Rød laks – genetiske effekter (901642)

Faglig sluttrapport

Prosjektperiode: 08.05.2020 – 30.09.2022

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Oppdragsgiver:
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Stikkord:
Oppdrettslaks, filetfarge, genetikk, stress, *bco1-like*

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Hovedfunn:

- Knock-out (KO) - eksperimenter med CRISPR/CAS9 bekreftet at *bco1-like* er det genet som i størst grad påvirker filetfarge hos laks
- Analyser av genaktiviteten i KO-fiskene støtter hypotesen om at overgang til mer vegetabilske fett-kilder i fôret også påvirker astaxanthin-omsetningen
- Gjentatt håndteringsstress og hypoksi månedene før slakting førte ikke til tap av farge eller astaxanthin
- Laks som var utsatt for gjentatt stress og hypoksi hadde lavere kroppsvekt, lavere kondisjonsfaktor og dårligere hud- og øyehelse sammenlignet med laks som ikke var håndtert.

Prosjektpartnere:

Norges miljø- og biovitenskapelige universitet (NMBU)

AquaGen

Innhold

1.1 Sammendrag

Dårlig eller ujevn innfarging av pigment i laksefilet er en utfordring for norsk laksenæring. Økt tilsetning av pigment i fôret synes ikke å løse problemene. Tidligere studier har indikert at genetikk styrer så mye som 60% av variasjonen i filetfarge, men også fôrsammensetning og stress i forbindelse med håndtering antas å kunne påvirke dette. Målsettingen med dette prosjektet var å 1) bruke knock-out (KO) linjer av laks for å studere effekten av enkelt-kandidatgener på innfarging og identifisere metabolske nettverk som er påvirket av disse genene; 2) bruke selekterte avlslinjer av laks (rød/blek) i et sjøvannsforsøk for å kvantifisere opptak av pigment og undersøke hvorvidt gjentatt eksponering for hypoksi/stress påvirker innfarging. Forsøkene bekreftet at genet bco1-like trolig er det viktigste genet som styrer pigmentering. Genuttrykksanalyse avslørte også at astaxanthin-omsetningen og nettverket av underliggende gener er knyttet til kolesterol-byggende prosesser, vitamin D-syntese og fettomsetning. Med tanke på at vegetabilske olje-baserte dietter påvirker gener som kontrollerer lipid metabolisme, kan resultatene indikerer at overgangen fra marint til vegetabilsk fôr også kan ha påvirket astaxanthin-metabolismen.

Sjøvannsforsøket viste ingen tap av astaxanthin eller filetfarge etter gjentatt trenging og hypoksi i forkant av utslakting. Denne observasjonen gjaldt både rød og blek genetikk-linje. Fiskegrupper utsatt for stress hadde for øvrig lavere kroppsvekt og kondisjonsfaktor sammenlignet med ustressede kontroller. Fisk fra stress-gruppe hadde også redusert velferd i form av økt skjelltap, blødninger og ugjennomsiktig øyelinse. Den praktiske betydningen av resultatene kan være å belyse at gjentatt hypoksi/stress har en negativ effekt i produksjonssammenheng, men påvirker ikke pigmentering. Overlappende nettverk av gener som håndterer astaxanthin- og lipidmetabolisme understreker også viktigheten av ulike fettkilder i fôret for å oppnå tilfredsstillende pigmentering.

1.2 Summary

Weak or uneven colour distribution in salmon fillet is a problem for the Norwegian aquaculture industry. Increasing the amount of pigments added to the feed does not seem to solve this problem. Previous studies have indicated that genetics controls as much as 60% of the variation in fillet colour, but also the overall feed composition and stress during handling is believed to affect fillet colour. The aim of this project was to (i) use of knock out (KO) lines of salmon to investigate the effect of single candidate genes on pigmentation, and identify the metabolic networks affected by these genes and (ii) use selected lines of salmon (RED/PALE) in a seawater experiment to quantify uptake of pigment, and determine if repeated exposure of hypoxia/stress have an impact on pigmentation. Our experiments confirmed *bco1 like* as the most important gene for pigmentation. The gene expression analyses also revealed that astaxanthin metabolism and pathways of underlying genes are linked to

cholesterol biosynthetic processes, vitamin D synthesis and lipid metabolism. Knowing that vegetable oil-based diet does affect genes controlling lipid metabolism, our results may indicate that the change from fish-based oils to vegetable oils also have affected the astaxanthin metabolism.

The seawater experiment showed no loss of astaxanthin or fillet colour after repeated crowding and hypoxia prior to harvesting. This observation was true for salmon being from either the RED or PALE genetic lines. However, the 'stressed' fish had lower body weight and lower condition factor compared to non-stressed controls. Fish in the 'stress' group did also show lower welfare determined by increased scale loss, bleedings and eye lens opacity. The practical use of our results might be to highlight the negative impact of repeated hypoxia/stress in a production setting, although not related to pigmentation. The 'overlap' of networks handling astaxanthin metabolism and lipid metabolism also underscore the importance of the different fat sources in the feed in achieving satisfying pigmentation.

2. Introduction

Red flesh colour is the main quality advantage of salmon over other fish, and weak or uneven colour distribution is a problem for the Norwegian salmon industry. Salmon is not able to produce the pigment astaxanthin *in vivo*, so it must be supplied through the feed. The astaxanthin level in fillets of Norwegian farmed salmon was 7 mg/kg on average in 2005 [Mørkøre 2008], while today's farmed salmon often has an average content down to the lower limit value of 6mg/kg. We have also observed values as low as 4mg/kg. The fillet colour has become paler even though the feed's pigment level has increased from 35mg/kg in 2005 to an average level of 50-70 mg/kg in today's feed. Even diets with 100 mg/kg have been tested without solving the colour challenges.

Previous studies have shown that genetics controls as much as 60% of the variation in fillet colour. A targeted use of this genetic variation can improve the selection of fish with the preferred characteristics. This will give significantly faster progress than traditional family-based breeding. An essential aim of this project was therefore to use established knowledge of genetics that affect pigment uptake to expand knowledge of basic mechanisms that control uptake and retention of pigment. To achieve this, we have used two different fish materials with individuals that have had one of the three pigment-related genes *bco1*, *bco1-like* or *abcg2* inactivated (knocked out using CRISPR/CAS9) and two lines of salmon that were strongly selected for high and low fillet colour, respectively. By studying variation in gene expression between salmon with different genetic ability

to deposit pigment, we will gain valuable knowledge about factors of general importance for pigment uptake.

In recent years, the salmon's feed composition has changed radically in the direction of a more vegetable based ingredients. Several experiments have pointed to the risk of pale fillet colour when fish oil is replaced with high inclusion of rapeseed oil [Mørkøre, 2012; FHF#900653], but the mechanisms behind these observations have not been clarified. The combination of changed feed composition with lower bioavailability of pigment, and possible consumption of astaxanthin as an antioxidant during stress and diseases, are proposed as important reasons for reduced colouring of Norwegian salmon. It is therefore important to increase the knowledge about bioavailability and metabolism of astaxanthin and get an overview of factors that can contribute to better and more consistent pigmentation of salmon muscle.

Storage of astaxanthin in muscle starts in the freshwater phase, but first during the seawater phase the salmon become deep red. Problems related to stress during handling and illness are also mainly experienced during the seawater phase. Hypoxia during crowding or due to infections causes oxidative stress that is presumably counteracted by the antioxidant astaxanthin. By exposing salmon to repeated hypoxia, we wanted to estimate the effect of oxidative stress on fillet colour and pigment concentration in muscle of salmon with a wide range in colouration; I.e. salmon selected for extra redness and for paleness. This will provide quantitative measures of astaxanthin as an antioxidant in salmon muscle.

- Scope of project

Project period: 08.05.2020-30.09.2022.

The project has had a financial framework of NOK 4 010 000, NOK 3 360 000 to NMBU and NOK 650 000 to AquaGen (of which NOK 325,000 is own contribution).

- Project organisation (roles/responsibilities: project group, steering group, others?)

The project has been carried out in collaboration between NMBU and AquaGen. The project leader has been Prof. Dag Inge Våge (NMBU). Others involved from NMBU have been Prof. Turid Mørkøre. Project participants from AquaGen have been Dr. Jacob Seilø Torgersen and Dr. Fabian Grammes.

The reference group for the project has consisted of:

Magnus Åsli (Cermaq Norway), Karina Daae Nilsen (Skretting Norway), Hanne Morkemo (Norway Royal Salmon and later Columbi Salmon).

