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Changes in daylength and temperature from April until August for Atlantic salmon (*Salmo salar*) reared in sea cages, increase growth, and may cause consumption of antioxidants, onset of cataracts and increased oxidation of fillet astaxanthin

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ABSTRACT

The hypothesis of the present study was that increased growth in spring, stimulated by increasing temperature and daylength, leads to oxidative stress in Atlantic salmon with accumulation of oxidation products in the tissues and increased utilization of antioxidants. The drop in fillet pigmentation and astaxanthin, often observed in spring by the industry, could be explained by oxidative stress. Furthermore, oxidative stress may cause production related diseases such as development of cataracts and melanin spots in the fillet. We sampled Atlantic salmon from two cages in a commercial scale experiment in Northern Norway (67°N), every month from April until August and then every second month until December (510 ± 160–3060 ± 510 g, mean weight ± std). The specific growth rate (SGR) increased with increasing temperature until midsummer and decreased thereafter. We found that vitamin E in the fillet and vitamin C in the liver were depleted in the spring and were restored in the autumn, even though the dietary concentrations were stable. Astaxanthin concentration in the muscle was constant during the spring and summer and increased in the autumn, concomitant with an increase in astaxanthin supplementation. Cataract increased from zero in May until July, when 90% of the fish were affected. The glutathione based redox-potential in the lenses became more reduced from June, indicating a protective mechanism against oxidative stress and cataract. The number of fish with melanin spots was high in June and decreased in August and October, but the size and intensity of the remaining spots increased in the same period. The change in vitamin C and E concentrations, cataract and glutathione metabolism during spring and early summer, indicate that the fish became oxidized in this period, while malon-di-aldehyde (MDA) and astaxanthin concentrations did not support the hypothesis. There are too few data to draw conclusions on possible effects of oxidative stress on melanin spots.

1. Introduction

Atlantic salmon farmed in netpens in the sea are exposed to seasonal variations of many kinds, the most important being changes in daylength and temperature. This has been addressed in several papers since the beginning of commercial salmon farming and we refer to (Dessen et al., 2017) for summary and references. Alne et al. (2011) and Oppedal et al. (2006) found that during the first autumn in the sea both 1+ and 0+ smolts under a natural photoperiod had high growth rates, lipid accumulation and increasing condition factor. In spring and early summer, the condition factor, lipid level and lipid retention were declining, concomitant with an increase in specific growth rate (SGR). The increase in SGR lasted until midsummer and with the declining light and temperature in the autumn, the fish started to retain lipid and

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increased the condition factor, while the SGR declined (Alne et al., 2011; Oppedal et al., 2006). In June the concentration of astaxanthin in the muscle of fish exposed to natural light was lower than in December and also compared fish at continuous light in June (Oppedal et al., 2006).

The background of the present study was the findings by Nordgarden et al. (2003) indicating that Atlantic salmon fillet had elevated thiobarbituric acid reactive substances (TBARS) and lowered fillet concentration of astaxanthin and α-tocohpherol (α-TOH) during imcreasing water temperatures and rapid increase in growth in spring and early summer. Furthermore, Hamre et al. (2016) found that Atlantic salmon reared in sea cages in Northern Norway from January until the end of June had extraordinary low tissue retention of dietary vitamin C, indicating increased utilization and breakdown of this vitamin. Both these studies indicated increased oxidative stress during the period of strongly increasing growth rates in Atlantic salmon.

It is now well established that redox signalling is at the basis of regulation of metabolism in all living organisms (Jones and Sies, 2015). On one hand, physiological levels of singlet oxygen (O_2^-) and hydrogen peroxide (H_2O_2) act as second messengers to reversibly oxidise cysteine thiols in proteins, changing their conformation and activity (Jones and Sies, 2015). On the other hand, redox couples like reduced and oxidized glutathione (GSH/GSSG), thioredoxins and glutaredoxins establish the redox potentials of cells, organelles and cellular microenvironments, which in turn determine the redox state of cysteine thiols (Huseby et al., 2009). Redox regulation of cysteine thiols in proteins open and shut protein redox switches, determining if metabolic pathways are going to be open or not. An example is that a more oxidized cellular environment induces a redox switch that releases the nuclear factor-erythroid 2-related factor 2 (NRF2) transcription factor from a complex with another protein, KEAP1. Once released, NRF2 migrates to the nucleus and induces the transcription of at least 50 mammalian genes; many of which code for antioxidants, thiol oxidoreductases and glutathione synthesis/recycling genes; that are involved in maintaining the redox balance and/or are involved in redox signalling Ma (2013).

The integrated network of antioxidants/redox couples that are produced endogenously is very complex, but generally, when a compound is oxidized, it is recycled to the reduced form by other antioxidants in a chain reaction or network, and ultimately by reductants such as NADH/NADPH produced by energy metabolism (Jones and Sies, 2015). These reactions are catalysed by an array of antioxidant enzymes, such as glutathione peroxidases. The externally provided antioxidants vitamins C and E from the diet are also recycled in this network upon oxidation, probably by GSH and NADPH (Hamre et al., 1997; Meister, 1994; Mrtensson and Meister, 1991).

The purpose of the present research was to study the potentially increased oxidative stress during the period of increasing growth rates in spring in Atlantic salmon, focusing on the endogenous redox regulation system and measuring the GSH/GSSG dependent redox potential. Furthermore, it was hypothesized a connection between the apparent oxidative stress in spring and the occurrence of production related diseases such as cataracts and dark melano-macrophage invaded spots in the fillet.

2. Materials and methods

2.1. Fish trial

Experiments were conducted in accordance with Norwegian laws and regulations concerning experiments with live animals, as regulated by the Norwegian Food Safety Authority. The present study only involved sampling from a commercial scale feeding experiment. The sampling were end-point samplings and the fish were euthanized in a humane and legally approved way and therefore did not need approval.

A large scale fish trial was started by Biomar and GIFAS in autumn 2016, where fish in two of 10 sea cages (90 m diameter), each containing 150,000 fish, were fed a plant based diet derived from the ARRAINA project (Hemre et al., 2016). The main aim of this project was to develop micronutrient recommendations for fish fed plant-based diets. Key nutrients in the diet applied here (analysed) are given in Table 1. The experimental fish shared the same genetic background and were purchased from Sundsfjord Smolt AS. Table 2 provides a full description of the 0+ fish groups in the trial cages. Except for flushing and change of netpens, there were no known potential stressful events for the fish until September when the delousing was applied (diet with SLICE). In each of October, November and December there was one 1–2 days storm and mechanical delousing was performed in October and December (Table 3). One smolt group was transferred to sea in September (cage A) and one in October (Cage B, Fig. 1). The sampling period lasted from April until December 2017 as marked in the Fig.. The SGR was high in the autumn, decreased in January, stayed low until April, increased from April until July, thereafter it decreased again, in parallel to changes in temperature (Fig. 1). The survival was higher in cage A than in cage B (Fig. 1). The temperature at 4 m depth reached a minimum of approximately 4 °C in January/February and a maximum of approximately 13 °C in August (Fig. 1). The time point of maximum temperature is given as a pink triangle in all fig.s.

2.2. Fish feeding and husbandry

The trial was carried out in one of GIFAS' commercial sites, Leirvika Nord (67°N). The site is equipped with an RH MultiFeeder barge, which has a capacity of 240 t (divided between 4 silos). Feed was delivered to the cages by feed blowers (AkvaGroup AS, Norway). The feeding rate in the trial cages ranged from 0 to 2.89% of BW day^{-1}. The feeding ration was determined by biomass, seawater temperature and average fish weight. Furthermore, surface feeding behaviour was observed by onsite employees, and the feeding intensity was adjusted accordingly.

The dead fish hauls were inspected daily during the breaks between meals. The number of dead fish per cage was recorded.

2.3. Sampling

Fish were sampled from cages A and B every month from April 5th until August 16th, and then every second month until December 6th. Sample fish were collected by amassing a cohort of fish using a purse seine and then netting the required number of sample fish using a landing net. This method ensured that the sample fish were

Table 1
Levels of key nutrients in the 7 batches of diet used during the experimental period (mean and SD in % of mean (CV%). A 200 g sample of each of the 7 diet batches was homogenized and distributed to the different analyses with 0.5–5 g and two technical replicates per analyte and sample.)

