Norges miljø- og biovitenskapelige universitet
Faculty of Biosciences
Institute of Animal- and Aquaculture Sciences

2023
ISBN:
978-82-575-2150-9

Project report

Skin detachment from salmon and rainbow trout

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Technical report: Skin detachment from farmed salmon and rainbow trout / Kartlegging av årsaker til at skinn løsner fra laks og regnbueørret	*Report number* *Availability* *Open*
Authors Turid Mørkøre, Lene Sveen, Eunice E. Boahemaa-Kobil, Thomas Larsson, Arnaud Lefrancois, Bjørn Roth, Maria B. Rojo, Timotè Moinhos, Izumi Sone, Marte Røsvik, Julia Formanowicz, Helena M. M. Conde	*Date* 31/12-2023
Faculty of Biosciences Department of Animal and Aquacultural Sciences	*Pages* 42
Client: FHF – Norwegian Seafood Research Fund Fiskeri- og havbruksnæringens forskningsfinansiering	*Client reference number* 901737
Key words Fish skin, loose skin, farmed salmon, rainbow trout, welfare, stress, feed, quality	*NMBU project number* 3551000113

Oppsummering:
En spørreundersøkelse blant aktører i oppdrettsnæringen viste at problemer med skinn som løsner fra laks- og ørretmuskel oppstod i 2019. Problemene oppdages typisk etter lagring, med indikasjoner på høyere forekomst i slutten av vår-sommer på Sørvestlandet, og sensommer- høst i Nordlige regioner. Problemene synes å være forbigående, det vil si at huden fester seg igjen etter en periode. Stressforebygging er avgjørende for fiskevelferden, også gitt at underhudsfettet viste seg å kollapse og bli flytende da skinnet ble utsatt for trykk. I samtlige tilfeller av løst skinn ble en blank væske funnet mellom skinn og muskel, hovedsakelig bestående av fett (69%), med høyere innhold av 18:1n-9 og 18:2n-6 (typiske for planteoljer), og lavere innhold av 22:6n-3 (typisk for fiskeolje), enn i filét. Også sammensetningen av aminosyrer varierte, noe som indikerer selektiv lekkasje. Vi fant overraskende store forskjeller i kollagenet mellom laks og regnbueørret, men vi klarte ikke å skaffe regnbueørret med løst skinn. Laks med løst skinn hadde ikke økt aktivitet av nedbrytende enzymer, men høyere grad av harskning (lipidperoksydasjon). Laks med løst skinn hadde gjennomgående mye filetspalting og bløt muskel, som også hadde høyere grad av harskning, selv om fisken var nyslaktet. Mikroskopering viste unormalt utseende av muskelcellene, men ikke for bindevevstrukturen i laks med løst skinn. Genanalyser (transkriptom) viste tydelige endringer, spesielt knyttet til betennelsesprosesser i muskelvevet hos laks med løst skinn. Konklusjonen er at problemet med løst skinn oppstår mens fisken lever, at det er koblet til bløt muskel, og at det oppdages en viss tid etter slakting. I tillegg til kunnskap om løst skinn, ga prosjektet også nyttig kunnskap som kan benyttes i fremtidig forskning på bløt tekstur og filetspalting.

Summary:
A survey aimed at relevant stakeholders highlighted loose skin as a quality concern since 2019. The issue is typically detected after storage, with indications of higher prevalence during late spring-summer in Southern-West regions, while during late summer-autumn in Northern regions. The issue seems transient, i.e. skin seems to reattach after a period. Industry statistics and experiments found no correlation between common delousing methods and loose skin. Stress prevention is crucial for fish welfare, also given the observed vulnerability of the adipose tissue under the skin to collapsing under mechanical pressure. In each case of loose skin, a glossy liquid was found between the skin and muscle, being mainly composed of fat (69%), with a higher content of 18:1n-9 and 18:2n-6 (found in plant oils), and a lower content of 22:6n-3 (found in fish oil), compared with the skeletal muscle. Also, the amino acid composition varied, indicating selective leakage. We were unable to obtain rainbow trout with loose skin, but surprisingly large differences in collagen composition were found between the species. No indications of increased activity of degrading enzymes were detected, but the extent of lipid peroxidation was higher in salmon with loose skin compared with salmon with reattached skin, as well as in soft muscle in contrast to firm muscle. Microscopic examinations did not reveal any explicit signs of abnormal connective tissue structure in salmon with loose skin. Instead, variations in the morphology of myofibers were observed. Further, transcriptomics revealed substantial changes in the muscle transcriptome, including a large array of inflammatory genes in fish with loose skin. It is concluded that the issue of loose skin arises while the fish is alive, is associated with soft muscle, and becomes apparent after a certain period post-slaughter. In addition to novel knowledge on loose skin, the project provided useful knowledge that can be utilized in future research on soft texture and fillet gaping.

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HOVEDFUNN

- Problemet med løst skinn oppstår mens fisken er i live, men det antas at ugunstige forhold under slakting, lagring og prosessering (slik som «descaling») kan forverre problemet.
- Problemet oppdages etter slakting, ofte etter flere dagers lagring, og er sammenfallende med bløt filet og filetspalting.
- Oppdrettslaks er mer utsatt for å få løst skinn til visse årstider, avhengig av anleggenes geografiske beliggenhet
- Løst skinn synes å være midlertidig – skinnet fester seg etter en viss tid
- Laks med løst skinn er kjennetegnet ved en blank fettrik væske under skinnet med høyt innhold av typiske planteoljer – høyere enn i muskelen.
- Muskelen i laks med løst skinn har økt grad av harskning, avvikende utseende av muskelcellene, noe endret sammensetning av bindevevet og antydning til pågående betennelsesprosesser.
- Prosjektet gav også nyttig kunnskap som kan benyttes i fremtidig forskning på bløt tekstur og spalting i filet.

MAJOR FINDINGS

- The issue of loose skin arises while the fish is alive, but adverse conditions during slaughter, storage and processing (such as “descaling”) are believed to exacerbate the problem.
- The problem is detected after slaughter, often several days into storage, and coincides with soft fillet texture and gaping.
- Farmed salmon is more susceptible to developing loose skin during specific seasons, depending on the geographical location of the farm.
- Loose skin appears to be temporary – the skin reattaches after a certain period.
- Salmon with loose skin is characterized by a glossy, fatty liquid under the skin with a high content of typical plant oils – higher than in the muscle.
- The muscle in salmon with loose skin shows increased levels of rancidity, altered muscle cells morphology, slightly changed composition of the connective tissue of the muscle, and indications of ongoing inflammatory processes.
- The project provided also useful knowledge that can be utilized in future research on soft texture and fillet gaping.

Norsk sammendrag

Skinn som løsner fra fileten er et problem som kjøpere av oppdrettslaks og ørret rapporterer om. Siden det mangler kunnskap om omfang og årsaksforhold, tok prosjektet en bred tilnærming. Målet var å finne kjennetegn ved fisk med løst skinn, kartlegge omfanget, og vurdere eventuelle sesongmessige og geografiske variasjoner. Prosjektet ønsket også å undersøke om problemet skyldes forhold hos den levende fisken eller om det er knyttet til oppdrettspraksis, slakting eller lagringsbetingelser.

Fiskematerialene som ble undersøkt hadde bred bakgrunn – takket være velvillig deling og samarbeid med næringen. Vi fikk også tilgang på tilpasset, relevant statistikk og vi gjennomførte en spørreundersøkelse blant næringsaktører. Vi lyktes ikke å få tak i regnbueørret med løst skinn.

Spørreundersøkelsen viste at løst skinn ble anerkjent som et kvalitetsproblem i 2019, og blir typisk oppdaget etter flere dagers lagring. Det betyr at problemet har oppstått nylig og at det vanskelig å oppdage etter slakting. En tredjedel av de spurte rapporterte om problemer med løst skinn.

Sesongvariasjoner: Statistikk som bygger på reklamasjoner fra oppdrettsområder på Sørvestlandet viste at mer enn 90% av tilfellene skjedde i perioden fra juni til august, uten noen rapporterte tilfeller fra september til februar. I nord observerte vi problemet sent på sommeren og tidlig på høsten. Problemer med løst skinn er vanligvis midlertidige, og vi registrerte at skinnet hadde festet seg etter tre måneder. De utfordrende periodene sammenfaller med tider med rask vekst og i alle de undersøkte tilfellene, hadde laks med løst skinn også bløt muskel og uttalt filetspalting.

Avlusing/ handtering: Statistikk fra bransjen viste ingen sammenheng mellom løst skinn og antall avlusinger med standard avlusingsmetoder. Forsøk i prosjektet viste heller ingen effekt av pumping eller oppbevaring av fisk ved lav (RSW) eller høy temperatur (simulert termisk avlusing). Resultater fra modellstudier (mikroskop) viste imidlertid at underhudsfettet hadde lett for å kollapse og bli flytende når det ble utsatt for trykk. Derfor er det viktig å unngå at fisken utsettes for slag og klemming, både av hensyn til dyrevelferden og for å beskytte skinnet og underhudsfettet.

Egenskapene til laks med løst skinn ble nøye studert ved å analysere flere partier med laks. Resultatene viste at laks med løst skinn hadde varierende mengder blank væske mellom skinn og muskel. Denne væsken besto hovedsakelig av fett (69%), med mer av fettsyrer fra planteoljer (18:1n-9 og 18:2n-6), men mindre av typiske marine fettsyrer (spesielt 22:6n-3), enn muskelen. Noen aminosyrer (histidin, arginin, metionin, glysin) var høyere i væsken enn i muskelen. Resultatene tyder på selektiv lekkasje fra muskelen. Histidin i hel muskel var koblet til filetspalting, også i fileter uten løst skinn.

Bindevevets aminosyrer hos laks med løst skinn var annerledes enn hos laks der skinnet var godt festet til muskelen (glysin, metionin, leucin, lysin, arginin). Siden laks med løst skinn også hadde bløt filet, ble kollagenet i fast og bløt muskel sammenlignet, og der fant vi forskjeller i aminosyrene treonin, serin, glysin, alanin, valin, tyrosin og hydroksylysin. Kollagenstabiliteten i bløt muskel var lavere enn i fast muskel. Vi fant uventet stor forskjell i bindevevssammensetningen i muskelen mellom laks og ørret.

Enzymer som bryter ned muskelen var ikke forhøyet, men graden av harskning var større i laks med løst skinn og i muskel med bløt tekstur. Mikroskopering av muskelen viste ingen synlige endringer av bindevevet, men muskelcellene hadde avvikende morfologi. Genanalyser (transkriptom) viste store forskjeller i betennelsesprosesser i laks med løst skinn.

Oppsummering: Problemet med løst skinn oppstår mens fisken er i live, men ugunstige forhold under slakting og lagring kan antakelig forverre problemet. Løst skinn synes å være midlertidig, knyttet til sesong. Problemet oppdages ofte etter flere dagers lagring, og er sammenfallende med bløt filet og filetspalting. Laks med løst skinn er kjennetegnet ved en blank fettrik væske under skinnet med høyt innhold av typiske planteoljer – høyere enn i muskelen. Muskelen i laks med løst skinn har økt grad av harskning, avvikende utseende av muskelcellene, noe endret sammensetning av bindevevet og antydning til pågående betennelsesprosesser.

Summary

Loose skin conditions in Atlantic salmon and rainbow trout raise concerns given their adverse effects on product quality and potential impairment of fish welfare. Hence, the aim of the project was to describe the extent, and characterize and uncover potential causes for the skin detaching from the muscle.

A survey aimed at relevant stakeholders revealed that loose skin has been recognized as a quality concern since 2019, typically being detected several days after storage. While the survey didn't show any correlation with the season or geographical location of the farms, the analysis of fish materials in the project suggested that the likelihood of encountering loose skin is higher during specific seasons, likely depending on the farm's location. Statistics from the West-Southern farming regions demonstrated that over 90% of the claims occurred during the period from June to August, with no claims reported from September to February. In the North, we observed the issue during late summer-early autumn. The loose skin issue tends to be present for a relatively short period, after which the skin reattaches — we observed reattachment after three months.

Statistics obtained from the industry showed no association between loose skin and the number of common delousing methods, and experiments in the project showed no effect of pumping or holding fish at low (RSW) or high temperature (simulating thermal delousing). Nevertheless, care should be taken to avoid stress to ensure good fish welfare, and because the adipose tissue under the skin seems susceptible to collapsing when exposed to mechanical pressure (microscope observation, model study).

Numerous batches of salmon identified with loose skin were examined but obtaining rainbow trout with loose skin proved unsuccessful. In each case of loose skin, a glossy liquid was found between the skin and muscle, being predominantly composed of fat (69%), with a higher content of 18:1n-9 and 18:2n-6 (found in plant oils), and a lower content of 22:6n-3 (found in fish oil) compared with the muscle. Also, certain amino acids (aa) were higher in the liquid, indicating selective leakage (his, arg, val, gly).

Analyses of the connective tissue of muscle revealed an unexpected, significant difference in the content of most aa’s of rainbow trout collagen compared with salmon. Aa’s differing between salmon with loose skin and reattached skin were gly, met, leu, lys and arg. Because salmon with loose skin also had soft texture and gaping (mostly severe issues), the collagen of firm and soft muscle was compared, showing difference in the aa’s thr, ser, gly, ala, val, tyr, hyl. Also lower collagen stability of soft muscle was determined as lower temperature of thermal transition and enthalpy of transition. No indications of increased activity of degrading enzymes was detected, but the extent of lipid peroxidation was higher in salmon with loose skin compared with salmon with reattached skin, as well as in soft muscle in contrast to firm muscle

Microscopic examinations did not reveal any explicit signs of abnormal connective tissue structure in salmon with loose skin. Instead, variations in the morphology of myofibers were observed. Further, transcriptomics revealed large scale changes in the muscle transcriptome, including a large array of inflammatory genes in fish with loose skin.

It is concluded that the problem with loose skin originates during the live phase of the fish, although typically identified after days of storage.

