; open circles represent L-selenomethionine groups (SM). Each data point is presented as mean ± SD (n = 3), the error bars are not visible for some data points. Test of significance between groups were analysed using one-way ANOVA following Tukey's multiple comparison test; groups with different letters within each graph differ significantly. The line represents the linear or non-linear regression of the five groups (basal + 4 SS groups). The regression equations were, whole body ($Y = 0.14X + 0.09$), plasma ($Y = 0.05X^2 + 0.18X - 0.05$), muscle ($Y = 0.06X + 0.08$), liver ($Y = 3.9X - 0.06$), kidney ($Y = 0.26X^2 + 1.29X - 0.5$) and liver/kidney ratio ($Y = 1.9X^2 - 0.4X + 2.1$). The response of Se concentration in kidney and liver/kidney ratio plateaued or not significantly different at or beyond 0.65 mg Se kg^{-1} diet, respectively.

Table 6
Slope-ratio comparison on the efficacy of sodium selenite (SS) and selenomethionine (SM) based on different response criteria assessed.

Response	Regression eq. (Y = bX + a) ¥		Slope ratio (SM/SS)	P-value (SM *vs* SS)
	Selenomethionine (SM)	Sodium selenite (SS)		
Whole body Se	y = 0.34× + 0.04	y = 0.16× + 0.08	2.16	< 0.0001
Plasma Se	y = 0.38× – 0.004	y = 0.15× + 0.05	2.49	< 0.0001
Fillet Se	y = 0.37× + 0.005	y = 0.04× + 0.09	9.74	< 0.0001
Hematocrit	y = 7.0× + 40.0	y = 8.6× + 38.6	0.81	ns
Liver Se	y = 1.9× + 0.6	y = 2.9× + 0.33	0.64	< 0.01
Kidney Se	y = 0.83× + 0.33	y = 0.87× + 0.33	0.95	ns
Liver/kidney Se	y = 0.23× + 1.92	y = 1.29× + 1.65	0.18	0.01
Faeces Se	y = 0.06× + 0.02	y = 0.24× – 0.03	0.27	< 0.0001

¥ Linear regression (b, slope; a, intercept) of the response to three graded dietary Se levels from each Se source representing diet groups A, B and C for sodium selenite, and diets A, F and G for selenomethionine. The test of significance (at 0.05 level) to determine if the slope of the two regressions were significantly different was performed using GraphPad prism 8. ns, not statistically significant ($p > .05$).

Table 7
Dietary selenium (Se) to reach saturation in response criteria, Se requirement of Atlantic salmon post-smolt and the contribution of each diet tested in meeting the requirement.

Response criteria	X: Dietary total Se (as SS) to reach saturation or homeostasis$^{(estimated)}$, mg kg^{-1} diet		Y: Respective Se requirement of Atlantic salmon$^{(calculated)}$, mg kg^{-1} diet	Z: Available Se supply (mg kg^{-1} diet); and proportion of Se requirement met by each of the experimental diets$^{(calculated)}$, (%)						
				A	B	C	D	E	F	G
	ANOVA[1]	Regression[2]		0.14	0.19	0.27	0.24	0.32	0.24	0.46
Body Se balance	–	0.66	0.27	51	68	99	88	115	89	166
Plasma Se	0.65	–	0.27	52	69	100	89	117	90	169
Hematocrit	0.65	0.65	0.27	54	71	103	92	120	93	174
Kidney Se	0.65	0.81	0.22–0.27	52–63	69–83	100–120	89–108	117–141	90–109	169–203
Liver/kidney ratio	0.65	0.89	0.25–0.27	52–57	69–75	100–110	89–98	117–129	90–99	169–185
AAC	0.65	0.92	0.25–0.27	52–55	69–73	100–106	89–96	117–124	90–96	169–179
Faecal loss	0.65	0.93	0.26–0.27	52–55	69–72	100–105	89–94	117–123	90–95	169–177
Non-faecal loss	0.65	0.87	0.25–0.27	52–55	69–73	100–106	89–96	117–124	90–96	169–179

X, estimated values obtained using the five treatments (basal + 4 SS supplemented groups); [1]based on post-hoc difference between treatments in respective response criteria; [2]parameter estimate from regression analysis. Y, calculated using the formula X*AAC%, the AAC values from diet respective to the Se level 42% for 0.65 or 27%, 0.8–0.9. Z, available Se level in diets A-G calculated as total Se concentration in diet*AAC% of the respective diets; proportion of Se requirement contributed by each experimental diet was calculated as follows, Z/Y*100.

best fit of the data moving from simple to more complex relationships, was used. In figures with first order polynomials, p indicates the probability that the slope is equal to 0. In figures with second order fits, p gives the probability that the first order equation fits the data better than the second order equation. All the data analysis was performed in GraphPad Prism (ver. 8.05).

3. Results

3.1. Growth and zoo-technical indices

Atlantic salmon post-smolt fed the different experimental diets grew more than twice during the experimental period from a mean initial body weight of 216 ± 27 g to reach 527 ± 72 g. The weight gain between treatments was not significantly different, neither was there an impact of the experimental diets on feed intake, feed conversion ratio, specific growth rate or hepato-somatic index (Table 5, $p > .05$). Hematocrit percentage was significantly higher in fish fed the 0.4 mg Se supplemented feeds, irrespective of the source; the hematocrit of fish fed 0.15 SS diet was significantly lower (Table 5, $p = .02$).

3.2. Proximate and mineral composition of body

The chemical composition of the whole fish were not affected by the dietary treatments. The mean dry matter, protein, total fat, ash and gross energy content of the fish across all groups were 36.3 ± 0.4%, 17.5 ± 0.5%, 16.1 ± 0.5%, 1.9 ± 0.1% and 10.3 ± 0.2 kJ/g.