Nutrient	Average	CV%
Crude protein (500-1000 g)	41.3	1
Crude protein (>1000 g)	37.3	1
Crude lipid (500-1000 g)	30.6	3
Crude lipid (>1000 g)	34.8	3
Ash	5.2	8
Moisture	5.7	7
Total AA	335	6
Mn	46	23
Fe	301	19
Cu	7,74	7
Zn	215	7
Se	1,23	4
Cobalamin	0,08	20
Folate	5,3	12
Niacin	90	5
Vitamin B6	12,6	6
Vitamin C	409	9
Vitamin E	175	4
Astaxanthin (Apr-Aug)	48	17
AstaXanthin (Aug-Dec)	113	14

Table 2
Key information on the smolt groups utilised in the study.

Smolt producer	Sundsfjord Smolt AS	
Breed	Aquagen Atlantic QTL-InnOva IPN (Green Track)	
Generation	Autumn 16G	
Delivery date to commercial site Leirvika	22.09.2016	19.10.2016
Average weight on delivery	89 g	99 g
No. of fish per cage	151.000	149.000
Cage	406/A	420/B
Hatching date	10.12.2015	
Start feeding (date / dd)	25.02.2016 / 900	
Vaccination (date / dd / weight)	12.08.2016 / 530 / 47 g	02.09.2019 / 577 / 56 g
Vaccine	Pentium Forte Plus	
Feed type at delivery	Polarfeed	

representative of the population in the cage. In the first sampling, 12 fish per cage were collected for visual inspection and pooled samples of 2 × 6 fish per cage were taken for analysis. In the second sample, May 3rd, 6 fish per cage were collected for both visual inspection and individual chemical analyses. For the rest of the sampling points, 10 fish per cage were collected and subjected to visual inspection and 5 fish were analysed individually. The sampled fish were killed by a blow to the head and the round weight and fork length were measured and recorded. The eyes of the fish were inspected for cataracts using a slit lamp (Heine HSK150, Heine Veterinary, Germany). The scores for each eye (ranging from 0 to 4, depending on the percentage coverage of the eye by the cataract, Fig. 7) were given and the total score for each fish (0–8) was calculated, based on the procedure given by Wall and Bjerkas (1999).

The fish were filleted and both fillets were inspected for melanin spots. The right panel in Fig. 8 shows the two types of spots that are commonly found, one type is red, possibly bleeding, the other type is grey to black indicating invasion of melano-macrophages (Bjørgen et al., 2015). Spots were scored according to their size and their location (Mørkøre et al., 2015). Number of fish with spots out of the 20 fish per sampling point, 10 fish per cage, and the intensity/size of spots per fish were determined. Score 0 represents no spots, 1; grey shadow, 2; spots of less than 3 cm diameter, 4; spots 3–6 cm, 8; spots more than 6 cm.

The right side of the muscle corresponding to the Norwegian Quality Cut (NQC, between the posterior end of the dorsal fin and the anus) was dissected. The skin was removed, the fillet was homogenized using a kitchen blender, distributed to tubes for the different analyses and snap-frozen on dry ice. The left side of the NQC was used for visual assessment of fat content and pigmentation (astaxanthin concentration and Salmofan coloration score). This was carried out using PhotoFish (AkvaGroup, Norway) according to the manufacturer's instructions. The liver was homogenized and distributed to tubes using the same procedure as for muscle. The samples were shipped to the Institute of Marine Research (IMR) on dry ice and stored at −80 °C until analyses.

2.4. Malon di-aldehyde (MDA) measurement

The MDA measurements were performed with a method newly established in the laboratory of IMR. All the chemicals and reagents used were analytical grade of the highest purity. Chloroform (CHCl3), methanol (CH3OH), ethylenediaminetetracetic acid (EDTA, C10H16N2O8), triacetic acid (TCA, CCl3COOH), 2-thiobarbituric acid (2-TBA, C4H4N2O2S), hydrochloric acid fuming 37% (HCl), natrium-dihydrogenphosphate (NaH2PO4), di-natriumhydrogenphosphate (Na2HPO4) were purchased from Merck (Darmstadt, Germany, ACS

Table 3
Potentially stressful events during the sampling period.

		apr.17	may.17	jun.17	jul.17	aug.17	sep.17	oct.17	nov.17	dec.17
Sampling	Date/s	05.apr	03.may	07.jun;28.jun		16.aug		04.oct		06.dec
	Cages	406, 420	406, 420	406, 420		406, 420		406,420		406,420
Flushing of netpens	Date/s		16.may							
	Cages		all							
Change of netpens	Date/s				19.jul	02.aug				
	Cages				406	420				
SLICE feeding w/ 2d starvation	Date/s						18.-24.sep			
	Cages						all			
Storms – no feeding	Date/s							02.-03.oct	23.-24.nov	07.dec
	Cages							all	all	all
Hydrolicer w/starvation	Date/s							27.oct - 01.nov		
	Cages							all		
Optilicer w/starvation	Date/s									06.-11.dec
	Cages									420,420

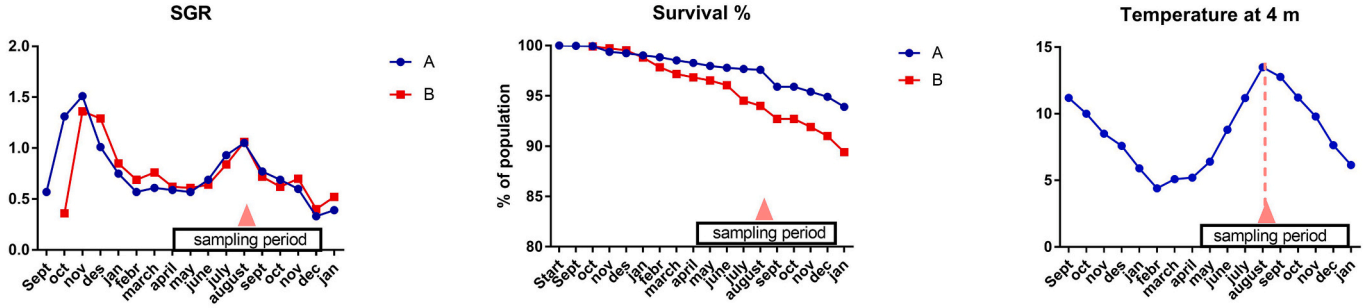

Fig. 1. Specific growth rate (SGR, % per day) and survival of the two Atlantic salmon smolt groups produced at a commercial scale (150,000 fish per cage) in Leirvika (67°N) from September and October 2016 until December 2017 in cages A and B, together with average temperature per month at 4 m depth. These are registrations on the whole population level by the fish farm. The maximum temperature in 2017 is indicated by a vertical line and a pink triangle at the x-axis. This triangle is reproduced in all fig.s. The sampling period is indicated at the x-axis. (For interpretation of the references to colour in this fig. legend, the reader is referred to the web version of this article.)

grade). Butylated hydroxytoluene (BHT, C15H24O, ≥96%) and 1,1,3,3-tetraethoxypropane (TEP, C11H24O4, ≥96%) were from Sigma (St. Louis, MO, USA). De-ionized water was purified in a Milli-Q system (Milli-Q system Millipore, Milford, MA).

MDA extraction was performed as in Hamre et al. (2001). Briefly, a portion (0.5 g) of homogenized fish tissue was weighed in a 10 ml Sovirel-tube and 4.0 ml chloroform:methanol (2:1, containing 0.005% BHT) were added. The air in the tube was exchanged with N2 for 10 s, the tube was closed with a screwcap, shaken for 30 min and immediately after, 2 ml of saturated EDTA solution were added. The tube was vortex-mixed and centrifuged for 20 min at 1500 x*g*. 2.0 ml of the methanol: water phase supernatant was transferred to a new tube and 2.0 ml TBA reagent was added. This system was vortex-mixed, warmed at 100 °C for 30 min, cooled in cold water and centrifuged for 10 min at 1500 x*g*. Finally, an aliquot of 1 ml was collected from the tube and placed in an autosampler vial. A 50 ml MDA stock solution was prepared by diluting 50 μl of TEP in HCl (0.1 N), which was used for preparing a MDA (0.1 mM) solution in water. The MDA aqueous solution was used to fit a regression line between 0.00 and 16.00 nmol. The limit of quantification (LOQ = 0.01 nmol) was calculated mathematically by the relationship between the standard deviation of the calibration curve and its slope using the multiplier suggested by the ICH standard (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) (Singh, 2015).

The quantitative determination of MDA by HPLC was performed based on the method described by Junghans et al. (2000) and Martin et al. (2009). The HPLC system was equipped with pump, degasser, autosampler, spectrofluorometric detector and system controller with a PC control program. Separation of MDA was done using a LiChrospher 100 RP-18 HPLC column (5 μm, 250 × 4 mm id), operated isocratically with a mobile phase consisting of a mixture sodium phosphate buffer (50 mM) and methanol in the proportion 6:4 (*v*/v). The MDA retention time and the total analysis time were 7 and 15 min respectively. Spectrofluorometric detector wavelengths were set at 513 nm (excitation) and 550 nm (emission). Results were expressed as nanomoles MDA present in 1 g of muscle or liver.

2.5. Other chemical analyses

Total amino acids in diets were determined after hydrolysis in 6 N HCl at 22 °C using an ultra performance liquid chromatography (UPLC) method as described by Andersen et al. (2013). L-histidine (His) and *N*-acetyl L-His (NAH) concentrations were determined in individual lenses by reversed-phase HPLC (Waters Corporation), based on the method described by O'Dowd et al. (1990), slightly modified by Breck et al. (2005). The B-vitamins niacin, folate and cobalamin were all determined by microbiological methods (Feldsine et al., 2002; Mæland et al., 2000). Vitamin B6 was determined by HPLC (CEN, 2005). Vitamin C and E were measured by HPLC according to Mæland and Waagbø (1998) and Hamre et al. (2010a), respectively. Microminerals were analysed in muscle and liver tissues (Julshamn et al., 2004). The method for astaxanthin analyses is given by Ørnsrud et al. (2004). For the analysis of total (tGSH) and oxidized (GSSG) glutathione, supernatants were prepared from samples using a commercial kit (Prod. No. GT40, Oxford Biomedical Research, Oxford, UK) and then analysed for absorbance at 405 nm in a microplate reader (iEMS Reader Ms., Labsystems, Finland) as previously described (Skjærven et al., 2013).

2.6. Calculations and statistics

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

$$E_h = E_0' - RT/nF\ ln\left([GSH]^2/[GSSG]\right)$$

where the GSH and GSSG concentrations are in M and E_h is given in volts. E_0' is the standard reduction potential at pH 7 and 25 °C and was assumed to be −0.240 V. The measurements are the average of whole tissue and do not take into account that the reduction potential varies between tissue types, and between organelles within the cells (Bruce et al., 2012; Kemp et al., 2008; Schafer and Buettner, 2001).

Data were analysed using Statistica (ver. 13.4.0.14, TIBCO Software Inc.) and are given as mean ± pooled SE. Factorial ANOVAs, with cage and time as independent variables, were applied to all datasets and Tukey's post hoc test for different sample sizes was used to analyse differences between means. When there were no significant differences between cages the data from cage A and B were pooled and reanalysed by one way ANOVA. When Levene's test showed non-homogenous variances between time points, effects of time were analysed with Kruskal-Wallis multiple comparison test on combined data from cage A and B. Differences between cages gave non-significant Levene's tests in all cases.

3. Results

3.1. Feed samples

The diet fed to fish between 500 and 1000 g contained 41.5% crude protein and 31.3% lipid, while the fig.s for the diet fed to fish above 1000 g were 37.2 and 35.2, respectively. Other key nutrients, except for astaxanthin, were reproduceable between feed batches and therefore stable during the experiment. Astaxanthin supplementation was increased from approximately 50 to approximately 100 mg/kg in August (Table 1).

3.2. Morphometry and somatic indices

The fork-length, weight, condition factor and Salmofan pigment evaluation results from the sampled fish are given in Fig. 2. Fish in cage A were significantly larger ($p = 10^{-5}$) and had more fillet pigment ($p = 0.0007$) than those in cage B. Length showed an almost linear increase with time ($p \langle 10^{-3}$), while the condition factor was stable during spring and summer ($p > 0.1$) and increased after the temperature maximum in early August ($p < 0.05$). In August and September, there was also a difference in condition factor between cage A and B ($p = 0.013$), which had disappeared in December. This led to an exponential increase in weight from August ($p < 10^{-3}$). The fillet pigment was stable from April until July ($p = 0.9$–1) and increased thereafter ($p < 0.0004$). The fillet lipid level, as measured by Salmofan, was similar in the two cages ($p = 0.6$), was stable from April until June ($p > 0.055$) and increased thereafter ($p < 0.006$).

3.3. Tissue antioxidant status

The antioxidant status was generally higher in fish from cage A than in those from cage B, decreased during spring and summer and increased again after the temperature maximum in early August (Fig. 3). This was true for vitamin C in liver where there was a difference between cages ($p = 0.007$) and an effect of time ($p = 0.002$ in cage A and 0.0002 in cage B). For vitamin E in muscle, differences between cages ($p = 0.004$) and the effect of time ($p < 10^{-6}$) were statistically significant. Similarly, for astaxanthin in the muscle, differences between the cages ($p = 0.001$) and an effect of time ($p < 10^{-6}$) were significant. Similar to the visual Salmofan evaluation, the concentration of astaxanthin was stable from April until July ($p > 0.10$) and increased thereafter ($p < 0.002$). Like in the liver, muscle vitamin C concentration was higher in cage A than in cage B ($p = 0.0002$), but the difference was pronounced only in July. There was no effect of time on muscle vitamin C level in cage A ($p > 0.08$), whereas in cage B the effect of time was significant ($p = 10^{-4}$), with July as the low point and December as the high point. Liver and muscle MDA were similar in the two cages ($p = 0.14$ and 0.08, respectively). MDA in the liver was higher in May than in June, October and

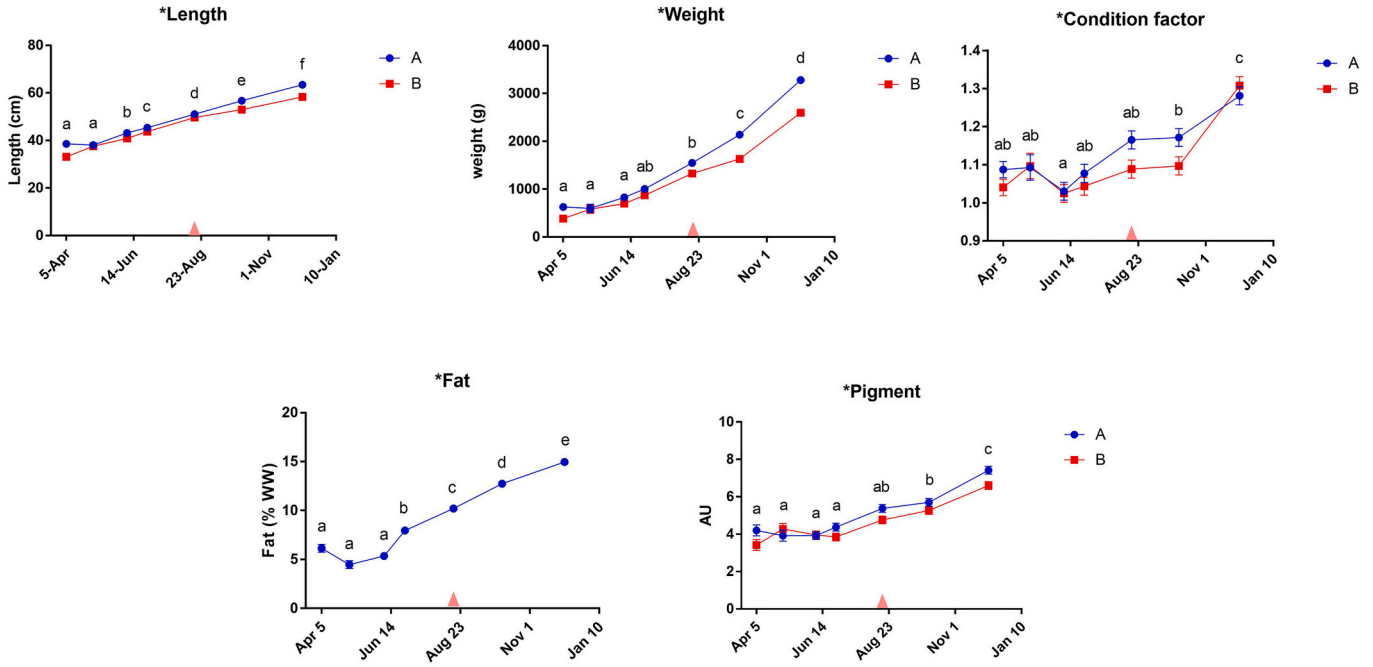

Fig. 2. Fork-length, weight, condition factor and Salmofan pigment evaluation results (mean ± SEM) from Atlantic salmon sampled from sea cages A and B in Leirvika once or every second month between April 5th and December 6th, 2017. Separate curves for cages A and B indicate statistical differences between the cages and different letters indicate differences between sampling points ($p < 0.05$).

