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Dietary zinc, selenium and water temperature during early seawater phase influences the development of vertebral deformities and cataract in adult Atlantic salmon

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ABSTRACT

The impact of dietary trace minerals (TM; zinc and selenium) and water temperature during early seawater phase on the development of vertebral deformities and cataracts in adult Atlantic salmon was studied. Two experimental feeds (control and High TM) and two water temperatures (12 and 16 °C) were designed in a 2 × 2 factorial arrangement. The Zn and Se levels in the control and high TM diet corresponded to 150 or 200 mg Zn/kg and 0.5 or 0.7 mg Se/kg diet, respectively. Atlantic salmon post-smolts (mean weight, 138 g) were distributed in 12 tanks and were randomly assigned to one of the treatments in triplicate groups. The experimental regime lasted for 12 weeks and thereafter the fish were sampled or transferred to three net pens comprising all four treatments in a common garden. The fish were further grown on a common commercial feed and assessed for vertebral deformities and cataracts as adults (4 to 4.5 kg). Atlantic salmon post-smolt fed the control diet or reared at 16 °C grew more than those fed the high TM diet or reared at 12 °C at the end of the 12-week experimental feeding period. Vertebral mineral density, concentration of Zn and Se in the whole body, plasma and bile were influenced by the dietary treatments. The fast growth rates observed in the control diet and 16 °C exposed fish were associated with increased risk of vertebral deformities and cataract at adult stage. The preventive effect of the high TM diet on incidence and severity of vertebral deformities and cataracts was highly effective if the fish were reared at 12 °C during early seawater phase, but was reduced or absent if reared at 16 °C. Cataract inducing effect of high temperature at 16 °C was much stronger than the cataract mitigating effect of high TM diet at 16 °C. Overall, feeding Atlantic salmon post-smolts a high TM diet (Zn: 200 mg/kg; Se: 0.7 mg/kg diet) during early seawater phase for 12 weeks reduced the incidence of vertebral deformity and cataracts in adult fish, albeit subject to water temperature.

1. Introduction

Zinc (Zn) and selenium (Se) are vital trace minerals required for the growth and health of Atlantic salmon, alike other fish species (Watanabe et al., 1997). Reduction in fish meal and increased use of plant ingredients meant that Zn and Se are the most limiting trace minerals in present day salmonid feeds (Antony Jesu Prabhu et al., 2016). Plant ingredients are naturally low in Zn and Se, and their availability is further reduced by the presence of anti-nutritional factors (Sugiura et al., 1999; Sugiura et al., 1998). High inclusion of plant ingredients in Atlantic salmon feeds had reduced the dietary supply and availability of Zn and Se (Antony Jesu Prabhu et al., 2019; Antony Jesu Prabhu et al., 2018; Vera et al., 2020). Further, the present EU regulation exerts a maximum limit of 0.5 mg Se/kg and 180 mg Zn/kg in the feed, with a recommendation for further reduction to 150 mg Zn/kg diet (EFSA, FEEDAP, 2014; European Commission, 2016; 2013). The optimal dietary levels for Zn (180 mg/kg diet) and Se (0.65 mg/kg diet) in low fish meal feeds were only recently determined in post-smolt Atlantic salmon

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(Antony Jesu Prabhu et al., 2020; Sartipi Yarahmadi et al., 2022). Suboptimal supply of Zn resulted in poor mineral status and cataracts (Sartipi Yarahmadi et al., 2022); while Se deprivation compromised body Se homeostasis, oxidative metabolism and anti-inflammatory responses (Antony Jesu Prabhu et al., 2020). Optimal nutrition at critical life stages such as smoltification and early seawater phase are important to ensure the health and welfare of farmed Atlantic salmon through challenging conditions (Holen et al., 2022; Sissener et al., 2021). Therefore, it is important to understand and take into consideration the susceptibility of critical life stages to sub-optimal trace mineral nutrition and their long-term effects to improve the health of farmed Atlantic salmon.

Skeletal deformities are a major fish health issue in Atlantic salmon farming (Fjelldal et al., 2009; Fjelldal et al., 2012a, 2012b). Vertebral mineral status is pivotal in the etiology of vertebral deformities in Atlantic salmon (Fjelldal et al., 2009). The onset and development of these disorders are influenced by multifactorial elements, namely environmental (photoperiod or temperature); nutritional (vitamins, minerals or amino acids); and genetic (mutations or ploidy) (Fjelldal et al., 2012a, 2012b; Waagbø, 2006). Further, smoltification and early seawater phase are critical life stages of Atlantic salmon more susceptible to environmental and nutritional challenges. Accordingly, induction of mineral deficiency related bone deformities has been reported during smoltification (Fjelldal et al., 2006) and the early seawater phase (Fjelldal et al., 2007; Fjelldal et al., 2009; Grini et al., 2011). Stressful or congenial encounters at critical stages of an animal's life can imprint respective long-lasting impacts in later life stages. In accordance, exposing Atlantic salmon smolts to elevated temperature or sub-optimal dietary minerals predisposed the adults to increased prevalence or severity of vertebral deformities later (Fjelldal et al., 2012a, 2012b; Grini et al., 2011). Earlier studies in Atlantic salmon investigating the role of minerals in vertebral deformities have focused largely on phosphorus (Fjelldal et al., 2016; Fjelldal et al., 2012a, 2012b; Fjelldal et al., 2009; Fraser et al., 2019). Zinc and selenium are vital trace minerals of significance to bone metabolism and their impact on long term bone health in Atlantic salmon requires investigation.

Cataracts are reversible or irreversible damage to the lens resulting respectively from oxidative or osmotic imbalance (Waagbø et al., 2003). Elevated temperature and sub-optimal nutrition are important drivers of cataract development in Atlantic salmon (Bjerkås et al., 2001; Bjerkås and Sveier, 2004; Ersdal et al., 2001; Waagbø et al., 2003). Oxidative damage to lens tissue is considered one of the major causes of cataract development (Bjerkås et al., 2001). Further, as oxidative metabolism during rapid growth periods can induce cataract formation in Atlantic salmon, it has been suggested that dietary antioxidants can have a protective effect (Hamre et al., 2022). Zinc and selenium being respectively part of superoxide dismutase (Cu/Zn SOD) and glutathione peroxidase (Se-GPx) are effective anti-oxidants (Lall, 2022). In humans and in fish, trace minerals like Zn is known to play a role in prevention of cataracts (Ketola, 1979; Srivastava et al., 1992). Deficient or sub-optimal dietary Zn during smoltification resulted in increased cataracts after seawater transfer (Sartipi Yarahmadi et al., 2022). However, the effect of dietary Zn on further development of cataracts in a later life stage remains to be studied. Plasma Se-GPx activity was reduced in Atlantic salmon with cataracts indicating an indirect relation between Se nutrition or metabolism and cataracts (Bjerkås et al., 2001). Therefore, the aim of this study was to investigate how temperature and dietary Zn/Se supply during early seawater phase interact to influence the development of vertebral deformities and cataracts in adult Atlantic salmon.