3. Problem statement and purpose

The fillet colour has become paler even though the feed's pigment level has increased from 35mg / kg in 2005 to an average level of 50-70mg / kg in today's feed for slaughter fish. The purpose of this project was to provide knowledge about basic mechanisms that control the uptake and retention of pigment – an understanding that is essential for the formulation of ingredients in a feed that gives salmon fillets stable colour intensity in accordance with market requirements. Furthermore, the project's results were to provide insight into how repeated oxidative stress affects colouring, and whether fish with favourable genetics for pigmentation were better protected against the negative effects of oxidative stress.

Main goal:

Ensure good fillet colour of Norwegian farmed salmon by describing and understanding genetic and molecular bottlenecks, and secondly understand the effects of stress during handling of salmon prior to harvesting.

Milestones:

A. Mapping genetic and metabolic bottlenecks for astaxanthin uptake in intestinal epithelium using established *abcg2*, *bco1*, and *bco1-like* CRISPR/CAS9 knock-out (KO) lines.

B. Produce quantitative data on astaxanthin uptake and degradation in intestinal epithelium from salmon with superior and standard genetics.

C. Investigate the significance of salmon genetics for pale and uneven colour of salmon fillets and link redness to crowding and oxidative stress.

4. Project realisation

This project was initiated to elucidate molecular control of astaxanthin metabolism and availability for muscle deposition, and investigate how a stressful farming environment may affect muscle redness.

Genetic selection for improved fillet colour is applied in all salmon breeding programmes, and AquaGen has been using marker assisted selection for muscle redness over several generations. In addition, we have created a unique red line for study purposes. Here, redness has been the only selection parameter and we see that muscle colour is getting close to what can be observed in Rainbow trout. In an RCN project (GeneInnovate) AquaGen has created three CRISPR/CAS9 knockout lines to study muscle redness. The target genes for KO were all underlying major muscle colour QTLs. The use of gene edited lines to study candidate gene function is currently the method of choice in model organisms and may also help the understanding of genotype – phenotype – environment interactions in Atlantic salmon. The KO lines used in this project was created using the best targeting CRISPR/CAS9 design identified in preliminary studies. In this FHF project we have carried out phenotype studies on the specimens featuring the highest possible knockout percentage. Compared to model organisms where KO phenotypes are studied in homozygous offspring, phenotypes in salmon are commonly carried out in the founder generation because of the long generation interval. This is of importance since gene edited founders display a mosaic appearance of the edit, meaning that some cells have been edited and others have a normal functional copy of the target gene (wild type). Thus, only specimens with a high percentage of edited cells should be included in downstream studies. Analysis of the KO phenotypes was carried out using RNA sequencing and image analysis of sectioned intestine and liver tissue. The transcriptome data from the KO fish have also been analysed together with data from salmon fed different diets and of different genetic background to elucidate how dietary composition affects astaxanthin metabolism.

5. Results achieved, discussion and conclusion

In this project two main research questions have been investigated. In the first part, we knocked out single candidate genes to observe the effect on fillet colour. How inactivation (knock out) of these genes affected the underlying metabolic networks were investigated by gene expression analyses (RNAseq). To achieve this, fertilised salmon eggs were gene-edited to make abcg2, bco1 and bco1-like knock out (KO) individuals, respectively. These individuals were raised in freshwater tanks (13 ^{o}C) at the Centre for Sustainable Aquaculture at NMBU and fed commercial feed containing astaxanthin. When the individuals reached approximately 200 g, they were slaughtered, and muscle samples were collected for measuring redness by colorimetric analyses. Samples were also taken from intestine, liver and muscle for gene expression analyses (RNAseq) and microscopy.

In the second part, a seawater experiment was conducted at LetSea, Dønna to evaluate the effect of repeated crowding and hypoxia on filet colour. Two different lines of salmon were used, one line selected for high pigment content and one line selected for low pigment content. The fish were kept from sea transfer until slaughter at approximately 4 kilos. The fish were fed standard commercial feed, with 40 or 50 mg/kg astaxanthin. During the last months before slaughter, fish were exposed to crowding and hypoxia 1, 2 or 3 times respectively, with approximately 1 month between the exposures. For the hypoxia test, fish were transferred to a tank with 200L seawater (ambient temperature, 90% oxygen saturation) without the supply of oxygen. The oxygen level was continuously monitored and ended when the oxygen level in the tank reached 35% saturation. The fish were then anaesthetised before length and weight were recorded, and then transferred back to sea.

5.1 Molecular mechanisms underlying salmon redness

5.1.1 Redness phenotypes and knockout efficacy

To assess the effect of CRISPR KO on salmon fillet colour (Figure 5.1.1), a total of 75 non-edited control fish were phenotyped and sampled for analysis (mean weight 202 g). For the KO lines, 116 *abcg2* (mean weight 249 g), 200 *bco1* (mean weight 189 g) and 187 *bco1 like* (mean weight 183 g) tentative KO fish were sampled. Mature dwarf males were excluded from further analysis. Since many of the potential KO fish are wild type because of mosaicism and potential mishaps in the microinjection, we chose to analyse a subset of fish from each group, the top 30 most intensely red in each line. As visualized in the plot, knockout of *abcg2*, and *bco1* have a modest but significant positive effect upon redness. Knockout of *bco1 like* however did have a dramatic effect upon muscle redness. Using DNA from the intestine and one primer spanning the Cas9 cut site, qPCR analysis revealed mean KO efficacy of 64, 59 and 91% for *abcg2*, *bco1* and *bco1 like*, respectively. Surprisingly, a subset of the specimens in the *bco1* KO lines showed redness values below the lowest control fish (data not shown).

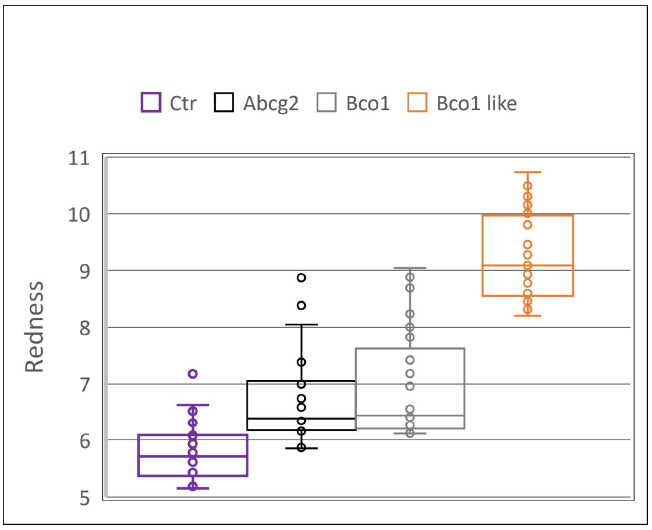


Figure 5.1.1. Muscle redness phenotypes (Minolta A values, Y axis) for the 30 specimens with most intense redness of each KO line compared to the most intensely red control fish. All KO lines were significantly more red compared to the control group, however, bco1 like KO resulted in a pronounced increase in muscle redness. All fish were raised in a similar environment, on a diet with the same astaxanthin content (70ppm) and have the same parents.

5.1.2 Cellular responses

The link between *abcg2* and lipid /cholesterol metabolism has been known and intensively studied for many years (Hegedüs et al., 2015). New findings on *bco* function also suggest that the encoded proteins are involved in hepatic lipid metabolism (Lim et al., 2018). Using LipidTox staining, formalin fixated intestinal and liver sections (300 µm) from the *abcg2 and bco1* KO lines were stained for lipid droplets (Figure 5.1.2). The *abcg2* KOs were also analysed for cholesterol content using Cholera toxin B subunit. Intestinal tissue lipid droplet analysis, using image segmentation and analysis, revealed that both *bco1* and *abcg2* KOs described statistically significant increase in lipid content and number of lipid droplets. Remarkably, there was no differences between specimens belonging to the *bco1* KOs showing stronger or paler colour versus all control fish. Cholesterol staining was conducted for the *abcg2* KOs only and verified the importance of Abcg2 for cholesterol export, thus more cholesterol was visible in the intestine of KO fish. In the intestinal villi, cholesterol was evident in enterocyte cytosol and also nucleus. And in the lipid droplets, cholesterol was mainly located at the surface.