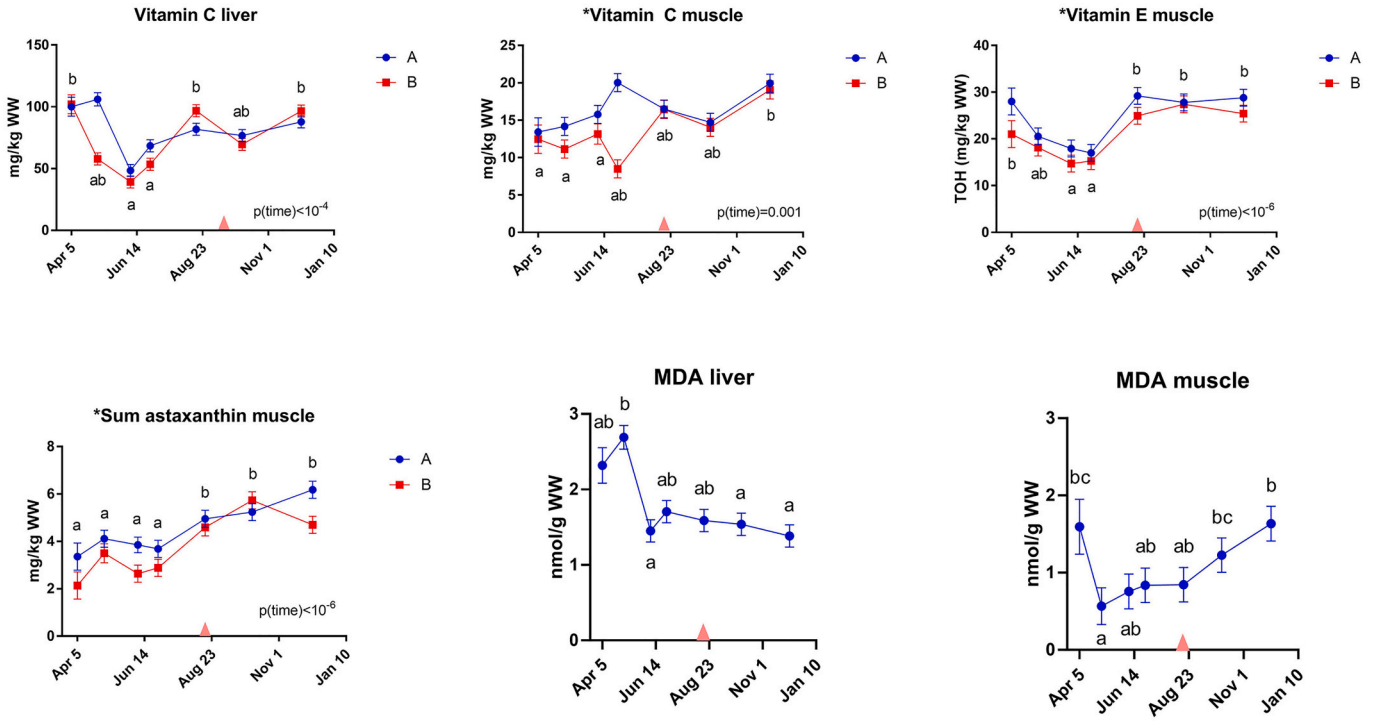

Fig. 3. Vitamin C, E, astaxanthin and malon-di-aldehyde (MDA, mean ± SEM) in tissues of Atlantic salmon sampled from sea cages A and B in Leirvika once or every second month between April 5th and December 6th, 2017. Separate curves for cages A and B indicate statistical differences between the cages and different letters indicate differences between sampling points ($p < 0.05$).

December ($p < 0.05$) while the other sampling points were intermediate. The effect of time was significant ($p = 0.0004$). MDA in muscle was higher in weeks April and December compared toMay ($p < 0.05$). The other sampling points were intermediate. The effect of time had a $p = 0.0001$.

Micro-mineral concentrations (Mn, Fe, Cu, Zn and Se) were analysed in the muscle (Fig. 4) and liver (Fig. 5). In the muscle, levels of Mn ($p < 10^{-4}$), Fe ($p = 0.03$), Cu ($p = 0.01$) and Zn ($p < 10^{-4}$) gradually decreased with time with statistically significant high and low values recorded in the April and Oct-Dec samplings. The muscle Se

concentrations decreased initially with time ($p = 5*10^{-4}$) reaching the minimum in July, thereafter, increased to maximal levels in August and remained stable until December. The response was similar between the cages ($p > 0.05$) and the results in the graphs are merged per sampling. In the liver, concentrations of Mn ($p = 0.003$), Cu ($p < 10^{-6}$), and Se ($p < 10^{-6}$) changed with time, peaking during the Oct -Dec period, while Fe and Zn were unaffected by time ($p > 0.05$). The response of Zn and Se was different between the cages, with lower Zn or Se levels in fish from cage B compared to cage A ($p < 0.05$).

Liver, muscle and lens reduced glutathione (GSH), oxidized glutathione (GSSG) and redox potential (E_h) were similar between the two cages ($p > 0.05$), except for E_h in muscle ($p = 0.005$, Fig. 6). Liver GSH was stable from April until August ($p > 0.5$), where there was a sharp decrease in October ($p = 10^{-4}$), then stability until December ($p = 0.5$). Liver GSSG was stable from April until August, except for the point in June, which was lower than that in April ($p = 0.02$). Liver GSSG in October and December was lower than at all points earlier in the sampling period ($p < 0.02$). The effect of time on Liver E_h had $p = 0.04$. Muscle GSH appeared to have a peak in July, but due to large variation, there was no significant effect of time on this variable ($p > 0.6$, Fig. 4). Muscle GSSG had a peak in July, higher than all other time points ($p < 0.0008$). There was a difference in Muscle E_h between the cages, but this was only true for the point in April, where the muscle from fish in cage B was more reduced than that in cage A ($p = 0.008$). Except for this point, there was no change in Muscle E_h with time ($p > 0.4$). Lens GSH was similar in April and May ($p = 0.5$), but increased sharply in June ($p = 0.002$) and then stabilised for the rest of the sampling period ($p > 0.6$). Lens GSSG was stable in April and May ($p = 0.6$), then decreased in June ($p = 0.0006$) and was again stable for the rest of the period ($p > 0.7$). Accordingly, lens E_h was stable in April and May ($p = 0.5$), deceased in June ($p = 0.0006$) and was then stable until December ($p > 0.7$).

Cataract (Fig. 7) was not detected until June, The fraction of the sampled fish with cataract was 90% in June in cage B and in August in cage A, while after October, it was 50% and 30% in cage A and B, respectively.

Cataract was not detected in May. The score of affected fish increased sharply in July in cage B and was significantly higher than for fish in cage A ($p = 0.006$). In fish from cage A, the score increased in August ($p = 0.001$). Scores in both cages showed a tendency to decrease in October and December, but were not significantly different from peak scores ($p > 0.05$). The fraction of fish with cataract showed a similar development as the cataract score (Fig. 7). There was a continuous decrease in L-histidine (HIS) and Na-Acetyl-L-histidine (NAH) in lens from fish in both cages between May and October ($p < 10^{-6}$) and a stabilisation of both compounds from October to December (Fig. 7).

The number of fish with pigment spots in the fillet out of the 20 fish taken from both cages was counted and their size and colour intensity were classified on a scale from 0 to 8 (Fig. 8). The proportion of fish with pigment spots appeared to decrease from June (30%) until August (10%) and then stabilize. The average intensity of the spots was low at 1.3 in June and increased ($p = 0.04$) to 4.3 in October.

4. Discussion

The specific growth rate of fish sampled in the present study was stable from February until May, then increased to a maximum in August and decreased again until December. The minimum and maximum growth rates correlated with minimum and maximum temperatures.

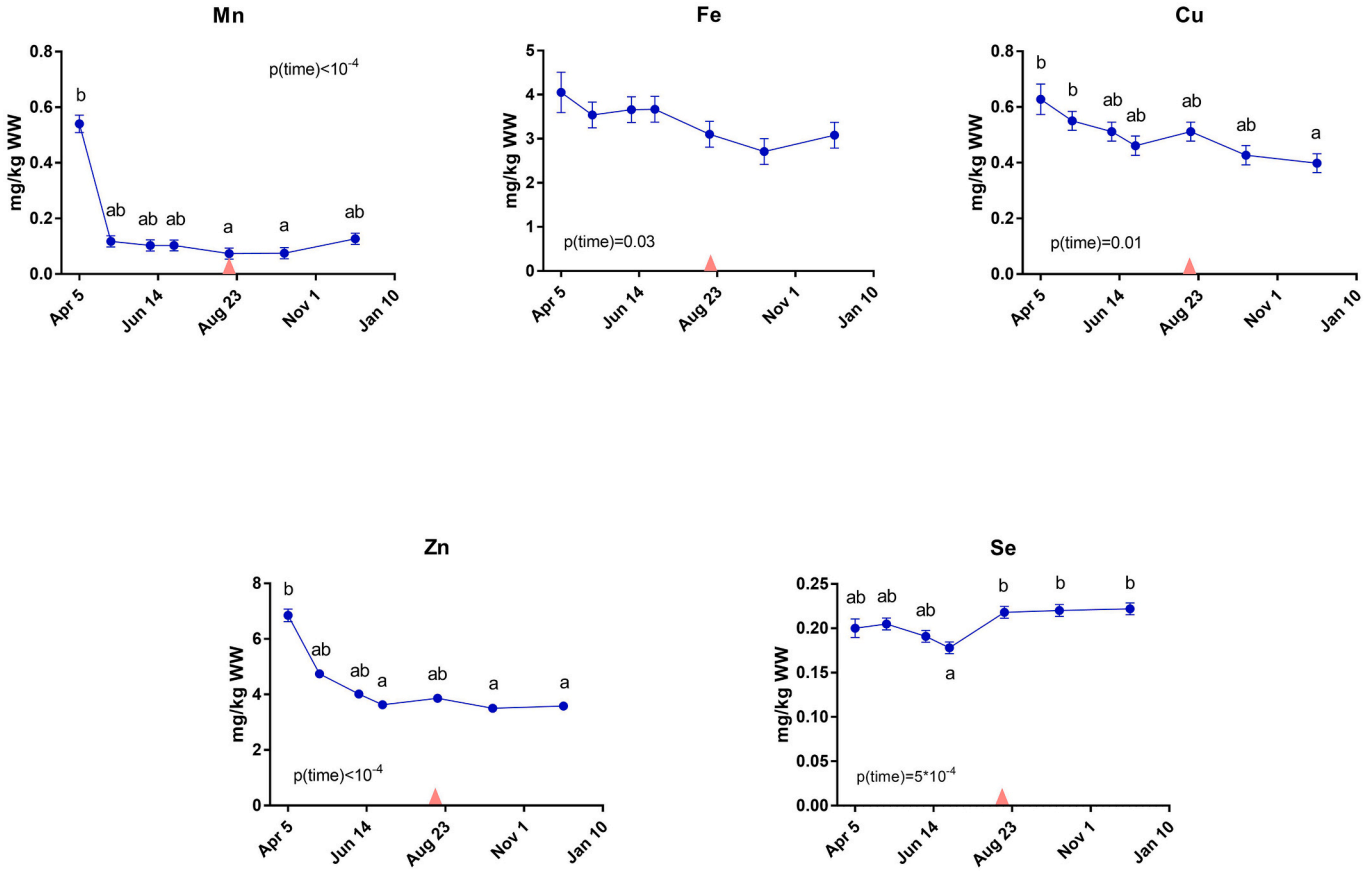

Fig. 4. Microminerals (mg/kg wet weight, mean ± SEM) in muscle of Atlantic salmon sampled from sea cages A and B in Leirvika once or every second month between April 5th and December 6th, 2017. Since there were no differences between cage A and B ($p > 0.05$), the data were combined. Different letters indicate differences between sampling points ($p < 0.05$).

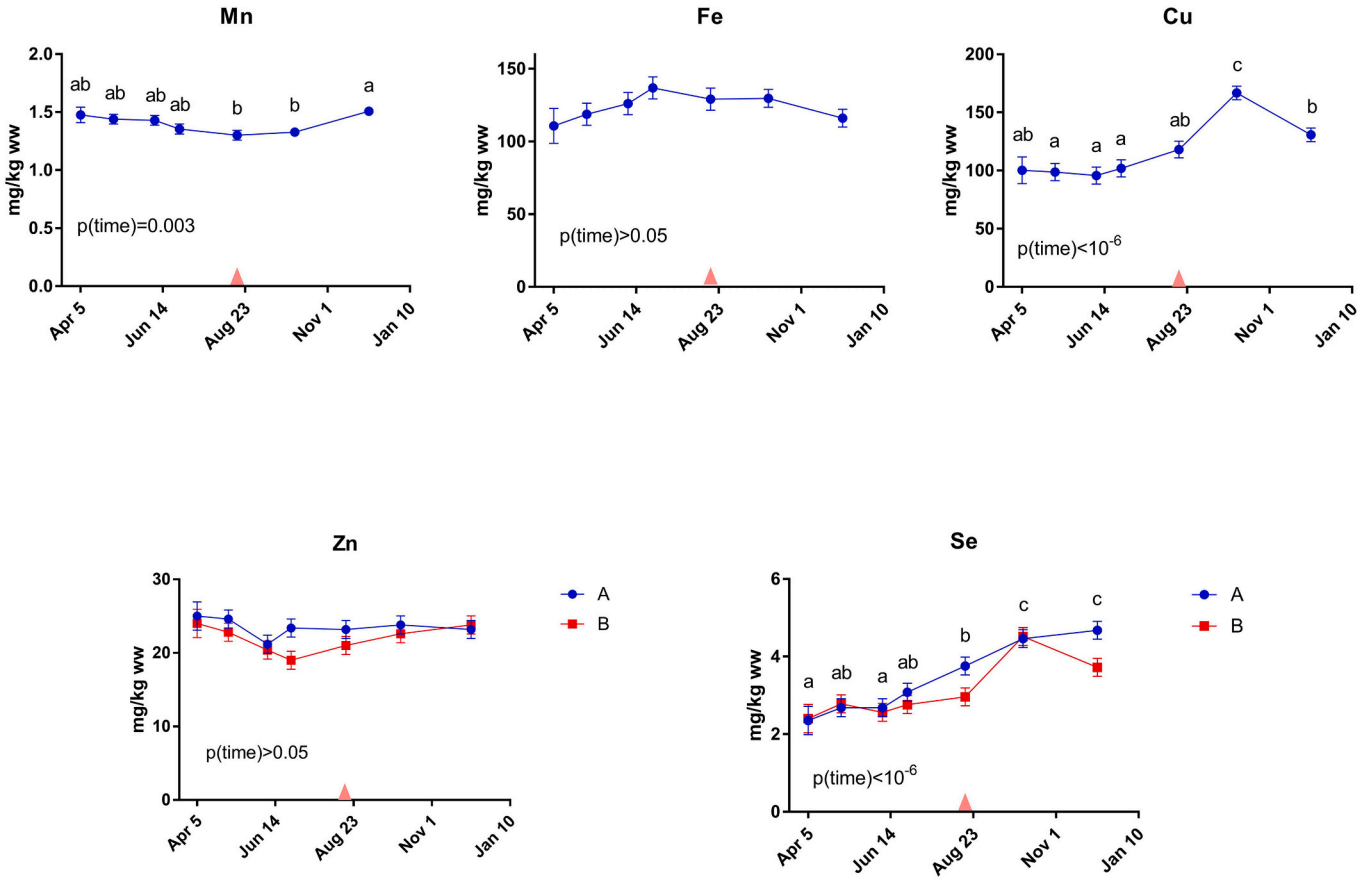

Fig. 5. Microminerals (mg/kg wet weight, mean ± SEM) in liver of Atlantic salmon sampled from sea cages A and B in Leirvika once or every second month between April 5th and December 6th, 2017. Separate curves for cages A and B indicate statistical differences between the cages and different letters indicate differences between sampling points ($p < 0.05$).

The changes in SGR are similar to the results of (Oppedal et al., 2006). Changes in lipid levels and condition factor which show that the fish are relatively lean in spring and summer and retain lipid and condition in autumn are also in line with previous results (Alne et al., 2011; Oppedal et al., 2006). There was no difference in SGR in the sampling period between the two cages. Fish in cage A and B were from two different smolt groups and were transferred to sea in September and October, respectively. Sampled fish in cage B were slightly smaller, had a period with lower condition factor and had lower survival rate than fish in cage A. Salmofan measurement showed that fish in cage A was better pigmented than fish in cage B, while fat content was similar. Vitamin C and E and analysed astaxanthin in the muscle and vitamin C in the liver were also higher in fish from cage A compared to B. Therefore, it seems that the smolt group in cage A was more robust than that in cage B and that this affected the tissue oxidation state and levels of antioxidants.

In the present study, there was a decline in vitamin C concentration in the liver and vitamin E concentration in the muscle to approximately 50% from April until June. The concentrations were restored again in August. This is in accordance with the data from Nordgarden et al. (2003) and Hamre et al. (2016) and could be due to lowered supply and/or increased consumption of these nutrients in the fish. Vitamin E is fat soluble, it is thought to be absorbed by passive diffusion, and tissue concentrations in balance with the diet in Atlantic salmon increase linearly with increasing dietary concentrations when conditions are stable (Hamre, 2011). The dietary concentrations were similar in the different feed batches, the amount delivered corresponded to the increasing feed intake, and the steady state concentration would not be changed if other factors, such as oxidation, did not affect tissue levels. For vitamin C we showed that retention during the spring was very low and negative at dietary concentrations below 70 mg/kg (Hamre et al., 2016). These considerations point to consumption being an important reason for the lowered tissue-concentrations of the antioxidant vitamins. It indicats that the fish were exposed to increased oxidation in spring and early summer, corresponding to the period of increasing day length, temperature and growth rate. This was not reflected in MDA concentrations though, which could indicate that redox signalling is not readily transferred between ROS and lipid oxidation, where MDA is an end-product. Metabolic rate in poikilotherm organisms increases with increase in temperature. It can be quantified by the temperature quotient (Q10), which indicates the change in metabolic rate when the temperature rise 10 °C and varies between 2 and 3 times in most biological systems (Reyes et al., 2008). This results in increased growth up to a certain limit when the maximal capacity of growth and metabolism is reached. In Atlantic salmon post smolt, this limit is approximately 13 °C (Handeland et al., 2008; Hevroy et al., 2012), e.g. similar to the maximum temperature in the present study. A possible explanation of increase in tissue oxidation state is that growth is accompanied by increased metabolism that would lead to higher production of ROS in mitochondria (Diebold and Chandel, 2016). Transgene Atlantic salmon (Almroth et al., 2012) and zebrafish (Rosa et al., 2008) with extra copies of the gene for growth hormone have more oxidized tissues than the wild types, supporting this hypothesis. However, in plants, hormones that regulate growth, development and stress response use ROS as second messengers (Bartoli et al., 2013). If this is also true in animals, the higher oxidation in the tissues of Atlantic salmon found here, could be a result of growth hormone signalling, independent of increased metabolism. In contrast to the present findings, α-tocopherol was increased and oxidative markers decreased in a study where Atlantic salmon was loaded with vitamin E and

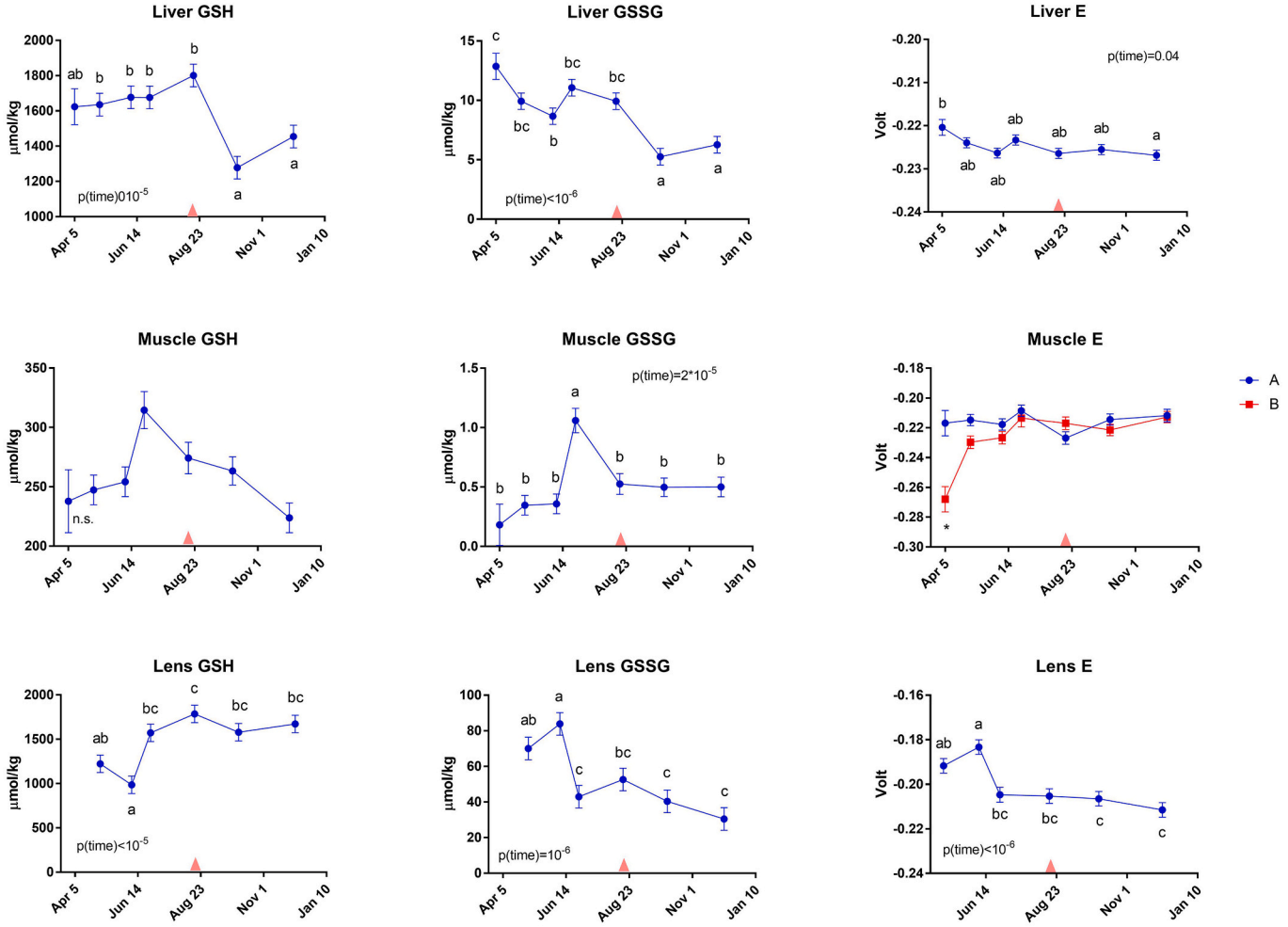

Fig. 6. Reduced and oxidized glutathion (GSH and GSSG) and the redox potential (E_h) (mean ± SEM) in liver, muscle and lens of Atlantic salmon sampled from sea cages A and B in Leirvika once or every second month between April 5th and December 6th, 2017. Separate curves for cages A and B indicate statistical differences between the cages and different letters indicate differences between sampling points ($p < 0.05$).

astaxanthin and then exposed to 19.5 °C and starvation for 4 weeks (Grunenwald et al., 2019). Therefore, feeding and perhaps increase in daylight may be necessary to induce the oxidative stress found here.

It is unknown how much of the growth stimulation in spring is caused by increase in day length and how much depends on the temperature rise. There is a connection between the circadian clock and redox signalling (Edgar et al., 2012; Putker et al., 2018), which manifests itself in oscillation of overoxidized peroxiredoxine, H_2O_2 and cell redox state in parallel with expression of clock genes in all aerobic organisms (Edgar et al., 2012). Similar oscillations can be seen in glucose metabolism and oxygen consumption rate in fibroblast cell cultures (Putker et al., 2018). Maximum output is at the maximum expression of *period,* a key gene in the circadian clock. Increasing day length in spring will increase the daily period of high metabolic rate, which may be part of the reason for the increased growth and oxidative load found in the present study. Therefore, both higher temperature and increasing day length may give higher metabolism and growth. Accordingly, the more oxidized tissues in spring and early summer could be caused by increased metabolism and resulting higher production of ROS in mitochondria or by redox signalling through other pathways, such as GH or circadian clock signalling.

Oxidized and reduced glutathione (GSH and GSSG), form a red-ox couple with very high intracellular concentration (0.5-5 mM in salmon muscle and liver) (Hamre et al., 2010b). Their relative concentrations are thought to be important for maintaining the redox potential (E_h) of cells and cellular compartments. E_h is instrumental for opening and closing of redox switches in proteins and therefore for the directions of metabolism (Hoffman et al., 2008; Kemp et al., 2008). Furthermore, GSH is an important endogenous antioxidant that may be produced in excess to protect the organism against oxidative stress. If the oxidative stress is severe, steady state GSH concentration will decline, as seen in zebrafish transgene for GH (Rosa et al., 2008). The protective function of GSH is confirmed by the measurements of GSH in liver and muscle in the present study, with high and increasing GSH concentrations in the spring and early summer and lowered concentrations from august in both organs. In muscle, GSSG concentrations followed the concentrations of GSH, resulting in a relatively constant redox potential. Perhaps we also see an adjustment of E_h from relative reduced to more oxidized values in cage B in May, possibly to facilitate muscle growth, which, seems to correlate with more oxidized tissue (Almroth et al., 2012). In liver, the development of GSSG and the E_h are towards a more reduced tissue. It can be speculated that increased metabolism and growth requires higher biosynthesis in the liver and that a more reduced cellular environment would facilitate this. It is of course contradictory that vitamin C in the liver is consumed, indicating oxidation, while GSH has a trend of increased concentration in spring concomitant with GSSG and E_h decline, indicating a more reduced liver. This could perhaps be explained by the fact that vitamin C is available at a constant rate from the diet, while GSH can be produced by the fish body on demand, for example as antioxidant protection when the tissue becomes oxidized.

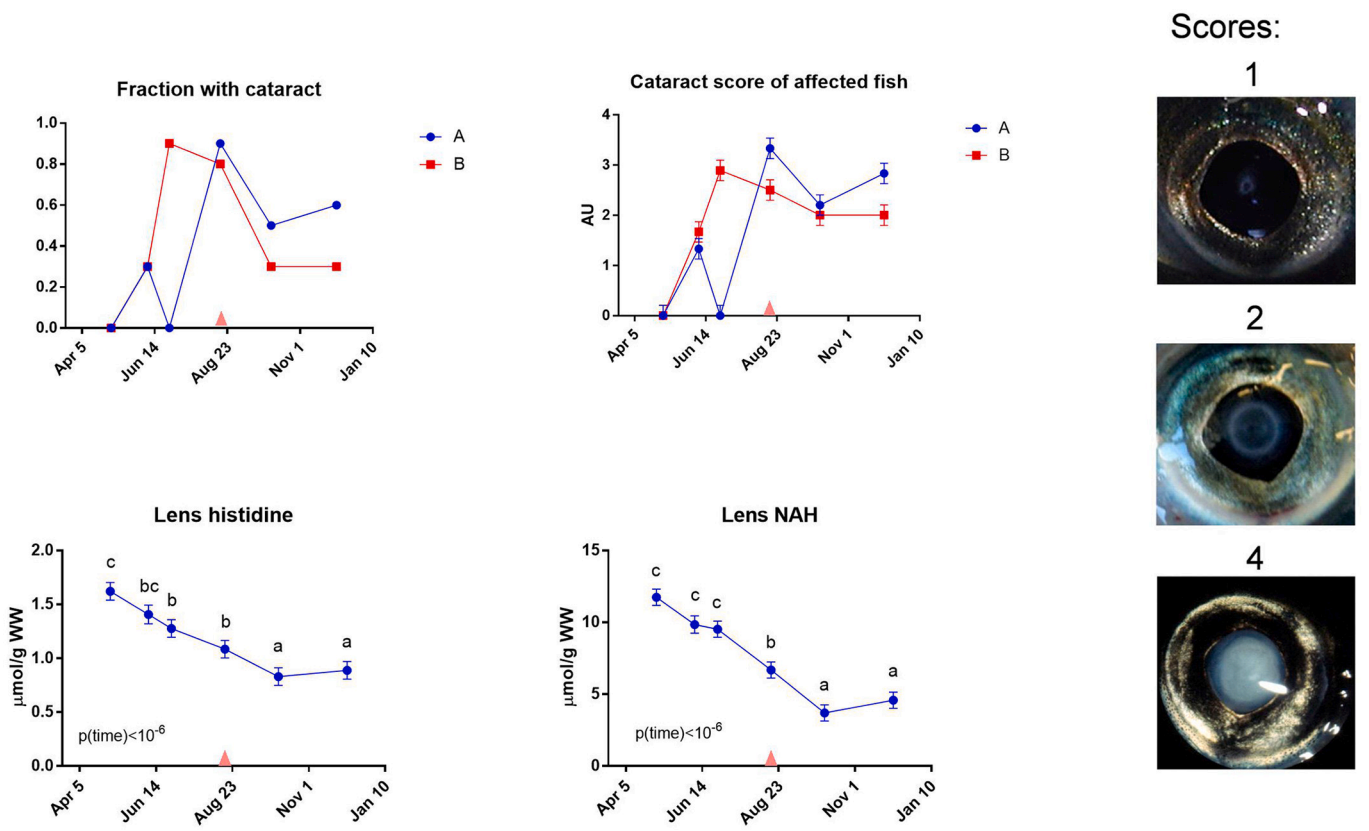

Fig. 7. Cataract (fraction of fish with cataract and cataract score of affected fish), lens histidine and lens *N*-acetyl L-histidine (NAH) (mean ± SEM) in Atlantic salmon sampled from sea cages A and B in Leirvika once or every second month between April 5th and December 6th, 2017. The panel on the right indicates the scoring system, which ranges from 1 to 4 for each eye so that maximum score per fish is 8. Separate curves for cages A and B indicate statistical differences between the cages and different letters indicate differences between sampling points ($p < 0.05$).

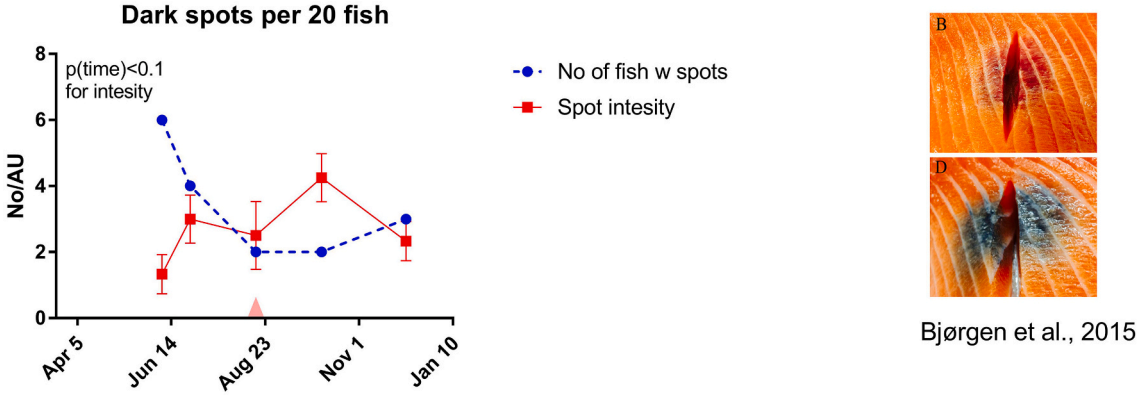

Fig. 8. Dark spots in fillet of Atlantic salmon sampled from sea cages A and B in Leirvika once or every second month between April 5th and December 6th, 2017. The panel on the right shows the two types of spots that are commonly found, one type is red, possibly bleeding, the other type is grey or black indicating invasion of melano-macrophages. Number of fish with spots out of the 20 fish per sampling point is shown in blue and the intensity of spots per fish in red. Score 0 represents no spots, 1; grey shadow, 2; spots of less than 3 cm diameter, 4; spots 3–6 cm, 8; spots more than 6 cm (Mørkøre et al., 2015) The scores were summed per fish, both fillets, and data are from 20 fish, 10 per cage (mean ± SEM). (For interpretation of the references to colour in this fig. legend, the reader is referred to the web version of this article.)

There is clearly a need for more research to understand the redox regulation of fish tissues.

Microminerals have a vital role in various biological processes impacting animal health and tissue specific changes in their concentrations mediate metabolic processes and their impacts (Underwood et al., 1979). Microminerals like Mn, Se, Fe or Cu on their own or by interaction with other antioxidants influenced performance, lipid and astaxanthin levels in liver, muscle lipid or plasma of Atlantic salmon (Hamre et al., 2004). Copper is essential for the activity of the enzyme lysyl peroxidase facilitating collagen and elastin synthesis, and its deficiency leads to porcine muscle defects (Stryer, 1988). Increased gaping of the fillet coincided with decreasing serum and muscle Cu concentrations from March–April (Mørkøre and Austreng, 2004) and a micronutrient package supplementation affected fillet texture associated with low whole body Cu levels in Atlantic salmon (Hamre et al., 2020). Vaccination, dietary Zn and vitamin E supplementation stimulated the

immune response in Atlantic salmon increasing melanin deposition in the fillet and other organs (Jafelice, 2014). In Atlantic salmon the melanogenesis occurs in muscle-located granulomas, which represents an association between the immune and pigmentary systems (Larsen et al., 2012). In the present study, increasing intensity of the dark spots correspond with decreasing muscle micro-mineral concentrations (Cu, Fe, Mn and Zn), among other causative agents discussed earlier. Regarding the redox status, dietary Se and tissue Se status influence the GSH/GSSG redox couple in Atlantic salmon (Hamre et al., 2016; Prabhu et al., 2020). In accordance, we observed that the GSSG concentration in muscle peaked during July which corresponded with the significant drop in muscle Se concentration. Further, the increasing levels of Cu and Se also corresponded with decreasing trend in hepatic E_h. Hepatic Cu and Se concentrations increased alike peaking during Oct-Dec, confirming a positive correlation observed previously in juvenile Atlantic salmon (Lorentzen et al., 1998). The micro-mineral levels observed in the muscle and liver are within the normal range reported for Atlantic salmon (Lall, 1995; Prabhu et al., 2016). However, the temporal changes might hold significance in relation to the peroxidation, red-ox and pigmentation differences observed.

Lowered fillet pigmentation and the production related diseases, cataract and dark spots in the fillets, are all great challenges in the salmon industry (Grunenwald et al., 2019; Koppang et al., 2005; Nordgarden et al., 2003; Waagbø et al., 2020). A hypothesis of the present study was that oxidized tissues in spring and summer would negatively affect fillet quality, fish health and welfare by worsening these conditions.

Fillet astaxanthin was stable in spring and summer and increased from August until the end of the experiment. The increase in August coincided with increase in dietary astaxanthin supplementation from ~50 to ~100 mg/kg. Generally, astaxanthin concentration in the muscle follows an asymptotic relationship (Torrissen et al., 1995; Ytrestoyl et al., 2004), where the dietary level of astaxanthin determines 90% of the variation in the muscle of a homogeneous group of fish. The muscle becomes saturated with astaxanthin at a dietary concentration of approximately 60 mg/kg (Torrissen et al., 1995; Ytrestoyl et al., 2005), and according to Torrissen et al. (1995), there was no significant difference in muscle astaxanthin in fish fed 40 and 150 mg/kg. Other factors such as differences in fish weight, metabolism and digestion have effects, but much less that dietary astaxanthin. According to Torrissen et al. (1995), the length of the feeding period has a large effect on the muscle concentration of astaxanthin. Ytrestoyl et al. (2004) found lower muscle concentration in 1+ that in 0+ smolts. This could be the result of shorter feeding period with an astaxanthin supplemented diet in the 1+ smolts. There are large losses of astaxanthin due to metabolism as addressed by Ytrestoyl et al. (2004), but this phenomenon has not yet been explained. The constant to slightly decreasing (insignificant) trend of muscle astaxanthin in spring in the present study could indicate that it was consumed, possibly by oxidation. Lowered fillet astaxanthin in spring was found by Nordgarden et al. (2003). Absorption and retention of astaxanthin seems to become more efficient as the fish grow (Bjerkeng et al., 1992; Grunenwald et al., 2019; Torrissen et al., 1989) and this effect, rather that increased dietary concentration from 50 to 100 mg/kg, could explain the increasing astaxanthin concentration in autumn found here.

Cataract was not detected until June, and in the period after, the fraction of salmon with cataracts increased rapidly to around 90%. The occurrence of cataract coincided with the increase in temperature and growth, and systemic oxidative stress as indicated by the decrease in vitamin E and vitamin C, the GSH/GSSG concentrations in liver and muscle, and the reduction in His and NAH. Oxidative stress is considered a major risk factor for cataract development in both animals and humans (Spector, 1995; Williams, 2006), and in the period before cataract was seen the low levels of GSH and higher levels of GSSG indicate oxidative stress in the lens. GSH is an important lens antioxidant, and lower GSH levels have also been found in Atlantic salmon lenses exposed to oxidative stress in ex vivo lens cultures (Remo et al., 2011). Previous studies have shown that water temperature influences the lens GSH synthesis and turn-over in Atlantic salmon and rainbow trout (Bjerkas et al., 2001; Remo et al., 2017). Thus, both the increase in temperature, increased growth rates, metabolic changes in the fish and oxidative stress during the spring may have resulted in cataract development in the present study. These changes may also have triggered an increased GSH synthesis in the lens, resulting in higher lens GSH concentrations and stable redox potential during the fall. In the fall, both the fraction of fish and severity of cataracts in the affected fish were constant or lower compared to the summer, indicating that the cataract development in the affected fish did not increase in severity during the fall.

The concentration of lens His and NAH decreased steadily during the summer until the fall, from 12 to 3 μmol/g. NAH is suggested to cover several functional roles in the lens, including osmolyte (Rhodes et al., 2010), buffer (Breck et al., 2005) and antioxidant (Remo et al., 2011), therefore being vital to maintain water balance and cell integrity. The lens concentration of both histidine and NAH are highly dependent on the dietary level of histidine (Remo et al., 2014), and a lens NAH concentration above 8.8 μmol/g has been suggested to be a safe level for minimizing severity of cataracts in Atlantic salmon smolt after SW transfer. At temperatures above optimum, a higher dietary level of histidine is needed to mitigate cataract severity and prevalence in Atlantic salmon post-smolts (Sambraus et al., 2017) and adult salmon (Waagbo et al., 2010), possibly due to underlying changes in growth, metabolism and oxidative stress, as shown in the present study. The reported NAH concentrations below the safe level of 8.8 μmol/g clearly increased the susceptibility to cataract development in this period. The steady reduction in lens His and NAH status during the summer indicate insufficient His supply to cover both rapid growth and eye health.

The data on dark spots in the fillet are quite weak, but one can use them to formulate an hypothesis. It appears that the spots were small but the number of fish with spots were relatively high at the first point of registration in June, corresponding with the lowest levels of vitamin E in muscle and vitamin C in liver. At the same time, the concentration of Se in the muscle was at its minimum. The intensity and size of spots increased while the number of fish with spots decreased from June until October. It has been shown that vitamin E deficiency may cause muscular dystrophy in salmonids (Bell et al., 1985; Poston et al., 1976), but an examination of the literature showed that muscle damage due to oxidation most often occurs at combined deficiency of dietary selenium and vitamin E (Gatlin et al., 1986; Hamre, 2011). In the present study, lysis of muscle cells and blood vessels may have been caused by oxidative stress combined with lowered vitamin E and Se to produce many small haemorrhages. Most of the spots may have been healed, while the remaining ones developed into larger spots by infiltration of virus and melanomacrophages as described by Bjørgen et al. (2015).

5. Conclusions

The hypothesis of the present study was that increasing growth rate during spring and early summer causes Atlantic salmon to have more oxidized tissues. The decrease in vitamin C and E concentrations and regulation of glutathione metabolism during spring and early summer, correlate with growth rate and indicate that the fish became oxidized in this period. The development of Astaxanthin and MDA concentrations do not directly support the hypothesis, but the absence of increase in astaxanthin concentration during spring, compared to a large increase in august, could be an indication of astaxanthin consumption due to oxidation. The concentration of micro-minerals in the liver (Se) and muscle (Mn, Zn and Cu) varied with season and over time, but to understand if it was a cause or consequence of growth linked oxidative metabolism needs further study. Cataract development seems to be correlated to the changes in antioxidant metabolism and possible growth linked oxidative stress in spring, while there are too few data to conclude on whether development of melanin spots is related to these

changes.

Redox regulation is very complex, and it can well be that some markers respond as expected, while there is lack of response in others, even if the hypothesis is valid. Growth has been connected to increased oxidation in many organisms, including fish, but further studies are needed to understand these interactions in detail in Atlantic salmon. Overall, the lowered vitamin C and E tissue concentrations during spring and summer support our hypothesis, while understanding the lack of expected changes in astaxanthin and MDA concentrations needs further research.

Author statement

Name of the author	Types of contribution
Kristin Hamre	Conceptualization, Funding aquisition, Project administration, Investigation, Data curation, Writing - original draft.
Giulia Micallef	Investigation, Methodology, Writing - first draft and review
Marie Hillestad	Investigation, Methodology, Writing - review
Johan Johansen	Investigation, Methodology, Writing - review
Sofie Remø	Investigation, Methodology, Writing - first draft and review
Wuxiao Zang	Investigation, Methodology, Writing - review
Elisabeth Ødegård	Investigation, Methodology, Writing - review
Pedro Araujo	Investigation, Methodology, Writing - first draft and review
Antony Philip	Investigation, Methodology, Writing - first draft and review
Rune Waagbø	Conceptualization, Project administration, Writing - review

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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