1. Introduction

Recent market reports have raised concerns regarding the detachment of skin of farmed salmon and rainbow trout occasionally detaching from the skeletal muscle following a period of ice storage. It is unfortunate for the aquaculture industry that fish are sent to the market with serious quality defects, such as fish with skin lacking attachment to the muscle. Quality deviations have negative economic consequences as batches with severe quality defects lead to complaints, undermining the reputation of the aquaculture industry. The timing of the issue with loose skin is unknown. Therefore, questions may arise concerning the welfare of fish with skin detaching from the flesh, as it cannot be ruled out that the problem occurs while the fish are alive. If the problem arises while the fish are alive, it is likely that the salmon will experience reduced growth and compromised health and resilience. Based on the above, it is considered beneficial for the industry to gain knowledge about the extent of the problem and to identify the causes of its occurrence.

The challenge arises as the issue with loose skin becomes noticeable only to customers, rather than during the phases of harvesting and packaging. This limited visibility hampers the accurate assessment of the problem's severity. Furthermore, the difficulty in tracing the underlying causes complicates the implementation of effective mitigation measures.

It has been indicated that the problem of skin detachment from the muscle displays seasonal variation, potentially with a geographical component, although this remains unverified. Moreover, there is insufficient information on whether the skin loosening from the muscle is linked to other quality-related problems, such as soft muscle and gaping. There seems however to be evidence indicating that the mid-posterior region of the fillets is more prone to encountering problems with loose skin, while the anterior fillet region is rarely affected. Moreover, the region along the midline appears to pose the highest risk of skin loosening (Figure 1).

There is a total absence of documentation concerning factors contributing to problems with skin detachment from the fillets. Nonetheless, the primary hypothesis proposes that stressful events during the harvesting process, coupled with delousing operations play a significant role (including stressful crowding, pumping, and exposure to high temperatures). The potential contributions of genetic background, and fish health issues are seldom mentioned.

Due to the overall deficiency in knowledge, this project has adopted a comprehensive approach, examining operational factors during farming and harvesting, delousing, and feed. Additionally, thorough analyses of fish with skin detachment from the fillets have been conducted. Moreover, a survey has been carried out to assess the extent of the issue concerning skin loosening from the fillet.

Figure 1. Images illustrating salmon skin detaching from the flesh. Image A) Salmon originally graded as superior quality – skin problems occurred after storage in the mid-posterior region of the fish. Image B) Cutlets of slaughtered fish with skin detaching particularly along the mid-line.

2. Objectives

To describe the extent, geographical distribution, and uncover possible causes for the skin detaching from farmed salmon and rainbow trout.

Secondary aims

1) To collect information regarding prevalence and geographical distribution of the problem with skin detaching from the fillet
2) To develop objective measurements of skin attachment to the skeletal muscle.
3) To assess the importance of farming conditions and handling during farming and harvesting.
4) To search for underlying causes of weak attachment between skin and fillet.

3. Overall approach and project group

The scientific partners in the project were (participants listed under WPs):

- Norwegian University of Life Sciences, NMBU (project leader)
- Nofima
- Universidad Complutense, Madrid, Spania.

Reference group:

- Sekkingstad, Anne Karethe,
- MOWI, Åse Hodnefjell
- Cermaq, Magnus Åsli
- Lerøy, Line Rønning
- Aller Aqua, Sofie Barsøe

FHF responsible

- Sven Martin Jørgensen

The project was divided into four work packages (WP 1-4)

WP1: Prevalence of skin detachment and objective measurements of skin attachment

Responsible Dr. Thomas Larsson, Nofima.

Participants MSc Arnaud Lefrancois, Nofima

Reference group, and industry significant contributors

WP2: Fish handling and harvesting

Responsible: Dr. Bjørn Roth, Nofima

Participants: Prof Turid Mørkøre, NMBU; Dr. Izumi Sone, Nofima

Reference group, and industry significant contributors

WP3: Underlying causes of skin detachment and development

Responsible: Prof Turid Mørkøre

Participants: Helena M. M. Conde and Maria B. Rojo, Universidad Complutense; MSc Eunice Efua Boahemaa-Kobil, MSc Marte Røsvik and MSc Julia Formanowicz, MSc Sumeng Sumeng and Dr. Hanne K. Hustoft, NMBU

WP4: Histolgy and gene expression

Responsible: Dr. Lene Sveen, Nofima

Participant: MSc Timotè Moinhos, Nofima

An overview of the project approach, scientific participants and industry involved is shown in Figure 2.

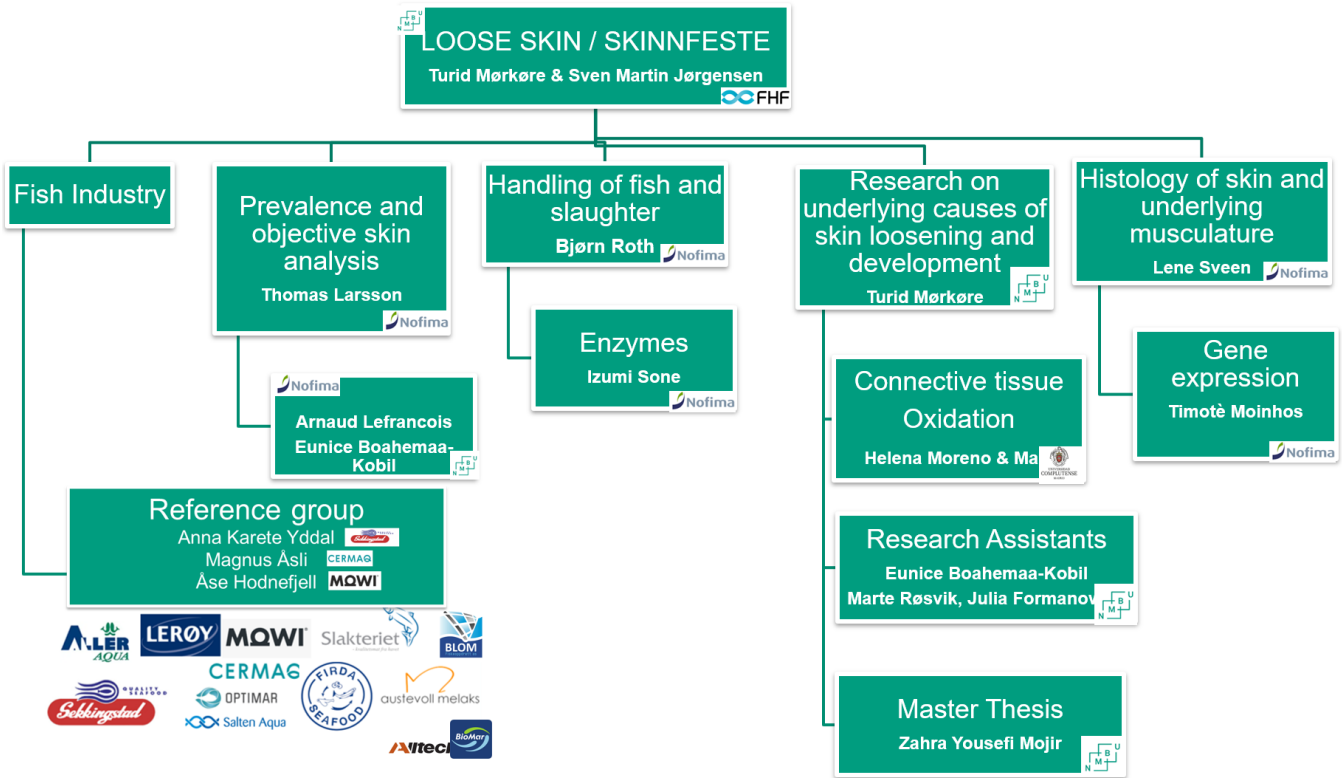

Figure 2. Overview of the project activities and participants

3.1. Project execution and methods

In the project we managed to obtain a wide range of relevant biological fish material for analyses due to a close cooperation with the industry and ongoing projects financed by FHF and others (particularly Ministry of Fisheries; R&D Licenses: Arctic Salmon Research Centre and Aller Aqua). Additionally, the reference group was deeply involved in both the strategic and the practical activities in the projects, contributing significantly with knowledge, relevant fish material and customized statistics.

In a project of the current nature, where foundational knowledge is notably restricted, collaborative efforts with the industry and a willingness to share and contribute fish material and knowledge are imperative for achieving meaningful advancements in knowledge. Without this close collaboration and the immediate sharing of pertinent fish materials and knowledge, the impact of the present project would be limited, particularly since the project did not involve tailored, specific seawater trials, considering the current knowledge status of the issue at hand.

Therefore, we wish to express our gratitude to the industry participants who contributed to the project with fish material, knowledge, and facilitation in various ways, including tailored, relevant statistics.

An overview of the main fish materials used in the project is given below, in Table 1

Table 1. An overview of the fish material used in the project.

Test description	Region	Date	Analyses
Effects of pumping at harvest	North	2022	Predefined soft A. salmon was exposed to handling/ pumping during harvesting, with sensors determining exposure of fish to extreme >20G. Effects on skin attachment or fillet texture were not found, although skin damages/injuries were observed at high G forces.
Slaughter boat (bleeding, "bløggebåt")	West	Jan 2023	Skin detachment of salmon obtained from a commercial slaughterhouse were analysed. The fish originated from the same rearing unit but were transported/handled either on a slaughter boat or well-boat. Also, comprehensive statistic related to skin/fillet quality were obtained for fish from wellboat vs slaughterboat
Fish population reported to have loose skin	West	Nov 2022	Fish were analysed for skin detachment and fillet quality. Skin and skeletal muscle samples were taken for histology examination, chemical analyses and collagen characterization
RSW simulation	South	Oct 2023	Fish were exposed to Resirculated Sea Water
Salmon with loose skin	West	Feb, 2022	Fish were analysed for skin detachment and fillet quality. Skin and skeletal muscle samples were taken for histology examination, chemical analyses and collagen characterization
Salmon with loose skin	North	Aug, 2021	Fish were analysed for skin detachment and fillet quality. Skin and skeletal muscle samples were taken for histology examination, chemical analyses and collagen characterization
Salmon with re-attached skin	North	Oct, 2021	Salmon were analysed for fillet quality and skin characteristics (detachment), and collagen properties
Salmon with loose skin	North	Oct 2022	Salmon were analysed for fillet quality and skin characteristics (detachment), and collagen properties and histology
Warm temperature exposure	Small scale	Oct 2023	Fish muscle pieces (20) from four salmon individuals were exposed to temperatures up to 34 degrees. Samples were examined macro- and microscopically
Sea-lice treatment/ delousing	Statistics	2023	Relationship between claims on loose skin and number of delousing and delousing method by an industry collaborator
Skin and muscle of control fish	West	2023	Salmon and rainbow trout considered to be of high quality (skin and muscle) according to laboratory analyses and market responses were analysed for skin/skeletal muscle collagen characterization and oxidation products. A dietary group fed black soldier fly meal was also included (collagen thermal stability)

4. Methods

4.1. Survey

To gather information on frequency and geographical distribution of the loose skin problem, a survey was conducted using a digital questionnaire created in Microsoft Forms. The content of the survey was developed based on input from the project- and reference group, as well as in collaboration with the FHF-project «Kunnskapskartlegging pigmentering» (FHF# 901745). The survey was distributed to 173 recipients in agreement with the General Data Protection Regulation. All collected information was treated confidentially and the results have been anonymized.

The questionnaire contained questions related to the following topics,

1. Information about the respondents (company, field of activity, location).
2. Experience, frequency, and importance of the loose skin issue.
3. Origin of the fish material and relevance of production area for loose skin issue.
4. Seasonality of the loose skin issue.
5. Association with other quality deviations (e.g. wounds / ulcers, fillet color, fillet gaping).

For questions related to regional effects, the 13 Atlantic salmon aquaculture production zones in Norway were used (Figure 3; Norwegian Ministry of Trade, Industry and Fisheries, 2017). The 13 regions were not individually analyzed in the interpretation of the results but were combined into 4 regions: region 1-4; 5-6; 7-8; 9-13.

Figure 3. The 13 Atlantic salmon aquaculture production zones in Norway used for questions related to fish and production origin in the survey on the loose skin issue.

4.2. Dry matter, fat, fatty acids, protein, and amino acids

Dry matter, content of fat, fatty acids, protein and amino acids were determined in the skeletal muscle (area between the posterior end of the dorsal fin and the gut; Norwegian Quality Cut, NQC), and in liquid collected under the skin of fish with loose skin (no or minor liquid present in fish with the skin being well attached to the skeletal muscle). Generally, fish belonging to the same group (e.g. same net pen or treatment) were pooled and homogenized. Standard methods were used (Mørkøre et al., 2020); O'Fallon et al. (2007).

4.3. Collagen and MDA

Amino Acid profile of Connective Tissue

Connective tissue (CT) from skeletal muscles (NQC) was isolated according to the method described by (Moreno et al., 2012), with slight modifications. In short, the procedure was as follows: The samples were homogenized for 15 seconds near NaCl 8‰ at 0-5 °C. The resulting solution was filtrated with a strainer, and the mixture was washed out with consecutive rinses of cold tap water. This process was repeated until whitish CT was obtained. Fat was removed using a solution of 1:10 butanol: water (v:v) was added to connective tissue for two hours. The resulting CT was rewashed with distilled water. Finally, the CT was dried and stabilized at -80 °C. The amino acid profile was analysed using HPLC. Each sample was weighted and hydrolyzed using constant boiling 6 N HCl containing 0.1% phenol and using norleucine as an internal standard (Sigma–Aldrich, Inc., St. Louis, MO, USA). After the hydrolysis, samples were vacuum-dried, dissolved in application buffer and injected into a Biochrom 30+ AminoAcid Analyser (Biochrom, Kaysville, USA).

Differential Scanning Calorimeter

A differential scanning calorimeter (DSC Q1000, TA Instruments,New Castle, USA) was used to determine the thermal behavior of the CT (Moreno et al., 2012). Samples were placed in hermetically sealed aluminum cups. A drop of 8‰-NaCl solution was added to samples to prevent the collagen from melting due to a lack of water. Under a dry nitrogen purge at a 50 mL/min rate, the samples were scanned in two replicates at a rate of 10 °C/min from 5 to 90°C. After cooling to 5°C with a rate of 30 °C/min, second scans were taken to search for any residual/new effects. To normalize thermal data to dry matter content, the water content of each individually encapsulated sample was calculated using desiccation at 105°C. Temperature, Tpeak (°C), and enthalpy of transition DH (J/gdm) data are presented as mean values with their standard deviations.