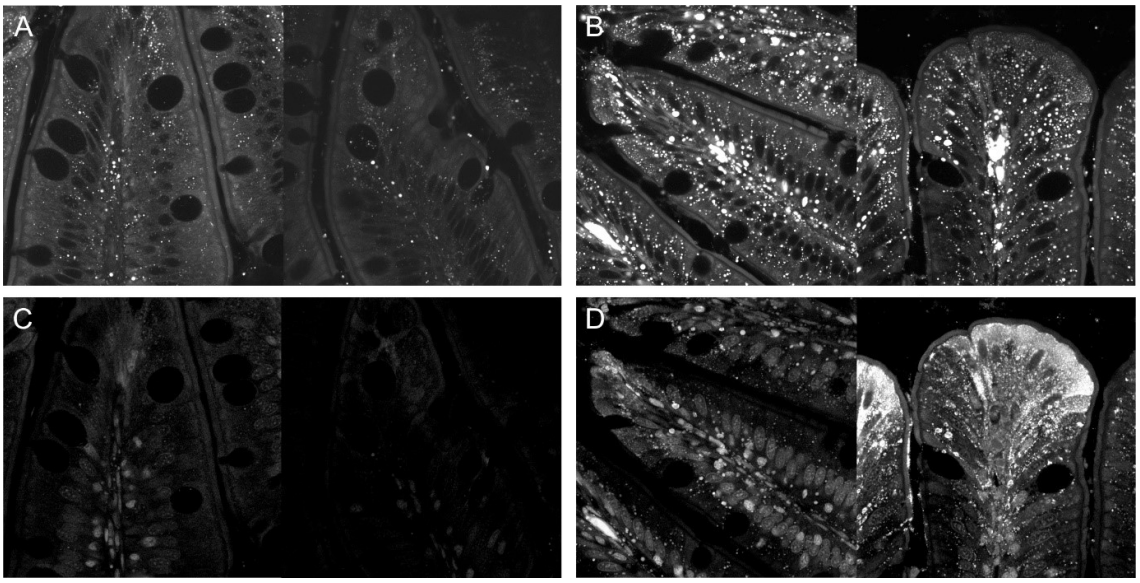


Figure 5.1.2. Lipid droplets (A and B) and cholesterol staining (C and D) in wild type and abcg2 KOs (16 g). Two images from representative specimens are shown for each image subset. A) Lipid droplets in the mid intestine of two control salmon describe very few lipid droplets compared to the abcg2 KO (B) and similarly less cholesterol in the enterocytes (C versus D). All images were captured using the same settings and post processed similarly.

In the liver, both *abcg2* and *bco1* highly red KO fish showed increased lipid contents (p=0.017), whereas the *bco1 KOs* with lower than average redness were not significantly different from the controls. The number of lipid droplets were also higher for the *abcg2* KOs, whereas *bco1* high were not statistically different. On the other hand, the pale *bco1* KO fish had significantly more lipid droplets. Median lipid droplet size was also found significantly larger for all the KO fish of all groups. Analysis of *abcg2* KOs was also carried out on 16 g salmon in a preliminary experiment and here even more striking differences in lipid load and intracellular cholesterol was evident compared to the larger ~200g fish.

5.1.3 Transcriptome analysis

To identify gene expression affected by CRISPR KO we carried out RNA sequencing and differential gene expression (DEG) analysis on specimens with the highest possible KO efficiency (Table 5.1.1). Gene edited specimens were compared to the control group grown up from the very same batch of eggs, whereas the specimens from the RED and PALE lines selected by marker assisted selection were compared to each other.

Table 5.1.1. Samples included in the DEG analysis comprise genotyped specimens from the three KO groups and salmon selected for presence or absence of the favourable QTLs. Wt: Non edited wild type specimens.

Sample	Tissue	N=	KO eff.
Ctr's	Intestine	5	-Wt
Ctr's	Liver	5	- Wt
abcg2 KO	Intestine	8	60-83%
abcg2 KO	Liver	8	60-83%
bco1 KO	Intestine	8	68-88%
bco1 KO	Liver	8	68-88%
bco1 low KO*	Intestine	8	68-88%
bco1 low KO*	Liver	8	68-88%
bco1 like KO	Intestine	8	86-100%
bco1 like KO	Liver	8	86-100%
Pale genetics	Intestine	8	- Wt
Pale genetics	Liver	8	- Wt
Red genetics	Intestine	8	- Wt
Red genetics	Liver	8	- Wt

abcg2 KO

The number of differentially expressed genes (DEGs) was most prominent in the intestine of *abcg2* KOs, describing 1726 genes with altered expression. Clustering network analysis revealed that the most significantly upregulated networks included cholesterol biosynthetic process, sterol biosynthetic process, and GTPase activity (Figure 5.1.3 and 5.1.4). There were four genes to be directly involved in carotenoid metabolism (logFold2 change 1.0-1.3; 1-*apoA1, carm1, jun and rdh12*).

Figure 5.1.3. Complete cluster of intestinal DEGs in abcg2 KOs. Coloured dots represent the individual genes, where different subnetworks are represented by their respective colours. The red and blue outlines around the genes corresponds to upregulation or downregulation, respectively. The more intense the outline colour, the stronger the up/downregulation.

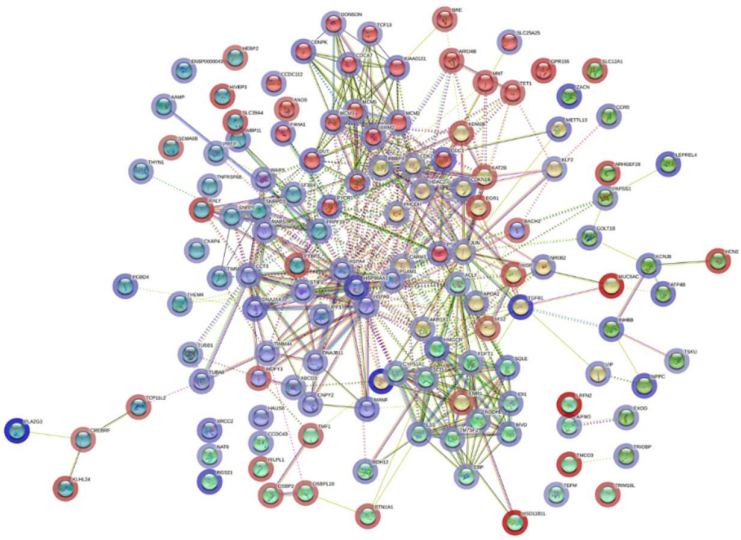
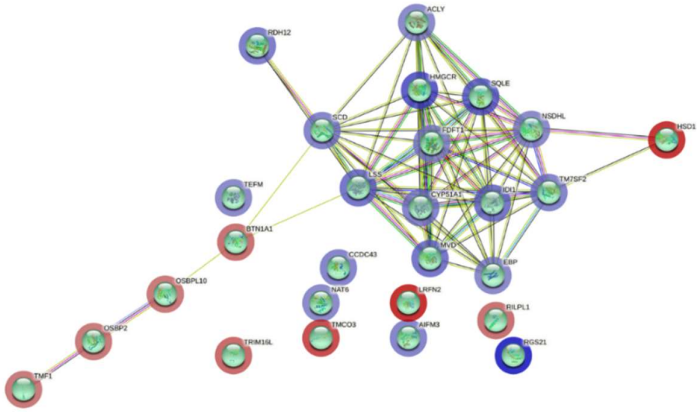


Figure 5.1.4. The intestinal cholesterol metabolism and steroid subnetwork in abcg2 KO is mainly upregulated, with two exceptions. Disconnected nodes correspond to unique DEG, but without a link to the cholesterol metabolism pathway.



In the liver of *abcg2* KO fish, many downregulated genes were involved vitamin D synthesis, such as positive regulation of vitamin D 24-hydroxylase activity, vitamin D3 metabolic process, and positive regulation of cholesterol efflux.

bco1 KO

In the intestine, there was no distinct differences between the intensely red and very pale *bco1* KO salmon. However, in the liver of the former group of specimens, 258 genes were differentially expressed versus the control samples. Upregulation of lipid metabolism (GO:0019216), downregulation of positive regulation of cellular process were detected, as well as genes involving response to hypoxia (Figure 5.1.5).