2. Material and methods

2.1. Ethical statement

The experiment were performed at the Institute of Marine Research (IMR), Matre Research Station (60° N, 5° E, Western Norway), which is authorized for animal experimentation (Norwegian Food Safety Authority, facility 110) in accordance with national and international guidelines, certified using Norwegian research permit number 12827.

2.2. Experimental design

The experiment was designed and executed with a 2 × 2 factorial arrangement with triplicate groups in each factor combination. Dietary trace mineral level (diet) and temperature of water (temperature) were the main factors studied. Each factor was investigated at two levels namely 'control' and 'high'. In the diet factor, the control level corresponded to the dietary levels of EFSA scientific opinion for reduction of total Zn in salmonid feeds i.e. 150 mg/kg (EFSA and FEEDAP, 2014) and existing EU maximum limit for Se i.e. 0.5 mg/kg (European Commission, 2013). The high level corresponded to the Zn (200 mg/kg) and Se (0.7 mg/kg) levels as estimated to be optimal based on our recent findings in Atlantic salmon post-smolts (Antony Jesu Prabhu et al., 2020; Sartipi Yarahmadi et al., 2022). In the temperature factor, the control and high levels corresponded to 12 °C and 16 °C reflecting the optimal and high range of water temperature for Atlantic salmon post-smolts during early seawater phase.

2.3. Experimental diets

Two experimental diets namely 'control' and 'high TM' were formulated from similar basal ingredient mix with low fish meal inclusion. The control diet was formulated to contain 150 mg Zn/kg and 0.5 mg Se/kg diet. The high diet was formulated to contain Zn and Se to contain 200 mg Zn/kg and 0.7 mg Se/kg diet. The supplementation of Zn and Se was achieved through respective inorganic sources zinc sulphate and sodium selenite. Formulation of 'control' and 'high TM' diets along with their analysed proximate and nutrient composition is provided in Table 1.

2.4. Experimental animals, husbandry and sampling

The Atlantic salmon used in this study belonged to the Aqua Gen

Table 1
Feed formulation and nutrient composition of the experimental diets.

	Control	High TM
Ingredient (%)		
Wheat	9.70	9.70
Corn gluten	20.0	20.0
Wheat gluten	20.0	20.0
Soy Protein Concentrate	20.0	20.0
Fababean dehulled	1.00	1.00
Fishmeal North-Atlantic	2.50	2.50
Rapeseed oil	12.1	12.1
Fishoil North-Atlantic	9.9	9.9
Premixes/amino acids	4.60	4.60
Yttrium premix	0.10	0.10
Zn-Se premix (control)*	0.10	–
Zn-Se premix (high)**	–	0.01
Analysed composition		
Dry matter, %	93.4	93.5
Crude protein, %	46.6	46.3
Crude lipid, %	25.2	24.6
Ash, %	3.3	3.3
Phosphorus, %	0.92	0.91
Zinc, mg/kg	149	196
Selenium, mg/kg	0.49	0.71

* Zn—Se (control), g/kg premix: Zinc sulphate, 400 g; Sodium selenite, 6.76 g; α-cellulose, 593.24 g.

** Zn—Se (high), g/kg premix: Zinc sulphate, 500 g; Sodium selenite, 13.42 g; α-cellulose, 486.58 g.

Atlantic QTL-innOva IPN/PD/CMS strain (AquaGen AS, Trondheim, Norway) and were purchased as eyed eggs (375° days) from a commercial supplier (Aqua Gen AS Hemne, Norway). The fish reared under standard condition for production of yearling smolts (Fjelldal et al., 2006), were PIT-tagged (Glass tag 2, 12 mm, TrackID AS, Stavanger, Norway) on 15 February 2018 and were transferred to 9 °C seawater on 08 May 2018. On 04 June 2018, a total of 540 Atlantic salmon smolts (138 ± 8 g; 23 ± 0.1 cm) had their pit tags registered and weights and lengths recorded before they were randomly allocated between 12 square white covered fiberglass tanks (1 × 1 × 0.43 m) at the environmental research lab, Matre research station, Institute of Marine Research, with 45 fish per tank. The tanks were supplied with 9 °C seawater with a salinity of 34 ppt. In the period 04 to 07 June, water temperature was adjusted to 12 °C in 6 tanks and 16 °C in 6 tanks. Within each temperature, the fish in triplicate tanks were fed one of the two experimental diets for twelve weeks. Feeding was performed to satiation through automated feeders twice a day between 08:00–09:30 AM and 12:30–14:00 PM. Excess feed was not collected and hence feed efficiency estimates were not calculated. The fish were kept on simulated natural photoperiod during the experimental feeding period of 12 weeks. The water flow was adjusted so that the oxygen saturation of the outlet water was always above 80%.

At the end of the 12-week feeding period, on 20th August 2018, a sampling was performed wherein 12 fish were randomly sampled from each tank. The sampled fish were anesthetized in tricaine methanesulfonate (Finquel, MS-222, 0.1 g/l) before reading of the pit tag. The sampled fish were scored for cataract after registration of length and weight. After scored for cataract, six fish meant for whole body composition analysis were pooled and homogenized in a blender. The other six fish were sampled for blood using heparinized syringe and plasma recovered after centrifugation at 3000*g* for 5 min. After sampled for blood, the fish was dissected to collect liver, bile and vertebrae samples. The remaining fish (weight, 227 to 260 g, depending on the group, Table 2) were transferred to a net pen (12 × 12 m) in a common garden. The fish were fed a common commercial feed during this period (Skretting Norway). During this period in the net pen, on 5th Dec 2018 a mid-term sampling for length and weight were performed on all the fish. The final sampling and termination of the trial was performed on 4th June 2019. All the fish were euthanized by an overdose of anesthetics (Finquel, MS-222, 0.5 g/l), killed by a sharp blow to the head, had their pit-tag recorded, and length and weight measured. Thereafter, the fish were scored for cataract and x-rayed for radiographic analysis of vertebral deformities as described previously.

2.5. Analytical methods

The proximate composition (dry matter, crude protein, crude lipid, and ash) of experimental feeds and homogenized whole fish samples were analysed by the following standard methods. In brief, dry matter was measured after oven drying to constant weight at 105 °C for 24 h; ash content determined by combustion in a muffle furnace at 550 °C for 18 h (NMKL, 1991). Crude protein was estimated by kjeldahl method (6.25 x N), total lipid in fish tissue and feeds respectively by Soxhlet method following ethyl-acetate and acid-extraction extraction (NS 9402, 1994). The concentration of Zn and Se, in feeds, fish and tissues were analysed through microwave assisted digestion and inductively coupled plasma mass spectrometry (ICP-MS) as described elsewhere (Silva et al., 2019). Vertebrae mineral density (VMD) was estimated as the percentage of ash in the dry weight of the vertebrae. Vertebrae nos. 40 to 43 from each fish were pooled and defatted in a 2:1 volume mix of

Table 2
Growth performance of Atlantic salmon during the different phases and overall in the study.