3.3. Whole body and tissue selenium concentrations

The Se concentrations in the whole body and tissues of Atlantic salmon post-smolt were significantly affected by the levels and sources of Se (Fig. 1). The Se concentration in whole body (Fig. 1A, $p < .0001$), liver (Fig. 1B, $p < .0001$) and muscle (Fig. 1C, $p < .0001$) increased linearly with increasing dietary Se concentration. On the other hand, Se concentrations in plasma (Fig. 1D, $p < .0001$), kidney (Fig. 1E, $p < .0001$) and liver/kidney Se ratio (Fig. 1E, $p < .0001$) showed non-linear response, reaching saturation at or beyond dietary total Se (as SS) of 0.65 mg Se kg^{-1} feed. Slope-ratio analysis was performed to assess the relative efficiency of the two Se sources in improving the Se status of the whole body and tissues (Table 6). The efficiency (measured as slope ratio) of SM was significantly higher than SS in the whole body (2.16, $p < .0001$), muscle (9.74, $p < .0001$) and plasma Se concentrations (2.49, $p < .0001$). The contrary was significant (SM < SS, $p < .01$) in the liver (0.64, $p < .01$), liver/kidney ratio (0.18, $p < .001$) and faeces (0.27, $p < .001$); whereas, no significant difference was found in the slope ratio of hematocrit (0.85, $p > .05$) and kidney Se levels (0.95, $p > .05$). Dietary Se requirement of Atlantic salmon post smolt (on available basis) and the corresponding dietary Se levels (on total basis) estimated to meet the requireemnt as per these response parameters are provided in Table 7..

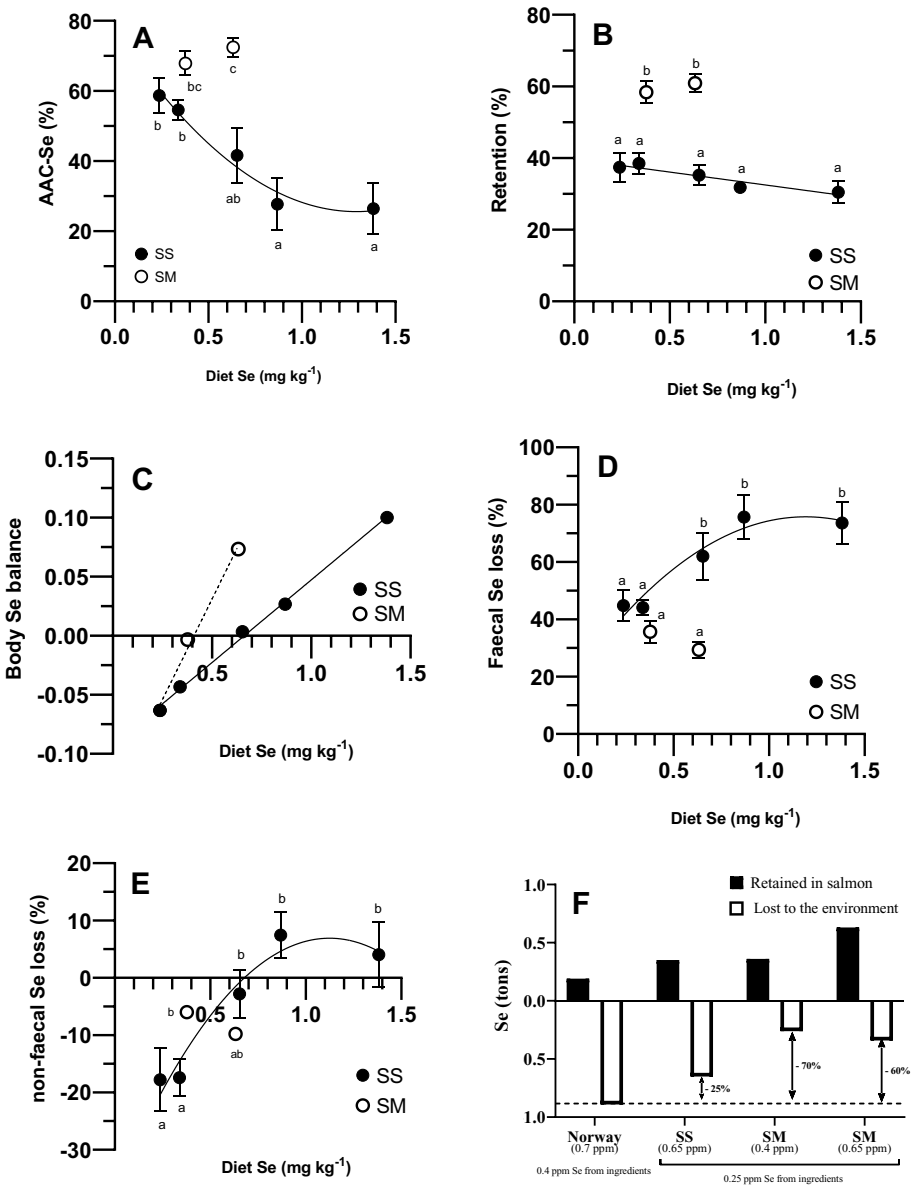


Fig. 2. Apparent availability of selenium (Se) (Fig. 2A), retention (Fig. 2B), body Se balance (Fig. 2C), faecal (Fig. 2D) and non-faecal Se loss (Fig. 2E) in Atlantic salmon post-smolt fed different levels and sources of Se are presented here. Dark circles and regression line represent sodium selenite (SS) fed groups, including the basal diet; open circles and dashed line represent L-selenomethionine (SM) groups. Each data point is presented as mean ± SD (n = 3), the error bars are not visible for some data points. Test of significance between groups were analysed using one-way ANOVA following Tukey's multiple comparison test; groups with different letters within each graph differ significantly. The regression equations were, AAC of Se ($Y = 77X^2 - 80X + 31$), retention ($Y = -7.2X + 40$), faecal Se loss ($Y = 22X^2 + 91X - 38$) and non-faecal Se loss ($Y = -37X^2 + 78X - 35$). In the body Se balance (SS, $Y = 0.14X - 0.09$; SM, $Y = 0.34X - 0.14$), X-intercept indicates the dietary Se concentration at which Se homeostasis was achieved with reference to the initial Se status of the fish (SS, 0.66; SM, 0.41 mg kg^{-1} diet). In Fig. 2F, the retention (black bars) and loss (white bars) of dietary Se (in tons) estimated from salmon produced and feed used in Norwegian salmon farming (Aas et al., 2019) and Se retention obtained data in this study. The dietary Se levels for Norway was obtained from the Norwegian feed surveillance program, mean dietary Se content of 0.7 mg kg^{-1} diet (Sanden et al., 2017). Compared to the estimated Se loss in Norwegian Atlantic salmon farming at present, the three proposed scenarios of dietary Se levels/source from this study had the potential to reduce Se loss to the environment by 25, 70 and 60%, respectively.

