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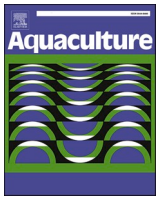

Impact of dietary zinc and seawater transfer on zinc status, availability, endogenous loss and osmoregulatory responses in Atlantic salmon smolt fed low fish meal feeds

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ABSTRACT

Atlantic salmon fed low fish meal feeds supplemented with zinc (Zn) were studied in two feeding trials. In trial I, Atlantic salmon parr were fed six graded Zn levels (40 to 249 mg kg^{-1} as $ZnSO_4$) for 8 weeks in freshwater followed by a 4-week seawater phase. In trial II, Atlantic salmon post-smolt were fed for 10 weeks in SW with 10 dietary Zn levels (45 to 280 mg kg^{-1}), either as $ZnSO_4$ or Zn-glycinate. Growth was unaffected by dietary Zn in both trials. Dietary Zn affected concentration of Na + and K+ ions in plasma, branchial and intestinal expression of sodium potassium ATPase, tissue and body Zn status, and cataracts. Seawater transfer significantly reduced apparent availability, body and tissue levels of Zn due to increased endogenous Zn loss. Atlantic salmon post-smolt in seawater improved body and tissue Zn status with increasing dietary Zn levels, irrespective of the Zn source. Body or tissue saturation of Zn occurred at dietary Zn levels between 137 and 156 mg kg^{-1} with smolts in freshwater and 181 to 218 mg kg^{-1} in SW post-smolts. Dietary Zn levels below 180 mg kg^{-1} in low fish meal feeds compromised the Zn status and welfare of Atlantic salmon in seawater.

1. Introduction

Zinc (Zn) is a micro-mineral indispensable to several physiological and biochemical processes in fish (Lall and Kaushik, 2021; Watanabe et al., 1997). The symptoms of Zn deficiency in fish include growth retardation, increased mortality, cataracts and dwarfism (Ketola, 1979; Maage and Julshamn, 1993; MacDonald, 2000; NRC, 2011; Richardson et al., 1985; Satoh et al., 1987a). Atlantic salmon (*Salmo salar*) parr in freshwater (FW) require 37–67 mg Zn kg^{-1} diet (Maage and Julshamn, 1993). However, in practical diets formulations, Zn supplementation level to meet the requirement is dependent on several factors. The dietary requirements of minerals in fish was predicted through factorial modelling, which included feed intake, mineral availability, aqueous mineral uptake, body mineral gain and endogenous loss (Shearer, 1995). Nowadays, in Atlantic salmon feeds, fish meal (FM) is being replaced largely by plant-derived ingredients. In Norway, the average FM inclusion was 18% during 2013 (Ytrestøyl et al., 2015) and further decreased to 13% in 2016 (Aas et al., 2019). Although Zn is naturally present in both FM and plant-derived ingredients, the latter is relatively low in Zn which is also less available due to the presence of phytic acid. Consequently, reduction and replacement of FM with plant-derived ingredients have affected the availability and supply of dietary Zn to salmonids (Antony Jesu Prabhu et al., 2014; Antony Jesu Prabhu et al., 2018; Antony Jesu Prabhu et al., 2019a). Increasing Zn supplementation is limited by the current upper limit for Zn in salmonid feeds (180 mg Zn kg^{-1} diet) (European Commission, 2016) and further reduction to 150 mg Zn kg^{-1} diet has been suggested (EFSA, FEEDAP, 2014). Alternatively, improving dietary Zn availability using organic sources hold good potential. However, large variation exists in their efficacy compared to commonly used inorganic sources in fish feeds (Antony Jesu Prabhu et al., 2016). Specifically, Zn-gluconate or Zn-glycinate had the same effect as $ZnSO_4$ in Atlantic salmon. While the former study was in FW parr with only two dietary Zn levels (Maage et al., 2001), the latter was a short-term digestibility trial in post-smolt with a solitary dietary Zn level in a multi-factorial design (Silva et al., 2019).

Salmonids in different environments or life stages might have

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different physiological constraints and demands, leading to different dietary needs (Antony Jesu Prabhu et al., 2015). Atlantic salmon parr move from FW to seawater (SW) after smoltification. Development of higher SW tolerance and hypoosmotic ability are important physiological traits of Atlantic salmon smolts achieved through smoltification (McCormick et al., 2013; Prunet et al., 1989). Specifically, higher activity of sodium potassium ATPase (NKA) and the switching of the expression from the FW isoform (NKA α1a) to SW isoform (NKA α1b) occurs during smoltification and in SW (McCormick et al., 2013; McCormick et al., 2009; Sundh et al., 2014). Aqueous Zn exposure influenced branchial NKA activity in response to elevated salinity in Atlantic killifish (*Fundulus heteroclitus*), an euryhaline species (Loro et al., 2014). Further, carbonic anhydrase, a well-known Zn metalloenzyme, is also an integral component of the osmoregulatory machinery in saltwater fishes (Wilson et al., 2002). Despite the physiological role of Zn in osmoregulatory mechanisms, the impact of dietary Zn in the development of hypoosmotic ability during and after smoltification in Atlantic salmon remains unexplored. Given that smoltification and SW transfer are critical stages determining the performance of Atlantic salmon at sea, it is important to generate knowledge on life stage specific Zn requirements to Atlantic salmon. Therefore, the present study aimed to (i) investigate the impact of dietary Zn supply on osmoregulatory responses during smoltification and early SW transfer phase; (ii) optimise dietary Zn levels in low fish meal diets to Atlantic salmon during smoltification in FW and in post-smolt stage in SW; and (iii) compare the efficacy of an organic source (Zn glycinate) with the normally used inorganic source (Zn sulphate) in Atlantic salmon post-smolt.

2. Material and methods

2.1. Experimental design and diets

The study consisted of two feeding trials, both based on regression designs in a dose–response manner with graded levels of dietary Zn. The first feeding trial was designed as the 'transfer trial', Atlantic salmon parr in FW were fed 6 graded levels of dietary Zn for 8 weeks followed by transfer to SW and fed the respective feeds for 4 weeks. In the second feeding trial, designed as the "post-smolt trial", Atlantic salmon post-smolt completely acclimatized to SW were fed ten experimental diets with different level and source of Zn. The basal diets were formulated to be low in fish meal (10%) in both the feeding trials (Table 1) and produced by Skretting Aquaculture Research Centre (Stavanger, Norway). Yttrium oxide was added as an inert external marker for apparent availability measurements. The Zn concentration of the unsupplemented basal diet in the transfer trial was 40 mg Zn kg^{-1} diet and five other diets with total Zn concentrations of 63, 93, 124, 182 and 249 mg Zn kg^{-1} diet were prepared using Zn sulphate monohydrate ($ZnSO_4.H_2O$, Zn 35%, Vilomix, Hønefoss, Norway) as the Zn source. The rationale underlying the selection of the Zn levels is presented in Table S1. In the post-smolt trial, the basal diet contained 45 mg Zn kg^{-1} and six other diets were produced which had 63, 113, 130, 150, 200 and 280 mg Zn kg^{-1} diet through $ZnSO_4$ supplementation. Further, three more diets were produced with Zn chelate of glycine hydrate ($Zn(x)1–3.nH_2O$, x = anion of glycine ($C_2H_4NO_2^-$), Zn 26%, Phytobiotics, Eltville, Germany) as the Zn source to contain 72, 110 and 130 mg Zn kg^{-1} diet.

Table 1
Formulation and analysed proximate composition of the basal diets.