	12 °C		16 °C		p-value			Effect	
	Control	High TM	Control	High TM	Diet	Temp	Diet*Temp	Diet	Temperature
Phase I (Experimental feeding, tanks, May-Aug 18′)									
FBL, cm	28.7 ± 0.7	27.5 ± 0.2	29.1 ± 0.2	28.6 ± 1.0	0.04	0.09	0.4	C > H	
FBW, g	262 ± 22	227 ± 7	278 ± 10	261 ± 31	0.05	0.05	0.4		
ΔBW, g	121 ± 18	89 ± 5	139 ± 14	127 ± 17	0.03	0.01	0.3	C > H	12 < 16
ΔBL, cm	5.6 ± 0.5	4.5 ± 0.01	5.9 ± 0.3	5.7 ± 0.2	0.01	0.003	0.05	C > H	12 < 16
SGR	0.75 ± 0.07	0.61 ± 0.03	0.84 ± 0.08	0.8 ± 0.03	0.03	0.002	0.2	C > H	12 < 16
CF	1.09 ± 0.02	1.08 ± 0.01	1.12 ± 0.03	1.1 ± 0.02	0.3	0.07	0.8		12 < 16
Phase II (Commercial feed, net pens, Aug-Dec 18′)									
FBL, cm	46.6 ± 0.4	45.1 ± 0.2	46.7 ± 0.6	46.2 ± 1	0.02	0.1	0.2	C > H	
FBW, g	1409 ± 55	1269 ± 35	1470 ± 55	1408 ± 98	0.03	0.03	0.3	C > H	12 < 16
ΔBW, g	1148 ± 38	1042 ± 42	1191 ± 47	1147 ± 67	0.03	0.03	0.3		
ΔBL, cm	17.8 ± 0.3	17.5 ± 0.4	17.7 ± 0.4	17.6 ± 0.2	0.8	0.6	0.5		
SGR	1.58 ± 0.05	1.6 ± 0.06	1.55 ± 0.03	1.57 ± 0.06	0.4	0.3	0.9		
CF	1.39 ± 0.02	1.36 ± 0.03	1.42 ± 0.01	1.41 ± 0.03	0.2	0.01	0.7		12 < 16
Phase III (Commercial feeds, net pens, Dec 18′ – June 19′)									
FBL, cm	69.2 ± 0.4^{b}	66.7 ± 0.3^{a}	68.4 ± 0.1^{b}	68.6 ± 1.6^{b}	0.04	0.3	0.02		
FBW, g	4632 ± 131	4092 ± 195	4500 ± 210	4591 ± 382	0.2	0.2	0.06		
ΔBW, g	3323 ± 97	2823 ± 175	3030 ± 220	3183 ± 288	0.3	0.5	0.05		
ΔBL, cm	22.6 ± 0.1^{b}	21.5 ± 0.2^{a}	21.6 ± 0.7^{a}	22.5 ± 0.7^{b}	0.6	0.8	0.01		
SGR	0.65 ± 0.02	0.64 ± 0.03	0.61 ± 0.03	0.65 ± 0.01	0.3	0.4	0.08		
CF	1.38 ± 0.02	1.36 ± 0.08	1.38 ± 0.04	1.4 ± 0.02	0.9	0.5	0.5		
Overall (May 18′ – June 19′)									
ΔBL, cm	46.0 ± 0.14^{b}	43.6 ± 0.47^{a}	45.2 ± 0.5^{b}	45.8 ± 0.82^{b}	0.02	0.06	0.001		
ΔBW, g	4492 ± 127^{b}	3954 ± 195^{a}	4360 ± 218^{b}	4457 ± 368^{b}	0.2	0.2	0.04		
SGR	0.19 ± 0.00	0.19 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.6	0.1	0.6		

Data presented as mean ± SD (n = 3). FBL, final body length; FBW, final body weight; ΔBW, body weight increment; ΔBL, body length increment; SGR, specific growth rate; CF, condition factor. Statistical analysis primarily performed was 2-way ANOVA. The p-values and the nature of the main effects are provided along the same row. In case of significant interaction, 1-way ANOVA was performed followed by tukey's multiple comparison test. Treatment groups with different superscripts along the same row are significantly different. All analyses were performed at 5% significance level and the exact *p*-values are reported wherever possible. The length, weight and CF at the start of the experiment was respectively 23 ± 0.1 cm, 138 ± 8 g and 1.1 ± 0.1.

acetone and chloroform for 24 h and dried overnight at 100C. The dry weight of each pooled sample was measured to the nearest 0.01 mg. The vertebrae were then incinerated for 10.5 h in a muffle furnace (Mod. L 40/11/P320; Nabertherm GmbH) (115C for 0.5 h, 540C for 4 h and 750C for 6 h). The ash was further diluted in 5% nitric acid and analysed for Zn and Se in ICP-MS as previously described. The x-ray radiographic analysis of the vertebrae were performed using a Direct Radiology System (Canon CXDI-410C Wireless, CANON INC., Kawasaki, Japan) with a portable x-ray unit (Portable X-ray Unit Hiray Plus, Model Porta 100 HF, JOB Corporation, Yokohama, Japan) at 88 cm distance with 40 kV and 10 mAs. Each fish was evaluated for vertebra deformities as per the classification described elsewhere (Witten et al., 2009), and type and location were recorded. The cataracts were scored with the help of a hand-held slit lamp microscope (HEINE® HSL 150, Optotechnik). Opthalmic examination on both eyes of the fish was performed under darkened conditions (Remø et al., 2017) 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., 2001; Bjerkås et al., 1996).

2.6. Data analysis and statistics

Tank was used as the experimental unit in the statistical analysis of the data ($n = 3$). In all cases, unless otherwise specified, tank means were calculated from individual fish data before subjecting to statistical evaluation. The tank mean data was then subjected to test of normality, and in cases where the data were non-normally distributed, rank transformation was performed. Statistical analysis for difference between groups was performed through 2-way ANOVA for diet, temperature and diet x temperature interaction effects. The level of significance was set at 5% ($p < 0.05$). Whenever, the interaction effect was significant, one-way ANOVA followed by Tukey's multiple comparison analysis was performed. In addition to ANOVA, relative risk ratio (RR) analysis was performed for data on the incidence of vertebral deformities and cataracts at the end of the study. The relative risk ratio (RR) is defined as the risk of an event (deformity or cataract, in this case) occurring in an experimental group relative to that in a control group. An RR of 1.00 indicates that the risk is comparable in the two groups. A value >1.00 indicates increased risk; a value lower than 1.00 indicates decreased risk. Further, the 95% confidence limits (CL) of the RR means were used to assess the statistical significance (Knol et al., 2011). The statistical analysis of the data and graphical representation of the results were performed respectively in Statistica and GraphPad Prism (version 8).

3. Results

3.1. Growth performance

The growth performance of Atlantic salmon throughout the different phases of this study is presented in Table 2. During the experimental feeding period of the study (Phase I), increment in weight, length and SGR were significantly higher in fish at 16 °C and in the control diet fed group ($p < 0.05$), and a tendency for higher CF in fish at 16 °C ($p = 0.07$). Final length was higher in control diet group ($p = 0.04$), while a weak tendency for higher final length was observed in 16 °C ($p = 0.09$). Both diet and temperature had a strong tendency for an effect on final body weight ($p = 0.05$) being higher in control diet or 16 °C group. Further, a strong tendency for an interaction effect was observed in length increment with 12 °C-High TM diet group exhibiting lowest length increment ($p = 0.05$). In the subsequent phase in the sea from Aug to Dec 2018 (Phase II), the effect of diet and temperature observed in Phase I persisted on weight gain (Control > high TM; 12 °C < 16 °C; $p = 0.03$), but was no more significant for length increment ($p > 0.05$). However, the effect of diet on increasing the final length in fish fed control diet further increased ($p = 0.02$). The impact of diet or temperature on final weight (Control > high TM; 12 °C < 16 °C), and that of temperature on CF (12 °C < 16 °C), increased from a tendency in Phase I to a significant effect at the end of phase II ($p < 0.05$). There were no interaction effects that were statistically significant or even had a tendency in phase II. On the contrary, in phase III between Dec 2018 and June 2019, none of the main effects of diet or temperature observed in the previous growth phases were significant anymore in phase III, except for control diet fed fish being lengthier than high TM diet fed fish ($p = 0.04$). All the growth indices measured except CF had either a tendency (final body weight, weight gain and SGR) or an effect (final length and length increment) for diet x temperature interaction. In all these interactions, it was the 12 °C-high diet group that was statistically different from the rest. Overall for the entire study period, length increment was significantly influenced by the interaction between diet and temperature, with 12 °C-high TM diet group having the least length increment and a strong tendency for least weight gain ($p = 0.05$). No effect was observed on the overall SGR.

3.2. Zinc and selenium status

The concentrations of Zn and Se in the whole body, plasma, bile and vertebrae at the end of the 12-week feeding period are presented in Table 3. Diet, as a factor did not affect body, plasma or bile Zn levels, but significantly influenced vertebrae Zn concentration (control < high TM, $p = 0.007$). Temperature as a factor significantly influenced plasma and bile Zn concentrations (12 °C < 16 °C; $p < 0.01$), but no effect on whole body and vertebrae Zn concentrations. Regarding Se, whole body, plasma and bile Se concentrations were significantly influenced by both diet (control < high TM, $p < 0.001$) and temperature (12 °C < 16 °C; $p < 0.001$), while a tendency for temperature effect was observed in vertebrae Se (12 °C < 16 °C; $p = 0.07$).

3.3. Bone mineralization and vertebral deformities

The vertebrae mineral density (VMD) in Atlantic salmon at the end of the 12-week experimental feeding period was influenced by temperature (12 °C < 16 °C; $p < 0.05$), but not by diet (Table 3). The total number of adult fish with one or more deformed vertebrae and total number of deformed vertebrae were influenced by dietary and temperature history during early seawater phase (Fig. 1). The least prevalence of deformities, both in terms of incidence and abundance of deformed vertebrae was recorded in fish with high TM diet-12 °C history, while the contrary was observed in fish with the history of control diet at 16 °C (Fig. 1). The risk of developing vertebral deformities as a factor of dietary TM and temperature exposure during early seawater phase was assessed by relative risk ratio analysis (Fig. 2). The risk of deformity in adult salmon exposed to 12 °C during early seawater phase was half as much compared to those expose to 16 °C (12.1% vs. 24.5%; RR ratio, 0.43; $p < 0.05$). Similarly, the risk of developing deformity was significantly higher with an RR ratio of 1.76 in fish fed the control diet compared to the high TM diet during early seawater phase. The effect of high TM was however dependent on the temperature. The effect of high TM diet on reducing the risk of deformities was more effective at 12 °C than at 16 °C as indicated by the RR ratio of 1.33 vs. 1.05 (Fig. 2). Overall, the effect of temperature during early seawater phase was much stronger and with less variation than the effect of dietary TM on inducing vertebral deformities in adult Atlantic salmon. Regarding the classification on the type of deformities (Table 4), no major differences were observed except for a diet effect in the occurrence of homogenous compressions 'Type 2'. The homogenous compressions were observed only in the fish fed the high TM diet and was not present in the control diet fed groups (Control, 0% < high TM, 16.8%; $p < 0.05$).

3.4. Cataract

Atlantic salmon were scored for cataracts as post-smolts after the end of the experimental feeding and as adults at the end of the study (Fig. 3).

Table 3
Vertebrae mineral density and concentration of Zn and Se in the vertebrae, whole body, plasma and bile of Atlantic salmon post-smolt after the 12 week feeding period.

		Vertebrae			Whole body		Plasma		Bile	
		VMD (%)	Zn[1]	Se[2]	Zn[1]	Se[1]	Zn[1]	Se[1]	Zn[1]	Se[1]
12 °C	Control	52.1 ± 0.7	158 ± 17	12 ± 8	27.5 ± 1.1	0.18 ± 0.003	175 ± 51	1.31 ± 0.3	3.3 ± 1.9[a]	0.66 ± 0.1
	High TM	51.7 ± 0.8	167 ± 16	15 ± 8	27.9 ± 1.6	0.20 ± 0.005	175 ± 64	1.60 ± 0.4	2.8 ± 1.6[a]	0.91 ± 0.2
16 °C	Control	52.5 ± 0.5	156 ± 19	17 ± 9	28.4 ± 1.9	0.16 ± 0.003	197 ± 46	1.46 ± 0.3	3.6 ± 1.9[a]	0.85 ± 0.2
	High TM	52.2 ± 0.8	180 ± 21	18 ± 7	29.4 ± 1.3	0.19 ± 0.006	237 ± 48	1.99 ± 0.2	5.2 ± 2.8[b]	1.15 ± 0.2
p-value										
Diet		0.19	0.007	0.36	0.43	0.001	0.22	< 0.001	0.33	< 0.001
Temp.		0.02	0.33	0.07	0.19	0.001	0.001	< 0.001	0.01	< 0.001
Diet*Temp		0.77	0.16	0.37	0.74	0.89	0.17	0.19	0.04	0.61
Effect										
Diet			C < H			C < H		C < H		C < H
Temp.		12 < 16				12 > 16	12 < 16	12 < 16		12 < 16

Data expressed as mean ± SD (n = 3); [1] expressed in mg/kg; [2] expressed in μg/kg. VMD, vertebrae mineral density; Temp., temperature. Statistical analysis primarily performed was 2-way ANOVA. The p-values and the nature of the main effects are provided along the same row. In case of significant interaction, 1-way ANOVA was performed followed by tukey's multiple comparison test. Treatment groups with different superscripts along the same row are significantly different. All analyses were performed at 5% significance level and the exact p-values are reported wherever possible.