Determination of Malondialdehyde content: Lipid peroxidation

Malondialdehide (MDA) content was determined using the thiobarbituric acid reactive species (TBARS) procedure described by (Palma et al., 2015), with some modifications. Salmon muscle was homogenized (1:4 w/v) in 20% (w/v) trichloroacetic acid (TCA), 0.8 mL of 4 % (w/v) butylated hydroxytoluene. The homogenate was centrifuged at 4ºC and 8000 rpm for 10 minutes. The supernatant was mixed with 0.5 % (w/v) thiobarbituric acid (TBA) in 20% TCA in proportion 1:4 (V/V). The mixture was heated at 94ºC in a water bath for 30 min, cooled immediately in ice to stop the reaction, and centrifuged at 4ºC and 8000 rpm for 10 min. Absorbance of the supernatant was measured at 532 and 600nm. TBARS were calculated by subtracting the non-specific absorption at 600nm from the absorption at 532 nm and using a standard curve of MDA (0-120µM). Results were expressed as µg MDAg-1 of fresh weight.

4.4. Enzyme analysis

Crude enzyme extract was obtained from each fish (Table 2) as described by (Yang et al., 2015) with modifications. The protein concentration of the enzyme extracts was determined by the Lowry method (Lowry & Tinsley, 1976). The standard curve was constructed in appropriate concentration range using 7-amino-4-methyl coumarin (AMC) in distilled water. The activities of cathepsin B/L like, collagenase and calpain (including control with EDTA) in the extracts were determined in duplicate as previously described (Hultmann & Rustad, 2002, 2004) with modifications and expressed as µM (for cathepsin) or nM AMC/mg protein in sample. For selected experiments, extraction and subcellular fractionation was performed according to (Ertbjerg, Larsen, & Moøller, 1999) and cathepsin B and L activity was measured fluorimetrically.

4.5. Histology

Histological analyses were performed on formalin-fixed skin and muscle samples taken from farmed fish in various geographic locations, as well as before and after different post-mortem treatments (Table 2) Skin samples were either cut horizontally, or parallel to the direction of the scales. In brief, samples for histological examination were stored in 10% buffered formalin and sent to the veterinary institute in Harstad for embedding sectioning and staining, with routine staining (Hematoxylin and Eosin) or special stains (Alcian Blue and PAS, and Picro Sirius Red (PSR). Special stains were used to identify and differentiate between mucins, glycogen, and other carbohydrate-rich substances. PSR is a stain that binds to collagen fibers, providing enhanced visibility of collagenous structures in tissues. The tissue sections were analyzed at Nofima by two trained histologists.

Table 2. Tissue samples used for histological analysis and / or microarray / enzyme activity.

Fisk	Characterized with loose skin	Treatment	Analysis
Fish fed ecological feed	No	Skin and muscle tissue samples from sea cage (n = 10), and after pumping (n = 10)	Histology and enzyme activity
Control fish	No	Normal production	Histology
Long term storage samples	No	From sea cage (n = 4) and after pumping (n = 4), in total 80 samples, skin from ten different positions on the body	Histology
South loose skin, Feb 2022	Yes	Skin and muscle tissue, and organ package, sampled from the sea cage	Histology, microA
North loose skin, Aug 2021	Yes	Skin, sampled from fish in sea cage	Histology, microA
North re-attached skin, October 2021	No	Skin, sampled from fish in sea cage	Histology, microA

4.6. Microarray

Transcriptome analyses were conducted on three different sample sets (Table 2). The sample set from the west coast, named (E-LooseSkin (E-LS). was taken from fish with confirmed loose skin. The samples from Finnmark, from the same location, where loose skin was detected in August 2021 (A-LS),

while the skin had normalized by October approximately two months later (A-Reattached Skin (RS)). All samples analyzed were collected directly at the production site.

RNA from the samples was processed using a 15k Microarray for both skin and muscle samples, prepared and analyzed according to (Sveen, Krasnov, et al., 2021). The gene expression profile of fish diagnosed with loose skin was compared to a pool of healthy samples from Nofima's STARS database, procedure according to (Ertbjerg, Larsen, & Moøller, 1999). Additionally, the sample set was compared to a variety of samples from diseased fish, including PD, HSMI, PRV, SAV, plasmids, melanin spots, and mechanical wounds in Nofima's STARS database (Krasnov et al., 2011).

4.7. Instrumental measurement of skin attachment strength

A method for objective analyses of skin attachment to the skeletal muscle was developed.

Skin attachment strength was measured instrumentally (Single Column Table Frame, series 5940, capacity 2 kN, Instron, Norwood, MA, USA) using the NQC cutlet, by cutting a strip, 2 cm deep and 2.5 cm wide, along the lateral line and 2.5 cm dorsally of that line. The skin from the posterior part of the strip (~4 cm) was released from the muscle to enable grip by instrument, then attached to grip probe (model 2713-004, Instron), and pulled at constant speed (5 mm/sec) in the rostro-caudal direction until whole the strip was pulled off the NQC. Results are presented as the total work required to pull the skin strip off the whole NQC, corrected for total distance. See Figure 4 for example pictures from the skin attachment analyses.

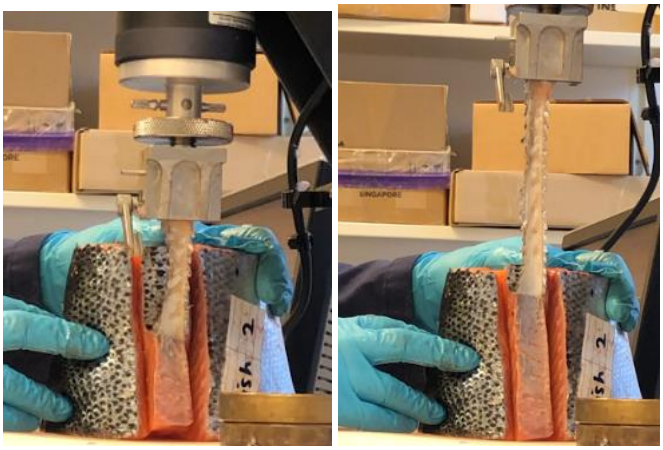

Figure 4. Pictures illustrating analyses of skin attachment of Atlantic salmon (Dec 2022 fish material)

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4.8. Fillet quality

Fillet quality was assessed by evaluating the degree of gaping (i.e., slits and holes in the fillet), using a scale from 0 to 5, where score 0 indicates no gaping and score 5 indicates extreme gaping (Andersen et al., 1994), and instrumental analysis of fillet firmness in the anterior and dorsal part of the fillet (TA-XT2, Stable Micro Systems Ltd., Surrey, England; (Mørkøre & Einen, 2003).

5. Results

5.1. Survey

The survey was distributed to 173 recipients, yielding 34 responses (Table 3). Of these, 33 responses originated from Norway, while one response was received from Denmark. The respondents covered employers engaged in farming of fish, fish processing, slaughterhouse, and purchaser.

The survey respondents included representatives from all major producers (in terms of volume) of both salmon and rainbow trout.

Among the respondents, 25 provided answers exclusively related to salmon, five addressed both rainbow trout and salmon, and two focused solely on rainbow trout. In total, 11 out of the 34 respondents (32%) reported experiencing loose skin.

Table 3. Overview of information concerning the 34 survey participants addressing the loose skin issue.

Business area	Responders business role	Response about salmon and / or trout	Experience of loose skin
Farming	Production leader	Salmon	No
Farming	Production leader	Salmon	No
Farming	Sales	Salmon and trout	No
Farming	Production leader	Salmon	No
Farming	Production leader	Salmon and trout	No
Farming	Veterinarian	Salmon	No
Farming	Quality manager	Salmon	No
Farming	CEO	Salmon	No
Farming	Production leader	Salmon and trout	No
Farming	Sales	Trout	No
Farming	CEO	Salmon	No
Farming	Production leader	Salmon	No
Farming	Veterinarian	Trout	No
Farming, slaughterhouse	Quality manager	Salmon	No
Farming, slaughterhouse	Quality manager	Salmon	No
Farming, slaughterhouse, processing	Quality manager	Salmon	No
Farming, slaughterhouse, processing	Quality manager	Salmon and trout	No
Farming, slaughterhouse, processing	Quality manager	Salmon	No
Processing	Quality manager	Salmon	No
Processing	CEO	Salmon and trout	No
Slaughterhouse	Quality manager	Salmon	No
Slaughterhouse	Quality manager	Salmon	No
Slaughterhouse	Quality manager	Salmon	No
Customer	Purchaser	Salmon and trout	Yes
Customer	Purchaser	Salmon and trout	Yes
Export	Sales	Salmon	Yes
Export	Quality manager	Salmon and trout	Yes
Farming	Sales	Salmon	Yes
Farming, slaughterhouse, processing	Biological analyses	Salmon	Yes

Processing	Production leader	Salmon and trout	Yes
Processing	Purchaser	Salmon and trout	Yes
Processing	Quality manager	Salmon	Yes
R&D	Production leader	Salmon	Yes
Slaughterhouse	Quality manager	Salmon	Yes

The occurrence of the loose skin problem was noted for the first time between 2019 and 2023 for all respondents except one, who encountered the issue in the 1990s (Figure 5A). The responses varied in terms on whether the problem increased, remained stable or decreased, and there appeared to be no clear consensus with regards to development during this relatively short period. Regarding production region, responses covered all regions specified in the questionnaire, in Norway. There was no clear difference between production regions with regards to experience of the loose skin issue (Figure 5B).

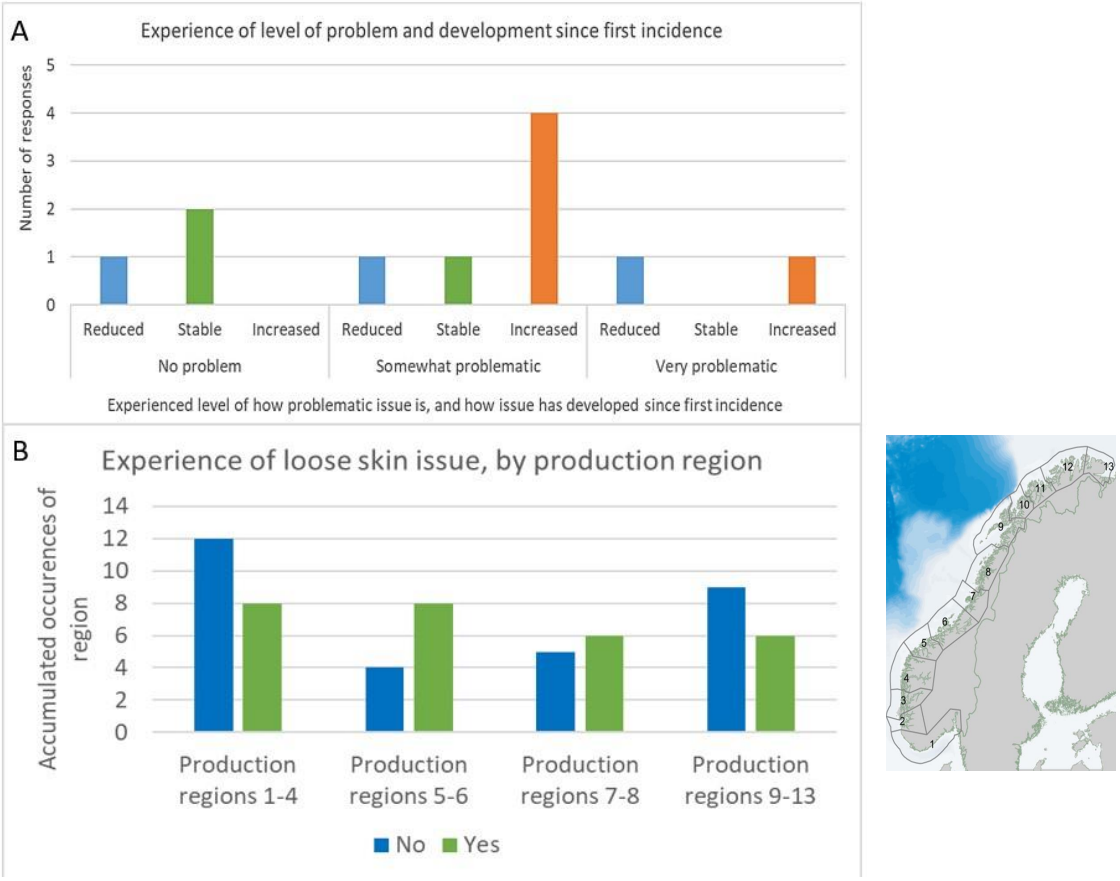

Figure 5. A. Responses on how the loose skin issue has developed since it was detected, based on the perceived severity of the issue. B. Experience of loose skin by accumulated occurrences of the different production region groups.

Regarding business sector, 13 respondents were farmers, and only one had encountered the issue of loose skin (Figure 6A). Moreover, when feasible, responses were classified based on the primary business sector in which the respondent was predominantly engaged.

In the pre-processing phase (farmers and harvesters), only 10% of the respondents had experienced problems with loose skin, whereas in the post-harvest phase (processing, export, and customer), 78% of the respondents' reported problems with loose skin. Additionally, two veterinarians took part in the survey, and both reported no encounters with the loose skin issue. Among the 11 respondents that had experienced loose skin, the identification of loose skin predominantly came from customer complaints (n = 5 out of 11). Others reported that loose skin was detected during or after processing (n = 3), during quality control in the production cycle, or at harvest (n = 3). Three participants mentioned receiving over 10 complaints annually related to the loose skin problem, revealing a discernible pattern associating a greater number of complaints with an increased perception of the issue's severity (Figure 6B). Furthermore, all 11 respondents who had encountered the loose skin quality issue reported an association with poor muscle quality, particularly fillet gaping and soft muscle. When survey participants were asked about potential causes of poorly attached skin to the fish, nutrition was the most frequently suggested cause, although responses varied significantly (Figure 6C).