Ingredients (%)	Transfer trial	Post-smolt trial
Wheat	13.5	14.5
Wheat gluten	17.5	14.5
Sunflower meal	3.0	5.0
Soya protein concentrate	31.0	31.0
Faba beans	5.0	5.0
Fish meal[a]	10.0	10.0
Fish oil[a]	10.0	9.0
Rapeseed oil	7.5	8.5
Microingredients and premixes[b]	2.5	2.5
Proximate Composition (Analysed, as is)		
Dry weight (g kg^{-1})	922	924
Lipid (g kg^{-1})	212	199
Protein, N* 6.25 (g kg^{-1})	480	440
Ash (g kg^{-1})	42	46
Zn (mg kg^{-1})	40.3	45.3

[a] North Atlantic.
[b] Contains zinc free mineral premix, vitamin premix, yttrium premix, monoamonium phosphate, histidine HCl, L-lysine and DL-methionine and astaxanthin.

2.2. Ethical statement

The feeding trials and sampling were conducted at Lerang experimental research station, Skretting ARC (Stavanger, Norway), an approved organization (Virksomhet) by the Norwegian Food Safety Authority (Mattilsynet). All the sampling procedures were performed on euthanized fish. Therefore, a specific permission from the animal experimentation administration (Forsøksdyrforvaltningen) was not necessary for the current study. The experimental procedures followed the Norwegian regulations on use of animals in research according to the EU directive 2010/63/EU.

2.3. Experimental conditions and animals

In the transfer trial, the Atlantic salmon parr (Stofnfiskur strain, Iceland; mean weight, 43 g) were fed 6 dietary Zn levels, in duplicate groups for 8 weeks in FW, transferred to SW and fed the respective diets for another 4 weeks. Initially, 1740 Atlantic salmon parr (mixed sex population) were distributed in to 12 tanks (145 fish $tank^{-1}$) filled by flow-through FW with a salinity of 0.5 g L^{-1} during the acclimatization period of 14 days, and fed with a commercial feed (Nutra Olympic, 3.0 mm, Skretting). During the start of the experiment, the number of fish was reduced to 120 fish per tank to account for increased biomass. The water temperature was 11 ± 1 °C and oxygen saturation at outlet was always above 80%, both were monitored continually during the experimental period. After the acclimation time and 24 h starving period, the six experimental diets were each randomly assigned to duplicate tanks. The fish were fed using automatic feeders twice per day until apparent satiation during the experiment period and feed intake monitored. After the FW phase of 8 weeks, all tanks were switched to SW, with a salinity of 34 ± 0.5 g L^{-1} for 4 weeks. The uneaten feed pellets were collected to estimate the actual feed intake. The photoperiod (light: dark) was 12:12 at the start of the experiment (first 2 weeks) and switched to 24:0 during smoltification (subsequent 6 weeks) and then switched back to 12:12 after SW transfer (last 4 weeks). In the post-smolt trial, Atlantic salmon post smolt (mean weight, 125 g) were fed 10 graded levels of Zn (1 basal diet, 6 diets with $ZnSO_4$ and 3 with Zn chelate of glycine) in triplicate groups (50 fish $tank^{-1}$) for a period of 10 weeks in SW (34 g L^{-1}). In our previous trial performed in the same facilities, the Zn concentration in the seawater was analysed as 3.5 ± 0.3 μg L^{-1} ($n = 3$) (Silva et al., 2019).

2.4. Sampling

In both the feeding trials, the weight and length of the fish were recorded at the end of the experimental feeding period from fish euthanized by an overdose (6 mL/L) of tricaine methane sulfonate (PharmaQ, Bergen, Norway). Samples for proximate composition and mineral analysis were taken at the start and end of the experiment in both feeding trials, with an additional sampling point at the end of FW phase in the transfer trial. In both the trials, ten fish per tank were sampled each for whole body and vertebrae analysis. Further, six fish were sampled for blood using heparinized (lithium heparin) vacutainers, centrifuged at 3000*g* for 5 min and the recovered plasma was stored at −20 °C. Opthalmic examination of the sampled fish (20 fish $tank^{-1}$ in

both feeding trials) was performed at the end of the experiment as described elsewhere (Waagbø et al., 2003). The examination was performed on both eyes under darkened conditions with a slit lamp microscope (HEINE® HSL 150 hand-held slit lamp, HEINE Optotechnik) and the cataracts were graded according to its severity on a scale from 0 to 4 for each eye and 0 to 8 for each fish as originally described (Bjerkås et al., 1996). The results are given as prevalence (% fish with cataract of 20 examined tank^{-1}) and severity (mean cataract score in the tank). Samples of tissues such as gill filaments (right side, from 2nd and 3rd gill arches) and different segments of the intestine, namely proximal, middle, and distal were sampled from 6 fish tank^{-1} at the end of FW phase, one week after SW transfer and at the end of SW phase in the transfer trial. The collected samples were flash-frozen in liquid nitrogen and further stored at −80 °C until analysis. Faeces samples were collected from 40 fish through stripping at the end of the experiment in both feeding trials, including an additional sampling point at the end of FW phase in the transfer trial. The faeces collected were pooled per tank, freeze-dried for 72 h, homogenized by pestle and mortar into a fine powder, and kept at room temperature until analysis to determine apparent availability coefficient (AAC).

2.5. Analytical methods

The diets were homogenized and analysed for estimating the dry matter, ash, lipid, protein following standard procedures. Briefly, dry matter was measured after drying to constant weight at 105 °C for 24 h; ash content determined by combustion in a muffle furnace at 550 °C for 16–18 h (NMKL, 1991). Total lipid was determined following ethyl-acetate and acid-extraction in fish tissue and feeds, respectively (NS 9402, 1994). Total nitrogen was measured with a nitrogen analyser (Vario Macro Cube, Elementar Analysensysteme GmbH, Germany), according to AOAC official methods of analysis and crude protein calculated as N x 6.25 (AOAC, 1995). The concentration of Zn, other minerals and yttrium in diets and faeces, and minerals in whole fish homogenate or tissue samples were analysed using a microwave assisted digestion and an inductively coupled plasma mass spectrometry (ICP-MS) as described elsewhere (Maage et al., 2001; Silva et al., 2019). Quantitative real-time polymerase chain reaction (q-PCR) was performed for evaluating the expression of target genes as described elsewhere (Prabhu et al., 2020). The 5′ → 3′ primer sequences for the analysed target genes Na^+,K^+ATPase α1a (Acc. No. AY319391) and Na^+,K^+ATPase α1b (Acc. No. AY319390) were as described elsewhere (Kiilerich et al., 2011). NKA α1a, F: CCCAGGATCACTCAATGTCAC; R: CCAAAGGCAAATGGGTTTAAT; NKAα1b, F: CTGCTACATCTCAACCAACAACAT T; R: CACCATCACAGTGTTCATTGGAT. The housekeeping genes used were as described in Prabhu et al. (2020), namely beta-actin (B-act, accession no. BG933897), 60S ribosomal protein L3 (RPL-13, accession no. AF321836) and Elongation factor 1 alpha (EF1α, accession no. NM_001141291). B-act, F: CCAAAGCCAACAGGGAGAAG; R: AGGGACAACACTGCCTGGAT; RPL13, F: CCAATGTACAGCGCCTGAAA; R: CGTGGCCATCTTGAGTTCCT; EF1a, F: CCCCTCCAGGACGTTTACAAA; R: CACACGGCCCACAGGTACA. The gene expression data were normalized by using reference genes in CFX Maestro 1.1 software (Bio robot EZ1 version 1.1Q04). The efficiency and ideal stability of applied reference genes for all group samples were confirmed by CFX Maestro 1.1 software.