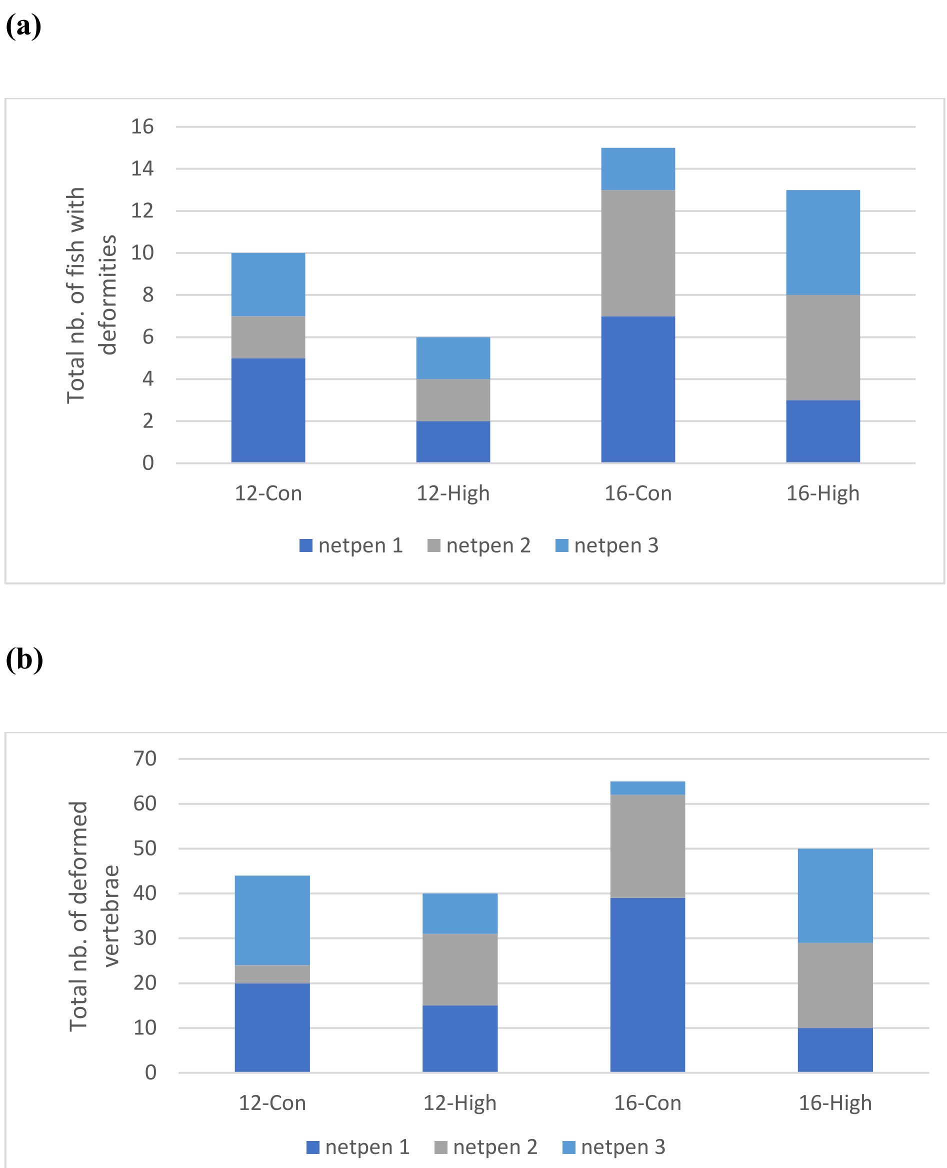

Fig. 1. Cumulative data on the prevalence of vertebral deformities in adult fish at the end of the study.
Legend: Impact of temperature and dietary trace minerals during early seawater phase on the (a) number of fish with deformities and (b) number of deformed vertebrae in each group. 12 and 16, water temperature in °C. Con and High, control and high TM diet.

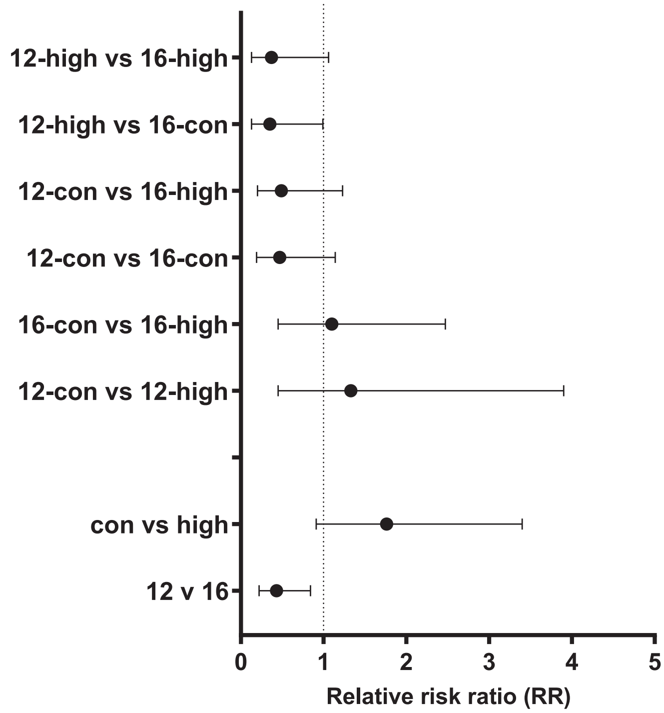

Fig. 2. Relative risk ratio for the impact of temperature and dietary trace minerals during early seawater phase on the incidence of vertebral deformities in adult fish.
Legend: The relative risk ratio (RR) of vertebral deformities in adult Atlantic salmon as a factor of their dietary and temperature history during early seawater phase is presented as a forest plot of different paired comparisons. In the X-axis is the RR ratio and in the Y-axis is the different paired comparisons. 12 and 16, water temperature in °C. Con and High, control and high TM diet. Data are presented as mean and 95% confidence intervals of the RR ratio. In all pairs, the RR ratio was computed considering the former as the control and the latter as experimental group (eg. 12 vs. 16, wherein 12 is the control and 16 is the experimental group). An RR ratio of 1 indicates that the risk is comparable in the two groups (dotted line). A value >1.00 indicates increased risk; a value lower than 1.00 indicates decreased risk in the control compared to the experimental group. The risk of vertebral deformity is (i) lower in post-smolts exposed to 12 °C compared to 16 °C (RR, 0.43) and (ii) higher in fish fed the control diet compared to the high TM diet (RR, 1.76).

At the end of the 12-week experimental feeding period in Aug 2018, all the fish investigated for cataract had a score of 1 or more, thereby no effect was found on the prevalence. In terms of severity, temperature had the major impact (12 °C < 16 °C; $p < 0.001$), with a strong tendency for interaction with the diet (Fig. 3A; $p = 0.06$). The mean cataract scores for the control and high diet groups were respectively 4.2 and 4.1; whereas the same for the temperature groups were 3.1 vs. 5.2 (12 °C vs. 16 °C). The strong tendency for an interaction appeared due to the difference in severity of the scores between 12 °C and 16 °C being higher in the control than the high TM group (2.6 vs. 1.6). In June 2019, at the end of the study, mean cataract scores were significantly influenced by the diet (control > high; $p = 0.03$) and temperature (12 < 16; $p = 0.004$) history during early seawater phase (Fig. 3B). Further, the increase in the mean cataract scores from Aug 18′ to June 19′ (Δ cataract score, Fig. 3C) was significantly higher in fish with 12 °C temperature history compared to 16 °C (1 vs −0.2; $p < 0.01$). Within the 12 °C group, significantly higher increase was found in the control diet fed fish compared to the high TM fed fish (1.7 vs. 0.5; interaction effect, $p < 0.05$). The risk of developing severe cataracts (score > 5) was assessed through relative risk ratio analysis (Fig. 4). Post-smolts exposed to 12 °C during early seawater phase had a lower risk of developing advanced stage cataracts as adults (RR, 0.39). Fish fed the control diet during early seawater phase had a higher risk of developing severe cataracts compared to those fed high TM diet (RR, 1.69). Further, the dietary effect was dependent on the temperature; the effect of high TM diet in reducing the severity of cataract in adult salmon was relatively more effective in fish with a temperature history of 12 °C compared to 16 °C (RR, 3.02 v 1.12).

4. Discussion

Prevalence of vertebral deformities and cataract in adult Atlantic salmon were influenced by elevated water temperature and dietary supply of Zn and Se during early seawater phase. Faster growth rates (Hamre et al., 2022; Hansen et al., 2010), increased temperature (Bjerkås et al., 2001; Fraser et al., 2019; Grini et al., 2011) and mineral deficiency (Fjelldal et al., 2016; Fjelldal et al., 2009; Sartipi Yarahmadi et al., 2022; Waagbø et al., 2003) among other factors induce one or more type of vertebral deformities and cataracts in Atlantic salmon. Among all the groups, slowest growth rate during the experimental feeding period, lowest final body length and weight was observed in the 12 °C, high TM fed fish (Table 2). Although not certainly clear, the possibility of higher supplementation levels of either Zn or Se being a reason for reduced growth in high TM groups cannot be overruled. High inclusion of inorganic Zn can reduce the uptake of other trace divalent metals like Mn or Cu. Indeed, the Mn status in the High TM fish especially at 12 °C were found to be the lowest. In addition, increased supplementation of Se as sodium selenite could also have negative effects on growth, although not observed in previous studies at similar levels (Antony Jesu Prabhu et al., 2020; Berntssen et al., 2018). Consequently, this group had the lowest incidence and severity of deformities and cataracts at adult stage. Elevated water temperature is known to induce early signs of vertebral pathologies and cataracts in Atlantic salmon (Bjerkås et al., 2001; Fraser et al., 2019; Grini et al., 2011). In this study, the proportion of fish with one or more deformed vertebrae and proportion of fish with advanced cataracts was twice as much in the 16 °C group compared to 12 °C. These results reaffirmed the potent effect of elevated water temperature on the development of vertebral deformities and cataracts in Atlantic salmon.

Increased prevalence of vertebral deformities was associated with low bone mineral density at an earlier life stage, especially in studies

Table 4
Effect of experimental feeding and temperature regimens during early seawater phase on the type of vertebral deformities in adult Atlantic salmon.

	12 °C		16 °C		Diet	Temp	D*T	Effect
Type of deformities	Control	High TM	Control	High TM				
Type 1: decreased intervertebral space	30 ± 28	7 ± 12	17 ± 15	28 ± 27	0.61	0.75	0.21	
Type 2: homogeneous compression	0 ± 0	27 ± 25	0 ± 0	6 ± 3	0.04	0.19	0.19	C < H
Type 5: one-sided compression	32 ± 24	16 ± 9	23 ± 13	28 ± 13	0.56	0.84	0.29	
Type 6: compression and fusion	23 ± 25	16 ± 9	37 ± 27	17 ± 6	0.50	0.87	0.36	
Type 7: complete fusion	0 ± 0	4 ± 7	0 ± 0	0 ± 0	0.34	0.34	0.34	
Type 8: fusion centre	7 ± 0 11	8 ± 14	22 ± 19	18 ± 16	0.91	0.21	0.76	
Type 13: hyper-radiodense with flat end plates	2 ± 3	0 ± 0	0 ± 0	0 ± 0	0.79	0.82	0.34	
Type 19: internal dorsal or ventral shift	7 ± 12	11 ± 19	1 ± 1	3 ± 5	0.62	0.88	0.33	

Data presented as mean percentage in each treatment group (mean ± SD, n = 3). The different type of deformities is classified based on Witten et al. (2009).