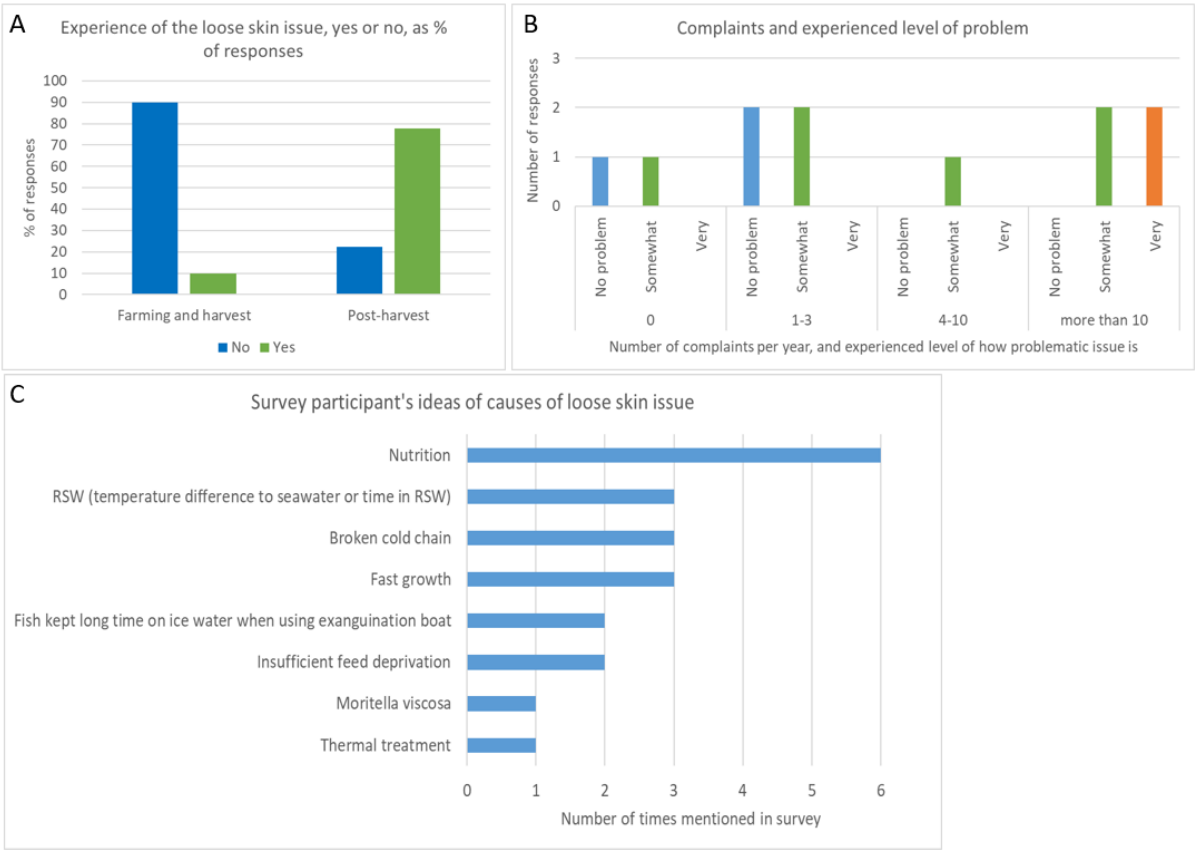

Figure 6 A. Experience of the loose skin issue, yes or no, as % of responses per business area grouped by value chain point of pre-processed and post-harvested fish. B Number of complaints per year, and experienced level of how problematic the loose skin issue is for the 11 responders who had experienced the quality issue C. A summary of survey participants' perspectives on the factors contributing to inadequate attachment of the skin to the muscle.

5.2. Customized statistics from the industry

Tailored statistics were supplied by the industry, documenting complaints about fish with loose skin along with a delousing history. The salmon originated from farms in the Southern and Western regions of Norway. No claims were reported from September to February, whereas nearly 90% of the claims were received between June and August. No consistent correlation was observed between the delousing method employed and the occurrence of claims related to skin detaching from the fillet.

5.3. Chemical composition of the liquid under the skin

We noticed that fish with loose skin displayed a relatively thick liquid between the skin and the muscle, whereas no such liquid was observed when the skin was tightly adhered to the muscle (fillet). Additionally, we observed an atypical appearance of the red muscle beneath the skin, resembling a heat-treated (cooked) texture. Consequently, liquid was collected from fish with loose skin, as well as muscle samples, to investigate their composition, including protein, amino acids, fat, and fatty acids. Samples were collected from different fish materials (Figure 7).

Figure 7. Pictures illustrating liquid under the skin of fish with loose skin. The liquid was collected by scraping the liquid beneath the skin after removing the skin.

The analysis of the amino acid and fatty acid content in the subcutaneous liquid of fish exhibiting loose skin was conducted in conjunction with examinations of the muscle composition. This was undertaken to determine if specific amino/fatty acids were being released from the muscle to a greater extent (selective loss).

The analyses of the gross composition revealed that the liquid was mainly composed by fat (69%) but also some protein (1%) was found in the liquid.

The amino acid analyses showed higher content of histidine, arginine, valine, and glycine in the liquid compared with the muscle. Glycine, a crucial amino acid contributing to collagen formation (the "glue" of the muscle), was notably higher in the liquid compared to the muscle. On the contrary, tyrosine, leucine, asparagine, serine, and isoleucine were consistently lower in the liquid compared with the muscle (Figure 8). These are observations are interesting and might add relevant insight not only to the problem with loose skin, but also salmon texture and gaping issues.

The fatty acid analyses likewise showed a different fatty acid composition of the liquid under the skin compared with the muscle – hence certain fatty acids were lost to a higher extent than others. Fatty acids that appeared to be significantly higher in the liquid resemble to a large extent those found in plant oils used in the feed for salmon and rainbow trout, i.e. 18:1n-9 and 18:2n-6. On the other hand, the long chained, marine fatty acid 22:6n-3 was consistently highest in the muscle. (Figure 9).

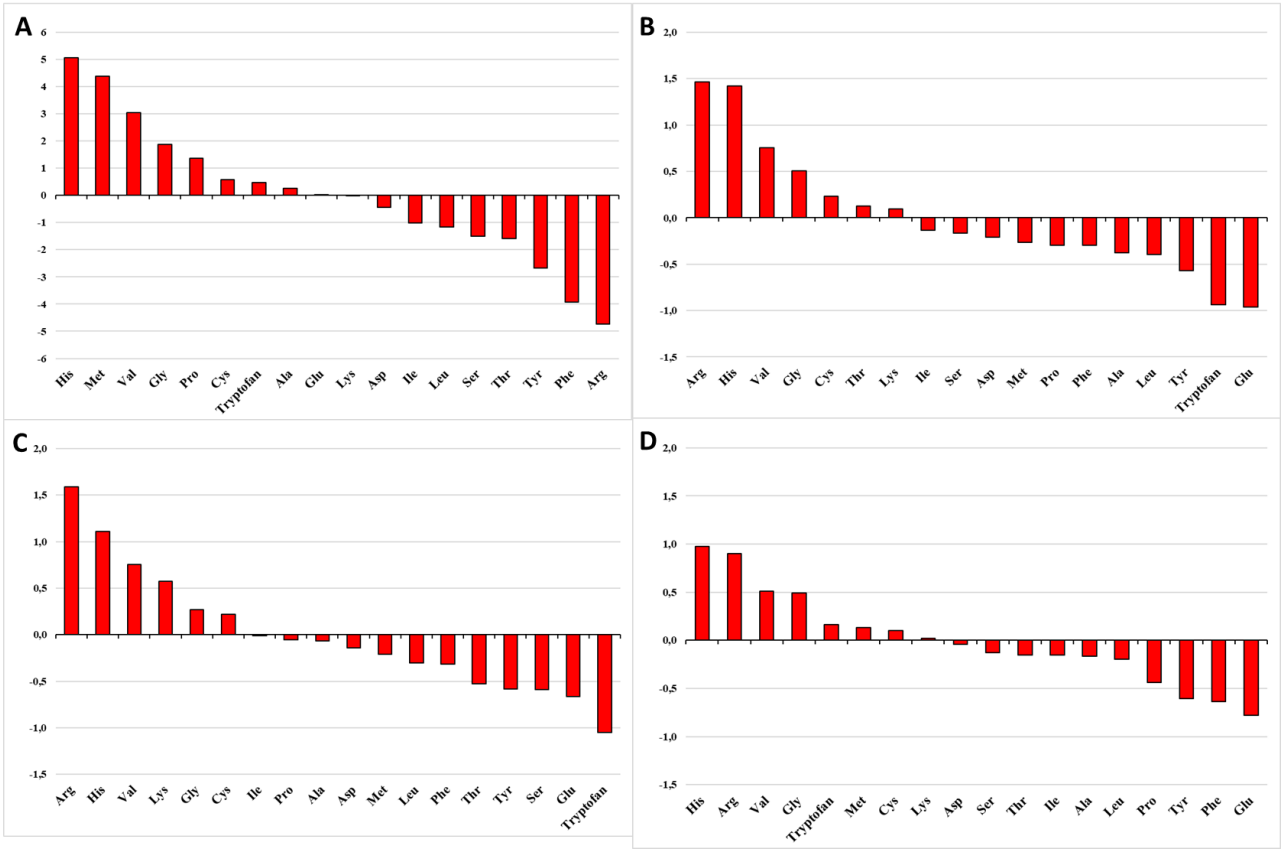

Figure 8. Relative (%) differences in amino acid concentration between the liquid sampled beneath salmon skin and muscle (positive values indicate higher concentration in the liquid under the skin, whereas negative values indicate lower concentration in liquid compared with the muscle). Results are shown for batches of salmon with loose skin, farmed under commercial conditions and fed standard commercial feeds A) August 2021, B) October 2022, and C) October 2022. D) illustrates fish fed a lean diet, but with similar amino acid and fatty acid profile.

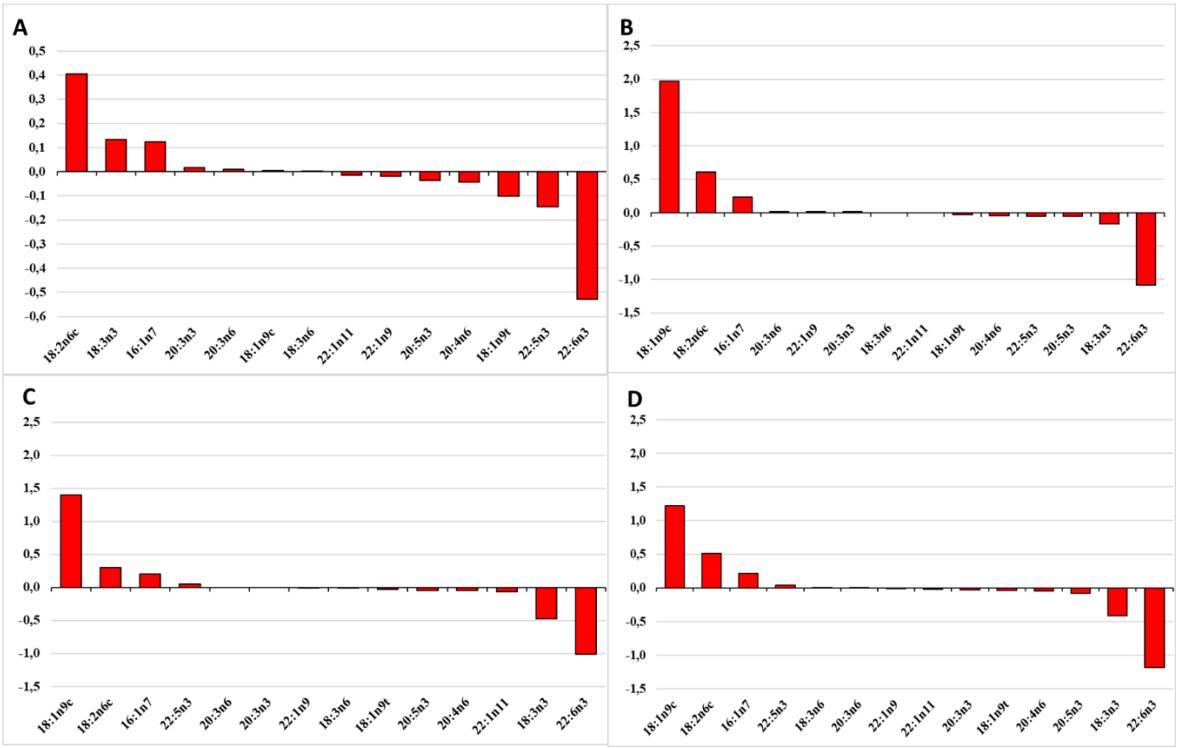

Figure 9. Relative (%) differences in fatty acid concentration between the liquid sampled beneath salmon skin and muscle (positive values indicate higher concentration in the liquid, whereas negative values indicate lower concentration in liquid compared with the muscle). Description of fish material, see text in Table 1

5.4. Connective tissue (collagen) and lipid oxidation (MDA)

Three fish populations were used for analyses of collagen properties and lipid peroxidation (Malondialdehide, MDA). All fish were farmed in commercial sea cages and all fish within the same population were fed with the same feed (standard commercial feed) (

Table 4):

Population A: Salmon (4.3kg) and rainbow trout (4.2kg) with firmly attached skin and firm muscle. i.e. fish in this population were intended to designate as *control's*, i.e. reference fish material (see Mojir 2023 for detailed information on the fish material)

Population B: Salmon with loose skin sampled in August (4.8kg) and salmon from the same population with firmly attached skin sampled in October three months later, in October (4.7kg).

Population C: Salmon (2kg) from one sampling point (July 2022), graded according to instrumental texture analyses (soft: breaking force <6N; firm: breaking force >7N).

Atlantic salmon vs. rainbow trout

The amino acid composition of the collagen of salmon and rainbow trout differed significantly for most amino acids.

Rainbow trout is generally less prone to issues with soft texture and gaping compared with salmon, but in the present study fillet firmness (determined instrumentally) and gaping severity (visual scoring) were similar for the species. Nevertheless, amino acids particularly associated with collagen characteristics differed significantly between the species, i.e. glycine (gly), proline (pro) and hydroxyproline (hyp), but in different directions. Whereas gly was higher in rainbow trout compared with salmon, hyp and pro were significantly lower.