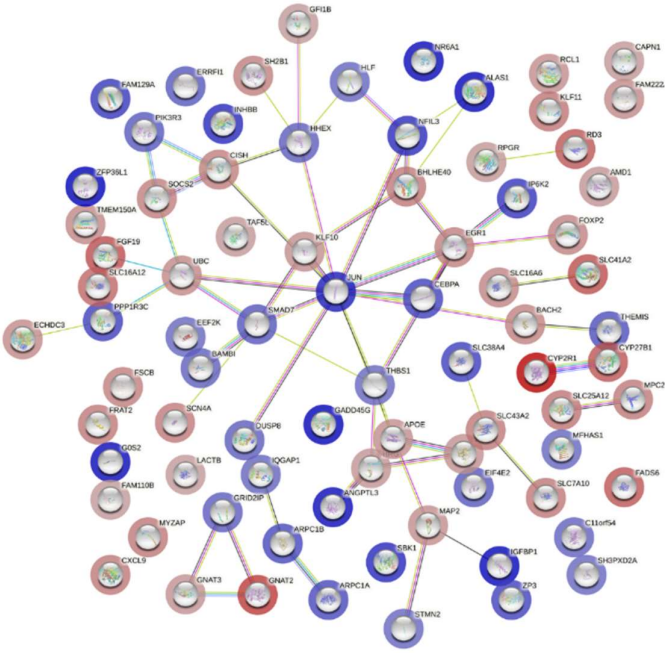


Figure 5.1.5. Cluster of differentially expressed genes in the liver of bco1 KOs. The coloured outlines around the genes tell whether the gene is upregulated or downregulated with red meaning downregulated and blue meaning upregulated. The more intense the colour, the strong the up/downregulation.

bco1 like KO and wild type lines selected for redness

In the intestine and liver of *bco1-like* KOs, very few DEGs were identified. This means that absence of the main astaxanthin cleaving enzyme does not affect expression of other genes, probably because retinol is provided in the diet (astaxanthin is a retinol precursor). Between the red and pale lines selected by marker assisted selection (QTLs), small changes to the transcriptome were identified in the liver (55 DEG), but no significantly enriched networks nor genes involved in the carotenoid metabolism were identified. In the intestine, 254 DEGs were identified. However, none of these genes could be linked to astaxanthin metabolism.

5.1.4 Conclusion

The results show that the bottleneck for a red salmon muscle is the intestine. The KO-experiments show that the gene *bco1 like* is a mjor controller of pigment uptake from the feed. Comparing gene activity between KO-fish and controls, differential expressed genes were related to cholesterol biosynthetic processes, vitamin D synthesis and lipid metabolism. Substitution of fish oil with vegetable oil-based diet does also affect genes controlling lipid metabolism. Increased amonts of vegetable oil in feed may therefore also affect astaxanthin uptake and deposition. We need more knowledge about how the differen lipid components in the feed affect pigment uptake.

5.2 Seawater experiment

5.2.1 Introduction

The red colour of farmed Atlantic salmon is characterized with deposition of carotenoids that originate from dietary supplemented pigments, as they cannot be synthesized *de novo*. In addition to dietary pigment concentration, there is a significant genetic component in variation in pigmentation and coloration. Thus, fish with improved pigmentation may be obtained through selective breeding (Gjedrem, 2000). Astaxanthin, the main pigment used in salmon diets, is a powerful antioxidant, hence it has been questioned whether the pigment is being utilized when salmon is exposed to stressful conditions. There is some evidence that stress during harvesting can have a negative impact on the fillet colour, but the knowledge about stress exposure during the farming phase is limited; including effects of crowding and hypoxia that may occur during for example delousing.

The aim of this experiment was to study effect of crowding and hypoxia stress during the harvesting phase on fillet pigmentation of two genetic lines being selected for low and high pigmentation - hence also facilitating the possibility to test interaction between pigment concentration in the flesh and robustness to stress and pigment losses.

5.2.2 The experiment

The use of experimental animals for this purpose was approved by Norwegian Food Safety Authority (FOTS ID 27541). The fish used were PIT-tagged Atlantic salmon (*Salmo salar* L.) smolts (120g) originating from two genetic lines (RED and PALE) from AquaGen's selective breeding program (Kyrkjesæterøra, Norway). The RED line was selected based exclusively on the flesh color intensity, whereas the PALE line was selected exclusively based on poor fillet pigmentation (termed PALE).

An equal number of salmon from each genetic line were distributed into two 125m^3 sea-cages equipped with feed collecting system (termed sea-cage 319 and sea-cage 419) in September 9th 2020 at LetSea aquaculture research facility in Dønna municipality, Northern Norway (Figure 5.2.1). The fish were kept in the sea-cages for 14 months and fed a standard commercial feed produced by Cargill (Ewos rapid, astaxanthin content 50 mg/kg). Uneaten pellets were collected and pumped up for quantification of feed intake and for adjustment of feeding rate. The amount of feed allocated to the sea-cages was adjusted to the appetite the day before.

Figure 5.2.1. LetSea Research station where the experiment was conducted in 125m^3 sea cages.

5.2.3 Stress test

The salmon in net pen number 419 were not exposed to crowding or hypoxia stress during the production phase, while salmon in net pen number 319 were netted and exposed to hypoxia stress either one, two or three times prior to harvesting November 14th – 15th 2021 (Table 2.1). Because the fish were PIT-tagged, it was possible to identify each individual fish.

The groups are referred to as P0, R0 (control fish, non-stressed), P1, R1 (stressed one time), P2, R2 (stressed two times), P3 and R3 (stressed three times), where P is the PALE genetic line and R is the RED genetic line. The fish were starved for three days before each stress exposure and before harvesting.

All the fish in sea cage 319 were crowded and 15 fish were transferred at a time to a tank with 200L seawater (ambient temperature, 90% oxygen saturation) without the supply of new seawater or oxygen. The oxygen level was continuously monitored (Handy Polaris 2, OxyGuard) and fish behaviour was registered (number of gasping, incidences of fish trying to jump out of the water) (Figure 5.2.2). When the oxygen level in the tank reached 35% saturation, after about 15 minutes, fish were transferred to a tank containing the anesthetic FINQUEL, trikainmesilat for about one minute, thereafter the anesthetized fish were registered for weight and length and transferred back to the sea-cage through a water-filled pipe.

The response to decreasing oxygen saturation showed similar responses between the repeated exposures, reaching the highest levels of air gasping, panic and flight responses at 40-50% oxygen saturation. At 35% oxygen saturation, air gasping and jumping out of the water were not visible.

Table 5.2.1. Design of the stress test

Stress 0 Sea-cage 319	Stress 1 August 2021 Sea-cage 419 2kg	Stress 2 Sept 2021 Sea-cage 419 3kg	Stress 3 Okotober 2021 Sea-cage 419 4kg
150 salmon	150 salmon stressed -75 RED line -75 PALE line 0 salmon not stressed	100 salmon stressed -50 RED genetic line -50 PALE genetic line 50 salmon not stressed	50 salmon stressed -25 RED genetic line -25 PALE genetic line 100 salmon not stressed
The design resulted in the following groups: Stress 0 25 RED (R0) + 25 PALE (P0) Stress 1 25 RED (R1) + 25 PALE (P1) Stress 2 25 RED (R2) + 25 PALE (P2) Stress 3 25 RED (R3) + 25 PALE (P3)			