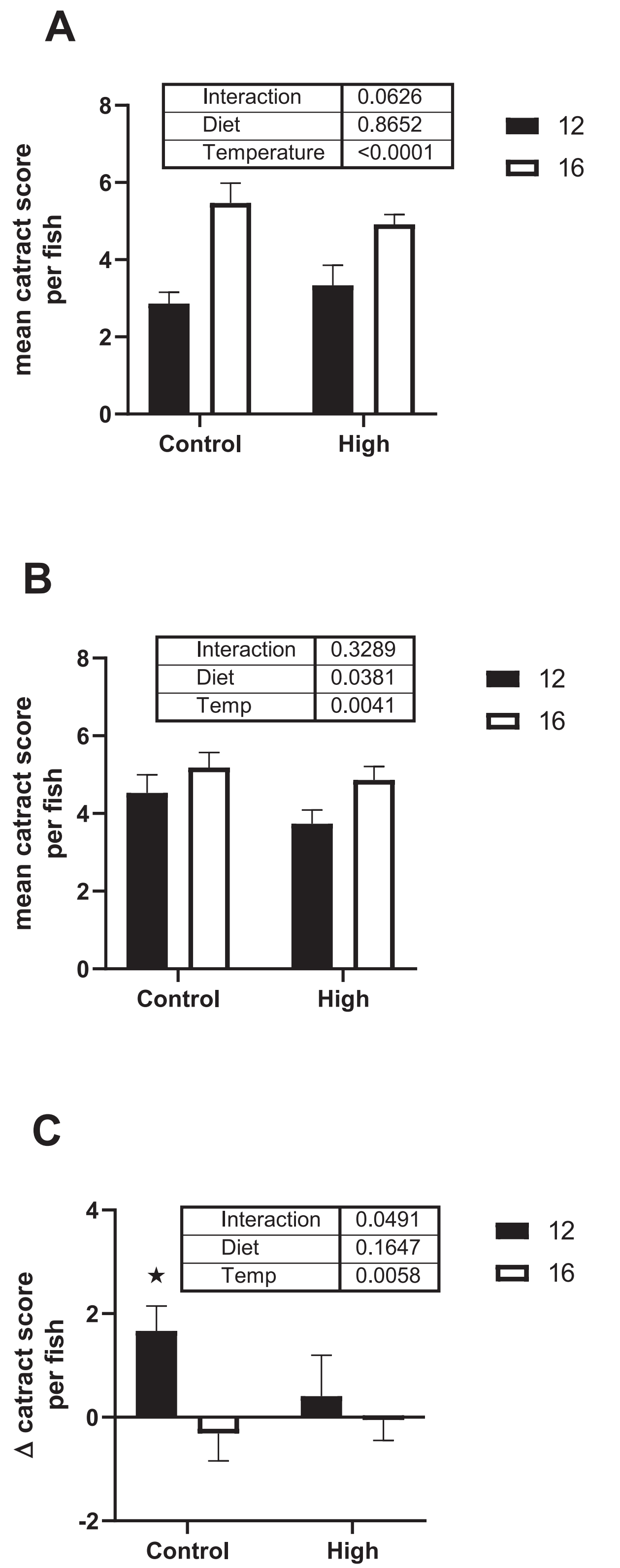

Fig. 3. Short- and long-term effects of temperature and dietary trace minerals during early seawater phase on the development of cataracts in adult fish.
Legend: Mean cataract scores in post-smolt (A), adult (B) and the difference between the two stages (C) are presented. Cataract scores are presented as mean ± SD as a 2 × 2 factorial dataset, grouped based on dietary factor (control or high TM) and temperature (12 or 16 °C, dark or white bars). The table inset within each graph provides the p-values of the main effects and their interaction following 2-way ANOVA.

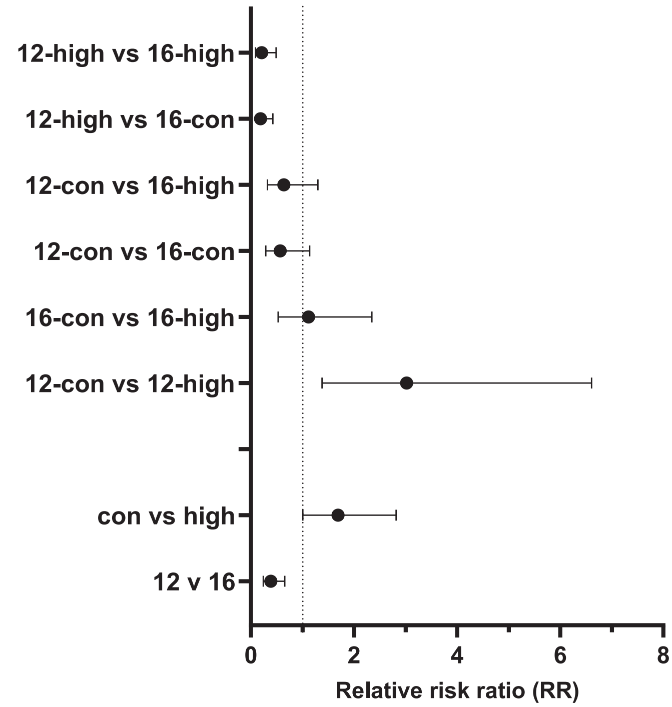

Fig. 4. Relative risk ratio for the impact of temperature and dietary trace minerals during early seawater phase on the incidence of severe cataracts (score > 5) in adult fish.
Legend: The relative risk ratio (RR) of severe cataracts (score > 5) in adult Atlantic salmon as a factor of their dietary and temperature history during early seawater phase is presented as a forest plot of different paired comparisons. In the X-axis is the RR ratio and in the Y-axis is the different paired comparisons. 12 and 16, water temperature in °C. Con and High, control and high TM diet. Data are presented as mean and 95% confidence intervals of the RR ratio. In all pairs, the RR ratio was computed considering the former as the control and the latter as experimental group (eg. 12 vs. 16 °C, wherein 12 °C is the control and 16 °C is the experimental group). An RR ratio of 1 indicates that the risk is comparable in the two groups (dotted line). A value >1.00 indicates increased risk; a value lower than 1.00 indicates decreased risk in the control compared to the experimental group. The risk of severe cataract is (i) lower in post-smolts exposed to 12 °C compared to 16 °C (RR, 0.39) and (ii) higher in fish fed the control diet compared to the high TM diet (RR, 1.69).

that investigated the role of dietary phosphorus (Fjelldal et al., 2009). In contrast, the increased prevalence of deformed fish in the 16 °C group was associated with a marginal, yet statistically higher vertebral mineral density compared to the 12 °C fish. It is unclear if elevated temperature induced conformational changes to the bone matrix might result in increased vertebral mineral density yet higher deformities. Phosphorus is a core structural component of the bone matrix and its depletion led to low mineral density, pre-disposing the fish to onset of deformities at a later stage (Fjelldal et al., 2012a, 2012b; Fraser et al., 2019). Apart from phosphorus, dietary supply of Zn and Se can be important for the prevention of bone deformities in Atlantic salmon (Baeverfjord et al., 2019). Zinc is the most abundant trace mineral in fish vertebrae (Antony Jesu Prabhu et al., 2016) and is involved in bone formation, development and remodeling (Baeverfjord et al., 2019). In the present study, a relative risk ratio of 1.76 for control vs. high TM diet (Fig. 2) showed that sub-optimal trace mineral supply during early seawater phase can increase the risk of developing deformities in adult Atlantic salmon. Zinc and selenium supplementation during early seawater stage reduced the overall risk of vertebral deformities in adult Atlantic salmon, only at 12 °C but not at 16 °C. Elevated water temperature as a factor had a much stronger effect than dietary TM in inducing deformities. Further, the wider confidence intervals in the relative risk ratio for the dietary effects suggested of other underlying confounding factors like genotype, sex etc., potentially interfering with the dietary effect. Genetic variation in vertebral deformities and mineral concentration is indeed reported in Atlantic salmon and some of these traits are considered heritable (Gjerde, 2007; Kvellestad et al., 2000; McKay and Gjerde, 1986; Thodesen et al., 2001; Vagsholm and Djupvik, 1998). Therefore, nutrient x genotype interaction studies could further advance the understanding on the role of minerals in vertebral deformities.