Collagen is a protein molecule made up of amino acids. It provides structural support to connective tissues, is rigid and resistant to stretching and is the perfect matrix for skin.

Collagen chains contain at least one domain composed of repeating Gly-Pro-Hyp sequence (triple helix stability).

Gly: Permits helix to twist - it is important for the configuration, so the collagen can withstand stress.
Pro: Avoid helix turning (rigidity)
Hyp: Helix stabilization by hydrogen bonds

} Important for collagen stability

Loose skin vs re-attached skin

A higher content of gly in the salmon with loose skin was observed, while the content of pro and hyp was similar of salmon with loose skin and in salmon with reattached skin. Amino acids being higher in salmon with reattached skin were met, leu, lys, and arg. In total the content of hydrophobic amino acids was significantly higher in salmon with reattached skin (

Table 4).

Soft texture vs firm texture

None of the main amino acids known for their involvement in collagen stabilization (gly, pro, lys, hyp) differed significantly between salmon with soft and firm texture. However, the collagen of firm muscle had higher content of thr and ser. On the other hand, a lower content was observed for the amino acids alanine (ala) valine (val), tyrosine (tyr), hydroxylysine (hyl) and total hydrophobic amino acids in firm compared with soft muscle (

Table 4).

Table 4. Amino acid profile of connective tissue samples obtained from salmon and trout. Values are expressed as means ± standard deviation. Different letters indicate significant differences (P <0.05) between the groups across populations.

	Population A		Population B		Population C	
AA	Salmon Control	Trout Control	Loose skin	Re-attached skin	Soft Texture	Firm Texture
Asp	64.99 ± 0.57 c	69.00 ±0.69 a	68.22±0.21a	68.85±1.10a	67.43±0.37a	67.74±0.36a
Thr	32.04 ± 0.6 a	26.70 ± 0.7 c	25.38±0.59a	25.94±0.93a	24.08±1.22a	26.59±0.40b
Ser	49.31 ± 0.6b	52.00 ± 0.7 a	50.69±0.35a	47.79±0.34a	47.16±0.64a	53.06±2.14b
Glu	84.36 ± 1.56 ab	86.19 ± 1.92 a	86.61±1.03a	90.22±0.03b	84.06±0.25a	84.16±1.37a
Gly	276.54 ± 4.24 c	305.14 ± 5.19 ab	313.88±2.89b	299.43±4.53a	312.87±2.97a	318.61±2.00a
Ala	106.31 ±0.71 ab	105.57 ±0.87 ab	112.12±1.19a	111.60±2.01a	113.47±1.22b	111.11±0.29a
Cys	4.42 ±0.21 b	6.82 ± 0.25 a	6.82±0.38a	6.41±0.57a	6.58±0.18a	6.46±0.19a
Val	27.53 ± 0.92 a	19.80 ± 1.13 c	18.42±0.26a	18.64±0.45a	19.43±0.20a	18.12±0.28b
Met	19.42 ± 0.46 b	21.70 ± 0.56 a	20.01±0.20a	20.90±0.47b	19.98±0.18a	19.92±0.40a
Ile	18.69 ± 0.6 a	13.93 ± 0.73 c	12.69±0.39a	13.13±0.65a	13.045±0.30a	12.13±0.30a
Leu	35.42 ± 0.88 a	30.83 ± 1.08 b	26.51±0.70a	29.37±1.92b	27.88±0.69a	26.33±1.27a
Tyr	10.62 ± 0.32 b	13.77 ± 0.39 a	14.11±0.10a	13.88±0.39a	13.79±0.55b	13.18±0.23a
Phe	20.49 ± 0.64 c	26.77 ± 0.79 a	28.14±2.28a	29.76±3.09a	24.78±0.85a	26.036±1.90a
Hyl	8.51 ± 0.11 a	8.03 ± 0.14 b	9.73±0.08a	9.55±0.17a	10.25±0.16b	9.74±0.12a
His	11.57 ± 0.27 a	9.30 ± 0.34 b	9.07±0.13a	9.42±0.38a	9.07±0.29a	9.05±0.38a
Lys	37.07 ± 0.7 a	33.74 ± 0.8 b	30.71±0.05a	32.88±0.12b	30.88±0.33a	29.38±0.13a
Arg	50.68 ± 0.47 a	45.77 ± 0.58 b	43.49±1.03a	46.20±1.55b	43.70±0.79a	42.84±1.38a
Hyp	51.85 ± 0.86 a	44.30 ± 1.06 b	43.99±0.93a	43.39±3.24a	44.77±0.38a	44.79±0.73a
Pro	90.17 ± 1.31 a	80.64 ± 1.61 c	79.32±2.44a	82.57±4.60a	86.44±5.39a	80.70±3.18a
Hydrophobic amino acids *	350.09 ± 2.44 a	325.93 ± 2.99 c	302.60±0.60a	311.03±1.24b	309.14±3.57a	301.03±0.93b
Imino acids (Pro + Hyp)	142.02 ± 1.96 a	124.95 ± 2.4 b	123.31±3.37a	125.96±7.83a	131.21±4.95a	125.49±5.44a
Pro hydroxylation (%)	36.50 ± 0.34 a	35.50 ± 0.42 ab	35.68±0.59a	34.42±0.59a	34.18±1.58a	35.71±0.53a
Lys hydroxylation (%)	18.73 ± 0.48 b	19.23 ± 0.59 ab	24.07±0.18b	22.52±0.24a	24.92±0.49a	24.10±1.05a

Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) is a thermal analysis technique that provides information about the heat flow into or out of a sample as a function of temperature or time. i.e. in our case it was interesting to study the thermal stability (low values indicate low stability).

Salmon and rainbow trout fed either a control feed or a feed supplemented with insects

The fish used were salmon and rainbow trout fed either a standard commercial feed (control) or a feed supplemented with black soldier fly meal (BSF). Since the issue of skin detachment coincided with the introduction of insect meal, an emerging raw material in Norwegian aquaculture, BSF fed fish were included in the investigation of collagen thermal stability.

The results showed no significant differences in thermal transition temperature between the salmon fed with BSF and the salmon control group (Table 5), indicating that incorporation of BSF meal did not affect the thermal properties of salmon connective tissue. Similar observations were made for trout, but the trout control group had a lower thermal transition temperature compared to the salmon.

The enthalpy of transition showed that the salmon and trout fed by BSF had a numerically higher value than the control group, but the differences were not significant (

Table 5).

Loose skin vs re-attached skin

The results showed no significant differences in thermal transition temperature or enthalpy of transition between lose skin and re-attached skin (

Table 5).

Soft texture vs firm texture

The results showed significantly higher values for the temperature of the thermal transition for salmon with firm muscle compared with salmon with soft muscle (higher thermal stability of firm fillets). In line, the enthalpy of transition was significantly lower for the soft salmon fillets. Firm texture also showed a higher content of gly, that is one of the major structural units of collagen, required to wrap around the three alpha chains of the tropocollagen molecule (Porfírio & Fanaro, 2016).

Table 5. Temperature of thermal transition (ºC) and the Enthalpy of transition (J/g) of connective tissue extracted from fillets (Norwegian quality cut, NQC) of Atlantic salmon and rainbow trout fed commercial feed or the same feed supplemented with 4% of black soldier fly larvae meal (Hermetia illucens). Samples from loose skin and re-attached skin, as well as soft and firm texture samples are also included. Values are expressed as means ± standard deviation. Different letters indicate significant differences between the groups (P≤0.05).

Samples	Temperature of thermal transition (ºC)	Enthalpy of transition (J/g)
Salmon Control	48.15±0.31a	3.30±1.38a
Salmon BSF	48.20±0.96a	4.71±1.75a
Trout Control	47.16±0.57a	4.30±1.75a
Trout BSF	47.40±0.84a	5.09±2.42a
Loose skin	46.75±0.31a	3.78±0.65a
Re-attached skin	46.93±0,095a	4.25±0.71a
Soft Texture	45.11±0.23a	4.05±0.06b
Firm Texture	45.79±0.05b	3.62±0.17a

Lipid peroxidation

The levels of MDA were analyzed in samples of salmon with loose skin and salmon sampled after skin reattachment (same fish population, approximately 3 months between the sampling time points - Aug, Oct). The results obtained indicate that the MDA levels detected in those salmon with loose skin were significantly higher than those obtained in fish that returned to their normal conditions with firmly attached skin (Figure 10).

Similarly, the determination of MDA was carried out in samples of salmon from a fish population, graded according to their fillet firmness, determined instrumentally. The results showed that the level of lipid oxidation was significantly higher for fillets with soft texture (Figure 10)

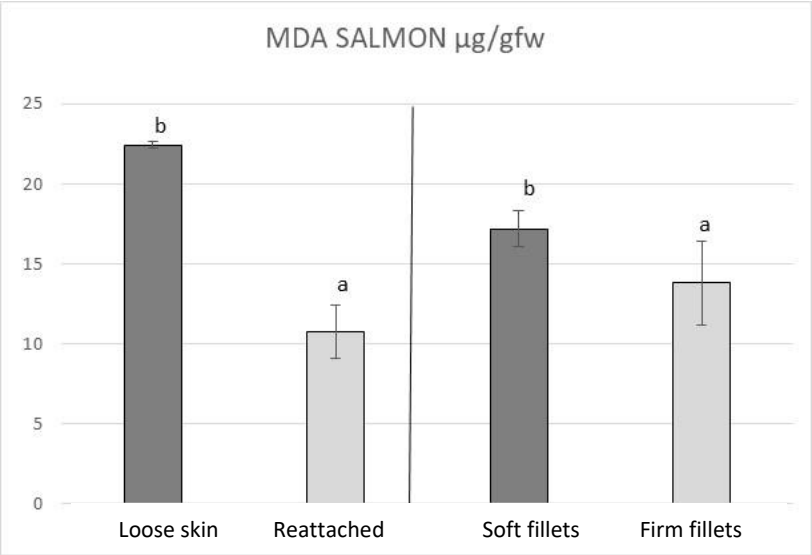

Figure 10. Changes in the content of thiobarbituric acid-reactive species (TBARS) expressed as MDA in salmon samples with loose skin or reattached skin, and salmon with soft or firm fillet texture. Different letters indicate significant differences within fish group according to Duncan's test ($p<0.05$)

5.5. Enzymatic analysis

Fish fed organic or inorganic minerals

The analyses were performed on salmon that had received feed with organic (75%) or inorganic minerals - both groups were considered to have loose skin and soft fillets, with indications of lower severity of skin and fillet issues of the fish fed organic minerals (farmed at the West coast, sampled March, 2022). Crude enzyme extracts were obtained, and respective enzyme activities were measured. The enzyme activity showed no significant differences between the dietary groups, implying that the feed did not affect the total enzyme activity in the white muscle (Figure 11).

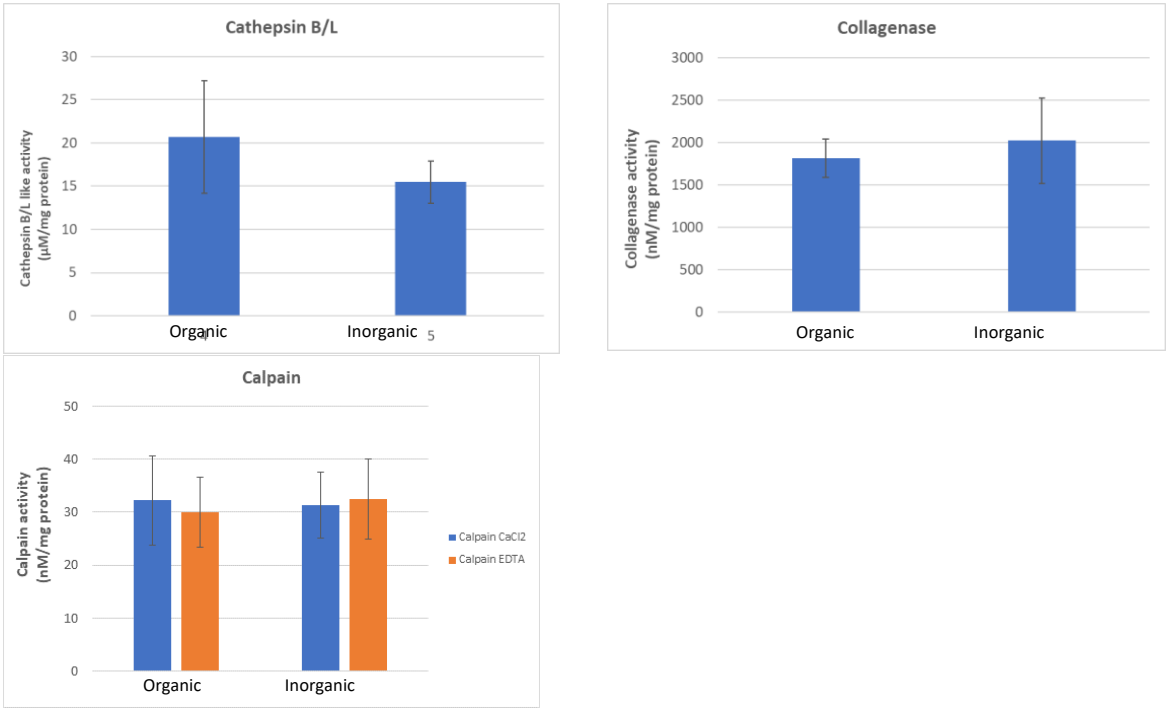

Figure 11. Average cathepsin B/L, collagenase and calpain activity with standard deviation (measured as µM (for cathepsin) or nM AMC/mg protein in sample).