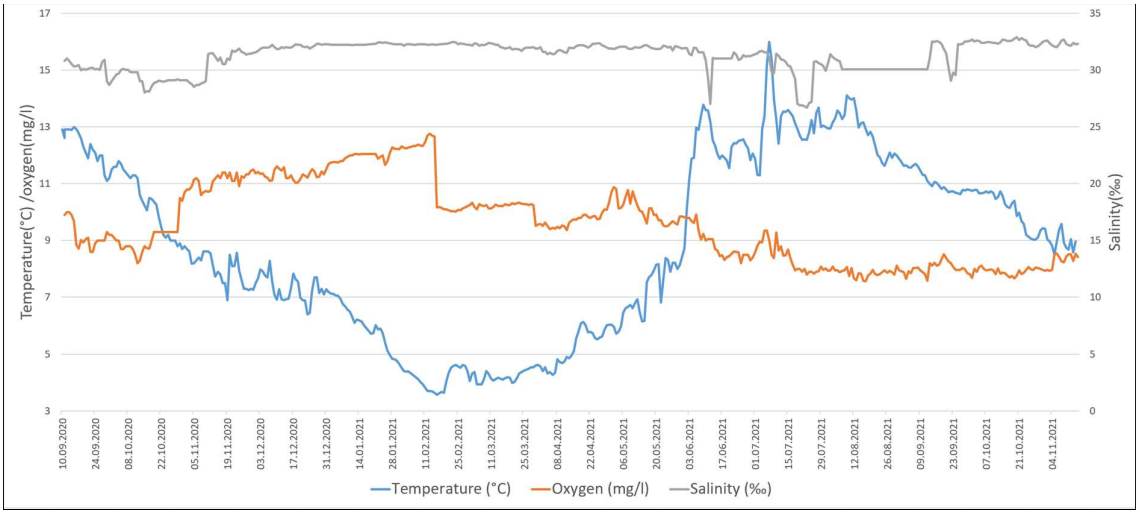
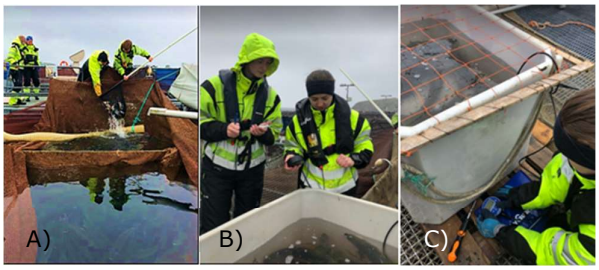
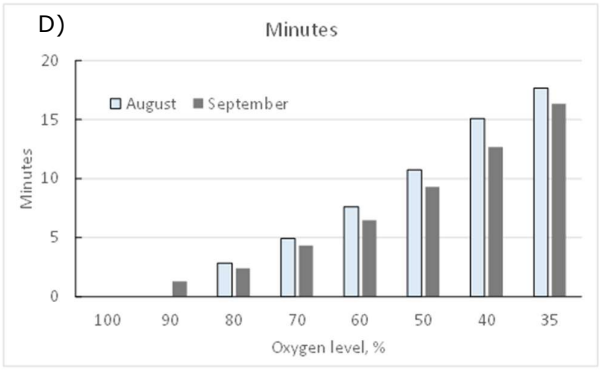
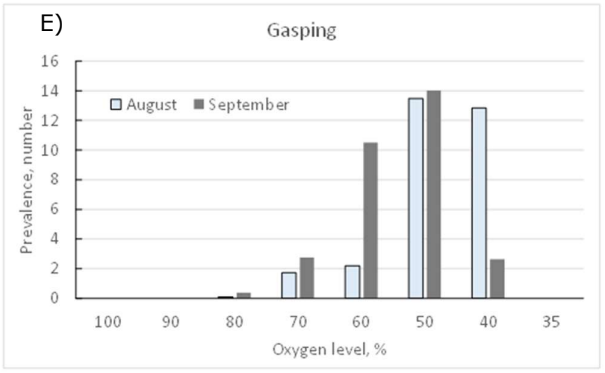


Figure 5.2.2. Average seawater temperature, oxygen level and salinity in measured in the sea-cages during the experimental period.







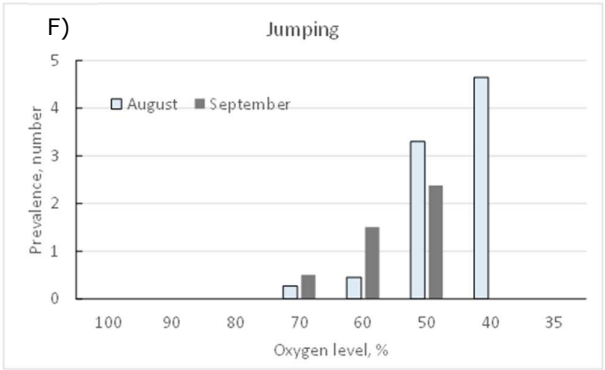


Figure 5.2.3. Fish were netted (A) three times (Aug., Sept. and Oct. 2021) prior to harvesting (Nov.) and transferred to a tank (200L seawater) without supply of oxygen (B). The oxygen level was continuously monitored (C-D) and frequency of air gasping (E) and jumping out of the water (F) were registered in Aug. and Sept. Fish were removed from the tank when the oxygen saturation reached 35%. 15 fish were kept in the tank at a time; n =10 in August (150 fish in total) and n = 7 in September (100 fish in total).

5.2.4 Harvesting and analyses

The fish were harvested November 14th and 15th 2021 by taking up fish randomly from the sea-cage, as genetic background and number of stress exposure were not possible to reveal from external appearance (ID linked to PIT-tag). The fish were anaesthetized to death using FINQUEL, trikainmesilat, thereafter both gill arches were cut, and the fish were bled out in a separate tank. PIT-tag, whole body weight and length were registered, in addition to eye lens opacity, before fish were photographed (left fish side) under standardized light condition for subsequent welfare scoring based on the images (scoring system mainly based on Nobel et al. 2018). Thereafter the fish were gutted, gender was determined, and organs scored for appearance: colour of the liver, visceral fat accumulation and visible fat deposits on the heart surface (Mørkøre et al. 2020). Weight of liver and hearts were recorded. The fish were filleted by hand, and the left fillet side was weighed and tagged, packed individually in plastic bags, stored on ice in Styrofoam boxes and transported the Norwegian University of Life Sciences (NMBU) for fillet quality analyses.

Colorimetric analyses were performed on skin and fillets using a Minolta chroma meter CR-400 (Minolta Sencing, INC Japan). Measurements of the skin were performed slightly above the lateral line anterior and posterior to the dorsal fin. Measurement on fillets were performed on the Norwegian quality cut. Sensory analyses of fillet colour were determined in a box with standardized light conditions, using a DSM SalmoFanTM (score 20-34). Gaping was scored according to (Andersen et al., 1994), and occurence and size of dark (melanin) spots were scored according to (Mørkøre et al. 2012).

Ten NQC cutlets from each group were homogenized, giving 80 samples in total for astaxanthin analysis in the muscle. The samples were frozen and sent to Nofima's BioLab in Bergen (Nofima, 2021; Zhou et al., 2011).

Statistical analyses and calculations

The model used for most of the measured and calculated values was

$$Y = stress + genetics + (stress * genetics) + gender + body\ weight + \varepsilon$$

Body weight and gender were removed from the model when non-significant.

Gutted yield was calculated from the body weight.

$$Gutted\ yield\ \% = \frac{Gutted\ weight}{body\ weight} * 100$$

Fillet yield was calculated using round weight and gutted weight.

$$Fillet\ yield\ of\ body\ weight\ \% = \frac{fillet\ weight}{body\ weight} * 100$$

$$Fillet\ yield\ of\ gutted\ weight\ \% = \frac{fillet\ weight}{gutted\ weight} * 100$$

Condition factor (CF) was calculated from the body weight in grams and length in cm.

$$Condition\ factor = \left(\frac{body\ weight}{lenght^3}\right) * 100$$

Cardio somatic index (CSI) was calculated from heart weight and body weight.

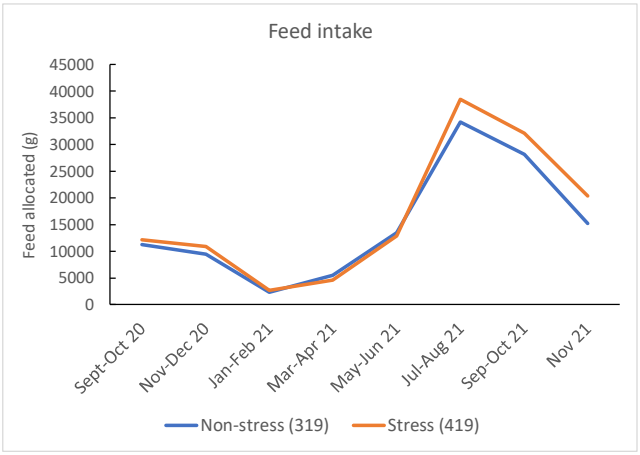
$$CSI\% = \frac{heart\ weight}{body\ weight} * 100$$

Statistical analyses were performed using SAS (statistical analysis software, version 9.4). The method of least squares to fit general linear models was used (the GLM procedure). This method includes analysis of variance, covariance, multivariate analysis of variance, partial correlation and regression (SAS, 2022). The significance level was set to $p \leq 0.05$.

5.2.5 Results and discussion

Production parameters

Feed intake was similar for the two net pens in the experiment from the point of sea-transfer (Sept 2020) until August 2021, at which time the fish were exposed to crowding and hypoxia stress for the first time. From August the feed intake of the stressed fish remained consistently higher until harvest November 2021. As the growth rate (TGC) was estimated relative to the feed allocated (appetite), TGC was also estimated to be slightly higher for the salmon exposed to stress (Figure 5.2.4). However, crowding and hypoxia stress tended to result in lower body weight at slaughter (P=0.10) as shown in Figure 5.2.5. Because all fish exposed to stress were kept together in the same sea-cage, it is not possible to calculate feed conversion ratio (FCR) for each of the groups, but the results indicate overestimating of the growth rate and that stress did not result in loss of appetite, but rather reduced feed utilization.



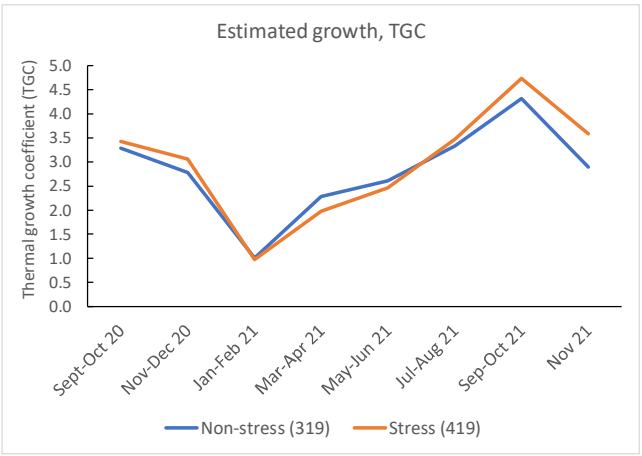


Figure 5.2.4. Amount of feed allocated according to feed eaten (A) and estimated growth rate (Thermal growth coefficient, TGC) (B) of salmon in the two net pens in the experiment.

Biometric traits

The body weight of the fish groups ranged from 3.65 – 4.36 kg on average (Figure 5.2.5). The body weight of the PALE genetic line was higher compared to the RED genetic line ($p = 0.04$). Stress did not have an overall effect on the body weight, although repeated stress exposure tended to reduce the overall fish growth ($p = 0.10$). Gutted weight and fillet weight showed a similar pattern as the whole-body weight. Interestingly, the fork length (range 62.5 - 64.7 cm) was higher of the RED genetic line compared with the PALE genetic line ($p < 0.0001$), thus indicating that exclusive selection for flesh coloration also altered the tissue growth pattern significantly. Whereas the salmon belonging the RED genetic line prioritized growth in skeletal length, muscle growth was prioritized by salmon belonging to the PALE genetic line. These different strategies resulted in a higher condition factor ($p < 0.0001$) and higher fillet yield ($p < 0.0001$) of the PALE genetic line (Figure 5.2.5 and 2.6). The gutted yield,

however, did not show a consistent variation pattern between the genetic lines, indicating that the relative amount of viscera was similar for the RED and PALE genetic lines.

Stress exposure did not affect the slaughter yield negatively, except that salmon from the RED genetic line exposed to 3X stress exposure, tended to have lower gutted yield (i.e. higher amount of viscera). Stress exposure resulted in a slightly decreased condition factor when considering the overall fish material (1.47 vs. 1.53; p = 0.05), but the fillet yield was not negatively affected by stress exposure; in fact the RED genetic line not exposed to stress had lowest fillet yield.

The relative liver weight (hepatosomatic index, HSI%) averaged 1.2% with no significant difference between the groups, but the relative heart weight (cardiosomatic index, CSI%) was consistently higher of the salmon belonging to the RED genetic line (CSI 0.14%) compared with the PALE genetic line (CSI% 1.137 vs. 0.118) (p < 0.0001). The CSI% was similar among the stress groups within each of the genetic lines.

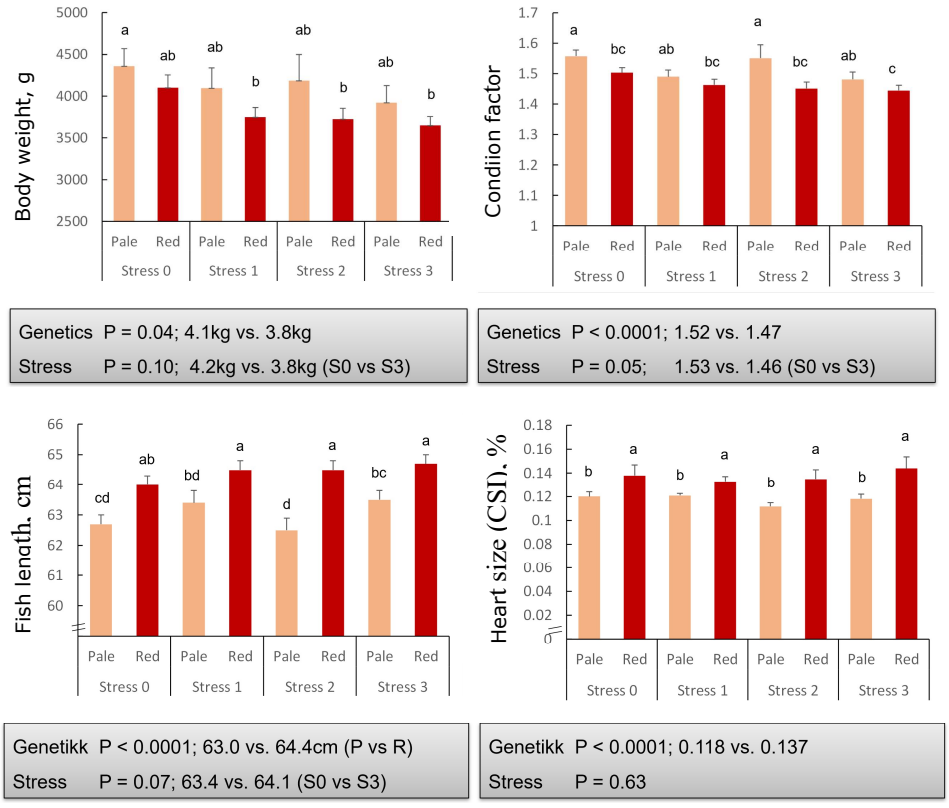


Figure 5.2.5. Whole body weight, fork length, condition factor and cardio somatic index of Atlantic salmon from two genetic lines (RED and PALE), exposed to crowding and hypoxia ("stress") 0, 1, 2 or 3 times prior to harvesting. Different letters above the standard error bars (SE) represent significant differences between groups (p < 0.05).

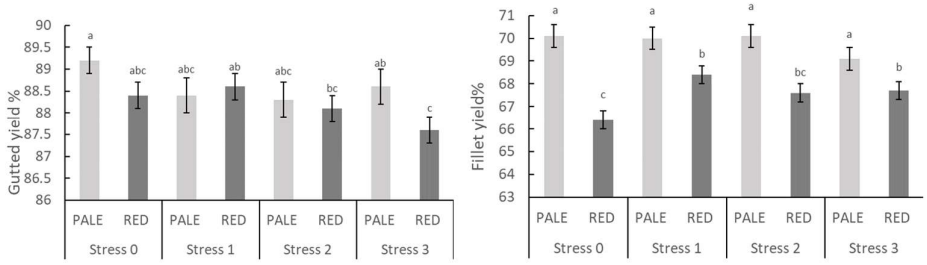


Figure 5.2.6. Gutted yield and fillet yield of Atlantic salmon from two genetic lines (RED and PALE), exposed to crowding and hypoxia ("stress") 0, 1, 2 or 3 times prior to harvesting. Different letters above the standard error bars (SE) represent significant differences between groups (p < 0.05).

Operational welfare indicators

The operational welfare indicators were similar for the genetic lines, but stress resulted in increased occurrence of scale loss, skin bleeding, eye bleeding and eye lens opacity (Figure 5.2.7). Colorimetric measurements of the skin showed that stress altered the skin colour. Salmon exposed to repeating crowding and hypoxia had darker skin that was less bluish/ more yellowish compared with fish that were not handled during the farming phase (Figure 5.2.8).

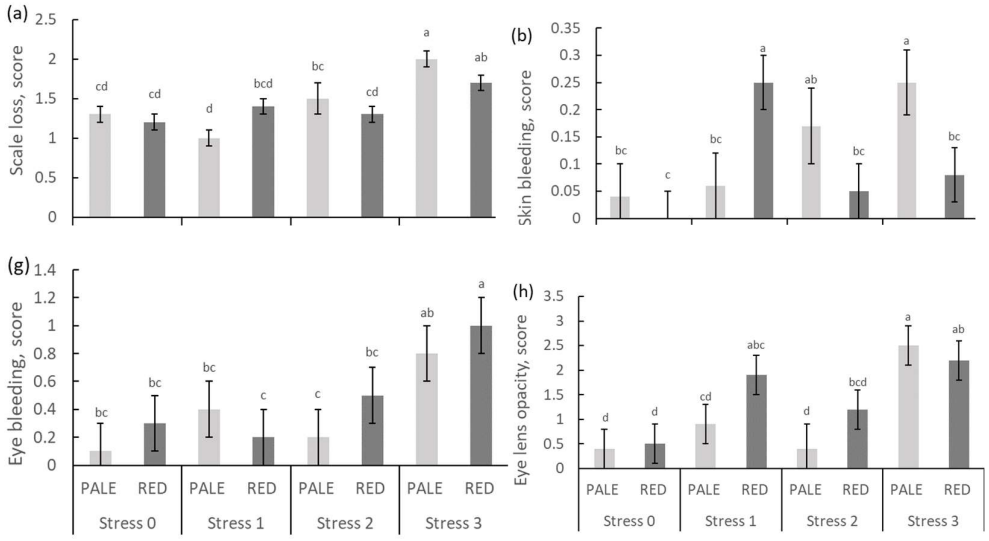


Figure 5.2.7. Operational welfare indicators (OWI) for scale loss (a), skin bleeding (b), eye bleeding (g) scored (0-3) and eye lens opacity (h) of Atlantic salmon from two genetic lines (RED and PALE), exposed to crowding and hypoxia ("stress") 0, 1, 2 or 3 times prior to harvesting. Different letters above the standard error bars (SE) represent significant differences between groups (p < 0.05).

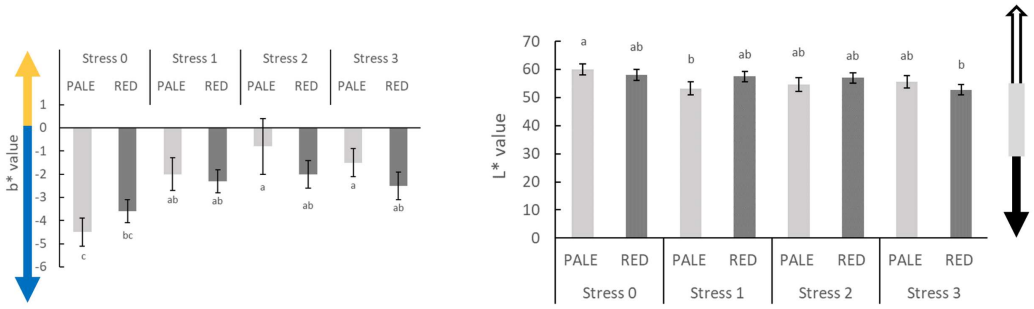


Figure 5.2.8. Colorimetric measurements of blue/yellow colour (b value) (A) and lightness/darkness of the skin of Atlantic salmon from two genetic lines (RED and PALE), exposed to crowding and hypoxia ("stress") 0, 1, 2 or 3 times prior to harvesting. Different letters above the standard error bars (SE) represent significant differences between groups (p < 0.05).*

Fillet colour and pigmentation

The astaxanthin concentration was significantly higher of the RED genetic line compared with the PALE genetic line ($p < 0.0001$) (Figure 5.2.9). The RED line had an average pigment concentration of 7.7mg/kg that is higher than generally found in Norwegian farmed salmon. The average concentration of astaxanthin in the muscle of the PALE genetic line was 4.3mg/kg, that is below the average values found in farmed Norwegian salmon. These results are interesting, pointing out the importance of the genetic background of the fish; i.e. at the same dietary pigment level, the RED line had a retention of pigments that was almost twice as high as that found in the PALE genetic line.

No reduction in pigment concentration was found in any of the groups due to stress exposure. These results are to some extent surprising, as we were expecting that crowding and hypoxia stress would result in deteriorated pigmentation. The results strongly indicate that additional factors than crowding and hypoxia stress should be explored with regards to problematic pale colour of salmon fillet.

The visual colour of the NQC corresponded with a SalmoColour score of 25.2 and 26.9 for the PALE and RED genetic lines, respectively. The visual colour intensity was slightly lower of the dorsal fillet part (loin) as expected, but the variation between the experimental groups was similar of the loin and NQC (Figure 5.2.10). It is however interesting that the visual colour score showed a higher variation among the groups than the pigment concentration, showing lower levels of the RED line that was not exposed to stress. Altered visual colour at similar pigment content in the muscle has been associated with altered light scattering reflection due to differences in protein structure, as in salmon fed krill meal (Mørkøre et al., 2020) and increased EPA and DHA levels (Lufti et al., 2022). Hence it is possible that muscle structural differences influenced the visual colour impression of the salmon belonging to the unstressed RED genetic line so that it appeared paler, although the pigment level was high. There

is evidence that fast growth can lead to altered muscle structure (softer fillets) (Mørkøre and Rørvik 2001). The RED genetic line, not exposed to stress had the highest numerical body weight among groups of the RED genetic line. However, our data cannot confirm such a relationship.

Instrumental colour analyses of fillet redness (a* value) were close to identical to the results on astaxanthin concentration (correlation r = 0.97), but the overall correlation between the a* value and visual SalmoFan score was also high (r = 0.84). The a* (redness) and b* value (yellowness) showed a similar variation pattern among the groups, but the instrumental lightness (L*-value) did not show an overall difference between the genetic lines (P=0.08).

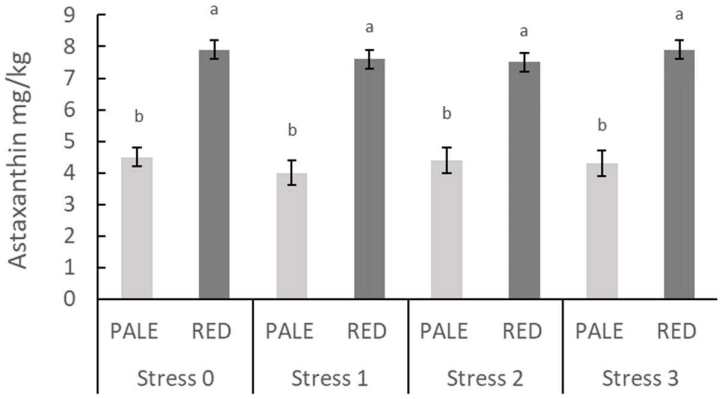


Figure 5.2.9. Astaxanthin concentration in the muscle (NQC) of Atlantic salmon from two genetic lines (RED and PALE), exposed to crowding and hypoxia ("stress") 0, 1, 2 or 3 times prior to harvesting. Different letters above the standard error bars (SE) represent significant differences between groups (p < 0.05).

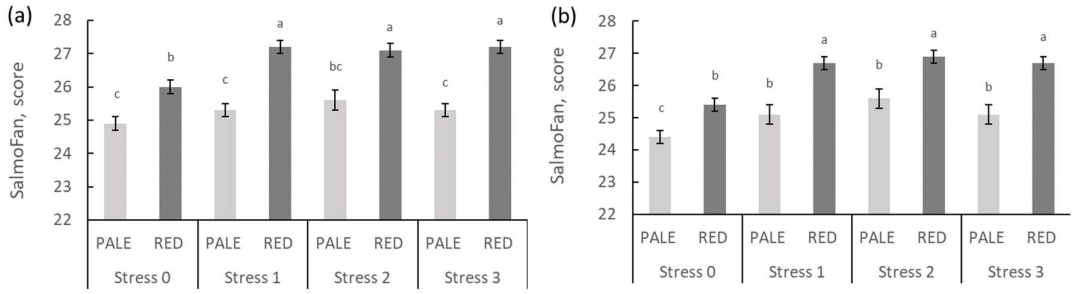
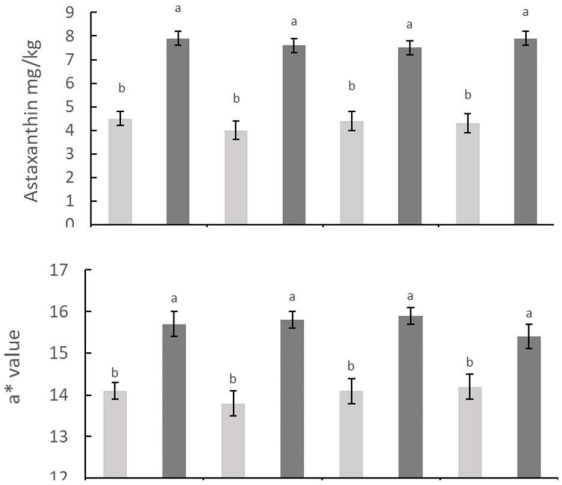


Figure 5.2.10. Visual color (SalmoFan score) of the NQC cutle (a) and the dorsal fillet part (loin) (b) of Atlantic salmon from two genetic lines (RED and PALE), exposed to crowding and hypoxia ("stress") 0, 1, 2 or 3 times prior to harvesting. Different letters above the standard error bars (SE) represent significant differences between groups (p < 0.05).

Figure 5.2.11. Picture illustrating the color of fillets from the PALE and RED genetic lines.



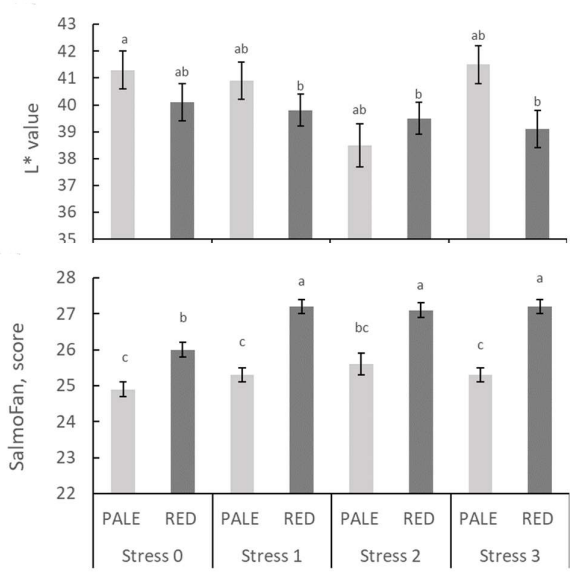


Figure 5.2.12. Different determination of the fillet colour of Atlantic salmon from the different genetic and stress groups: chemical analyses, instrumental analyses (a and L* values) and visual assessment (SalmoFan score). Different letters above the standard error bar (SE) represent significant differences between groups (p<0.05).*

The concentration of astaxanthin in the muscle of the PALE genetic line ranged from 2.9-6.1mg/kg whereas the astaxanthin concentration in the muscle of the RED line ranged from 6.0-10.1mg/kg (Figure 5.2.13).

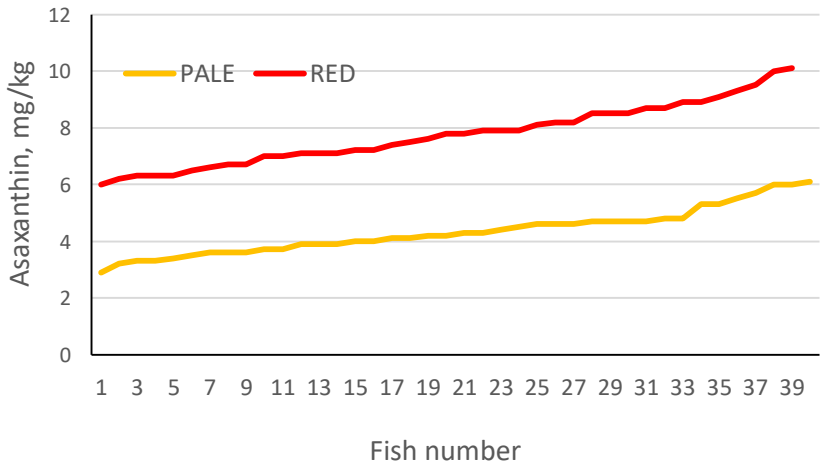


Figure 5.2.13. Astaxanthin concentration in muscle of individual fish of the PALE and RED line (n=80 in total).

5.2.6 Summary of seawater experiment

The total range in astaxanthin was 2.9-10.1mg/kg with an average of 4.3 and 7.7mg/kg for the PALE and RED genetic line, respectively. Hence, the fish material constituted an excellent population for

studying the effect of crowding and hypoxia stress on the pigment level and colour of salmon fillets, as well as possible interaction between fillet pigmentation and robustness to stress.

Exposing salmon to repeated crowding and hypoxia prior to harvesting did not negatively affect the fillet pigmentation or visual colour. Exposure to stress resulted in lower body weight at slaughter, lower condition factor, increased incidence of skin bleeding and scale loss, darker skin and higher score for eye bleedings and eye lens opacity. The operational welfare indicators (OWIs) were similar for the PALE and RED genetic lines and astaxanthin concentration in muscle did not correlate with any of the OWIs.

5.2.6 Conclusion

The experiment with large salmon in seawater showed no correlation between stressful farming conditions (handling and hypoxia) and pale muscle colour. In contrast, we found large color differences between salmon selected for high or low red colour. The experiments show that the fish's genetics are crucial for good colouring, and that we need more knowledge about how fatty substances in the feed affect the uptake and turnover of astaxanthin.

6. Main results

Three to five bullet points summing up main results in “Results achieved”.

Norsk:

- Knock-out (KO) -eksperimenter med CRISPR/CAS9 bekreftet at *bco1-like* er det genet som i størst grad påvirker filetfarge hos laks.
- Analyser av genaktiviteten i KO-fiskene støtter hypotesen om at overgang til mer vegetabilske fett-kilder i fôret også påvirker astaxanthin-omsetningen.
- Individer utsatt for gjentatt håndterings-stress og hypoksi viste ingen forskjell i filetfarge eller pigmentkonsentrasjon sammenlignet med ikke-stressede kontroller.
- Fisk utsatt for gjentatt stress og hypoksi hadde lavere kroppsvekt, lavere kondisjonsfaktor og dårligere hud- og øyehelse sammenlignet med ikke-stressede kontroller.

English:

- Knock out experiments using CRISPR/CAS9 confirmed that the *bco1-like* is the main causative gene underlying fillet colour.
- Gene expression analyses in the knock-out (KO) fish support the hypothesis that the increased use og vegetable oils in the feed also affect the astaxanthin metabolism.
- Fish exposed to repeated stress and hypoxia did not show any difference in fillet colour or pigment concentration compared to non-stressed controls.
- Fish exposed to repeated stress and hypoxia had lower body weight, lower condition factor and poorer skin and eye health compared to non-stressed controls.

7. Deliverables

Detailed overview over deliverables in the project.

Laksekjøttet blir bleikere. Nå skal de finne ut hvorfor. Intervju med prosjektdeltaker Jacob Seilø Torgersen (AquaGen) i Tekfish, 3. august 2020.

https://www.tekfisk.no/havbruk/laksekjottet-blir-bleikere-na-skal-de-finne-ut-hvorfor-/2-1-849390

AquaGen forsker på rødfargen til laksen. Nyhetsoppslag fra NCE Aquatech Cluster, 4. august 2020.

https://aquatechcluster.no/2020/08/aquagen-forsker-pa-rodfargen-til-laksen/

Aquagen og NMBU skal finne ut hvorfor laksekjøttet blir bleikere. Artikkel på «intrafish.no», 28. juli 2020.

https://www.intrafish.no/nyheter/aquagen-og-nmbu-skal-finne-ut-hvorfor-laksekjottet-blir-bleikere/2-1-848264

Two new projects on pigmentation in salmon, Fish Information and Services, 17. August 2020.

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Genetics to improve salmon pigmentation. Oppslag på "hatcheryfm.com", 20. august 2020.

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Norwegen: Projekte zur besseren Pigmentierung von Lachs. Oppslag i «fischmagazin.de», 20. august 2020.

https://www.fischmagazin.de/newsartikel-seriennummer-6057-Norwegen+Projekte+zur+besseren+Pigmentierung+von+Lachs.htm

Torgersen JS. Red salmon – genetic effects. Frisk fisk 2022 presentation.

Torgersen JS. Red salmon – genetic effects. Havbruk 2022 presentation.

Master theses:

Brandi Nicole Kuhn (2022) Phenotypic effects of knocking out the pigmentation related genes, abcg2, bco1, and bco1-like in Atlantic salmon. Master thesis NMBU, 2022.

https://nmbu.brage.unit.no/nmbu-xmlui/handle/11250/3012311

Marte Røsvik (2022) Fish welfare and color of skin and fillets of Atlantic Salmon from two genetic lines exposed to repeated hypoxia prior to harvesting. Master thesis NMBU, 2022.

Related master-thesis, not directly part of current project:

Johanna Henny Wagnerberger (2020) CRISPR based functional characterization of the abcg2b gene contribution to muscle pigmentation in Atlantic salmon. Master thesis NMBU, 2020.

https://nmbu.brage.unit.no/nmbu-xmlui/handle/11250/2726396

Not yet finalised deliverables:

Popular science papers:

Rød eller blek laks - betydningen av genetikk og miljø. *In preparation.*

Scientific papers:

- Abcg2 and intestinal carotenoid uptake in Atlantic salmon. *In preparation.*

- Genetic mechanisms controlling the red muscle of Atlantic salmon. *In preparation.*

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