2.6. Data analysis

The experiments were executed following a regression design. In the transfer trial, linear or non-linear regression analyses were performed to determine the trend and to estimate inflection points (i.e. requirement estimates based on specific response criteria), respectively. Further, one-way ANOVA or paired *t*-test were performed to assess statistical significance between dietary Zn levels or sampling points (8w-FW, 1w-SW and 4w-SW), respectively. A comparison between Zn sources ($ZnSO_4$ and ZnGly) was performed in the post-smolt trial by two-way ANOVA. The endogenous Zn loss (EL) and maintenance requirement (R_m) in FW and SW were determined through linear regression analysis and compared using the comparison of fits function in GraphPad Prism as described earlier (Antony Jesu Prabhu et al., 2015). Briefly, the method involved regression analysis of body Zn gain over dietary Zn intake in FW (trial 1) and in SW (trial 2), both expressed as μg kg^{-1} day^{-1}. The net body Zn gain, intercept of the model Y = aX+b on Y-axis when x = 0 (i.e. at zero dietary Zn intake) provided the EL of Zn. Whereas, the intercept of X-axis when, y = 0 (i.e. dietary Zn intake required to compensate for EL) provided an estimate of the maintenance requirement (Rm). Further, the non-linear regressions used to determine optimal dietary levels based on tissue Zn saturation as response criteria were either broken line or quadratic plateau model (Antony Jesu Prabhu et al., 2019b). The requirement estimates between FW and SW were compared of parameter estimates function in GraphPad Prism as described elsewhere (Antony Jesu Prabhu et al., 2017). Tanks were used as experimental units in both feeding trials to analyse data on growth, whole body proximate and mineral composition (n = 2, transfer trial; n = 3, post-smolt trial). Individual fish (n = 6 tank^{-1}) were used as observational units for data on plasma mineral concentration and gene expression analysis. The regression analysis and graphs were made in GraphPad Prism (version 8), whereas the ANOVA were performed in R (R Core Team, 2013). The AAC was determined using the ratio between the concentrations of Zn in diet and in faeces and the concentrations of an inert marker (IM) (i.e. yttrium oxide) in diet and faeces, as described by the following equation: AAC (%) = 100 – [100 * (IM in diet/IM in faeces)*(Zn in faeces/Zn in diet)].

3. Results

3.1. Transfer trial

Feed intake, weight gain, feed conversion and growth rate of Atlantic salmon was not differentially affected by dietary Zn (Table S2). Apparent availability of Zn, whole body Zn and vertebrae Zn

Table 2
Apparent availability coefficient (AAC) and concentration of Zn in whole body and vertebrae of Atlantic salmon fed different diets supplemented with Zn in the transfer trial.

Dietary Zn (mg kg^{-1})	AAC of Zn (%)		Whole body Zn (mg kg^{-1} ww)		Vertebrae Zn (mg kg^{-1} dw)	
	8w-FW	4w-SW	8w-FW	4w-SW	8w-FW	4w-SW
Zn level						
40	53.0^{ab}	15.5	31.5^{c}	24.5^{d}	88.5^{a}	58.0^{d}
63	46.5^{b}	17.5	33.5^{c}	25.0^{d}	96.5^{b}	59.5^{d}
93	45.5^{b}	10.0	34.0^{c}	29.0^{c}	93.5^{ab}	66.0^{cd}
124	59.5^{a}	24.5	39.0^{b}	29.5^{c}	109.0^{c}	76.0^{bc}
182	61.5^{a}	25.5	43.5^{a}	36.5^{b}	115.0^{c}	81.0^{b}
249	47.5^{b}	26.5	45.0^{a}	41.0^{a}	115.0^{c}	95.5^{a}
Pooled SD	6.9	4.3	1.7	0.6	2.5	1.3
p-value	*0.01*	*0.05*	*0.03*	*<0.001*	*0.05*	*<0.001*
FW vs SW						
Mean ± SD	52 ± 8^{x}	20 ± 7^{y}	38 ± 6^{x}	31 ± 6^{y}	103 ± 12^{x}	73 ± 13^{y}
p-value	*< 0.001*		*< 0.001*		*< 0.001*	

Data presented as mean per group (n = 2) and pooled standard deviation (SD) across all groups. AAC, apparent availability coefficient; 8w-FW, eight weeks feeding in freshwater; 1w-SW, one week after seawater transfer; 4w-SW, four weeks after seawater transfer. ww, wet weight; dw, dry weight. Data analysed by one-way ANOVA at 95% significance level (p < 0.05), followed by Tukey's multiple comparison post-hoc analysis. Post-hoc: Zn level, values within the same column bearing different superscripts (a, b, c) are significantly different. FW vs SW, values in the same row with different superscripts (x, y) are significantly different.

concentrations in the transfer trial are presented (Table 2). The AAC of Zn increased with Zn supplementation and was higher in fish fed dietary Zn levels of 124–182 mg Zn kg^{-1} diet at the end of FW phase (60–62%) or 124–249 mg Zn kg^{-1} diet in SW (25–27%), compared to fish fed lower dietary Zn levels. The mean AAC of Zn at the end of FW phase was 52.4% whereas the same at the end of SW phase was 20% ($p < 0.0001$). At the end of the FW phase, Zn concentrations in the whole body and vertebrae increased with increasing dietary Zn and reached the maximum when fed diet containing 124 mg Zn kg^{-1} or 182 mg Zn kg^{-1}, respectively. At the end of SW phase, the maximum concentration of Zn in whole body and vertebrae were achieved when fed 249 mg kg^{-1} dietary Zn. The mean whole body and vertebrae Zn concentrations at the end of SW phase was significantly lower compared to the levels recorded at the end of the FW phase ($p < 0.001$). Further, the magnitude of reduction was higher in fish fed lower dietary Zn levels.

The concentration of Zn and major electrolytes, namely sodium (Na^+) and potassium (K^+) were analysed in the plasma and the data are presented in Table 3. Plasma Zn concentration increased with dietary Zn levels fed with the minimal concentration recorded in the basal diet group and maximal response in 249 mg Zn kg^{-1} fed fish at all the three time points studied. The plasma Zn concentration at the end of FW was significantly higher compared to the concentrations observed 1 and 4 weeks after SW transfer ($p < 0.001$). The plasma Na^+ concentration was not affected by dietary Zn levels but increased significantly in SW compared to FW. The plasma K^+ concentration increased linearly with dietary Zn levels at the end of FW and after one week in SW transfer ($p < 0.001$), but not at 4 weeks after SW transfer. The impact of SW transfer lowered the plasma K^+ concentration compared to the FW phase ($p < 0.01$).

The concentration of Zn, Na^+ and K^+ were analysed in the gill filaments (soft tissue) and the data are presented in Table 4. The concentration of Zn in the gills were significantly lower in fish fed the basal diet, increased with dietary Zn and reached the maximum at the highest Zn diet tested ($p < 0.0001$). The linear relationship was observed at all three time points studied and there was no difference between FW and SW phases. The concentration of Na^+ in the gill was not affected by dietary Zn at the end of FW, but a linear decrease was observed one and 4 weeks after SW transfer. Gill K^+ concentration was not affected at any of the three time points by dietary Zn, whereas the mean K^+ concentration was significantly higher one week after SW transfer and declined after four weeks in SW compared to FW ($p = 0.0009$). The Na/K ratio in the gill was not responsive to dietary Zn in FW. However, it was significantly higher in the maximal Zn fed group compared to the basal diet fed group both at one week ($p = 0.02$) and 4 weeks ($p = 0.008$) in SW, exhibiting a linear relation. The Na/K ratio was significantly higher in SW phase compared to the end of FW ($p < 0.0001$).

The mRNA expression of sodium potassium ATPase isoforms (NKAα1a and NKAα1b) were analysed in the gills, mid-intestine and distal-intestine at the end of FW phase and one week after SW transfer (Fig. 1). In the gills, NKAα1b expression was higher than NKAα1a and had a significant linear trend in response to increasing dietary Zn at the end of FW ($Y = 0.001068*X + 0.71$; $p = 0.03$) and one week after SW ($Y = 0.0008878*X + 0.66$; $p = 0.01$). In the mid-intestine, NKAα1a responded with a linear increase to dietary Zn in FW ($Y = 0.001393*X + 0.09$; $p = 0.006$), but not NKAα1b. Nevertheless, NKAα1b expression was significantly higher than NKAα1a in FW but no difference was observed in SW. In the distal intestine, the expression of NKAα1b was significantly higher than the expression of NKAα1a at both time points. Dietary Zn increment resulted in a linear increase in NKα1b expression in the distal intestine at the end of FW ($Y = 0.0007796*X + 0.55$; $p = 0.03$), no such effect was observed after 1 week in SW.

At the end of the four-week SW phase, Atlantic salmon smolts fed the lowest Zn level had the most prevalence and severity of cataracts, which then declined with increasing Zn supplementation following a quadratic regression (Fig. 2). Based on the quadratic model, dietary Zn level of 169 and 173 mg Zn kg^{-1} diet resulted respectively in the lowest prevalence and severity of cataracts in Atlantic salmon smolts four weeks after SW transfer. Further increase in dietary Zn concentration up to 249 mg kg^{-1} diet tended to increase incidence of cataract.

3.2. Post-smolt trial

Feed intake, weight gain, feed conversion and growth rate of post-smolt Atlantic salmon was not differentially affected by dietary Zn levels or sources (Table S2). The AAC and concentrations of Zn in the whole body, vertebrae and plasma are presented in Table 5. Initially AAC of Zn increased, tended to be higher at dietary Zn concentration of 130 mg kg^{-1} diet ($p = 0.06$) and then decreased with further increase in dietary Zn, exhibiting a quadratic relation ($p = 0.007$). The concentration of Zn in the whole body and vertebrae were significantly affected by dietary Zn ($p < 0.0001$) and exhibited a linear relation ($p < 0.0001$). Plasma Zn concentration increased with increasing dietary Zn and reached a plateau beyond dietary Zn concentration of 200 mg kg^{-1} diet ($p < 0.0001$). None of the above responses were influenced differently by the two Zn sources namely $ZnSO_4$ or ZnGly. Lens examination in Atlantic salmon post-smolts revealed the presence of cataract in all examined fishes with no significant differences in prevalence or severity of cataract (mean score, 3.4 ± 1) in response to dietary Zn levels or sources.

Table 3
Zinc (Zn) and major electrolyte concentrations in the plasma of Atlantic salmon fed graded Zn levels in the transfer trial.

Dietary Zn (mg kg^{-1})	Plasma Zn (μmol L^{-1})			Plasma Na^+ (mmol L^{-1})			Plasma K^+ (mmol L^{-1})		
	8w-FW	1w-SW	4w-SW	8w-FW	1w-SW	4w-SW	8w-FW	1w-SW	4w-SW
Zn level									
40	99.4^{c}	42.6^{a}	24.7^{c}	161	169	173	6.1^{ab}	4.7^{a}	4.4
63	139.6^{bc}	60.4^{a}	35.3^{c}	161	172	180	5.5^{a}	5.4^{b}	4.5
93	209.8^{ab}	80.7^{ac}	84.6^{abc}	154	174	182	5.7^{a}	5.3^{b}	5.2
124	195.1^{ab}	80.7^{ac}	104.5^{abc}	160	172	185	6.2^{ab}	5.1^{ab}	4.8
182	247.1^{a}	97.2^{bc}	114.4^{ab}	163	176	179	5.2^{a}	5.2^{ab}	4.7
249	262.9^{a}	111.6^{b}	129.7^{a}	159	171	173	6.9^{b}	5.4^{b}	4.9
Pooled SD	44.1	37.1	45.9	8.4	5.9	11.5	0.6	0.4	0.6
p-value	*<0.0001*	*0.0006*	*0.002*	*ns*	*ns*	*ns*	*0.001*	*0.001*	*ns*
FW vs SW									
Mean ± SD	186 ± 61^{x}	79 ± 25^{y}	82 ± 43^{y}	160 ± 6^{x}	173 ± 3^{y}	179 ± 5^{y}	5.9 ± 0.6^{x}	5.2 ± 0.3^{y}	4.8 ± 0.3^{y}
p-value	*< 0.001*			*0.006*			*0.006*		

Data presented as mean per group (n = 2) and pooled standard deviation (SD) across all groups. 8w-FW, eight weeks feeding in freshwater; 1w-SW, one week after seawater transfer; 4w-SW, four weeks after seawater transfer. Data analysed by one-way ANOVA at 95% significance level ($p < 0.05$), followed by Tukey's multiple comparison post-hoc analysis. Post-hoc: Zn level, values within the same column bearing different superscripts (a, b, c) are significantly different. FW vs SW, values in the same row with different superscripts (x, y) are significantly different.

Table 4
Zinc and major electrolyte concentrations in the gill of Atlantic salmon fed graded Zn levels in the transfer trial.

Dietary Zn (mg kg^{-1})	Gill Zn (mg kg^{-1} ww)			Gill Na^+ (mg kg^{-1} ww)			Gill K^+ (mg kg^{-1} ww)			Gill Na/K ratio		
	8w-FW	1w-SW	4w-SW	8w-FW	1w-SW	4w-SW	8w-FW	1w-SW	4w-SW	8w-FW	1w-SW	4w-SW
Zn level												
40	86^{a}	90^{a}	85^{ab}	1.57	3.51	3.7	2.24	2.25	2.01	0.70	1.56	1.85
63	111^{ab}	92^{a}	68^{a}	1.55	3.41	3.79	2.21	2.27	2.07	0.70	1.51	1.83
93	103^{bc}	116^{b}	105^{b}	1.53	3.29	3.5	2.19	2.31	2.05	0.69	1.42	1.71
124	112^{bc}	124^{bd}	103^{ab}	1.51	3.23	3.48	2.17	2.28	2	0.69	1.43	1.74
182	131^{c}	134^{bd}	103^{ab}	1.52	3.41	3.47	2.22	2.31	2.13	0.68	1.48	1.64
249	150^{d}	153^{c}	163^{d}	1.52	3.16	3.18	2.23	2.25	2.21	0.68	1.41	1.54
Pooled SD	14.2	13.2	21.1	0.06	0.15	0.17	0.09	0.12	0.12	0.04	0.07	0.14
p-value	*<0.001*	*<0.001*	*<0.001*	*ns*	*0.005*	*<0.001*	*ns*	*ns*	*ns*	*ns*	*0.02*	*0.008*
FW vs SW												
Mean ± SD	116 ± 22	118 ± 24	105 ± 32	1.53 ± 0.02^{x}	3.3 ± 0.13^{y}	3.52 ± 0.21^{y}	2.2 ± 0.03^{x}	2.3 ± 0.03^{y}	2.1 ± 0.08^{z}	0.69 ± 0.01^{x}	1.5 ± 0.06^{y}	1.7 ± 0.1^{y}
p-value	*ns*			*<0.0001*			*0.006*			*<0.0001*		

Data presented as mean per group (n = 2) and pooled standard deviation (SD) across all groups. 8w-FW, eight weeks feeding in freshwater; 1w-SW, one week after seawater transfer; 4w-SW, four weeks after seawater transfer. Data analysed by one-way ANOVA at 95% significance level ($p < 0.05$), followed by Tukey's multiple comparison post-hoc analysis. Post-hoc: Zn level, values within the same column bearing different superscripts (a, b, c, d) are significantly different. FW vs SW, values in the same row with different superscripts (x, y, z) are significantly different.

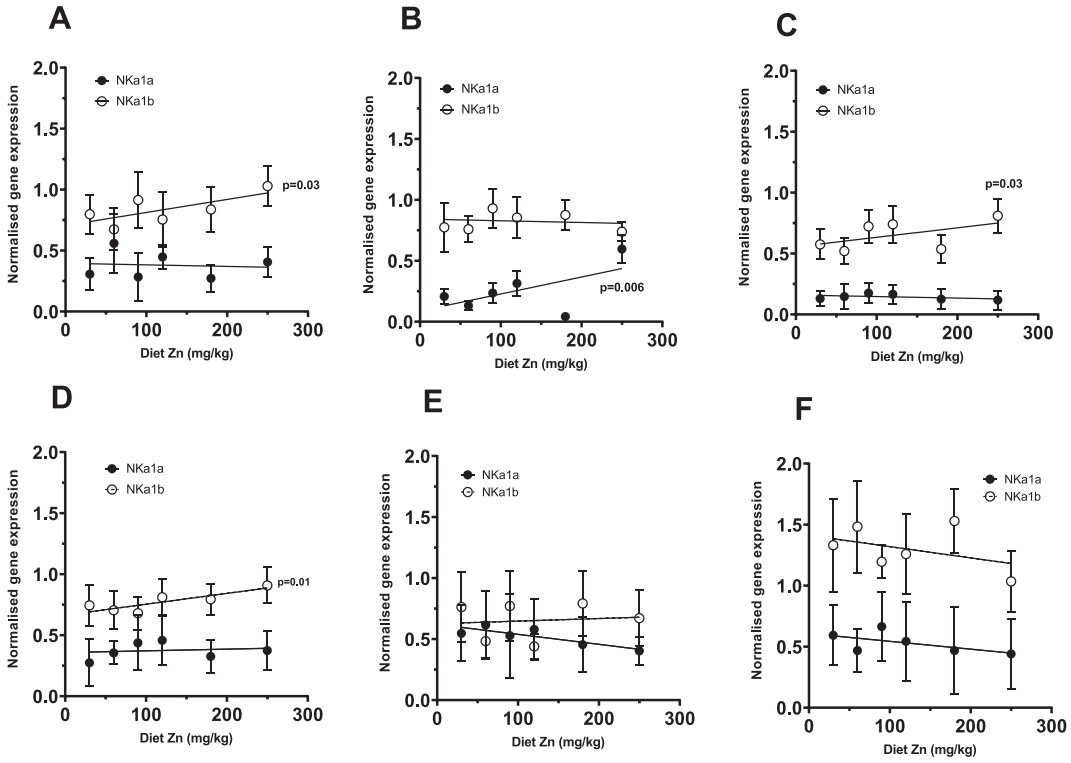


Fig. 1. Branchial and intestinal mRNA expression of sodium-potassium ATPase (NKA) α1a and α1b in Atlantic salmon fed different dietary zinc (Zn) levels before transfer and one week after seawater transfer.
Gill filaments, before and one week after seawater transfer (A and D); mid-intestine, before and one week after seawater transfer (B and E); distal intestine, before and after seawater transfer (C and F). Data are presented as mean ± SD ($n = 6$ fish per tank). Dark circles, NKA α1a and open circles, NKA α1b. The lines represent regression analysis of the respective mRNA expression (y-axis) as a function of dietary Zn level (x-axis). *P*-values adjacent to a regression line indicates significant deviation of the slope from zero (NKA α1b in A, C and D; NKA α1a in B).

3.3. Zinc requirement of smolt and post-smolt Atlantic salmon

Endogenous loss (EL) and maintenance requirement (R_m) for Zn in FW Atlantic salmon smolt and SW post-smolt were estimated and compared (Fig. 3). Based on total dietary Zn, the EL and R_m were respectively 24.1 ± 13.1 and 323.2 ± 128 μg kg^{-1} d^{-1} in FW; in SW it was 52 ± 6.2 and 998 ± 52 μg kg^{-1} d^{-1} respectively. The EL and R_m were significantly higher in SW post-smolt than in FW smolt by 116% and 209%, respectively ($p = 0.001$). However, there were no significant differences between SW post-smolt and FW smolt in EL (FW, 21.3 ± 13.1 vs. SW, 37.9 ± 10.8 μg kg^{-1} d^{-1}) or R_m (FW, 154 ± 64 vs. SW, 255 ± 37 μg kg^{-1} d^{-1}) on available basis, although they were 78% and 66% higher, respectively. Non-linear regression analysis was performed to estimate dietary Zn levels required to reach saturation in whole body Zn retention, vertebrae and plasma Zn levels (Fig. 4). Whole body Zn retention or gain saturated at 137 ± 25 mg kg^{-1} diet in FW smolt and the same in SW post-smolt was 181 ± 35 mg kg^{-1} diet. The dietary Zn levels to saturate vertebrae Zn levels were 167 ± 41 mg kg^{-1} diet in FW smolt

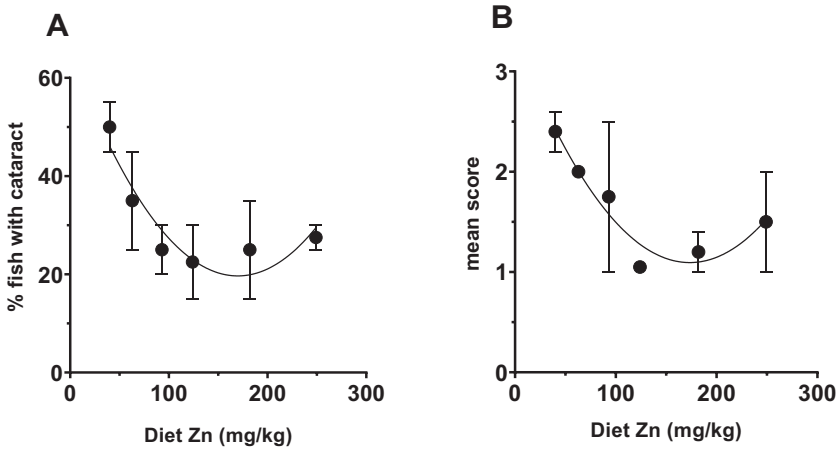


Fig. 2. Prevalence and severity of cataract in Atlantic salmon smolts at the end of the seawater phase in the transfer trial. A. Prevalence (% fish with cataract) and B. Severity (mean cataract score). Data presented as mean ± SD ($n = 20$ fish per tank). The line represents quadratic regression of the respective parameters (y-axis) as a function of dietary Zn levels (x-axis). The regression equations respectively were $y = 0.0016 \times {}^{2}–0.53\times + 64.9$ ($R^2 = 0.87$) and $y = 7E^{-05} \times {}^{2}–0.026\times + 3.34$ ($R^2 = 0.93$) for prevalence and severity of cataract. The respective estimations on the dietary Zn levels for minimal prevalence and severity of cataracts were 169 and 173 mg Zn kg^{-1} diet.

Table 5
Apparent availability coefficient (AAC), concentration of zinc (Zn) in whole body, vertebrae and plasma of Atlantic salmon fed with diets supplemented with different Zn levels and sources in seawater.

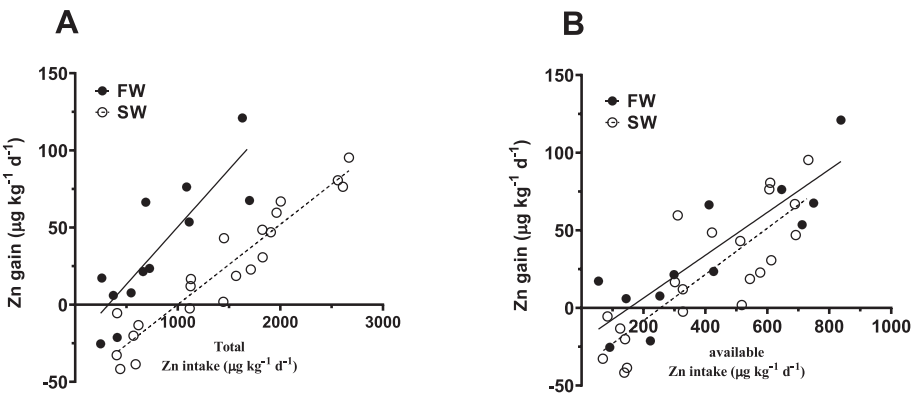
Zn source	Dietary Zn (mg kg^{-1} diet)	AAC Zn (%)	Whole body Zn (mg kg^{-1} ww)	Vertebrae Zn (mg kg^{-1} ww)	Plasma Zn (μmol L^{-1})
Inorganic source ($ZnSO_4$)	45	22.9	17.0^{e}	77.4^{d}	35.3^{f}
	63	23.3	17.3^{e}	85.1^{cd}	65.8^{ef}
	110	28.4	20.4^{cde}	99.7^{cd}	105.2^{de}
	130	35.3	23.8^{cd}	108^{bc}	154.5^{cd}
	150	30.1	25.2^{bc}	125.6^{ab}	183.4^{bc}
	200	28.8	28.1^{ab}	139.0^{a}	229.4^{ab}
	280	24.8	32.9^{a}	149.1^{a}	259.9^{a}
Organic source (Zn-Gly)	65	19.1	18.4^{de}	84.4^{cd}	70.8^{ef}
	110	24.6	21.2^{cde}	89.8^{cd}	109.2^{de}
	130	27.3	22.7^{cde}	100.4^{bcd}	130.9^{d}
Pooled SD		6.6	2.1	8.8	30.3
Statistics					
One-wayANOVA		*0.06*	*<0.0001*	*<0.0001*	*<0.0001*
Two-way ANOVA					
Zn level		*<0.05*	*<0.01*	*<0.01*	*<0.01*
Zn source		*ns*	*ns*	*ns*	*ns*
Interaction		*ns*	*ns*	*ns*	*ns*

Data presented as mean per group (n = 3) and pooled standard deviation (SD) across all groups. Data analysed by both one-way ANOVA (all 10 groups together) and two-way ANOVA (6 groups; Zn level and Zn source as main factors) at 95% significance level ($p < 0.05$), followed by Tukey's multiple comparison post-hoc analysis. Post-hoc: Zn level, values within the same column bearing different superscripts are significantly different.

and 218 ± 17 mg kg^{-1} in SW post-smolt. The plasma Zn levels saturated at dietary Zn of 156 ± 23 mg kg^{-1} in FW smolt and 198 ± 15 mg kg^{-1} in SW post-smolt. The magnitude of increase in the estimated dietary Zn levels required to reach saturation in measured responses were 27 to 38% higher in SW post-smolt compared to FW smolt, nevertheless, not statistically different.

4. Discussion

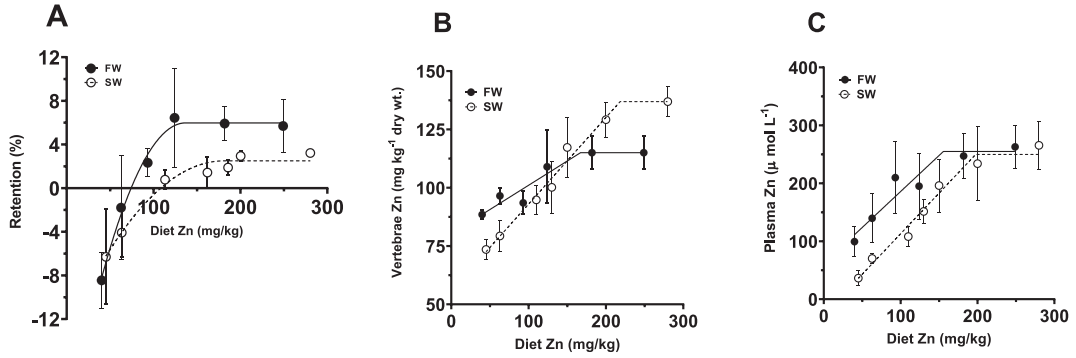
Sub-optimal supply of dietary Zn may not necessarily affect growth during an experimental duration, but, it has been shown to decrease body or tissue Zn status in salmonids (Antony Jesu Prabhu et al., 2019a; Antony Jesu Prabhu et al., 2018; Maage et al., 2001; Maage and Julshamn, 1993; Vera et al., 2020). Therefore, growth as a response criterion in the assessment of optimal dietary Zn supply has been questioned (NRC, 2011), and other stringent criteria based on tissue or body Zn status are proposed (Antony Jesu Prabhu et al., 2016). Post-prandial plasma Zn concentration can reflect on dietary availability and absorption (Antony Jesu Prabhu et al., 2014), while vertebrae and whole-body Zn levels are good indicators of long-term Zn status (Antony Jesu Prabhu et al., 2016; Maage et al., 2001). Differences in the whole body or tissue Zn concentrations between Atlantic salmon parr in FW and post-smolt in SW has been reported before (Antony Jesu Prabhu et al., 2019a; Shearer et al., 1994; Vera et al., 2020). The Zn status of smolts declined after SW transfer and the magnitude of decline was inversely proportional to dietary Zn level. The Zn status of the whole body was reduced by 64, 40 and 34% in the low (94 mg kg^{-1} diet), mid (156 mg kg^{-1} diet) and high (330 mg kg^{-1} diet) Zn dietary groups after a year in the sea (Vera et al., 2020). In the present study, the decline was about 23% and 9% in the lowest (40 mg kg^{-1} diet) and highest (249 mg kg^{-1} diet) dietary Zn levels tested, over a period of four weeks in SW. Zinc homeostasis can be affected by the different physiological and metabolic changes brought about by the shift from FW to SW (Hogstrand, 2013; Zhang and Wang, 2007). The apparent availability of Zn ranged between 45 and 62% in FW, in agreement with our previous report on Atlantic salmon (Antony Jesu Prabhu et al., 2019a). In the post-smolt trial, the AAC of Zn ranged from 23 to 35%, in fair agreement with our earlier report of 23 to 45% (Silva et al., 2019). The broad range of AAC values in Silva et al. (2019) were due to difference in Zn levels and interaction with other trace elements (Mn and Se). Comparison of specific diet groups with respect to Zn level (150 mg Zn kg^{-1} diet) and source (inorganic $ZnSO_4$) alike showed the AAC of Zn in post-smolt Atlantic salmon were not different between the two studies (30 vs 31%). However, much lower Zn AAC (10 to 27%) and reduction in body Zn levels four weeks after SW in the transfer trial suggest of an acclimation window characterised by a rapid decline during early days in SW. Differences in dietary Zn availability between FW and SW phases of Atlantic salmon has not been described before. However, Zn uptake as a whole and specifically by the gastrointestinal segment decreased as salinity increased in black seabream (*Acanthopagrus schlegeli*) (Zhang and Wang, 2007) and Atlantic killifish (Loro et al., 2014). The reduction can be attributed to changes in ionic speciation and physiological responses related to hypoosmotic regulation in SW (Glover and Hogstrand, 2003; Hogstrand, 2013; Loro et al., 2014; Prabhu et al., 2018). Active drinking of SW increases Ca^{2+}, Mg^{2+} and other cations in the intestinal lumen of marine or SW acclimated fish to many-fold higher than in FW fish (Bucking et al., 2011; Dabrowski et al., 1986; Loro et al., 2014). Most of the Ca^{2+} and Mg^{2+} are eliminated as amorphous $CaMgCO_3$ precipitates through HCO_3^- secretion (Foran et al., 2013; Wilson et al., 2002). Nevertheless, high luminal Ca^{2+}, Mg^{2+} and HCO_3^- concentrations reduce intestinal Zn uptake *in vitro* (Glover et al., 2004; Glover and Hogstrand, 2003; Prabhu et al., 2018) and dietary Zn availability *in vivo* (Antony Jesu Prabhu et al., 2014; McClain and Gatlin, 1988; Satoh et al.,



	A. Total Zn				B. Available Zn			
	FW	SW	p-value	%Δ_{FW-SW}	FW	SW	p-value	%Δ_{FW-SW}
EL	24.1 ± 13.1	52.0 ± 6.2	0.001	116%	21.3 ± 11.9	37.9 ± 10.8	ns	78%
R_m	323.2 ± 128	999.8 ± 52	0.001	209%	154.2 ± 64	255.1 ± 37	ns	66%
Units: µg kg^{-1} d^{-1}					Units: µg kg^{-1} d^{-1}			

Fig. 3. Regression analysis of daily zinc (Zn) gain over intake per unit body weight in freshwater (FW) and seawater (SW) Atlantic salmon.

Linear regression analysis of whole body Zn gain over Zn intake as total dietary Zn (A) and available Zn (B). Dark circles and solid line, feeding trial I in freshwater (FW, n = 12); Open circles and dotted line, feeding trial II in seawater (SW, n = 21). EL, Endogenous loss (−a, y-intercept) and R_m, maintenance requirement (−a/b, x-intercept) for Zn to Atlantic salmon in FW or SW expressed as microgram per kg body weight per day (µg kg^{-1} day^{-1}). Comparison of EL and R_m between FW and SW were performed using the comparison of fits function in GraphPad Prism version 8 for windows (GraphPad software, San Diego, CA, USA) and the p-value presented. The magnitude of difference in mean estimates of EL and R_m between FW and SW were calculated as (EL^{SW} or R_m^{SW}- EL^{FW} or R_m^{FW})/ (EL^{FW} or R_m^{FW}) and presented as %Δ_{FW-SW}.



Requirement estimates' (mg Zn kg^{-1} diet)				
	FW	SW	p-value	%Δ_{FW-SW}
Retention or gain	137 ± 25	181 ± 35	0.35	38%
Vertebrae Zn	167 ± 41	218 ± 17	0.16	34%
Plasma Zn	156 ± 23	198 ± 15	0.28	27%

Fig. 4. Non-linear regression analysis of plasma zinc (Zn), vertebrae Zn and Zn retention in Atlantic salmon fed graded dietary Zn concentration in freshwater and seawater.

Non-linear regression analysis of whole body Zn retention (A), vertebrae Zn concentration (B) and plasma Zn concentration (C) of Atlantic salmon in freshwater (dark circles and solid line, n = 12) and seawater (open circles and dotted line, n = 21). Whole body Zn retention followed a quadratic plateau model, while vertebrae and plasma Zn concentration followed broken line regression model. Comparison of the requirement estimates (expressed as, mg Zn kg^{-1} diet) between FW and SW were performed using the comparison of parameters function in GraphPad Prism version 8 for windows (GraphPad software, San Diego, CA, USA) and the p-value presented. The magnitude of difference in mean Zn requirement estimates between FW and SW were calculated as (Zn_{req}^{SW}- Zn_{req}^{FW})/ (Zn_{req}^{FW}) and presented as %Δ_{FW-SW}.

1993; Satoh et al., 1987b). Complementing the knowledge on reduced bioavailability of aqueous Zn to fish at increased salinities (Loro et al., 2014; Loro et al., 2014; Zhang and Wang, 2007), evidence for reduced dietary Zn availability in SW will lead to increased demand for dietary Zn to Atlantic salmon in SW.

Atlantic salmon, being an anadromous species undergoes smoltification before entering the SW phase to enable hypoosmotic ability (Maetz, 1971). In Atlantic salmon smolts ready for SW transfer, branchial and intestinal expression of NKAα1b, the SW isoform overrides the FW isoform, NKAα1a (McCormick et al., 2013; McCormick et al., 2009; Sundh et al., 2014). The upregulation of NKAα1b inactively occurs during smolt development in FW and becomes activated once smolts are transferred to SW; whereas, the abundance of NKAα1a is relatively stable and decreases with post-SW transfer (McCormick et al., 2013; Sundh et al., 2014). The above changes in the expression of NKA subunits were also observed in the transfer trial in response to smoltification and SW transfer. Interestingly, dietary Zn influenced the branchial NKAα1b expression during smoltification, and the impact persisted one week after SW transfer. Studies on the impact of dietary Zn on osmoregulatory responses in fish does not exist. However, gill ATPase activities were stimulated by aqueous Zn exposure *in vivo* and the increased activity of NKA enabled a relatively constant internal ionic environment in trout upon SW challenge (Watson and Beamish, 1980). The NKA activity in Atlantic killifish exposed to higher salinities in the presence of

Zn increased to about 30% and 90% at 10 and 35 g L^{-1}, respectively (Loro et al., 2014). In the SW, along with gills, the intestine also becomes an important osmoregulatory organ (Sundh et al., 2014; Whittamore, 2012; Wilson et al., 2002). As part of the osmoregulatory response to SW, the intestine of rainbow trout reabsorbed K^+, while Na^+ concentrated in the faeces (Bucking et al., 2011; Dabrowski et al., 1986). The NKA functions by pumping two K^+ ions and three Na^+ ions in and out of the cell, respectively (Marshall and Grosell, 2006). Although NKA activity was not measured in this study, plasma K^+ measured as a proxy for NKA activity mirrored the expression pattern of NKAα1a and NKAα1b in the gills and intestine. Further evidence was observed when increasing dietary Zn corresponded with changes in Na^+ and K^+ concentrations and their ratio in gills, also observed in Atlantic killifish upon exposure to higher salinities and Zn (Loro et al., 2014). Considering the impact of dietary Zn on the expression of NKA subunits in the gill, intestine and plasma ion levels, the impact of dietary Zn on Atlantic salmon smoltification requires further investigation.

Different factors like gain (G), endogenous loss (E), uptake from water (U), dietary availability (A) and feed intake (F) were used to predict the mineral requirement of rainbow trout using the multifactorial model C = [(G + *E*- U)/A])/F) (Shearer, 1995). According to the model, the Zn requirement of 10 g rainbow trout was predicted to be 20.6 mg kg^{-1} diet, assuming U = 0 and A = 1. Using the same model on our data provided an estimate of 18.3 mg Zn kg^{-1} diet in FW and 36 mg Zn kg^{-1} diet in SW (assuming U = 0). The Zn requirement estimate in FW was in close agreement with the estimate of 20.6 mg Zn kg^{-1} diet predicted by Shearer (1995). However, the estimate in SW post-smolt was 36 mg Zn kg^{-1}, higher than the estimate predicted by Shearer (1995). The dietary need for a nutrient under a given environment is determined by the physiological constraints faced by the animal. Changing from fishmeal-based feed to a complete plant-based feed decreased Zn availability, increased the EL and R_m for Zn in rainbow trout (Antony Jesu Prabhu et al., 2015). Similarly, in the present study, transition from FW to SW environment increased the dietary requirement for Zn in Atlantic salmon due to decreased Zn availability, increased the endogenous Zn loss and maintenance need. Drastic decrease in whole body Zn levels occur after the Atlantic salmon smolts are transferred to SW and the magnitude of reduction was inversely proportional to the dietary Zn levels fed (Vera et al., 2020). Osmoregulation in SW and stimulation of gill ATPases are known to augment metabolic expenditure in salmonids (Soengas et al., 2007; Watson and Beamish, 1980). Comparing the endogenous Zn loss between FW and SW indicated metabolic changes accounted for 2/3rd of the increased endogenous Zn loss in SW. Whereas, for maintenance need, a major and decisive part of the increase in SW was attributed to the difference in apparent availability of dietary Zn between FW and SW. Subsequently, the combined impact of changes in Zn metabolism and apparent availability led to an increase in the dietary Zn level required for Atlantic salmon in SW. Further, differences in water Zn concentrations and ability of Atlantic salmon to acquire Zn from FW or SW due to osmoregulatory challenges could also contribute to the differences as has been shown for Ca and Mg in fish (Hossain and Furuichi, 2000; Hossain and Furuichi, 1998; Lin et al., 2013; Robinson et al., 1986; Shearer, 1989). The Zn concentrations in Norwegian rivers in general is below 5 μg L^{-1}, except when affected my mining (Gundersen et al., 2019) and that of the SW analysed in the facility was 3–4 μg L^{-1} (Silva et al., 2019). Therefore, neither the absolute concentrations, nor the contrast between Zn concentrations in FW and SW could not have been large enough to induce significant differences in aqueous Zn uptake. Moreover, it is well accepted that diet is the major source of Zn to fish (Bury et al., 2003).

The NRC recommendation (NRC, 2011; 1993) states the Zn requirement of Atlantic salmon parr to be between 37 and 67 mg Zn kg^{-1} diet, following the findings reported elsewhere (Maage and Julshamn, 1993). Atlantic salmon parr fed 180 mg Zn kg^{-1} diet had a better body and tissue Zn status over 6 months, despite growth not being affected (Maage et al., 2001). In plant-based feeds, the dietary need for Zn to Atlantic salmon parr was found to range from 101 to 132 mg kg^{-1} total dietary Zn (corresponding 35 to 55 mg kg^{-1} available Zn), based on a multi-nutrient regression study (Antony Jesu Prabhu et al., 2019a). However, in the present study, Atlantic salmon smolts in FW which are ready for SW transfer required 137 to 167 mg kg^{-1} total Zn (80 to 100 mg kg^{-1} available Zn), higher than reported in parr. It is likely that Atlantic salmon smolts may have a higher physiological need for Zn than parr owing to the higher metabolic expenditure and osmotic changes discussed earlier. In Atlantic salmon post-smolt, the dietary Zn level required for maintaining body Zn status ranged from 140 to 177 mg kg^{-1} diet based on body Zn saturation (Antony Jesu Prabhu et al., 2019a; Vera et al., 2020), that is close to the range of 181 to 218 mg kg^{-1} diet in the present study. Current European legislation enforces a maximum limit of 180 mg Zn kg^{-1} for total Zn content in salmonid feeds, which was reduced from the previous limit of 200 mg Zn kg^{-1} diet mainly due to environmental concern (European Commission, 2016). Recent data on optimal dietary Zn levels in the present plant-based/low-fish meal feeds to Atlantic salmon post-smolt in SW are either close to or above the current maximum limit of 180 mg Zn kg^{-1} (Antony Jesu Prabhu et al., 2019a; Jensen et al., 2015; Vera et al., 2020). A further decrease in the total Zn content to 150 mg Zn kg^{-1} in salmon feeds as proposed (EFSA, FEEDAP, 2014) would challenge the Zn requirements of Atlantic salmon being met, especially in the SW phase.

The scientific opinion of the European Food Safety Authority states that the proposed 150 mg Zn kg^{-1} diet can ensure health, welfare and productivity of the target species, i.e. in this case, the salmonids. Skin health and wound healing was improved in Atlantic salmon post-smolts fed 240 mg Zn kg^{-1} compared to the control feed containing 92 mg Zn kg^{-1} (Jensen et al., 2015). Although, the present study did not examine skin health, prevalence and severity of cataracts, an important health and welfare indicator of farmed Atlantic salmon was affected by Zn supplementation. Atlantic salmon transferred from FW to SW are prone to develop a reversible form of opacity termed osmotic cataract, which may develop into irreversible cataracts with time (Bjerkås et al., 1996). Atlantic salmon smolts with cataracts had lower Zn status compared to healthy fish, nevertheless the incidence of cataracts could not be directly related to dietary Zn deficiency, but more to rapid growth of the smolts and smoltification process (Waagbø et al., 1996). Our data demonstrated that smoltification and SW transfer induced reduction in Zn status was inversly proportional to dietary Zn supply, thereby linking the incidence and severity of cataract to sub-optimal dietary Zn. Dietary trace elements (Cu, Fe and Mn) with similar properties to Zn were shown to affect cataract development in Atlantic salmon through changes in oxidative metabolism (Waagbø et al., 2003). The quadratic response in cataract development in relation to dietary Zn can be attributed to Zn deficiency or overload induced oxidative stress (Lee, 2018; Maret, 2019). It is suggested that dietary Zn levels not less than 180 mg kg^{-1} diet is required during smoltification and early SW phase to prevent predisposition of Atlantic salmon smolts to higher risk of cataracts during the rapid growth phase at sea.

The proposed dietary Zn levels can improve Atlantic salmon welfare but it would present a major challenge to the environment as less than 10% of the dietary Zn is retained in the body (Antony Jesu Prabhu et al., 2019a; Vera et al., 2020). Approaches to improve availability or utilisation of dietary Zn when fed plant-based diets have largely centred around the use of amino acid chelated organic Zn sources. Amino acids with low molecular weight (e.g. glycine) or with high affinity for Zn (e.g. histidine, cysteine, methionine and lysine) have the potential to increase Zn solubility or uptake *in vitro* (Glover and Hogstrand, 2002; Glover et al., 2003; Prabhu et al., 2018; Silva et al., 2020a, 2020b). However, the *in vivo* response in fish has been inconsistent (Antony Jesu Prabhu et al., 2016). Specifically, with salmonids, organic Zn sources tested were either no different (Gomes and Kaushik, 1992; Maage et al., 2001; Meiler and Kumar, 2021; Silva et al., 2019); or better than $ZnSO_4$ in improving Zn availability or status (Apines et al., 2001, 2003; Rider et al., 2010). Besides identifying an efficient source of Zn supplement,

dietary interactions that can affect Zn availability requires attention. A low or negative dietary electrolyte balance (dEB, −25 to −46 mEq/kg) in the freshwater feeds had a profound effect on the body and tissue Zn status of Atlantic salmon smolts (Philip et al., 2022). In rats, poly-unsaturated fatty acids (PUFA) improved Zn absorption and retention (Knudsen et al., 1990). In present day salmon feeds, combined substitution of FM and fish oil (FO) could be responsible for the decline in Zn status of slaughter size Atlantic salmon from 55 mg kg^{-1} body weight (Aas et al., 2019; Shearer et al., 1994) to 35 mg kg^{-1} body weight (Aas et al., 2019). Early nutritional programming of fish towards low Zn diets can provide insights into mechanisms for effective utilisation of dietary Zn. Better utilisation of dietary Zn would enable reduction in total Zn levels in diet, without compromising fish health and subsequently decrease environmental Zn load. However, without an effective means to improve dietary Zn availability or utilisation, restriction on dietary Zn levels below 180 mg kg^{-1} will compromise the Zn status and welfare of Atlantic salmon, especially in the sea.

Data sharing

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.737804.

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