Effect of pumping fish at harvest

Two groups, A and B (N=5), taken directly from netpen and after slaughtering and cooling respectively, arrived at Nofima, Stavanger on 23 March, 2022. The temperature of the fish upon arrival was 2.0°C. Speciments of red and white muscle were taken from NQC and subjected to subcellular fractionation and cathepsin B/L activity was measured in the respective fractions: «myofibrillar (Myo)», «lysosomal (Lys)», «heavy mitochondrian (Mit)» og «sarcoplasmic (Sarc)» fractions. There was a higher cathepsin B/L activity in the lysosomal fraction in the white muscle of A compared to B, indicating rupturing and leakage from the B muscle, possibly due to factors such as physical impact and/or stress (Bahuaud et al., 2008). There was a higher cathepsin B/L activity in red muscle than in white muscle, especially in the lysosomal and mitochondrial fractions, suggesting higher breakdown in red muscle. On the other hand, there was no group difference in red muscle (Figure 12). The values (regardless of white or red muscle) were much higher than what we have previously found in the previous feed trial (described above), with values ranging from 15 - 20 uM AMC/mg protein (Figure 11). The activities in the previous trial were measured in "crude enzyme extract," so the results are not directly comparable. Nevertheless, they may indicate a higher expression level of lysosomal cathepsin, perhaps related to the loose skin issue (Bahuaud et al., 2010; Zhang et al., 2019)

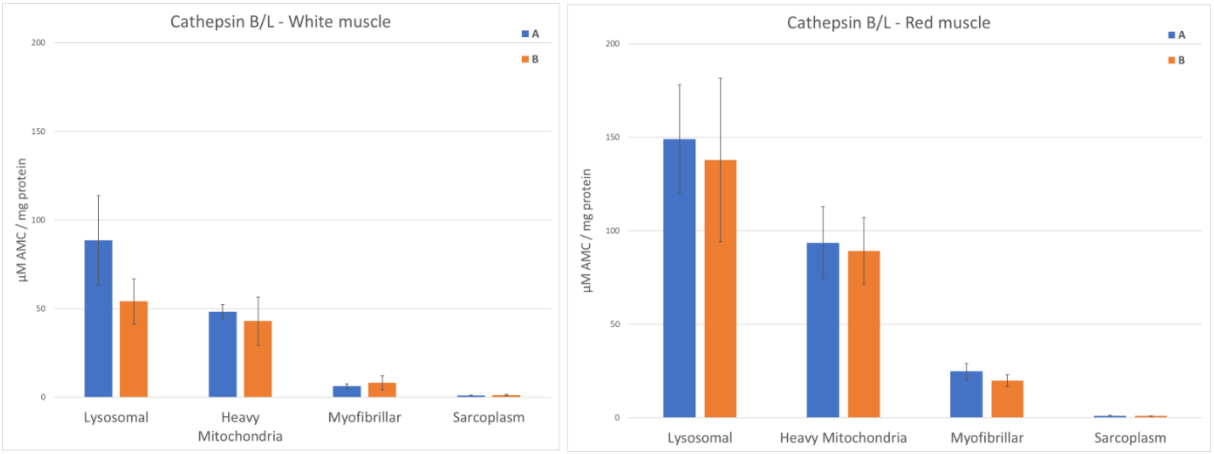

Figure 12. Enzyme analyses of fish with loose skin, from the same location but before and after slaughter (Blue (A – before slaughter) vs. Orange (B – after slaughter). In white (left panel) and red muscle (right panel).

5.6. Histology

To explore the structural composition of dense connective tissue in the skin, we conducted a series of methodological investigations into different skin sectioning techniques prior to embedding and staining. Our findings reveal that when we perform skin sectioning following the natural scale pattern (orientation of the scales), rather than a horizontal section along the anterior-posterior axis, we successfully generate tissue sections that mirror the alternating orientation of collagen fibers within the skin (Figure 13).

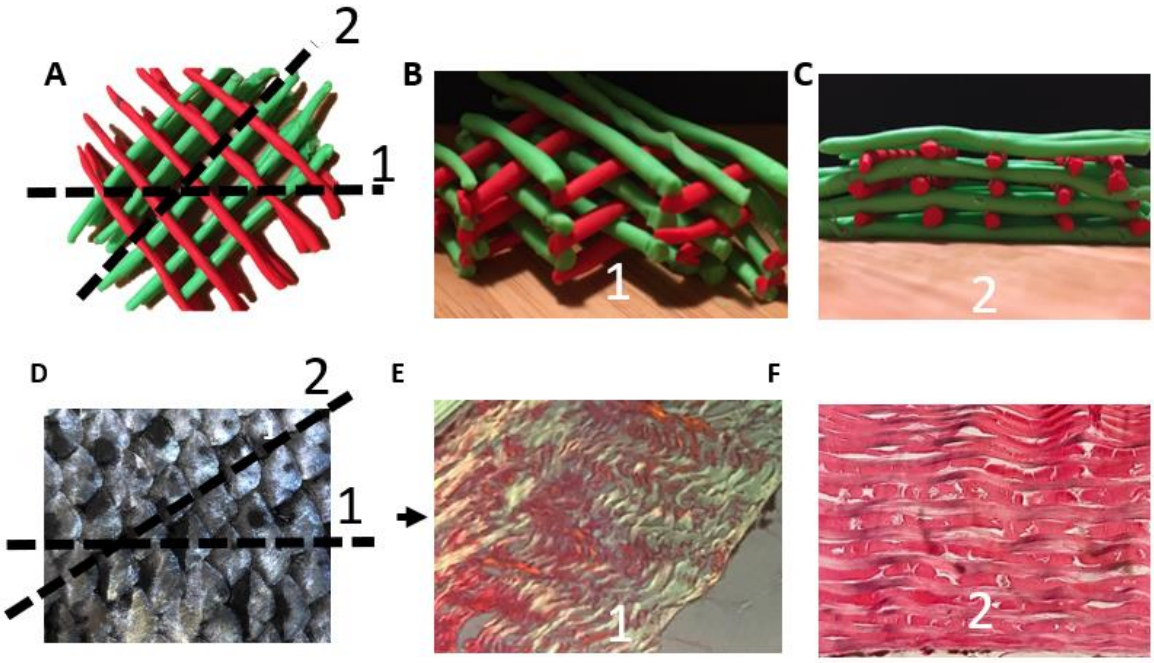

Figure 13. Collagen fibers in fish skin at different sectioning planes A – C Clay Model of Dense Connective Tissue in Skin. Red and green plastelina reflect the anticipated angle of collagen fibrils within the skin. B. Horizontal Collagen Network Section, section through the collagen network in a horizontal orientation. C. Section across the collagen network, revealing its structure of alternating fiber directions. D - F. Fish Skin and Histological Sections at Different Angles E. Cross section of fish skin, showcasing the "sock pattern" of collagen fibers. Section stained with Picro Sirius red and viewed under polarized light, with collagen fibers colored based on their orientation. F. Tissue section oriented "across," with every second fiber displaying an alternating direction, as illustrated in C. Tissue stained with Picro Sirius red and viewed under normal light.

To illustrate the alternating orientation of collagen fibers within the skin and explore potential connections between dense connective tissue in the skin and its separation from the underlying muscle tissue, we chose to section our samples across their structure as discussed in the above section. The analysis showed no discernible disparities in the histological characteristics of skin samples obtained from fish with either loose skin vs. firmly attached skin, or fish being handled (pumped) prior to analysis. Similarly, no alterations were noted in the white muscle tissue as a result of pumping (data not provided).

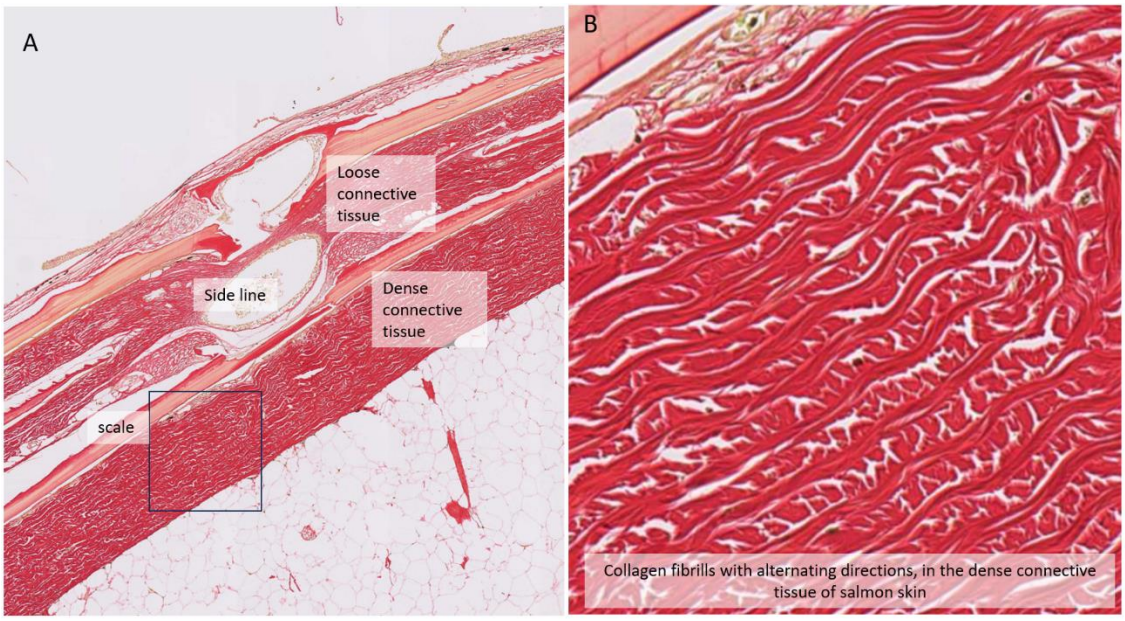

Figure 14. Histological appearance of fish skin and collagen fibrils. A. Representative sample of tissue section of fish skin sampled from fish population characterized with "loose skin". B. Enlarged area of A, the connective tissue of the skin looks normal, with densely packed collagen fibrills with alternating directions. Tissue stained with picro sirius red.

Given the observed variation in the ease of skin detachment from distinct body regions, particularly in the NQC area, we conducted a thorough examination of the skin and underlying tissues at various sampling sites (10 position per fish). The primary disparities were noted in the thickness of subcutaneous adipose tissue, which was more substantial in the mid-body regions (positions 2 - 4), and in the thickness of connective tissue within the myosepts, being thicker, in the tail region (position 5), as depicted in Figure 15. Further, the distance between the myoseptas were closer in the tail region, compared to the mid body, due to the organization of the myomeres.

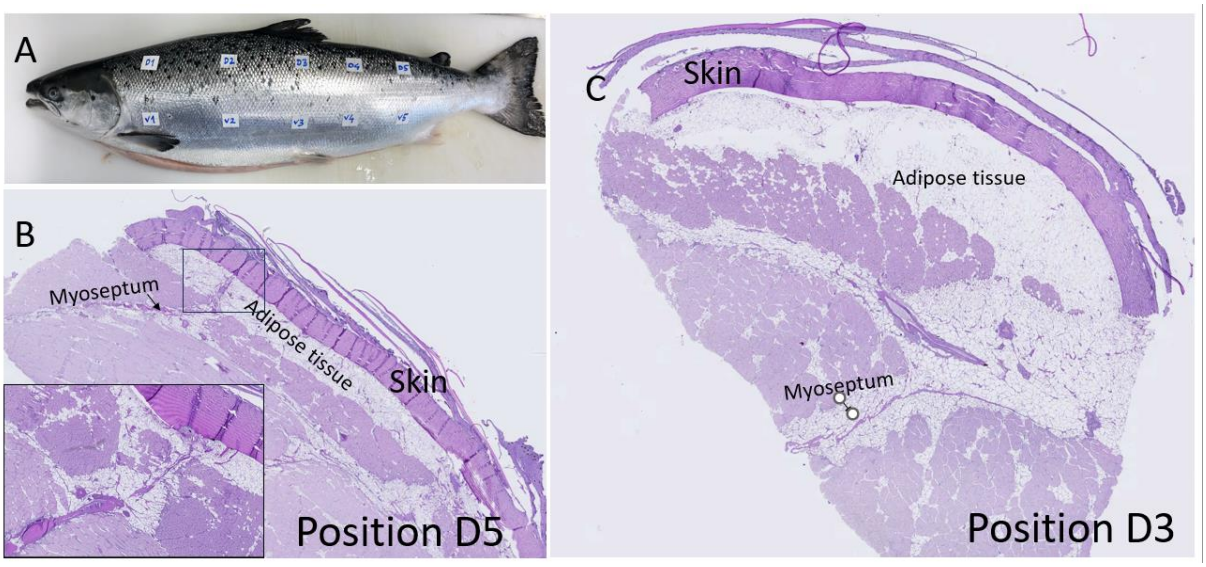

Figure 15. Skin differences at different sampling positions A. Skin sampled at 10 different positions. B. Skin in the tail region (D5 in A-dorsal tail region). The myosepta are thicker (insert photo), with thinner adipose tissue compared to C. skin sampled from the mid body, with thinner myosepts and thicker adipose tissue.

Skin samples from fish exhibiting loose skin characteristics were exclusively sourced from two production sites, denoted as "north" and "south." Notably, skin samples accompanied by red muscle tissue were obtained from the northern farming site, and only a solitary sample included white skeletal muscle tissue. Among these samples, there were no discernible alterations in the skin or red muscle tissue. However, the single sample featuring white muscle tissue displayed notable signs of tissue degeneration and hyalinization. To adopt a more comprehensive approach, we conducted extensive tissue sampling directly at a southern commercial production site where instances of loose skin were reported. Our sampling strategy went beyond just skin collection; we also gathered white muscle tissue and various organs, (intestine and gills) from ten fish. In this sample set, we observed pronounced signs of white muscle degeneration. This was evident through loosely packed myofibers of varying sizes, internalized nuclei, hyalinization around the fibers, and indications of atrophy. Further, an exudate was present in most samples, associated with the degenerated muscle fibers, and / or in the myoseptas (Figure 16). Also in these samples, the skin displayed no visible abnormalities, while red muscle tissue showed small inconsistent deviation in morphology, with vacuolization. Notably, within the same batch of samples from the southern production site, we identified muscular degeneration in the intestinal submucosal muscle tissue. Alterations in muscle tissue at multiple body sites may indicate a “systemic condition” affecting the muscle tissue. However, it is difficult to conclude as we only sampled multiple organs from one production site.