Compression and fusion-related deformities (Type 1 and Type 2) are commonly related to platyspondyly (Witten et al., 2009) and are more prevalent in farmed Atlantic salmon (Fjelldal et al., 2012a, 2012b). Platyspondyly related Type 2 homogenous compressions are characterized by homogeneously two-sided compressed vertebrae with shortening of the spine and inward bending of the edges of the vertebral end plates (Witten et al., 2009). Our data suggested that the Type 2 platyspondyly related homogenous compressions in Atlantic salmon can be linked to dietary trace minerals. In the present study, Type 2 homogenous compressions appeared only in Atlantic salmon that were fed high TM diet during early seawater phase (Table 4). In humans, platyspondyly is associated with a homozygous mutation that encodes for a membrane-bound zinc transport protein (SLC39A13, ZIP13), confirming the link between Zn or trace mineral metabolism and platyspondyly (Fukada et al., 2008; Giunta et al., 2008). In humans and other vertebrae models, it is suggested that negative interactions or competitive inhibition of other trace minerals eg. iron by zinc can induce platyspondyly related bone deformities (Giunta et al., 2008). Competitive Zn^{2+}-Fe^{2+} interaction are known to occur in fish and a tendency for which was observed in the vertebral Fe content (Control > high TM, $p = 0.08$; data not presented). High TM diet induced homogenous compressions could therefore be a consequence of this interaction. In this regard, organic trace mineral sources can reduce the negative effects on the uptake and utilization of other trace minerals (Kousoulaki et al., 2021; Silva et al., 2019).

Vertebral deformities are associated with oxidative stress and inflammation (Kupsco and Schlenk, 2016). The presence of inflammatory cells have been described in deformed Atlantic salmon vertebrae (Kvellestad et al., 2000). Inflammation is a potential risk for the onset and development of vertebral deformities in Atlantic salmon (Gil-Martens et al., 2010; Gil-Martens, 2010). Increasing plant ingredients in Atlantic salmon feeds can increase the risk of vertebral deformities (Fjelldal et al., 2010) and decreased dietary Se and Zn supply could be one among the several causative factors. Vertebral concentration of Zn and Se increased linearly with increasing dietary supply in Atlantic salmon post-smolts (Antony Jesu Prabhu et al., 2019). The antioxidant and anti-inflammatory properties of selenium can enhance bone health (Baeverfjord et al., 2019). Selenium deposited as selenoproteins or selenium containing proteins in the tissues provide improved antioxidant and anti-inflammatory responses during stressful conditions (Antony Jesu Prabhu et al., 2020; Khan et al., 2017; Rider et al., 2009). Atlantic salmon post-smolts fed dietary Se at the levels used in the high TM diet had higher tissue Se levels, favorable redox potential and were able to mount anti-inflammatory defenses in response to pro-inflammatory challenges (Antony Jesu Prabhu et al., 2020). Similarly, increased plasma, bile and whole body Se levels in fish fed the high TM diet in this study were associated with lower incidence of deformities.

Zinc deficient cataracts are prevalent in salmonids (Ketola, 1979) and sub-optimal levels (<180 mg Zn/kg) during freshwater increased the incidence and severity of cataracts in Atlantic salmon post-smolts after seawater transfer (Sartipi Yarahmadi et al., 2022). The role of Zn in cataract development was further emphasized by the disturbances in expression of genes regulating trace mineral or more specifically Zn metabolism (MT-B, MT-A and Cu/Zn SOD) in the lenses (Tröße et al., 2009). The influence of increased water temperature in the onset and development of cataracts is well documented in Atlantic salmon (Bjerkås et al., 2001). In accordance, the stronger effect of temperature overshadowed the dietary TM effect immediately after the experimental period, as reported earlier for other nutrients (Sambraus et al., 2017; Sissener et al., 2021). However, the interaction effects found at later stages indicated that dietary TM were able to reduce the severity of the cataracts, albeit subject to the temperature effect. Dietary pro-oxidants and oxidative metabolism during fast growth at increased temperature and daylength are potent drivers of cataract development in Atlantic salmon (Hamre et al., 2022; Waagbø et al., 2003). On the contrary, antioxidants like vitamin E and astaxanthin were able to reduce cataract development (Waagbø et al., 2003). Glutathione metabolism is central to the redox metabolism (Hamre et al., 2010) and selenium dependent glutathione peroxidase (Se-GPx) plays an important role in oxidative metabolism (Fontagne-Dicharry et al., 2020). Oxidative damage to lens tissue is considered one of the major causes of cataract development and was associated with lower plasma Se-GPx activity (Bjerkås et al., 2001) and altered expression of SeP, GPX, GST and TRX in the lenses (Tröße et al., 2009). Plasma and hepatic GPx activities are good markers of dietary Se supply and are responsive to dietary Se restriction or supplementation (Antony Jesu Prabhu et al., 2016; Fontagne-Dicharry et al., 2020). Despite the observation of reduced cataracts in high TM group at 12 °C, and compelling links with oxidative metabolism cataract development and selenium, direct evidence is still lacking. In this study, concerning the dietary effect, the part played by Zn and Se cannot be separated. Nevertheless, the role of dietary selenium as an antioxidant in reducing the development of cataract cannot be neglected and warrants further investigation.

To conclude, feeding Atlantic salmon post-smolts a high TM diet (Zn: 200 mg/kg; Se: 0.7 mg/kg diet) during early seawater phase for 12 weeks at 12 °C reduced the incidence of deformity and cataracts in adult fish, but the effect was less evident or absent if reared at 16 °C. The Zn and Se levels in the control diet increased the risk of vertebral deformities and cataracts in adult Atlantic salmon. The control dietary levels corresponded respectively to EFSA proposed further reduction for Zn (150 mg Zn/kg diet) and present maximum limit for Se (0.5 mg Se/kg diet) in complete feeds for salmonids in the EU. Our previous findings showed that the above limits can negatively affect the health of Atlantic salmon post-smolts and is now further emphasized for increasing the risk and severity of vertebral deformities and cataracts in adults, but subject to water temperature.

CRediT authorship contribution statement

Antony Jesu Prabhu Philip: Conceptualization, Methodology, Formal analysis, Validation, Investigation, Resources, Visualization, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Per Gunnar Fjelldal:** Conceptualization, Methodology, Formal analysis, Validation, Investigation, Writing – original draft, Writing – review & editing. **Sofie Remø:** Methodology, Investigation, Writing – review & editing. **Marta Silva:** Formal analysis, Investigation, Writing – review & editing. **May-Helen Holme:** Methodology, Resources, Writing – review & editing. **Erik-Jan Lock:** Methodology, Writing – review & editing, Project administration. **Rune Waagbo:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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