3.4. Apparent availability, retention and loss of selenium

Data on apparent availability and mass balance of dietary Se is presented in Fig. 2. The apparent availability of Se in the basal diet was 58.7%, which declined with SS supplementation and reached the lowest (26–27%) in the two highest SS supplemented groups (Fig. 2A; $p < .0001$). On the contrary, the apparent availability of Se increased up to 68–72% in the two SM supplemented groups. Retention of dietary Se was superior and Se loss was less in SM fed fish (Fig. 2B, $p < .0001$). The retention of dietary Se decreased linearly from 38% in the basal diet to 30% in the highest SS supplemented diet, while the retention increased up to 58–61% with Se supplementation as SM. Whole body Se balance as a function of dietary Se concentration indicated that fish fed SM were able to achieve whole body Se homeostasis with significantly lower Se supply than with SS (Fig. 2C, $p < .0001$). The dietary Se concentration to achieve body Se homeostasis with SS or SM were respectively 0.66 or 0.41 mg Se kg^{-1} diet. Selenium loss, faecal (Fig. 2D, $p < .0001$) and non-faecal (Fig. 2E, $p = .002$) increased with Se supplementation as SS, but not with SM. The increase was non-linear in both cases and reached saturation at 0.65 mg Se kg^{-1} diet; the broken-line regression estimates were 0.93 and 0.87 mg Se kg^{-1} diet, respectively. Dietary Se requirement of Atlantic salmon post smolt (on available basis) and the corresponding dietary Se levels (on total basis) estimated to meet the requireemnt as per these response parameters are provided in Table 7. The present and projected Se load to the environment is presented in Fig. 2F. The Se load to the environment in the business-as-usual scenario of Norwegian aquaculture was estimated to be 0.89 t per year (in 2016), which can be potentially reduced to 0.65 or 0.26–0.34 t per year by managing Se supplementation as sodium selenite or selenomethionien, respectively, a reduction by 25 to 70%.

3.5. Glutathione redox homeostasis

The level of reduced glutathione (GSH), oxidised glutathione (GSSG), their ratio (GSH/GSSG) and the redox potential (E_h) were determined in the liver and head kidney (Fig. 3). Level of GSH in liver and head kidney were not affected by dietary Se (Fig. 3A). Liver GSSG data were fitted to a quadratic function ($p = .04$), indicating that the concentrations were lower at intermediate SS supplementation of 0.4 and 0.65 mg Se kg^{-1} (total dietary Se, 0.65 and 0.87 mg Se kg^{-1}). A similar relationship between GSSG and SS supplementation was found in the head kidney (Fig. 3B). The liver GSH/GSSG ratio also followed a

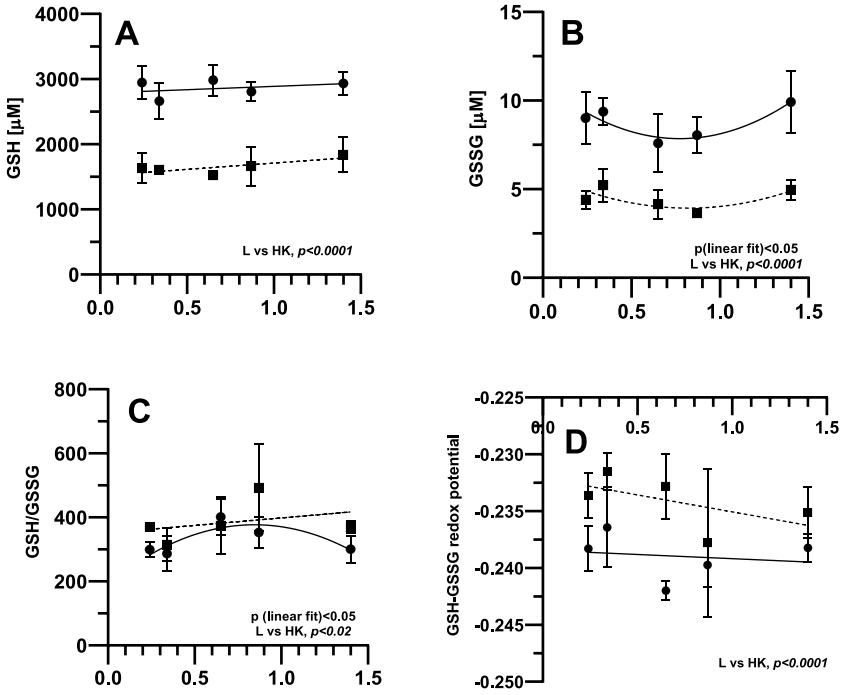


Fig. 3. Circle and dark line, liver (L); square and dotted line, head kidney (HK). Filled symbols, sodium selenite (SS) including the basal diet and open symbols, selenomethionine (SM). Reduced glutathione (GSH, Fig. 3A), oxidised glutathione (GSSG, Fig. 3B), GSH/GSSG ratio (Fig. 3C) and glutathione redox potential (E_h, Fig. 3D). Each data point is presented as mean ± SD (n = 3). Regression analyses was performed and none of the slopes of the linear models were different from zero. P-values indicate differences between organs and the probability that GSSG and GSH/GSSG models are linear instead of quadratic. Tissue comparison was performed using paired students' t-test (n = 21).

quadratic relationship with dietary Se, being maximum at intermediate dietary SS. The GSH/GSSG ratio in the head kidney was not affected by dietary Se (Fig. 3C). The redox potential (E_h) in the liver and kidney were not affected by the diets. The liver had higher levels of GSH and GSSG compared to head kidney (Fig. 3A, p < .0001; Fig. 3B, p < .0001). On the contrary, the GSH/GSSG ratio and redox potential were significantly higher in the head kidney compared to the liver (Fig. 3C, p < .02; 3D, p < .0001). The GSH, GSSG and redox potential of liver and kidney of fish fed the SM diets were similar to those fed the SS diets.

3.6. Dietary selenium induced apoptosis and inflammatory response in vivo

The mRNA expression of genes involved in oxidative, apoptotic and inflammatory pathways in the liver and head kidney tissues were studied (Fig. 4). The gene expressions of the treatment groups were analysed in comparison to the basal diet (control group). In the liver, Se supplementation at 0.4 ppm as SM significantly supressed *aif* expression (4A, p = .002). The expression of *bcl2* (Fig. 4B, p = .04) and *casp3* (Fig. 4C, p = .008) in 0.4 SS was significantly supressed in comparison to the basal diet group. Expression of *p38-MAPK* was not affected by dietary Se (Fig. 4D, p = .49). The expression of *gpx2* was significantly lower in 0.4 SS and 0.15 SM group when compared to basal diet (Fig. 4E, p = .008). The expression of *sod2* was unaffected by dietary Se (Fig. 4F, p = .09). In the head kidney, expression of *nfkβ* (Fig. 4a, p = .002) and *5lox* (Fig. 4b, p = .04) were significantly higher, while *tnfα* (Fig. 4c, p = .06) showed a tendency to be higher in 0.15 SS fed fish compared to the basal diet group. Compared to the basal diet group, the expression of *tlr7* (Fig. 4d, p = .09) showed a tendency to be higher and *cox2* (Fig. 4e, p = .06) had the contrary in 0.15 SS group. The expression of *gpx2* was higher in 0.15 SS group compared to the basal diet group (Fig. 4f, p = .01).

3.7. Apoptosis, inflammatory and antioxidant response in vitro

3.7.1. Liver cells

The liver cells were affected by the impact of dietary Se, but not by the treatment of hydrogen peroxide *in vitro* (Fig. 5). Liver cells isolated from fish fed the 0.4 SM diet had significantly downregulated mRNA expression of *aif* (Fig. 5A, p = .001) and *p38mapk* (Fig. 5B, p = .0.001) compared to the basal diet group. On the other hand, the expression of *cox2* (Fig. 5C, p = .02, Kruskal-Wallis) and *gpx3* (Fig. 5D, p = .04, Kruskal-Wallis) were significantly higher in 0.15 SS fed fish compared to the fish fed the basal diet. The expression of *bcl2, cat, sod2 and casp3* were not significantly affected by the treatments (Fig. 5E-H; p > .05).

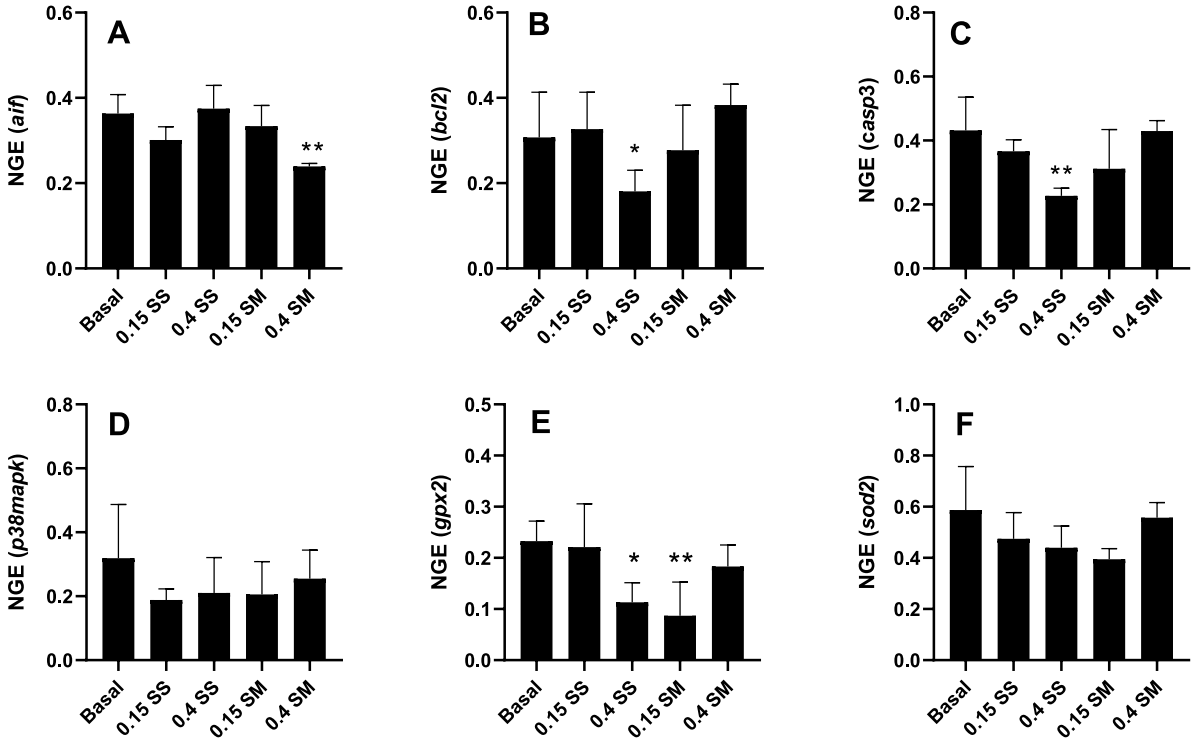
3.7.2. Head kidney leukocytes

Gene expression of markers in inflammatory, apoptotic and oxidative response pathway were significantly altered through dietary Se and LPS or PIC exposure to the HKL (Fig. 6). Exposing the cells to LPS significantly induced the expression of *il-1β* in all the groups except 0.4 SM (Fig. 6A). In cells exposed to LPS, Se supplied as SS tended to upregulate *cox2* and *5lox* expression, whereas Se supplied as SM tended to down regulate the expression of these genes. Consequently, *cox2* and *5lox* expressions in 0.4 SS were significantly higher than in 0.4 SM (Fig. 6B and C). Exposing the cells to PIC significantly supressed the expression of *nfkβ* in all the groups except 0.4 SM (Fig. 6D). The expression of *viperin* was induced by PIC exposure but unaffected by dietary Se (Fig. 6E). Significant upregulation of *ppara* expression was observed in 0.4 SS and 0.15 SM group compared to the basal diet (Fig. 6F). The expression of *gpx2* and *cat* were respectively downregulated in 0.4 SM and 0.15 SS compared to the basal diet, no effect of LPS or PIC was observed (Fig. 6G and H). An interaction effect for PIC indiced cat upregulation was observed in 0.4 SS group. The expression of *aif* was downregulated in 0.15 SS and 0.4 SM compared to the basal diet; no effect of LPS or PIC observed (Fig. 6I).

4. Discussion

Atlantic salmon, despite being a major aquaculture species, the requirement for dietary Se is unknown (NRC, 2011). The optimal dietary Se level in low fish meal diets for Atlantic salmon post smolt estimated using a wide array of response criteria ranged between 0.65 and 0.93 mg kg^{-1} diet, confirming our earlier estimate using a multi-nutrient regression design (Antony Jesu Prabhu et al., 2019). Besides, the estimate obtained using SS as the Se source here compared well with most other sea water fish species like grouper, 0.7 and 0.9 mg kg^{-1} diet (Lin, 2014; Lin and Shiau, 2005); cobia, 0.8 mg kg^{-1} diet (Liu et al.,

Liver



Head kidney

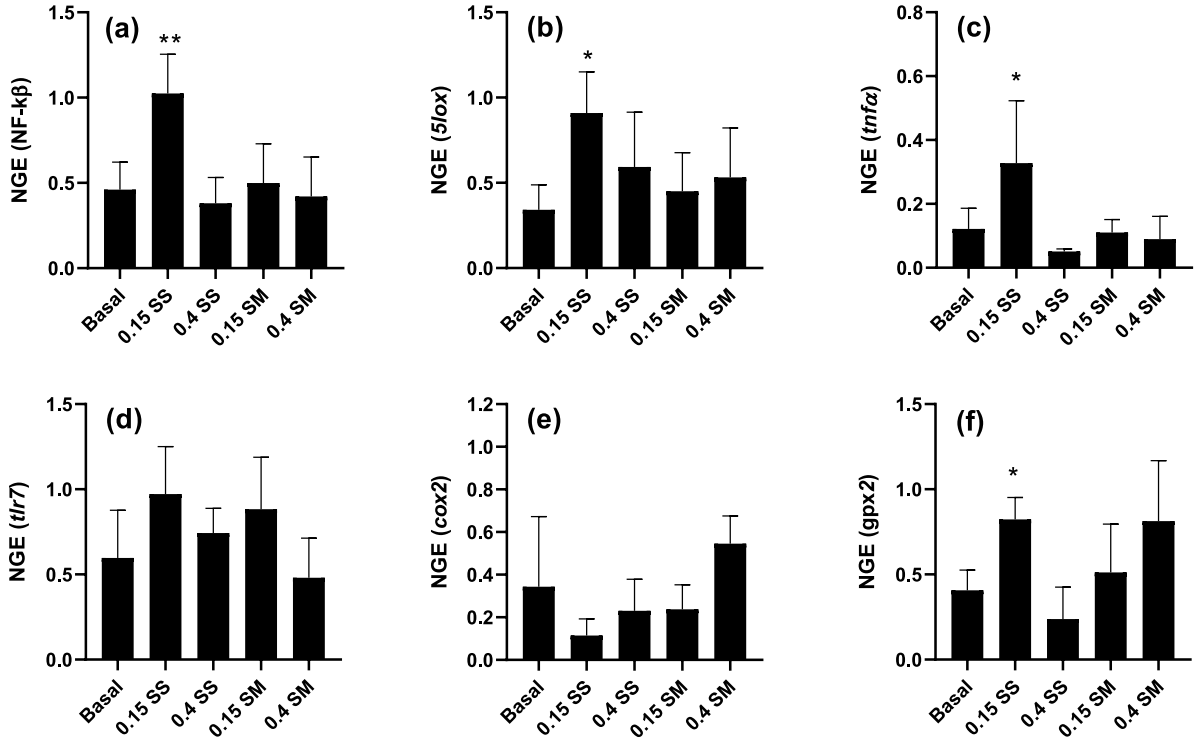


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Fig. 4. SS, sodium selenite; SM, selenomethionine. Upper case alphabets represent liver tissue (Fig. 4A-F); lower case alphabets represent head kidney tissue (Fig. 4a-f). Liver: *aif*, apoptosis-inducing factor (Fig. 4A); *bcl2*, B-cell lymphoma 2 (Fig. 4B); *casp3*, caspase 3 (Fig. 4C); *p38mapk*, p38 mitogen-activated protein kinases (Fig. 4D); *gpx2*, glutathione peroxidase 2 (Fig. 4E); *sod2*, superoxide dismutase2 (Fig. 4F). Head kidney: *nfkβ,* Nuclear Factor kappa-beta (Fig. 4a); *5lox*, Arachidonate 5-lipoxygenase (Fig. 4b); tnfα, tumor necrosis factor alpha (Fig. 4c); trl7, toll-like receptor7 (Fig. 4d); *cat*, catalase; *cox2*, cyclooxygenase 2 (Fig. 4e) and *gpx2*, glutathione peroxidase 2 (Fig. 4f). Data presented as mean and pooled standard deviation (n = 4). Treatments effects were considered significant when $p < 0.05$ upon ANOVA followed by Tukey's post-hoc multiple comparison analysis of each treatment with that of the control (basal diet). Asterix symbol above a treatment group within a graph indicate statistically significant difference of the respective treatment group compared to the basal diet group (*, $p < 0.05$; **, $p < 0.01$).

2010); black seabream, 0.86 mg kg^{-1} diet (Wang et al., 2019) and 0.94 mg kg^{-1} diet for gilthead seabream (Domínguez et al., 2019),. The estimates discussed above are on total Se basis as very few Se requirement studies in fish provide data on Se availability. The Se requirement of Atlantic salmon on available basis (Table 7: *ca.* 0.22 to 0.27 mg Se kg^{-1}) is comparable with rainbow trout (Hilton et al., 1980), channel catfish (Gatlin and Wilson, 1984) and a predicted relationship between the selenocysteine content of SEPP1 and Se requirements in vertebrates (Penglase et al., 2015). Therefore, variations in optimal dietary Se levels for different fish species is largely driven by changes in bioavailability (Antony Jesu Prabhu et al., 2016).

Bioavailability of a nutrient includes absorption, retention, and utilization (Fairweather-Tait and Southon, 2003). The better absorption of SM relative to SS at the GIT of Atlantic salmon post-smolt was evident through the AAC of Se, faeces Se concentration, plasma Se status, and the respective slope ratios. The concentration of Se excreted in the faeces was 4-fold higher in SS fed fish compared to those fed SM. Sodium selenite is absorbed in its selenite form ($HSeO^-$) and the process is dependent on intracellular glutathione levels (Misra et al., 2012). Anti-nutrient factors and other dietary additives can therefore interfere with selenite absorption at the GIT. Phytate-P and di-calcium phosphate reduced apparent availability and absorption of Se when suppled as SS (Antony Jesu Prabhu et al., 2014; Silva et al., 2019). On the contrary, both *in vitro* solubility and apparent availability *in vivo* were higher with SM compared to SS and not affected by phytate-P in the diet (Silva et al., 2019; Silva et al., 2020). Once absorbed, Se is transported in the plasma (Hilton et al., 1982) and hence post-prandial plasma Se levels are used to assess Se absorption and/or status (Antony Jesu Prabhu et al., 2014; Bell and Cowey, 1989; Hambidge, 2003; Lin and Shiau, 2005). In the present study, SM improved the plasma Se status more effectively than SS. Fish fed SM supplemented diets required only half the total Se in the diets to achieve similar plasma Se than those fed SS diets. For instance, plasma Se status in fish fed 0.36 mg Se kg^{-1} with SM was statistically similar with that of fish fed 0.65 mg Se kg^{-1} with SS and the same was true for fish from 0.65 SM *vs.* 1.4 SS. Moreover, the slope of plasma Se with SM supplementation was 2.6-fold higher compared to SS (SS, 0.15 *vs.* SM, 0.38; $p < .0001$). This corresponds well with the overall assessment that Se requirement was satisfied at 0.65 and 0.4 mg kg^{-1} of total dietary Se with Se supplementation as 0.4 ppm as SS and 0.17 ppm as SM, respectively.

Like mammals, liver and kidney are the major organs regulating Se metabolism in fish (Hilton et al., 1980; Hodson and Hilton, 1983). The selenium transported in the plasma is metabolised by liver, synthesised to selenoproteins, transported to extra-hepatic tissues and excreted by the kidney (Regina, 2015). The liver: kidney Se ratio (L:K) is thereby a useful measure to assess Se requirement and tolerance (Hilton et al., 1982). A steep increase in the L:K ratio signified excess dietary Se supply in rainbow trout (Hilton et al., 1982) and black seabream (Lee et al., 2008). Liver Se concentration increased linearly, while kindly Se levels plateaued at higher Se inclusion. Consequently, the L:K was significantly higher in fish fed 1.4 mg Se kg^{-1} diet, which falls in the maximum tolerance range for selenite supplementation in plant based Atlantic salmon feeds (Berntssen et al., 2018). The metabolism and homeostasis of Se is not only differentially regulated by dietary Se levels, but also by Se sources (Janz, 2012). The regulated and unregulated Se pools bring about tissue specific differential absorption and retention of selenite and SM (Burk and Hill, 2015). Selenite and SM are the main contributors to the regulated and unregulated Se pool, respectively. SM may also contribute to the regulated Se pool through transsulfuration pathway (Burk and Hill, 2015). The major non-specific Se pool is localized in the muscle, which understandably exhibited significantly higher retention with SM in many fish species (Cotter et al., 2008; Fontagne-Dicharry et al., 2015; Lin, 2014; Mechlaoui et al., 2019; Rider et al., 2010; Sele et al., 2018; Wischhusen et al., 2019). In contrast, liver Se concentration was higher with SS than SM; while kidney showed no source specific difference in Se deposition (Berntssen et al., 2018; Rider et al., 2010; Wischhusen et al., 2019). Atlantic salmon fed SM supplemented feeds attained body Se homeostasis at a lower dietary Se level due to higher availability and tissue deposition of SM over SS, as in channel catfish (Wang and Lovell, 1997). The markers of specific Se pool (eg. SEPP1, glutathione peroxidase) are considered to be more robust indicators of Se status than those associated with the non-specific Se pool (eg. muscle Se), both in mammals (Burk and Hill, 2015) and in fish (Antony Jesu Prabhu et al., 2016; Penglase et al., 2015). Thereby, Se balance and slope ratio analysis in SM group might be biased and driven by increased Se retention in the non-specific Se pool, particularly the muscle. Nevertheless, this precludes the possible health benefits that can be obtained from the Se stored in the non-specific Se pool during challenging conditions.

Tissue glutathione concentrations and redox status are good indicators of Se requirement and toxicity in salmonids (Berntssen et al., 2018; Fontagne-Dicharry et al., 2015; Hamre et al., 2010). Glutathione (reduced, GSH) concentration in the liver of Atlantic salmon was 2-fold higher than in head kidney and was not affected by dietary Se, as observed with other dietary pro- or anti-oxidants (Hamre et al., 2010). The concentration of GSH in a cell is high (*i.e.* 1–10 mM) and constitute > 98% of the total cellular glutathione pool. It is in steady state with the oxidised form, GSSG and their ratio expressed as the GSH/GSSG ratio or the reduction potential indicates the redox status of the cell. GSSG had a quadratic relation with increasing SS supplementation in both liver and head kidney. The liver GSSG tended to be higher in low and high SS diets, indicating that the tissues were slightly more oxidised at these supplementation levels. The GSSG level in liver and head kidney and the GSH/GSSG ratio in the liver had min and max levels, respectively, in the 0.4 to 0.6 SS supplemented groups (total Se, 0.65 to 0.87 mg kg^{-1} diet). This range corresponds well with the estimated optimal total Se range to satisfy the Se requirement of Atlantic salmon post smolt, based on hematocrit, tissue Se status and body Se homeostasis (Table 7). With regard to the impact of Se sources, both SS and SM lowered GSH levels in Atlantic salmon at Se concentrations of 15 mg kg^{-1} (Berntssen et al., 2018). In rainbow trout fry, organic Se improved GSH and GSH/GSSG ratio compared to SS (Fontagne-Dicharry et al., 2015), while the same effect was not reproduced in a subsequent study (Wischhusen et al., 2019). Source specific differences in glutathione metabolism arising from differential metabolic pathway of selenite or selenomethionine is suggested (Berntssen et al., 2017; Burk and Hill, 2015).

Primary cultures of Atlantic salmon liver cells and HK leukocytes are well established *in vitro* models to study tissue specific cellular responses to nutrient depletion or repletion *in vivo* (Espe and Holen, 2013; Espe et al., 2015; Holen et al., 2011; Martins et al., 2019). Grass carp fed Se-yeast *in vivo* exhibited anti-inflammatory effect of Se by modulating *nfkb, p38mapk* and *tnfα* signalling pathways (Zheng et al., 2018a; Zheng et al., 2018b). Similar anti-inflammatory responses were

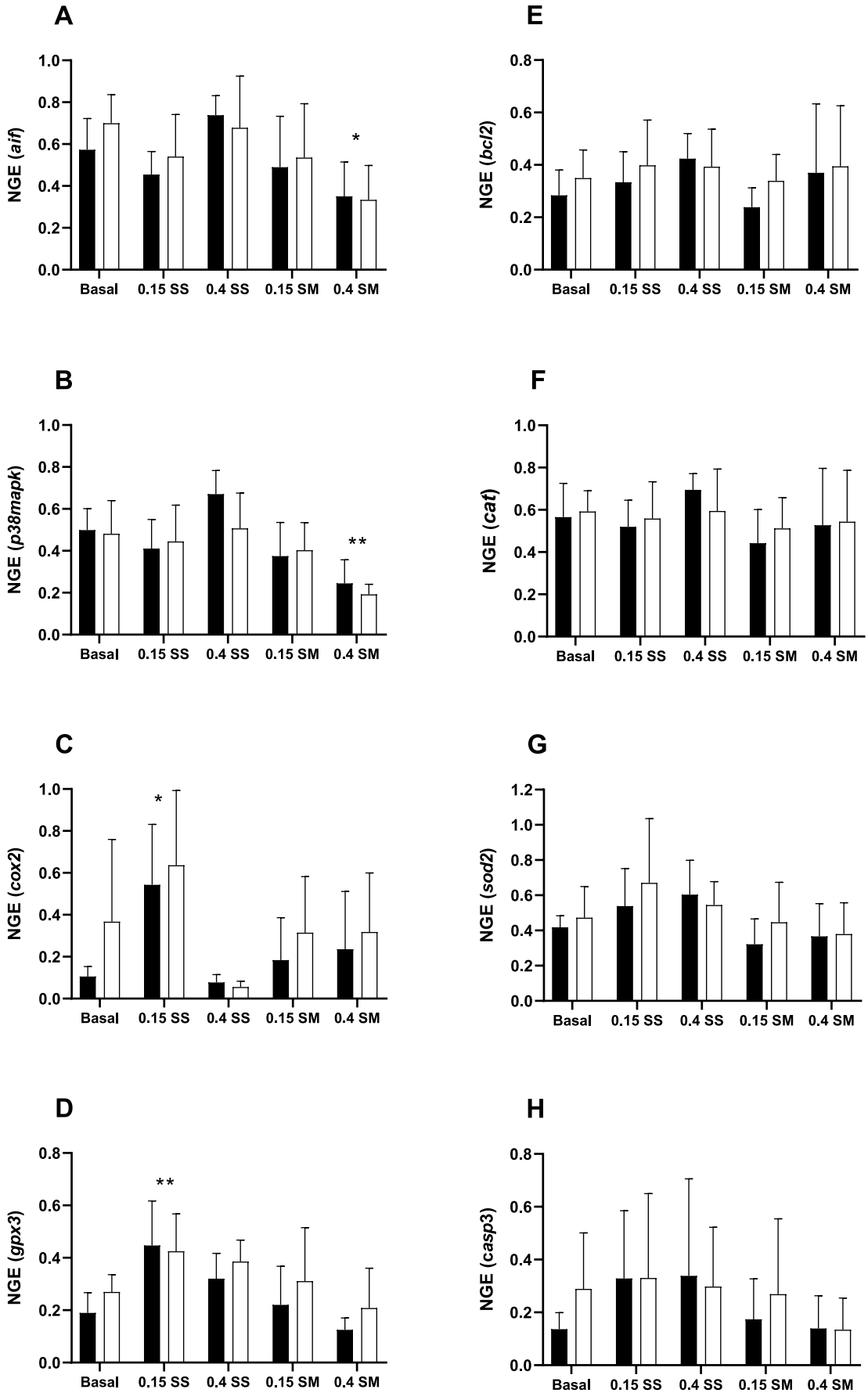

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Fig. 5. The black and white bars represent control and hydrogen peroxide (H_2O_2) treated cells. *aif*, apoptosis-inducing factor (Fig. 5A); *p38mapk*, p38 mitogen-activated protein kinases (Fig. 5B); *cox2*, cyclooxygenase 2 (Fig. 5C); *gpx3*, glutathione peroxidase 3 (Fig. 5D); *bcl2*, B-cell lymphoma 2 (Fig. 5E); *cat*, catalase (Fig. 5F); *sod2*, superoxide dismutase 2 (Fig. 5G); *casp3*, caspase 3 (Fig. 5H). Data presented as mean and standard deviation (n = 5). The test of significance performed by two-way ANOVA with diet and stimulus as main factors, followed by Tukey's multiple comparison of each treatment with that of the control (basal diet) for diet effect. Asterix symbol above a treatment group within a graph indicate statistically significant difference of the respective treatment group compared to the basal diet group (*, $p < 0.05$; **, $p < 0.01$).

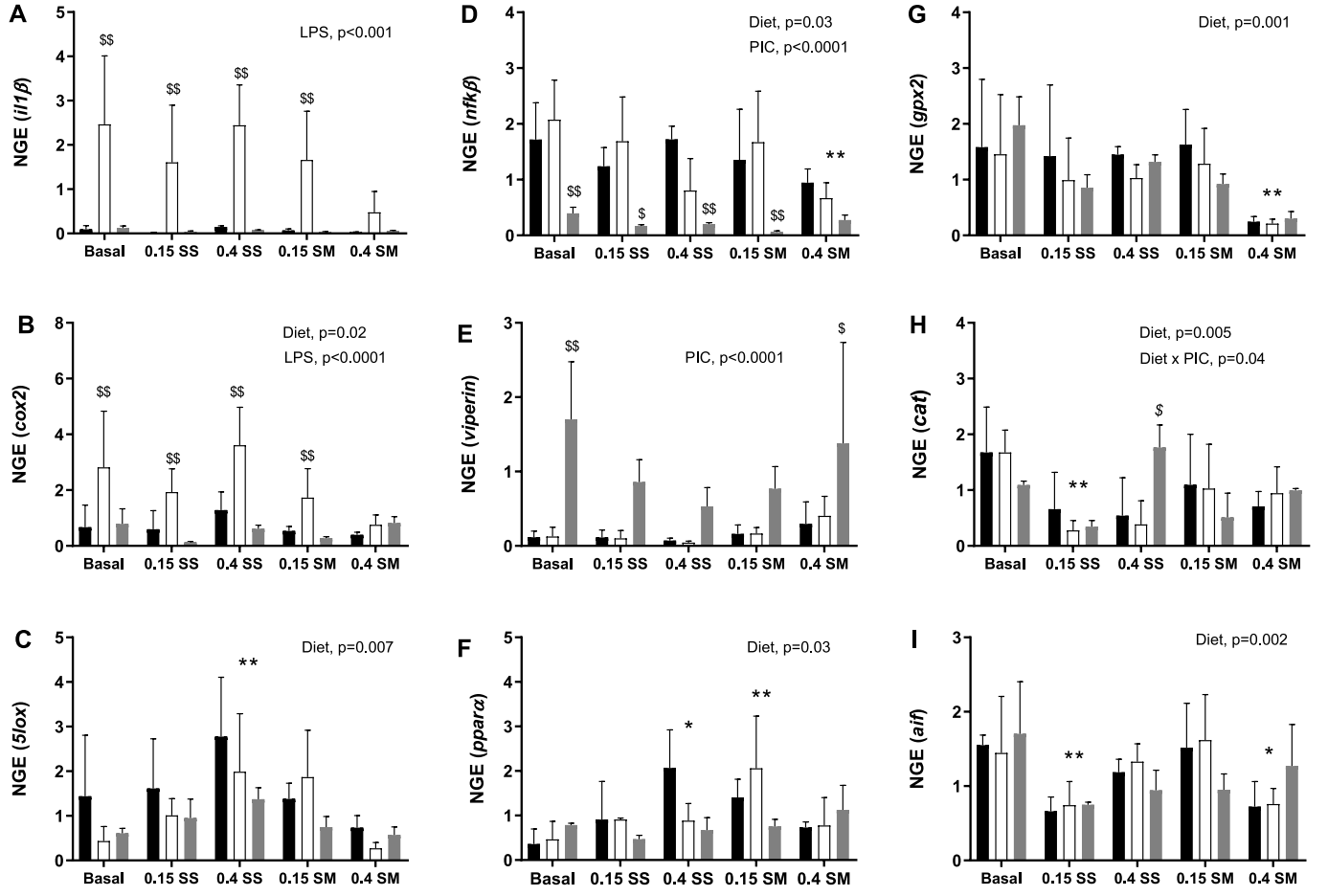

Fig. 6. The black, white and grey bars represent control, lipopolysaccharide (LPS) and Polyinosinic:polycytidylic acid (PIC) treatments, respectively. *il-1b*, interleukin 1 beta (Fig. 6A); *cox2*, cyclooxygenase2 (Fig. 6B); *5lox*, Arachidonate 5-lipoxygenase (Fig. 6C); *nfkβ*, nuclear factor kappa-beta (Fig. 6D); *viperin*, virus inhibitory protein, endoplasmic reticulum [ER]-associated, interferon [IN]-inducible (Fig. 6E); *ppara*, peroxisome proliferator activated receptor alpha (Fig. 6F); *gpx2*, glutathione peroxidase 2 (Fig. 6G); *cat*, catalase (Fig. 6H); *aif*, apoptosis-inducing factor (Fig. 6I). Data presented as mean and standard deviation (n = 3). The test of significance was performed by two-way ANOVA with diet and stimulus (LPS or PIC) as main factors, followed by Tukey's multiple comparison for diet or stimulus effect based on the ANOVA results. The asterix superscripts (*, $p < 0.05$; **, $p < 0.01$) indicate statistically significant differences between respective dietary group and the basal diet. The dollar superscripts ($, $p < 0.05$; $$, $p < 0.01$) indicates the effect of the stimulus (LPS or PIC) compared to the untreated control within each diet group.

reported in rainbow trout fed Se-yeast when subjected to intra-peritonial injection of PIC *in vivo* (Pacitti et al., 2016). In Atlantic salmon liver cells, suppression of *p38mapk* expression indicated protection against apoptosis (Espe and Holen, 2013). Se-deficiency and excess induced upregulation of *p38mapk* in spleen, kidney and skin of grass carp was supressed by optimal Se-yeast supplementation at 0.6 ppm (Zheng et al., 2018a; Zheng et al., 2018b). In the present study, the *in vitro* expression of *p38mapk* and *aif* in the liver cells were significantly reduced in 0.4 SM group compared to the basal and 0.4 SS group. Excess selenium induced apoptotic and oxidative stress in liver cells is associated with hydropic degeneration and other histological changes (He et al., 2014). In gilthead seabream, SM supplementation above requirement protected liver and muscle tissue against oxidative damage (Mechlaoui et al., 2019), while excess SS inclusion resulted in hydropic degeneration of hepatocytes (Domínguez et al., 2019). Glutathione peroxidase expression *in vivo* is a potent anti-oxidant marker responsive to dietary Se in rainbow trout (Fontagne-Dicharry et al., 2015). On the contrary, the reduced *in vivo* expression of *gpx2* by Se supplementation, irrespective of the source could be related to the decreased oxidation of glutathione in these groups. In the head kidney, *in vivo* expression of *nfkβ*, *5lox*, *tnfα* and *gpx2* were all higher in 0.15 SS fed fish compared to the basal diet group. Whereas in HKL cells *in vitro*, LPS or PIC activated induction of *il-1b*, *cox2*, *nfkb and gpx2* were attenuated in fish fed 0.4 SM diets. Similar effects of Se deficiency or repletion were reported in RAW 264.7 macrophages (Zamamiri-Davis et al., 2002), mammary epithelial cells in primary culture (Zhang et al., 2014) and murine macrophage cultures (Kim et al., 2004). The attenuation effect of Se was observed only in 0.4 SM group but not in fish from 0.15 SM and 0.4 SS groups that satisfied the requirement for body Se homeostasis. This is consistent with *in vivo* observations in rainbow trout (Pacitti et al., 2016), grass carp, (Zheng et al., 2018a, Zheng et al., 2018b), channel catfish (Wang et al., 1997) and hybrid striped bass (Jaramillo and

Gatlin, 2004) wherein supra-nutritional supplementation of organic Se was effective against viral or bacterial challenges, but not inorganic Se.

To conclude, the minimal Se requirement for Atlantic salmon on available basis was determined at 0.27 mg kg^{-1} diet. In plant-based feeds containing 0.24 ppm basal Se level, the minimal Se requirement was met by 0.41 ppm SS supplementation (total Se, 0.65 ppm) or by 0.17 ppm SM supplementation (total Se, 0.41 ppm). The SM supplementation level complied with the existing EU regulations, but SS did not [Regulations (EU) No 427/2013; 445/2013; 121/2014; 847/2014 and 2015/489; (EC) No 1831/2003 and amendments]. Increasing SM supplementation to 0.4 ppm (total Se, 0.65 ppm) indicated of health benefits to salmon assesed *in vitro*, however the feeds were then non-compliant with existing EU regulation. Further, use of SM was predicted to reduce the Se load to the environment from Norwegian salmon farming by about 60-70%. From the fish health perspective, dietary Se level required in plant-based feeds to maintain body Se homeostasis and improved health status of Atlantic salmon post-smolt exceed the existing EU maximum limit of 0.5 mg Se kg^{-1} complete feed.

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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