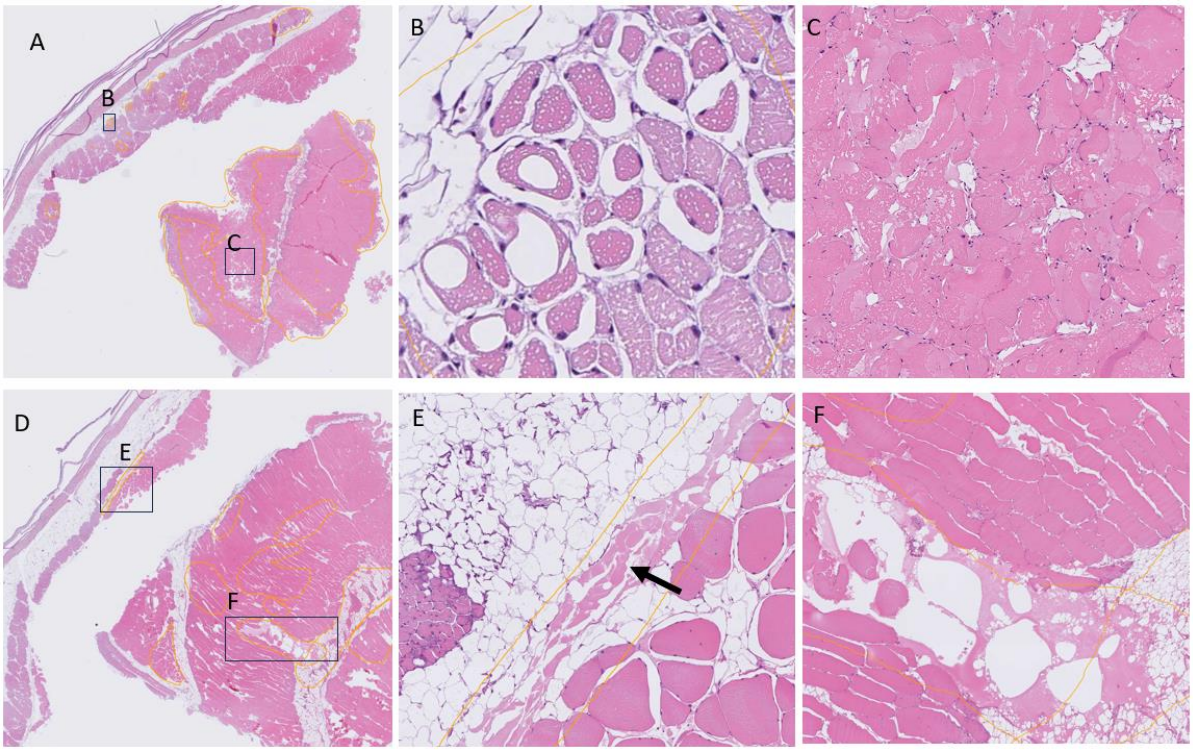

Figure 16. Tissue sections of skin with red and white muscle tissue In this figure, each row corresponds to a sample from an individual fish. A and D provide an overview of the complete tissue sections, with areas displaying degeneration marked by thin yellow lines. B, C, E, and F offer magnified views of specific regions within the primary samples, revealing distinct features: B showcases vacuolization within the red muscle tissue. C and F depict the degeneration of white muscle tissue, accompanied by exudate. E highlights muscle degeneration and exudate within the muscle tissue and myoseptum.

5.7. Transcriptomics, 15K microarray

Microarray analysis was performed on samples obtained from white muscle tissue collected at two distinct production sites, referred to as north and south. Muscle samples from the southern production site were labeled as south loose skin (SLS), while those from the northern region were sampled during observations of loose skin in August, termed north loose skin (NLS), and reattached skin (NRS) at the north.

The 15k microarray output unveiled a total of 1980 Differentially Expressed Genes (DEGs) across the three sample sets when compared to previous datasets of healthy samples. Among these, the white muscle tissue from NLS fish exhibited the highest degree of differential expression, with 1691 DEGs (Sum of Squares 3529.8). NRS fish displayed 742 DEGs (Sum of Squares 1575.3), while SLS fish exhibited the lowest degree of gene expression changes compared to the healthy sample set, amounting to 505 DEGs (Sum of Squares 827.6). Gene set enrichment analysis of the genes commonly expressed in the three sample sets revealed a noteworthy enrichment in immune response pathways, noticeable shifts in sugar and nuclease metabolism, and substantial alterations in cellular structure and tissue extracellular matrix (ECM) components across all fish samples (refer to Figure 17). Specifically, among the 80 differentially expressed genes (DEGs) commonly found in both sample sets diagnosed with loose skin (excluding reattached skin, refer to Figure 17), there was a significant enrichment of immune pathways, along with enrichments in cell apoptosis, cell ubiquitin, and the metabolism of amino acids and iron heme, as detailed in Figure 17. Compared to previous studies in the Nofima STARS database, this subgroup of genes exhibited a coinciding expression profile with fish infected with PD, HSMI, PRV, SAV, plasmids, and melanin spots. The gene expression also aligned with the wound healing process in mechanically induced wounds.

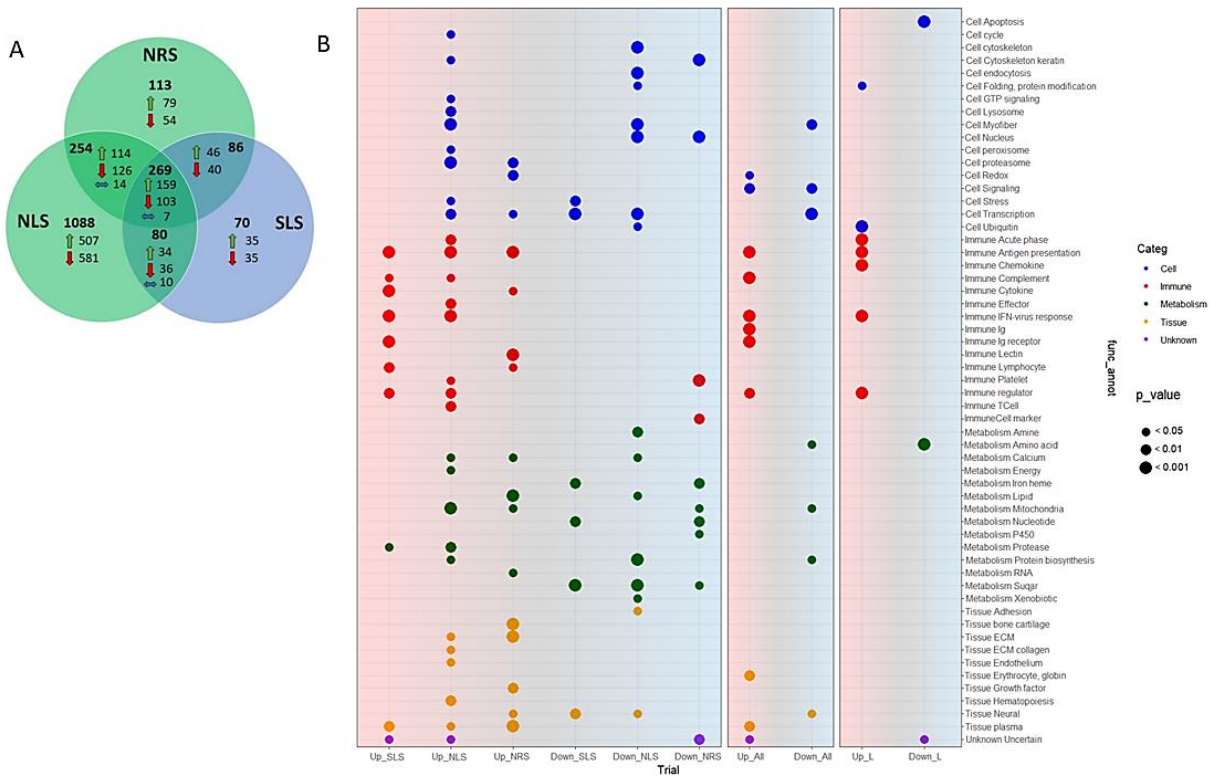

Figure 17. A Venn Diagram showing the number of exclusive and common DEG among the three datasets and their direction of expression. There were 1088, 113 and 70 DEG found exclusively in NLS, NRS and SLS respectively, 269 DEG to be commonly expressed in the white muscle all three groups. B up and downregulated functional pathways enriched in SLS, NLS and NRS independently as well as the enrichment of the commonly differentially expressed genes in the three set of samples (center of Venn Diagram) and in the loose skin only (-north re-attached) DEG (Bottom center of Venn Diagram).

5.8. Skin attachment strength

Relevance of direction of skin pull.

The first factor considered in the development of the method for objectively assessing on how firmly the skin is attached to the muscle was to determine the effect of pull direction. Hence determine if there was a difference in the force required to separate the skin from the muscle if pulling the skin off in the rostro-caudal direction (from “head to tail”, (HTT) or from “tail to head” (TTH). Measurements were performed on the NQC, left and right side. The HTT pull direction required significantly more work to pull the skin off the NQC compared to the TTH direction (Figure 18).

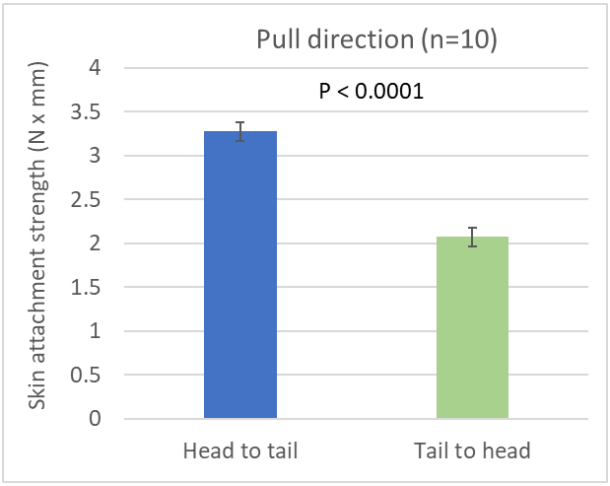

Figure 18. Skin attachment strength as affected by pull direction; where skin was pulled in the rostro-caudal ("head to tail") direction on one side of the NQC, and the opposite direction ("tail to head") on the other side (n=10).

Effect of pumping fish into the slaughterhouse

There was no significant difference (P = 0.7) between the skin attachment strength of salmon sampled directly from holding net pen outside slaughter facility and fish being pumped into slaughterhouse (Figure 19).

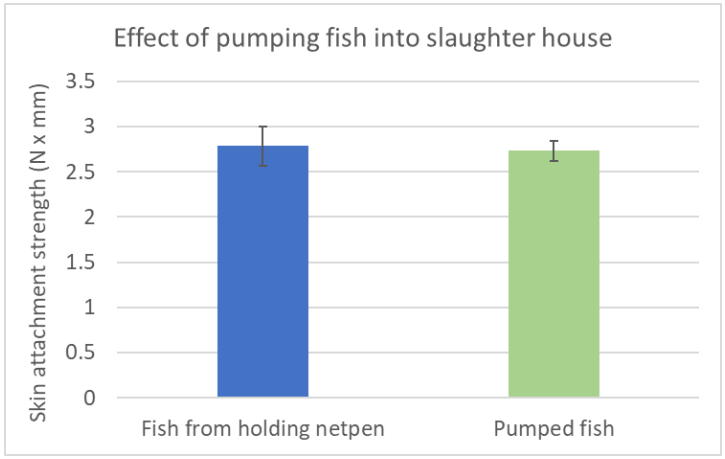

Figure 19. Skin attachment strength of fish pumped into slaughterhouse or sacrificed directly from the holding net pen outside slaughter facility.

Refrigerated seawater, RSW

RSW treatment of salmon prior to ice storage, as compared to storage on ice throughout the whole storage period, had no significant effect (P = 0.93) on skin attachment strength (Figure 20). When handling the fish there was an indication, from experience, that fish from the RSW group were more flexible and had less firm rigor state. Fillets from the RSW group were less firm as measured instrumentally, compared to the control group (P = 0.02; Figure 20). No fillet gaping was observed in any of the fish from the groups.

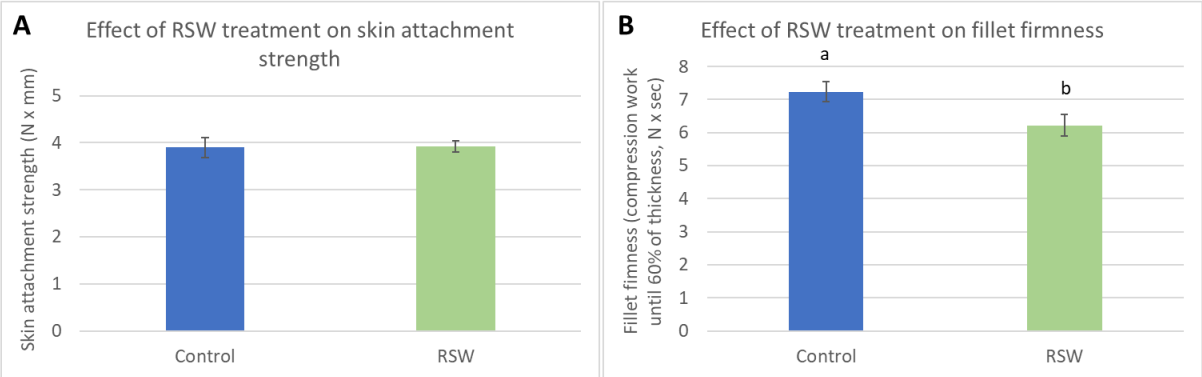

Figure 20. Skin attachment strength (A) and fillet firmness (B) of salmon stored in RSW and then on ice, compared to salmon stored on ice throughout the whole storage period («Control»).

Association with fillet firmness and gaping

For all fish materials that were analyzed for fillet quality, in addition to skin attachment strength, in the project, there was in total a significant positive correlation between skin attachment strength and firmness of the muscle (P = 0.007, n=36). No such association was found for fillet gaping (data not shown).

5.9. Exposure to high temperature, simulating thermal delousing

Different delousing methods are used in Norwegian seawater aquaculture, whereof thermal delousing is the most common method, involving short term exposure of the fish to temperatures up to 34°C. In the present small scale, model study we exposed muscle of salmon to temperatures ranging from 22 – 34% (22, 26, 30, 34°C with 30 second exposure and 34°C with 2 min exposure). I.e. our aim was to study the effect of temperature *per se* on skin characteristics. Samples were taken from five different locations of four 0.8kg salmon, farmed in freshwater (RAS, Fishlab, NMBU – samples were randomized relative to location). Heat treated muscle was compared with untreated muscle immediately after exposure under the microscope and in H&E-stained histology sections.

It was not possible to detect any clear temperature effects on the skin integrity, that coincides with statistics provided by the industry, showing no evidence of any relationship between thermal delousing or mechanical delousing and claims on loose skin. However, examining fresh skin samples under the microscope immediately after harvesting, revealed that the fatty layer under the skin is extremely vulnerable to mechanical stress. This was in our case illustrated by gently pressing a needle onto the fatty tissue (adipose layer), that instantly transformed to a foamy structure (Figure 21).

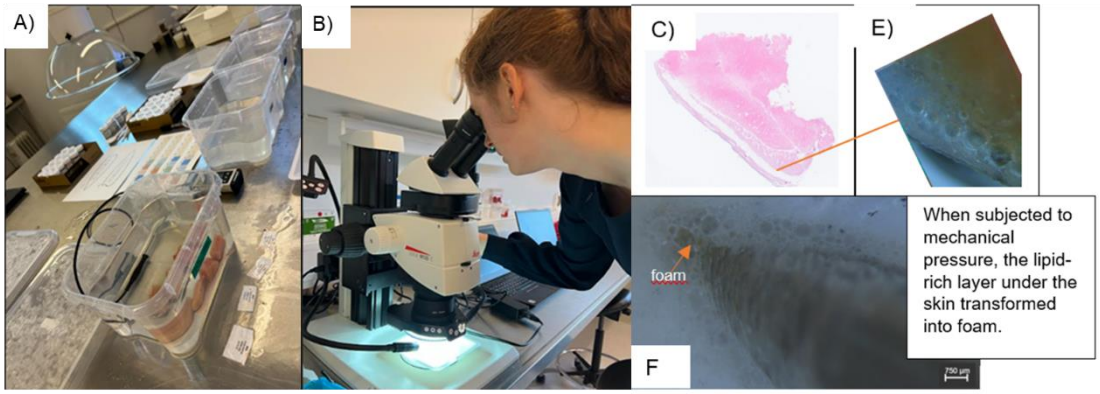

Figure 21. A) Treatment of salmon skin (including muscle) pieces in water bath with different temperatures (22-34°), B) examining the skin under the microscope immediately after treatment, C) histological section of skin and skeletal muscle, E) illustrating the visual appearance of the fatty layer under the skin, and F) illustrating transformation of the fatty layer to a foamy structure when exposed to gentle pressure by a needle (750 µm).

6. Discussion

Reports on loose skin conditions in Atlantic salmon and rainbow trout raise concerns given their adverse effects on product quality and potential impairment of fish welfare. Because the problem seldom is detected in the slaughter facilities, but rather after days of ice storage, it is challenging to detect loose skin issues before the fish reaches the market.

Since the problem with loose skin was acknowledged as a quality concern less than five years ago, in 2019, there has been limited research conducted and the hypotheses have been wide, considering incidents in the rearing phase, harvesting and improper storage conditions to be the main causes. Given the substantial lack of knowledge, this project had a broad approach, including a [I]survey among industry stakeholders, [II]sampling of fish with skin issues in ongoing feeding and slaughter handling projects, with the involvement of the project participants, [III]some minor tailored small-scale experiments, and importantly, [IV]close collaboration with the reference group and other industry stakeholders who contributed with relevant fish materials and knowledge.

In a project of the current nature, where foundational knowledge is notably restricted, collaborative efforts with the industry and a willingness to share and contribute fish material and knowledge are imperative for achieving meaningful advancements in knowledge. Without this close collaboration and the immediate sharing of pertinent fish materials and knowledge, the impact of the present project would be limited.

The survey conducted as part of the project involved respondents across the value chain, from farmers to purchasers, with 30 reporting on salmon and 7 on rainbow trout. One third of those responding to the questioners had experienced loose skin, and there was a broad agreement among them that the issue of loose skin occurred between 2019 and 2023. Interestingly, divergent opinions were given on how problematic the issue with loose skin is. Even some respondents who had experienced the issue did not perceive it as problematic. As expected, the survey indicated that loose skin is primarily identified post-harvest, with fewer occurrences during farming and harvesting. Complaints ranged from zero to more than 10 per year, with those experiencing over 10 complaints considering loose skin the most problematic. The survey did not reveal a specific high-risk period or geographic area for fish with loose skin. However, according to our observation and analyses of fish farmed in various regions, there seem to be certain periods of the year where the possibility of observing problems with loose skin is particularly high, such as late summer-early autumn in the North and late spring-summer on the West coast. These periods coincided with problems with soft fillets and gaping in those regions. Given the association of loose skin with other quality issues, there is a possibility that it is underreported, as customer claims may focus on different quality problems even when loose skin issues are present. Furthermore, companies involved in value-added products, like smoked salmon, are expected to have higher detection rates for loose skin. The survey did not provide precise prevalence information, leaving the exact percentage of fish with loose skin unknown at present.

Numerous batches of salmon identified with loose skin were examined but obtaining rainbow trout with loose skin for analysis proved unsuccessful. In each case of loose skin, a glossy liquid was observed between the skin and skeletal muscle upon fillet removal. The liquid predominantly comprised fat (69%), with different contents of specific fatty acids compared to the muscle. The fatty acids present in higher proportions closely resembled those found in plant oils, particularly 18:1n-9 and 18:2n-6, which are abundant in rapeseed oil—a primary oil source in salmon feed. Conversely, the long-chain marine fatty acid 22:6n-3 consistently exhibited the highest levels in the muscle. Therefore, this fatty acid (22:6n-3) appears to be preserved in the muscle and not selectively lost, as for the typical plant-based oils. Moreover, it is worth mentioning that we found evidence suggesting that the fatty tissue beneath

the skin is especially prone to "collapsing" under mechanical pressure (observed under the microscope of fresh muscle).

Although the protein content in the liquid collected under the skin was low (1%), a difference in the amino acid composition was likewise observed between the liquid and skeletal muscle. Higher content was observed for histidine, arginine, valine, and also glycine, a crucial amino acid contributing to collagen formation (the "glue" of the muscle). Additionally we found a significant correlation between histidine level and severity of gaping in a fish material were we studied effect of transportation. On the contrary, tyrosine, leucine, asparagine, serine, and isoleucine were lower in the liquid compared with the muscle. These consistent observations are interesting and might add relevant insight not only to the problem with loose skin, but also salmon texture and gaping issues.

The stability of amino acid composition in skeletal muscle is a well-established fact, minimally influenced by protein content or amino acid profile in the feed. Hence, it was somewhat unexpected to observe significant differences in the amino acid composition of the collagen between salmon and rainbow trout. These findings underscore the need for caution when extrapolating knowledge gained from one species to another, emphasizing the necessity for further investigations, particularly in the case of large rainbow trout farmed in seawater.

Gly is responsible of collagen stabilization throughout the formation of hydrogen bonds (Montero & Borderías, 1990). Hence the collagen of re-attached skin would, according to the results from the collagen stability analyses, be less stabilized by hydrogen bonds, but on the contrary seems to be more crosslinked due to the higher presence of lys involved in collagen strength and stability by the formation of covalent bonds (Moreno et al., 2016). Also, the higher presence of glutamic acid is an interesting finding. Amino acid residues having a molecular weight of 1000-10000, such as Glu among others, have been successfully studied as fibroblast growth promoting agent (Shimizu et al., 2015) Hence, it could be considered that in the re-attached skin there has been an occurrence of collagen biosynthesis. Moreover, it could be hypothesized that collagen of firm texture muscle is more stabilized than soft texture muscle. On the other hand, the lower presence of hydrophobic amino acids in firm texture muscle has been negatively correlated with breaking strength of Atlantic salmon muscle (Moreno et al., 2012).

Notably, amino acids being higher in salmon with reattached skin were met, leu, lys, and arg. In total the content of hydrophobic amino acids was significantly higher in salmon with reattached skin. These findings represent the first results showing differences in collagen amino acids related to the skin attachment, suggesting the need for further investigations, especially in cases of casualties. It can, however, be mentioned that *met* residues can provide a catalytically efficient antioxidant defense by reacting with oxidizing species, while *leu* (*leu* enriched diets) is shown to promote collagen remodeling with exercise, also indicating stimulated collagen synthesis. Lys has been shown to contribute significantly to collagen stability (gain in triple-helix stability in the presence of Gly-Pro-Lys-Gly-Asp/Glu-Hyp sequences (Persikov et al., 2005). Arg metabolism is an important pathway involved in collagen, and dietary supplementation is shown to enhance collagen synthesis. But again, there seem to be a comprehensive lack of knowledge regarding involvement of amino acids in collagen synthesis and stabilization, particularly in fish.

No indications of increased activity of degrading enzymes was detected, but the extent of lipid peroxidation was higher in salmon with loose skin compared with salmon with reattached skin, as well as in soft muscle in contrast to firm muscle. These findings are interesting, suggesting that oxidation should looked further into in future studies – also considering soft fillets.

Based on the transcriptional and histological analysis, it is suggested that the loose skin condition is caused by a component before slaughter, primarily affecting the musculature. Based on the histological analysis, it appears that loose skin may be associated with the degeneration of both white musculature and myosepts. Myoseptum, or myosepta, represents the connective tissue or membrane responsible for

separating muscle segments, known as myomeres, within the trunk of a fish. These myoseptal structures are firmly anchored to the fish's skin at multiple attachment points in the tail region, while they spread out at multiple smaller attachment points in the main torsoe. We also see that the adipose tissue is thicker, and the myoseptum thinner in the mid body positions of the fish, which are areas of the fish body which is reported to be most affected. Consequently, the weakening or degeneration of these myoseptal structures, and associated muscle tissue, seems like a plausible explanation explaining why the skin more easily detaches from the muscle tissue. Without any firm anchoring to internal structures, the skin, regardless of being intact, is likely to become easily detached from the muscle after filleting.

Further, the transcriptomic analysis with significant differential expression of genes linked to immune pathways, cellular structure, and myofibrillar structure suggests the presence of this condition in the seacages pre-slaughter. However, it may be a transient condition, given the absence of individuals with loose skin in the trial in the North, three months after its initial observation across all cages, potentially pointing to a seasonal effect. Like soft fillets, various factors, such as feed energy, body energy, metabolism, protease activity, stress, season, smolt type, and starvation impact fillet integrity, making it challenging to pinpoint a specific causative agent. It could be of relevance to investigate the presence of potential pathogens in fish with loose skin condition, as the transcriptomic profile showed similarities to fish affected by PD, HSMI, VirusResponsiveGenes (Krasnov et al., 2021). The transcriptomic profile observed shares similarities with the atrophying muscle of rainbow trout undergoing vitellogenesis, showing decreased amino acid metabolism as an energy conservation strategy (Salem et al., 2013).

Moreover, based on the questionnaire, it appears that the issue with the loose skin problem is relatively recent. Therefore, investigating changes in production practices, medications, or other factors may provide valuable starting points to identify the underlying cause leading to loose skin. Concerning lice treatments, mechanical interventions are increasingly employed after lice have developed resistance to multiple pharmaceuticals. Such mechanical treatments may cause to underlying muscle tissue (Sveen, Timmerhaus, et al., 2021). For example, new pharmaceuticals acting on lice, such as imidacloprid, received marketing authorization in 2021. The pharmaceuticals azamethiphos and imidacloprid act by disrupting the transmission of nerve impulses. In the context of histological analysis of muscle tissue in other animals, (de Souza et al., 2022; El-Garawani et al., 2022), both pharmaceuticals result in muscle tissue degeneration with exudate and minimal inflammation, with phenotypic resemblance to the muscle tissue observed in this study. Consequently, conducting short and long-term assessments of the effects of these pharmaceuticals could be performed to rule out these drugs as potential underlying causative agents.

It appears probable that the development of the loose skin condition results from multiple "stress" factors, although industry statistics and trials within this project indicate that traditional delousing treatments (thermal, mechanical, freshwater) and/or pumping during slaughter handling cannot be linked to skin detachments.

In general, our findings suggest that the condition is present in live fish in sea cages but is typically first identified at slaughter or primarily after post-processing of fillets.

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8. Popular Scientific Fact Sheet, FAQ

Frequently asked questions

“Loose skin” in salmon and rainbow trout

What is “loose skin”?

“Loose skin" typically refers to a condition where the skin of the fish becomes detached or separates from the underlying muscle tissue.

When was “loose skin” recognized as a quality concern?

The separation of skin from the fillets of Norwegian salmon and rainbow trout was acknowledged as a quality concern less than five years ago, in 2019, despite examples of fish with loose skin dates back two decades.

When does “loose skin” appear?

It may only become evident after the fish has been stored for several days, posing a challenge to identify this condition before reaching the market. The occurrence seems unpredictable, although it appears to happen more frequently during late spring to autumn than in winter.

What is the prevalence of “loose skin”?

In a recent survey (2022-2023) among farming companies, and slaughterhouses, one-third of the participants had experienced fish with "loose skin." There are no numbers available on exactly how often the problem occurs in Norwegian aquaculture.

Is the problem with “loose skin increasing?

Opinions among stakeholders vary, as some believe the problem is on the rise, while others consider it as either stable or decreasing.

Does fish with “loose skin” have other quality issues?

There appears to be a correlation between "loose skin" and soft flesh as well as gaping, but not with color and fillet yield.

What is the cause of “loose skin”?

Due to the recent emergence of the "loose skin" issue, there has been limited research conducted. However, recent research has unveiled that the problem originates during the live phase of the fish, although stressful handling during slaughter and suboptimal storage conditions may worsen the "loose skin" issue.

FAQ described by partners in the project (2021-2023): Skin detachment from salmon and rainbow trout (Norwegian: Skinnfeste). Please visit hht://############### or contact *turid.morkore@nmbu.no or sven.m.jorgensen@fhf.no for further information*

Project partners:

Project funding: