CONNECTIVE TISSUE AND THE ATTACHMENT OF PIN BONE IN SALMON AND COD

Why are the pin bones so firmly attached?

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What is the problem?

- The pin bones are difficult to remove early *post-mortem* from the filet
- When removed either the filet is damaged, or the pin bones break inside the muscle
- The pulling force of pin bones decreases *post mortem*, differ between anterior and posterior position in the fish, and is higher in cod compared to salmon (Leif Akse *et al*, Fiskeriforskning, Rapport 15/2002) .
- Little is known about how pinbones are attached to the muscle

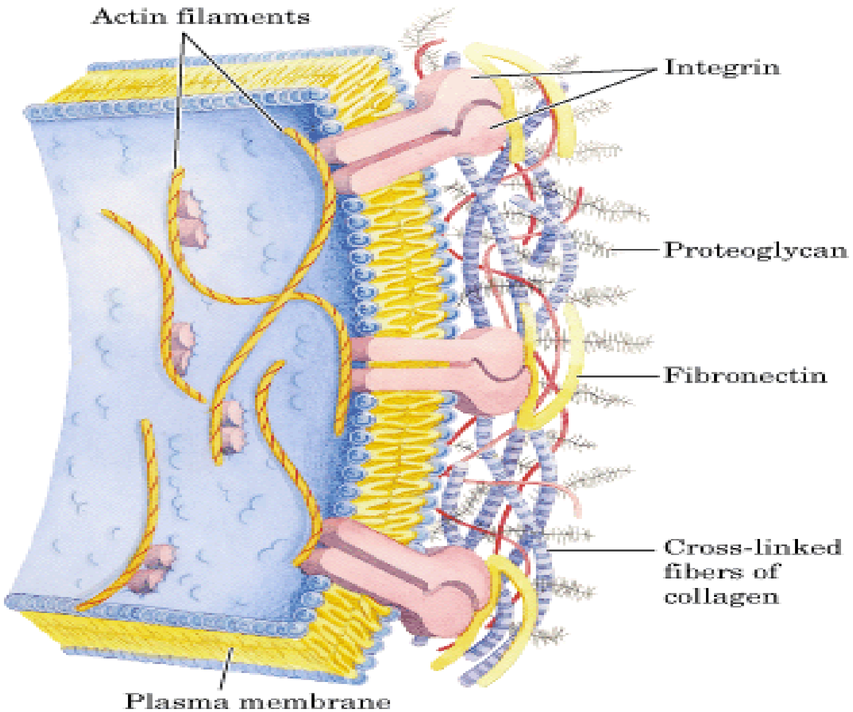
Connective tissue= a complex structural network

Extracellular matrix (proteoglycans, collagens and glycoproteins)

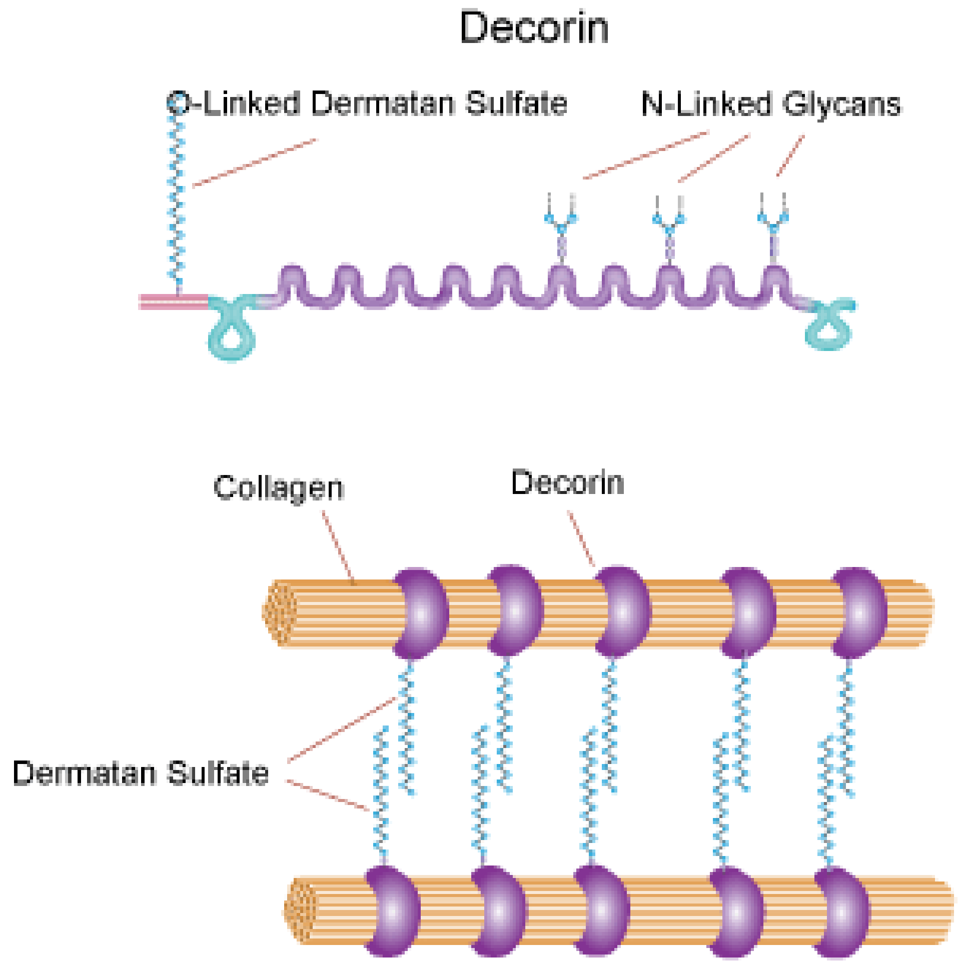
Adhesion proteins (syndecans, glypicans, integrins)

Cells (fibroblasts, fat cells, immune cells)

Enzymes (MMPs, serine proteases, aggrecanases, cathepsines etc.)



Strong interaction of carbohydrates and proteins



Aim of the study

- Characterize the structure of the attachement
- Identify connective tissue components in the structure
- Study enzymes and the degradation prosess *post mortem*

Sampling

- Salmon and cod
- 0h, 6h, 12h, 24h, 48h, 3 days and 5 days storage
- Dissected 6000 pinbones from anterior and posterior position in the filet
- Either fixed or frozen in liquid nitrogen before further analysis



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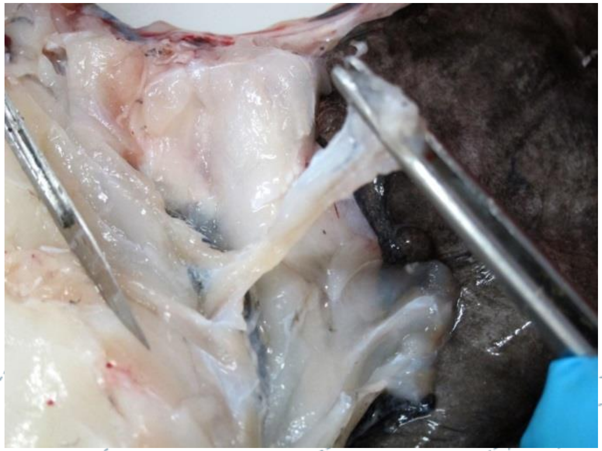
Methods used in the study

- **Microarray**: Screening of components in the structure.
  What is expressed of connective tissue components, adhesion proteins, enzymes ?

- **Histology:** Study structure, localization of relevant proteins and degradation of the structure *post mortem*

- **Zymografi:** Identify enzymes and their activity *post mortem*



- **Proteomics:** Identify relevant proteins that are changed (0h and 5d). Screening of proteins

- **Western blotting:** Verify changes of relevant single proteins during storage period

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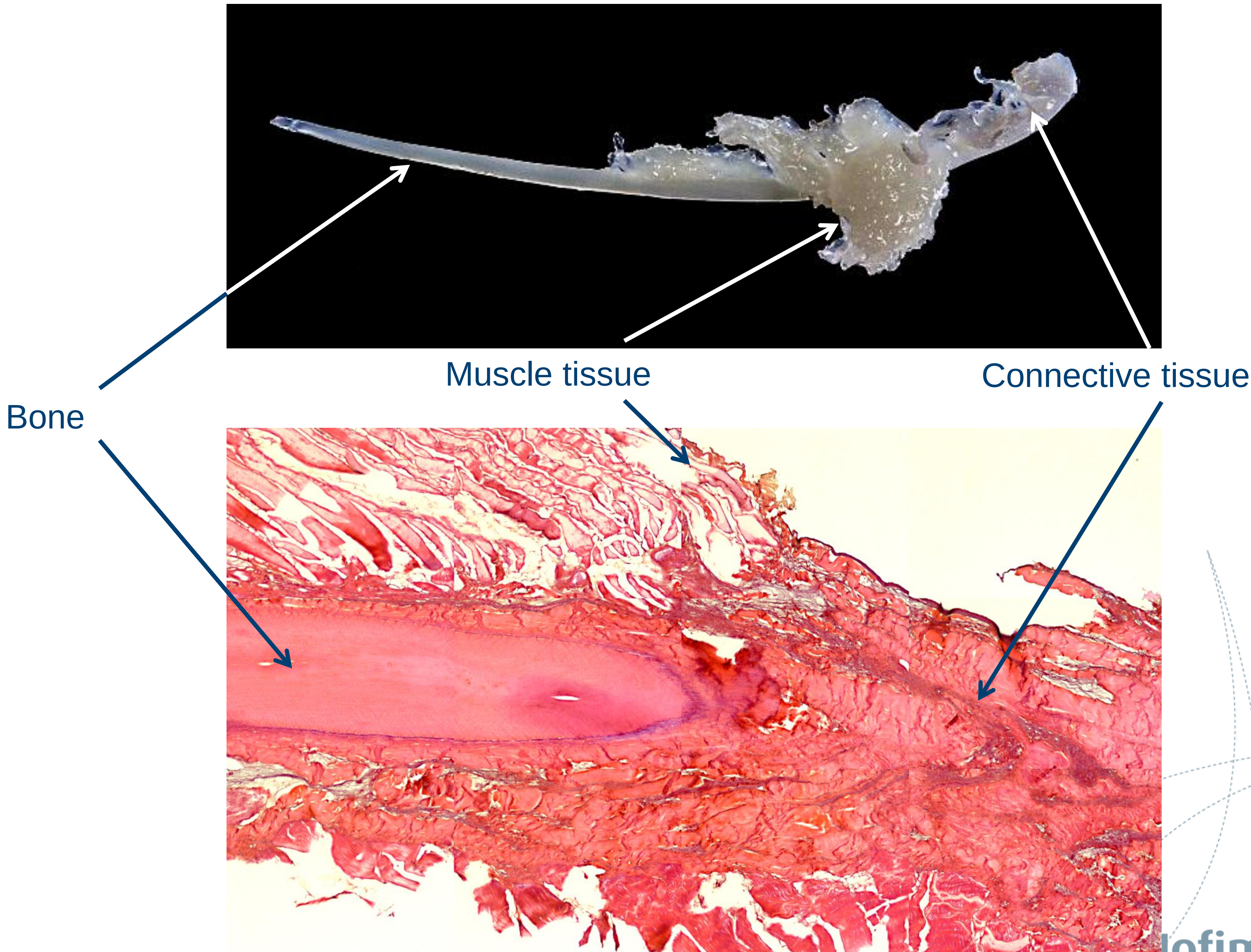
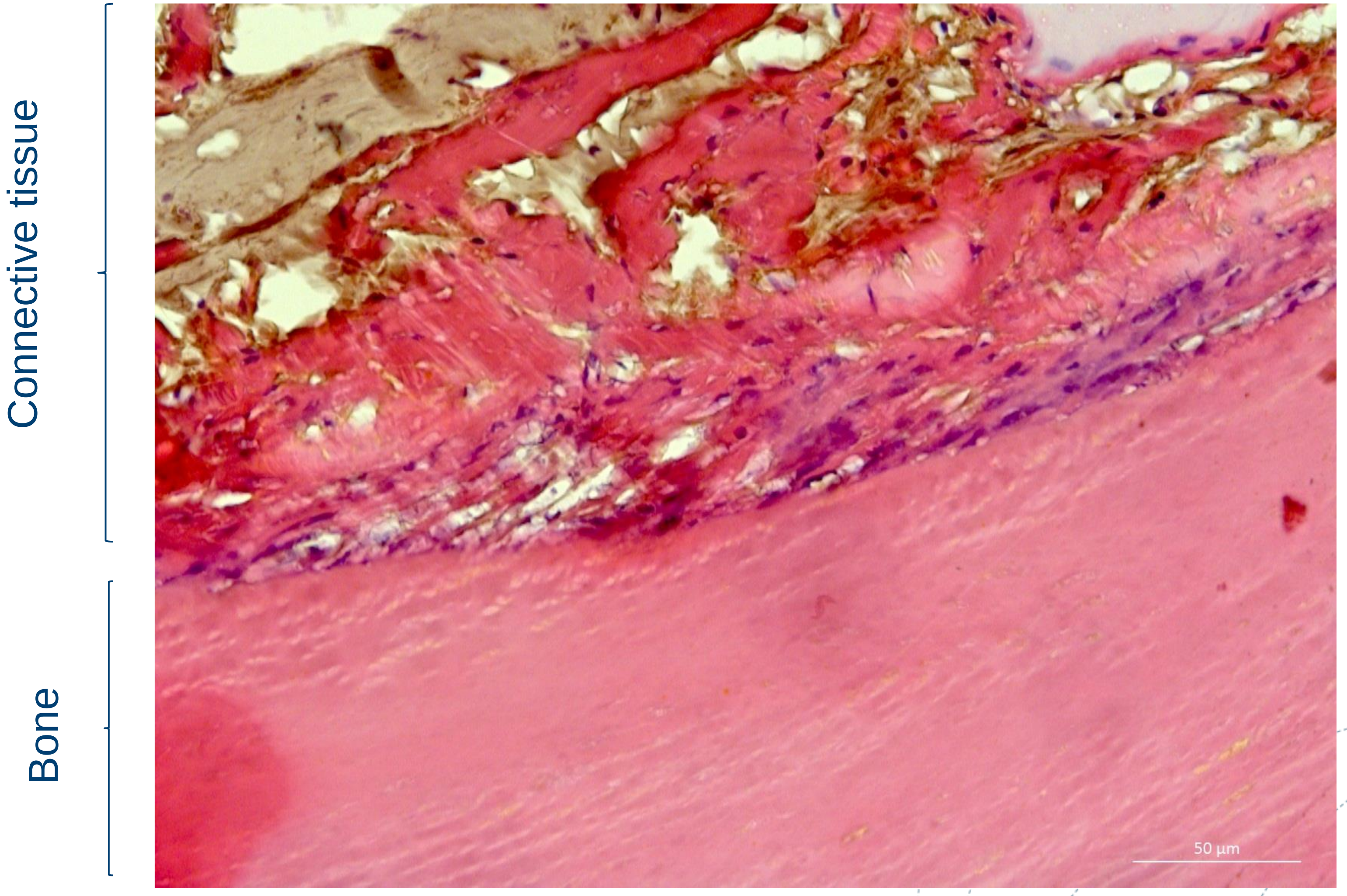
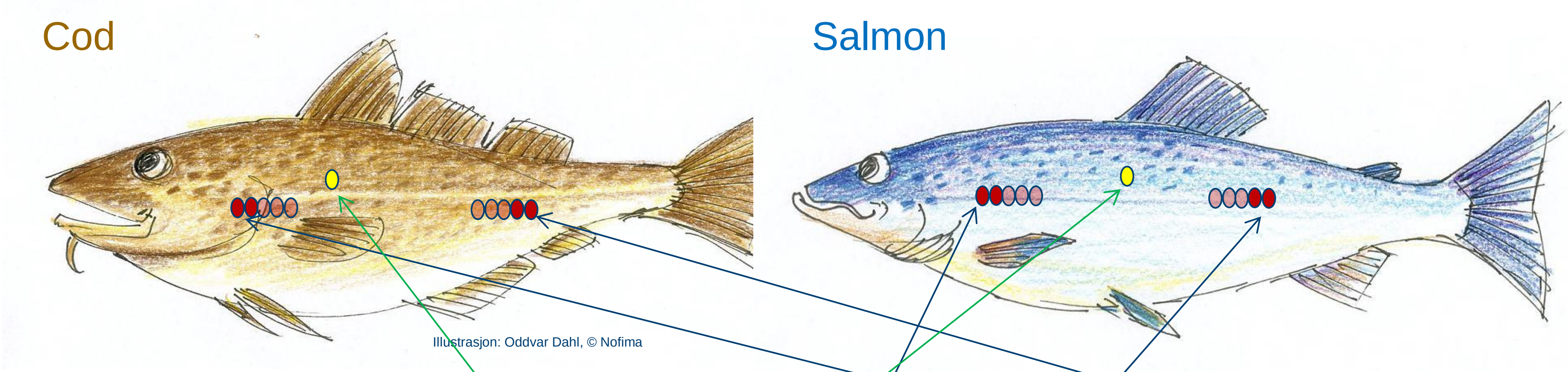

Bone
Muscle tissue
Connective tissue

Interphase connective tissue -bone



Gene expression analysis



- Pooled samples of the two most anterior and posterior pin bones from four fish were selected for microarray gene expression analysis.

- Pooled samples of muscle from all four fish were used as reference in the analysis.
- Comparison of gene expression profile:
  - Pin bone vs. muscle
  - Anterior pin bone vs. posterior pin bone

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Results – cod

Foto: © Frank Gregersen / Nofima

- > 2000 differentially expressed genes between pin bone and muscle
- Enrichment analysis of differentially expressed genes

Skeletal muscle	176
Immune	94
Metabolism-ribosome	50
Met-mitochondria	44
Differentiation	40
Metabolisme-lipid	21
Extracellular matrix	22
Stress	16
Metabolisme-proteosome	16
Neural	13
Chromosome	13
Metabolisme-protease	12
Cytoskeleton	11
Adhesion	8
Matabolisme-sugar	7
Metabolism-xenobiotic	6
Smooth muscle	5
Metabolisme-glycan	5
RBC	4
Metabolisme-amino acid	3

Examples of genes:

- Extracellular matrix: collagen I, IV collagen V, collagen XI, collagen XII, decorin, laminin
- Lipid metabolism: fabp, lipase, acyl CoA synthetase, acyl CoA dehydrogenase
- Protease: MMP13, calpain, cathepsin F,catepsin H, serine-protease, elastase
- Adhesion: Integrins

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Results – salmon

Foto: © Frank Gregersen / Nofima

- >193 differentially expressed genes in pin bone vs. muscle
- Examples of genes:
  - Extracellular matrix: collagen I, collagen III, collagen X, collagen XV, lumican, transgelin,
  - Proteases: collagenase, cathepsin K, MMP2, TIMP2, serine protease

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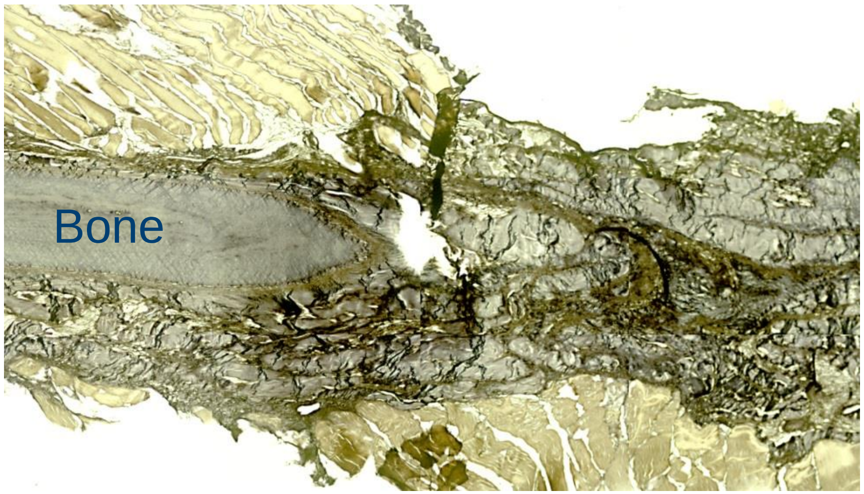
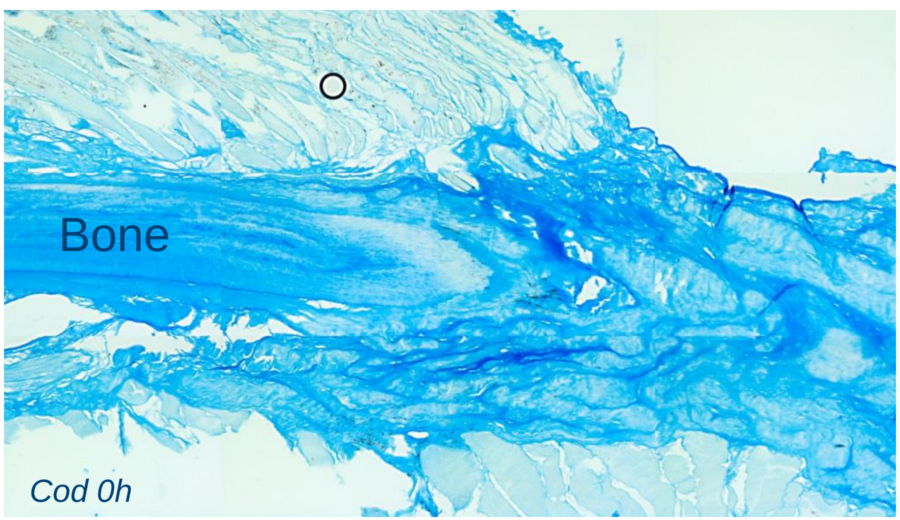
Results – cod and salmon

- Generally higher gene expression levels in anterior vs posterior pin bones of both species
- Different extracellular matrix composition between the two species

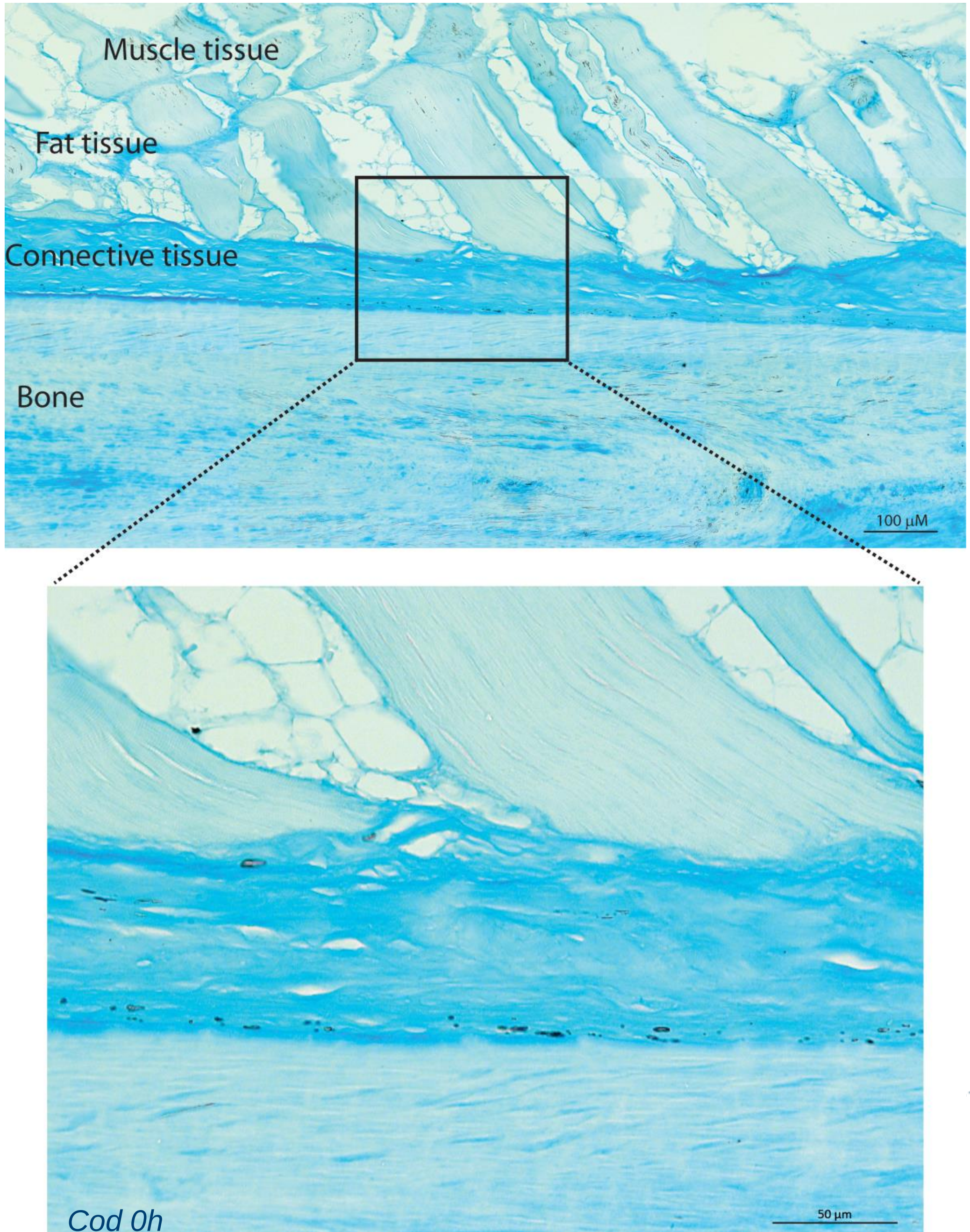
The connective tissue is rich of elastin, proteoglycans and collagens



Dark colour: Elastin
Brown: Muscle

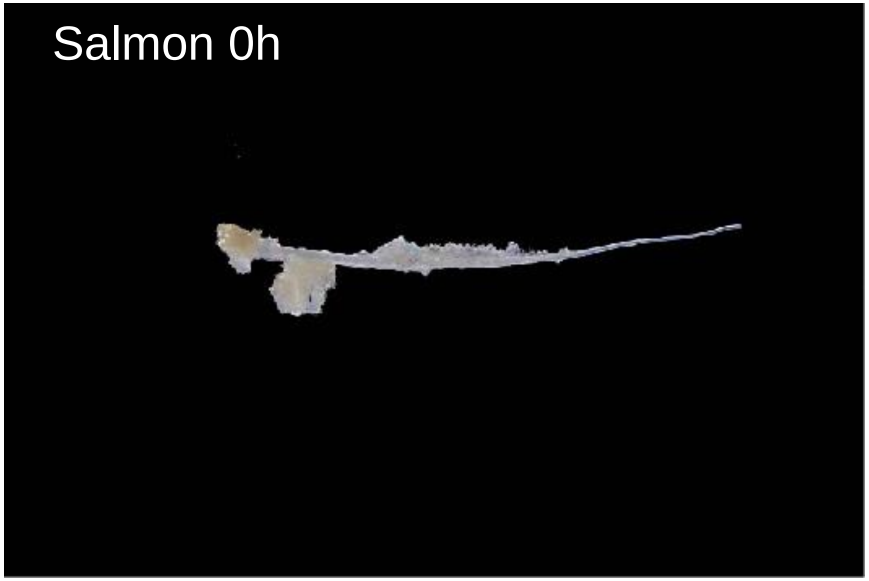


Dark colour: Proteoglycans
Light blue: Muscle



Tight connection between bone, connective tissue and muscle!!

But what happens during storage?

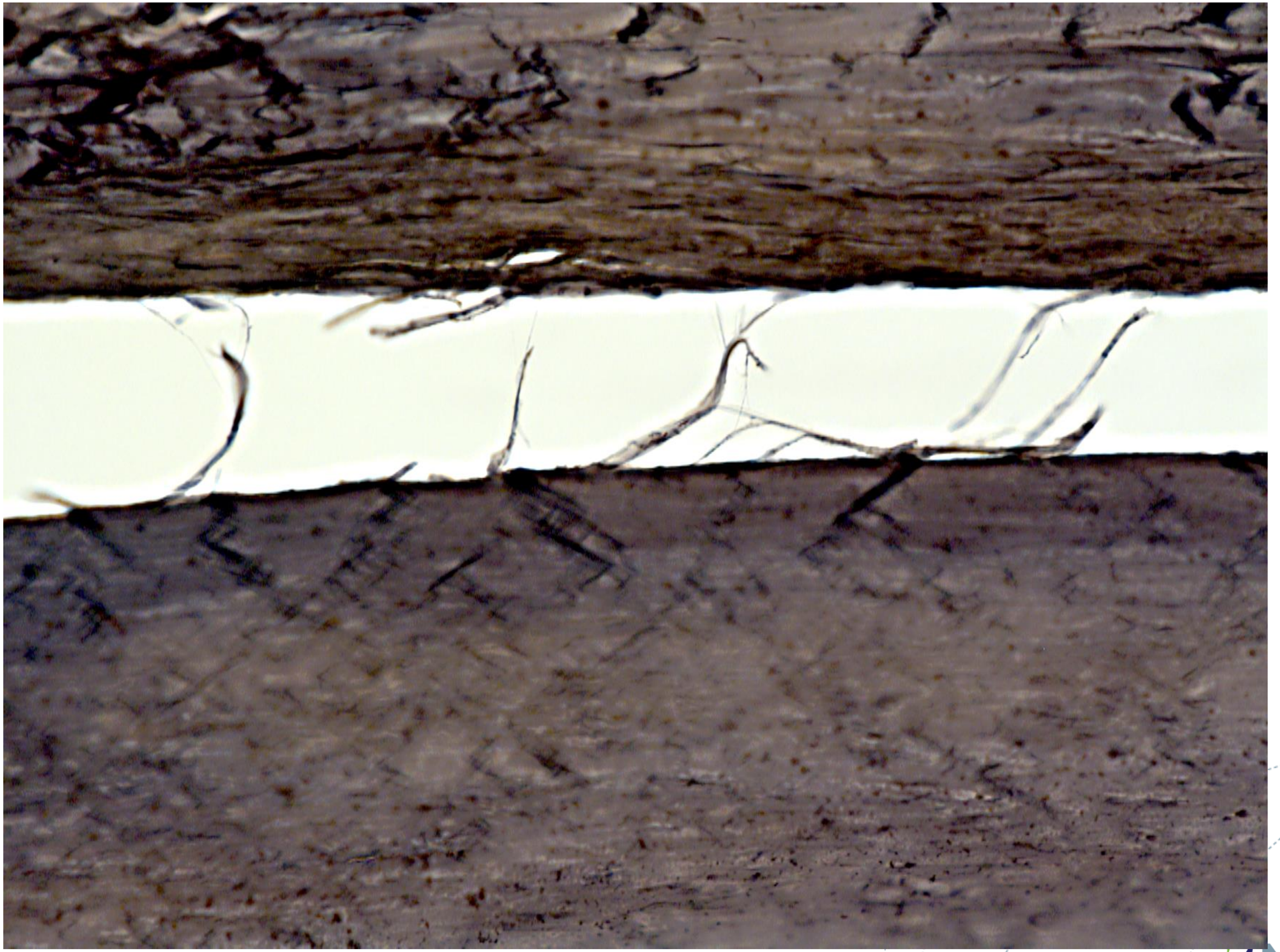

Salmon 0h


Cod 0h


Salmon 5 days


Cod 5 days

Elastin fibres are broken

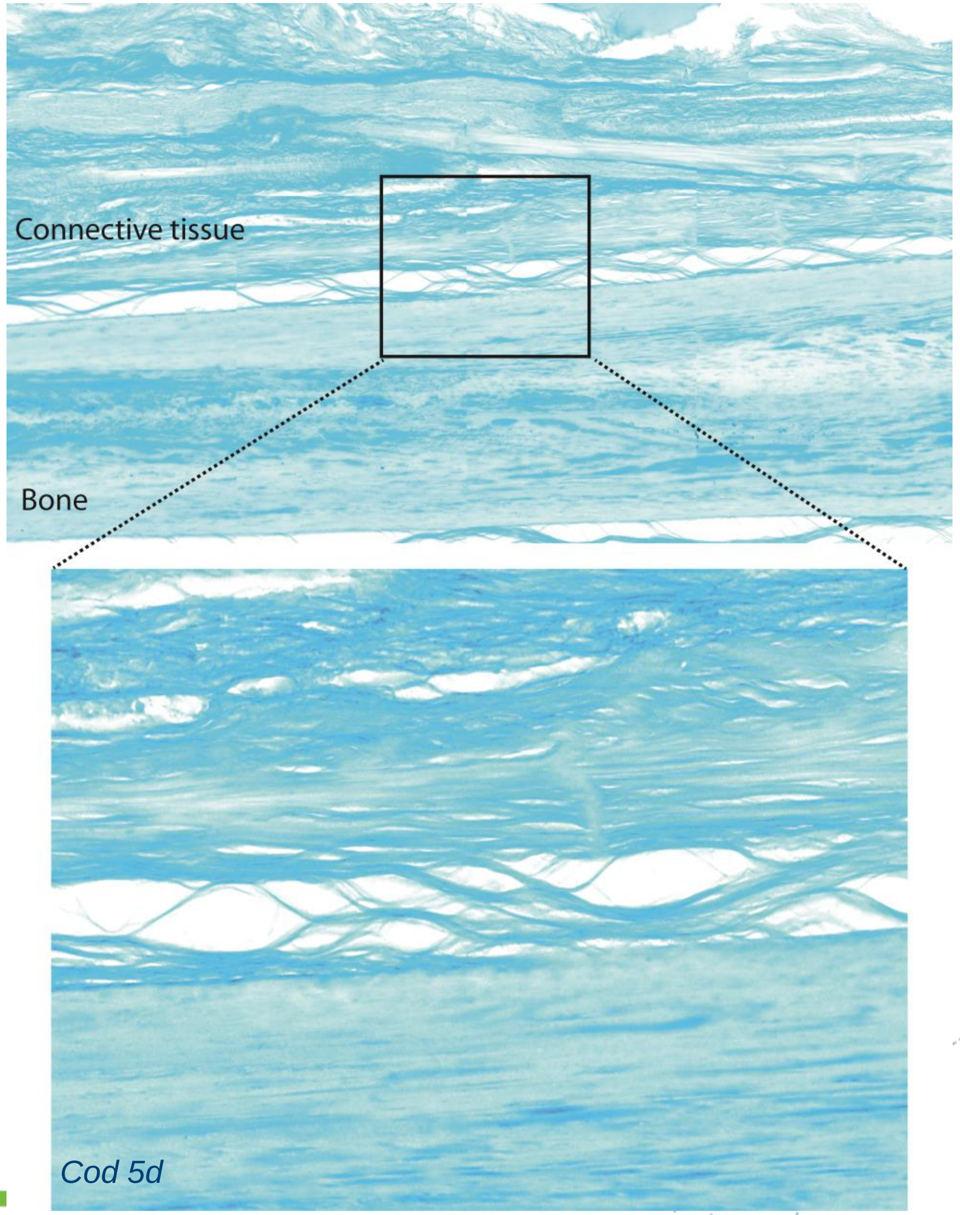


Connective tissue

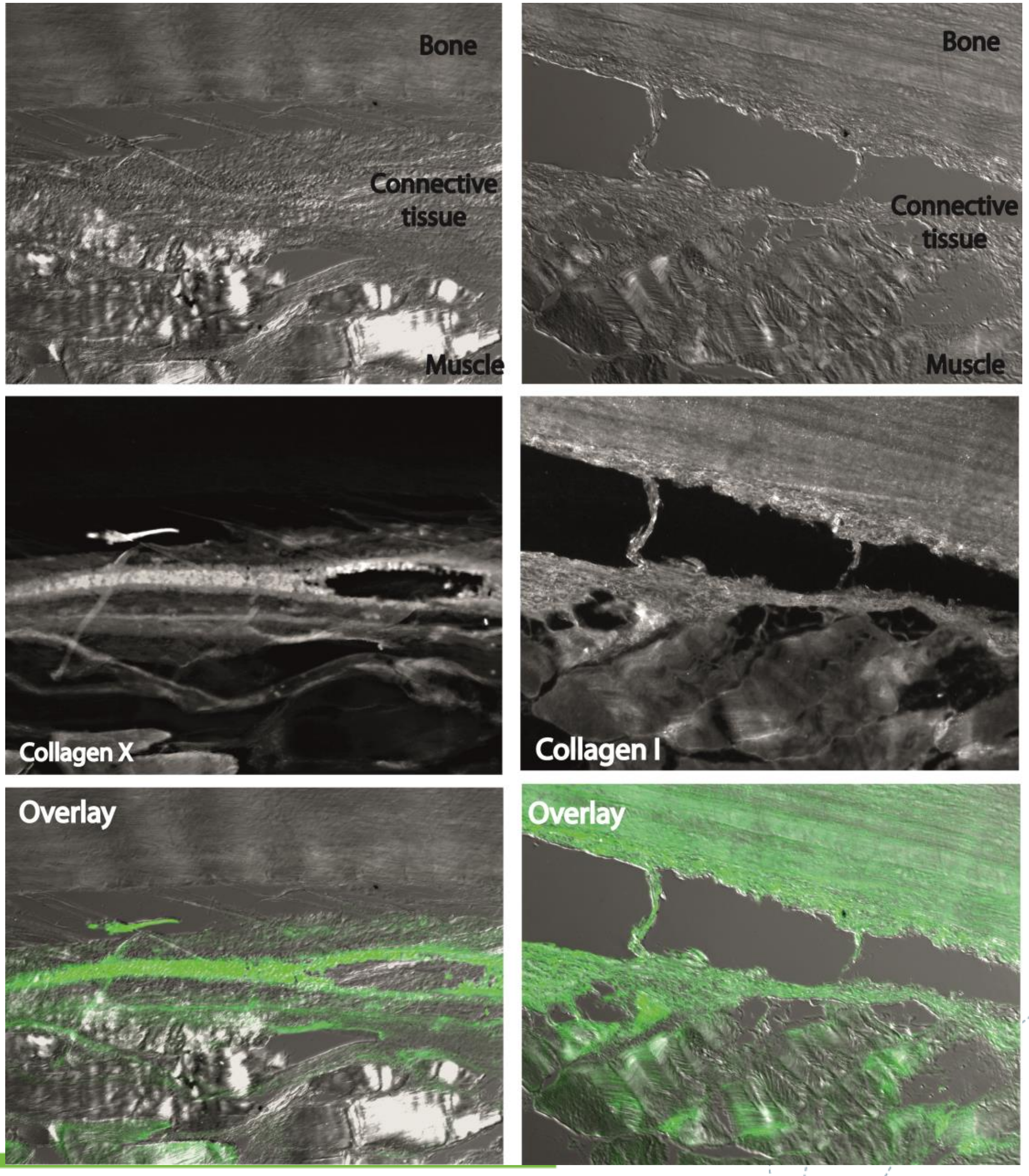
Bone

Cod 5 day.

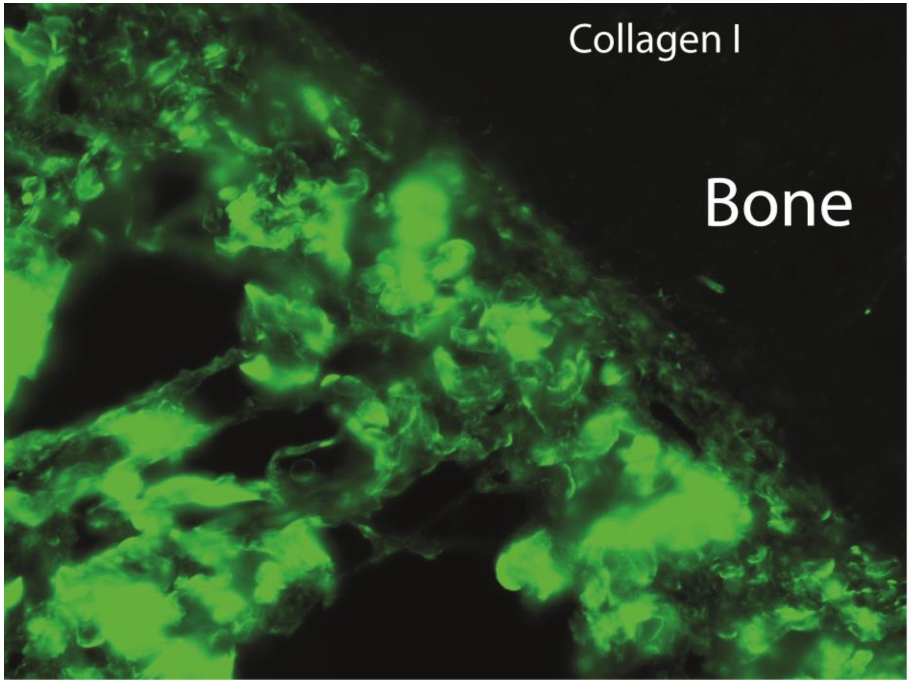
Threadlike structures containing proteoglycans

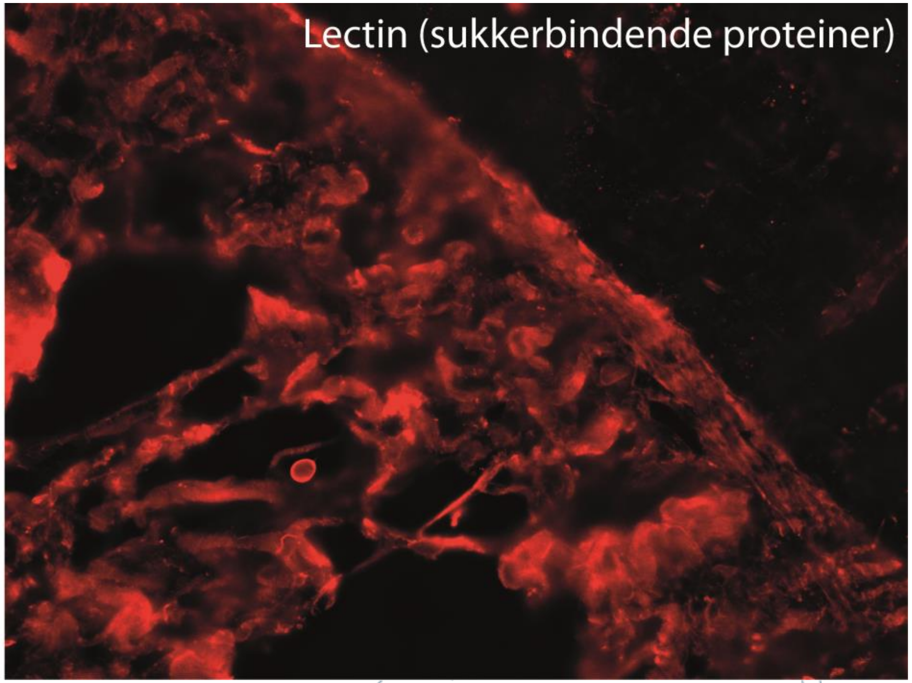


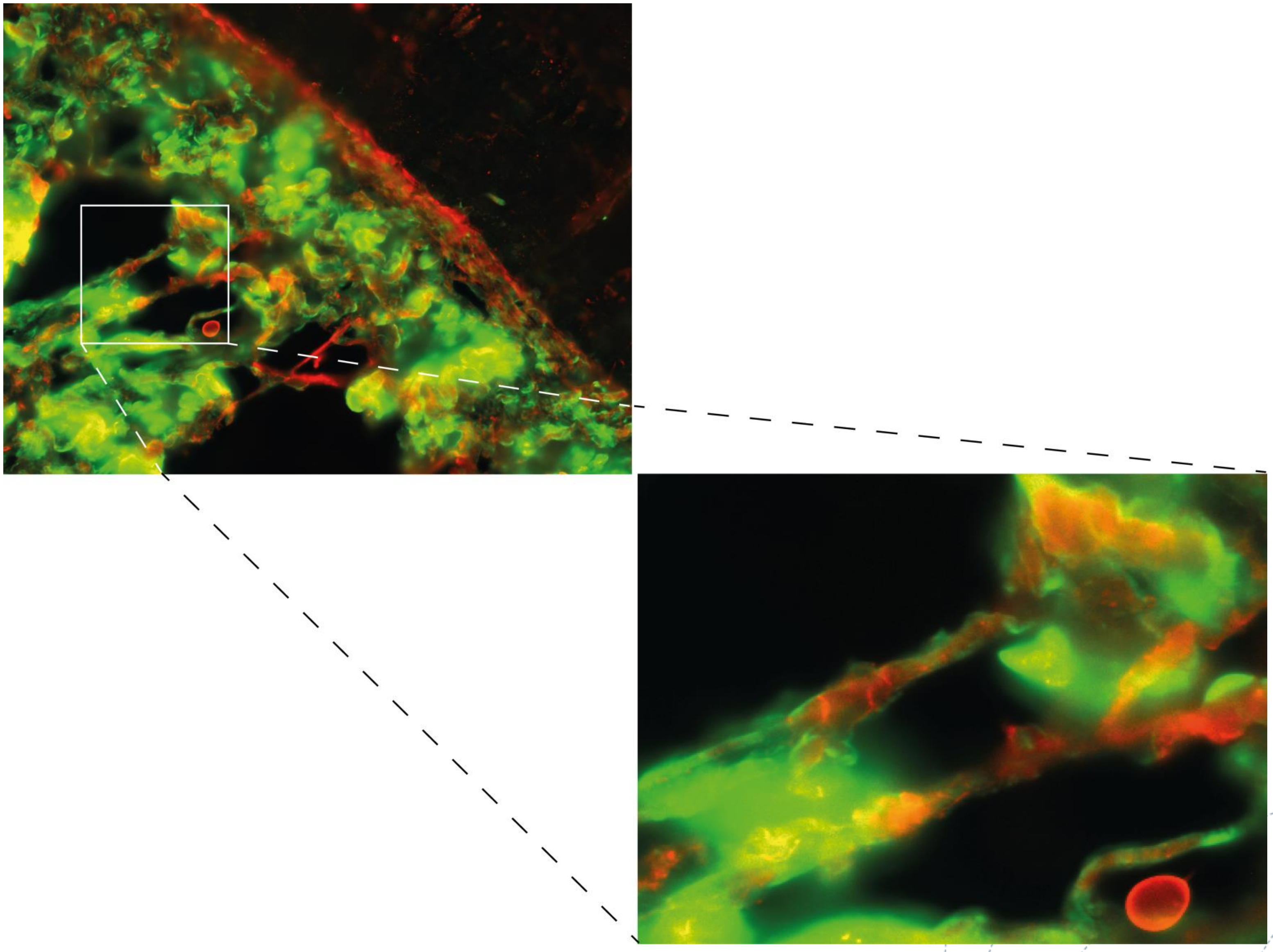
Localization of collagen X and I in the splitting area

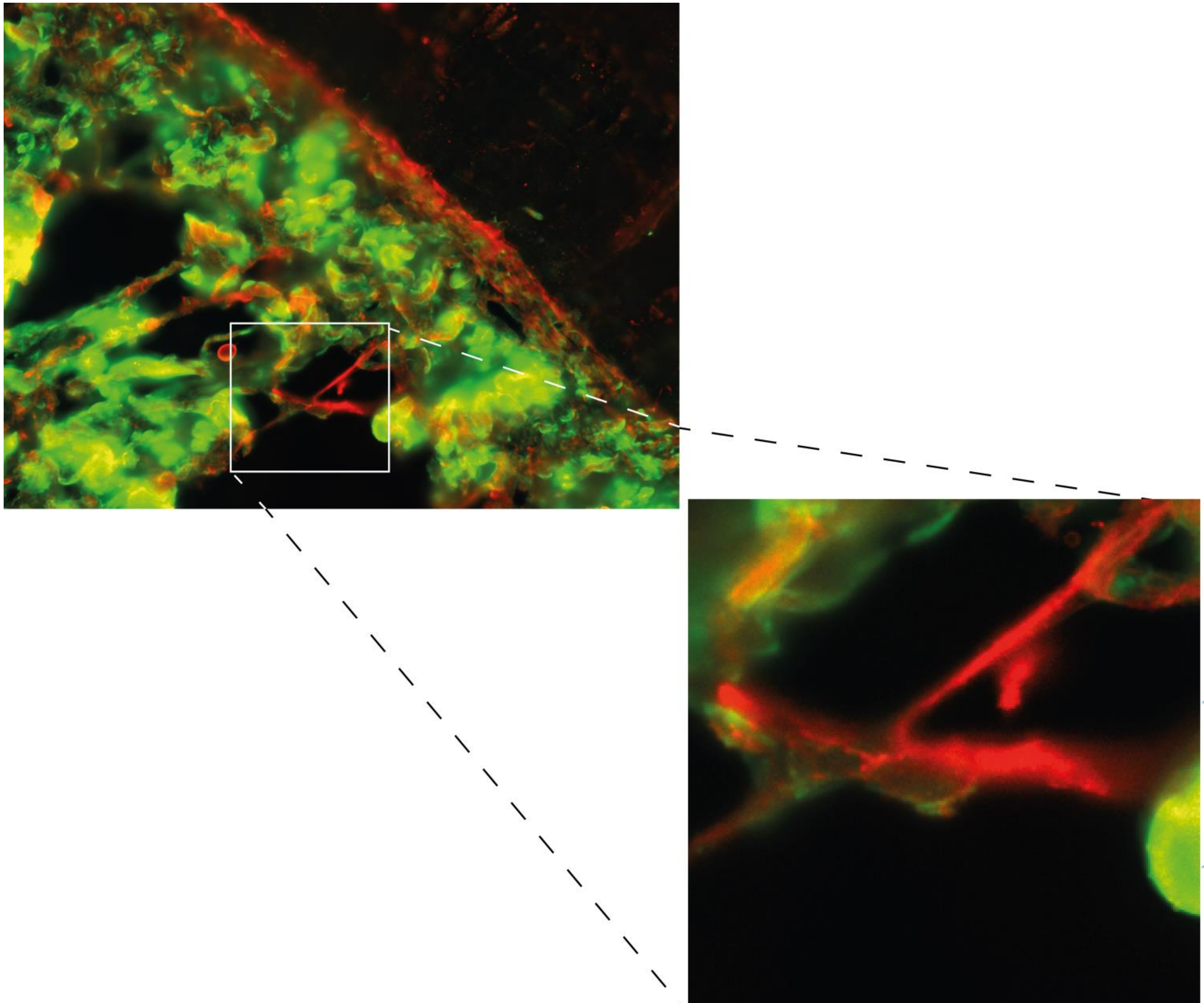


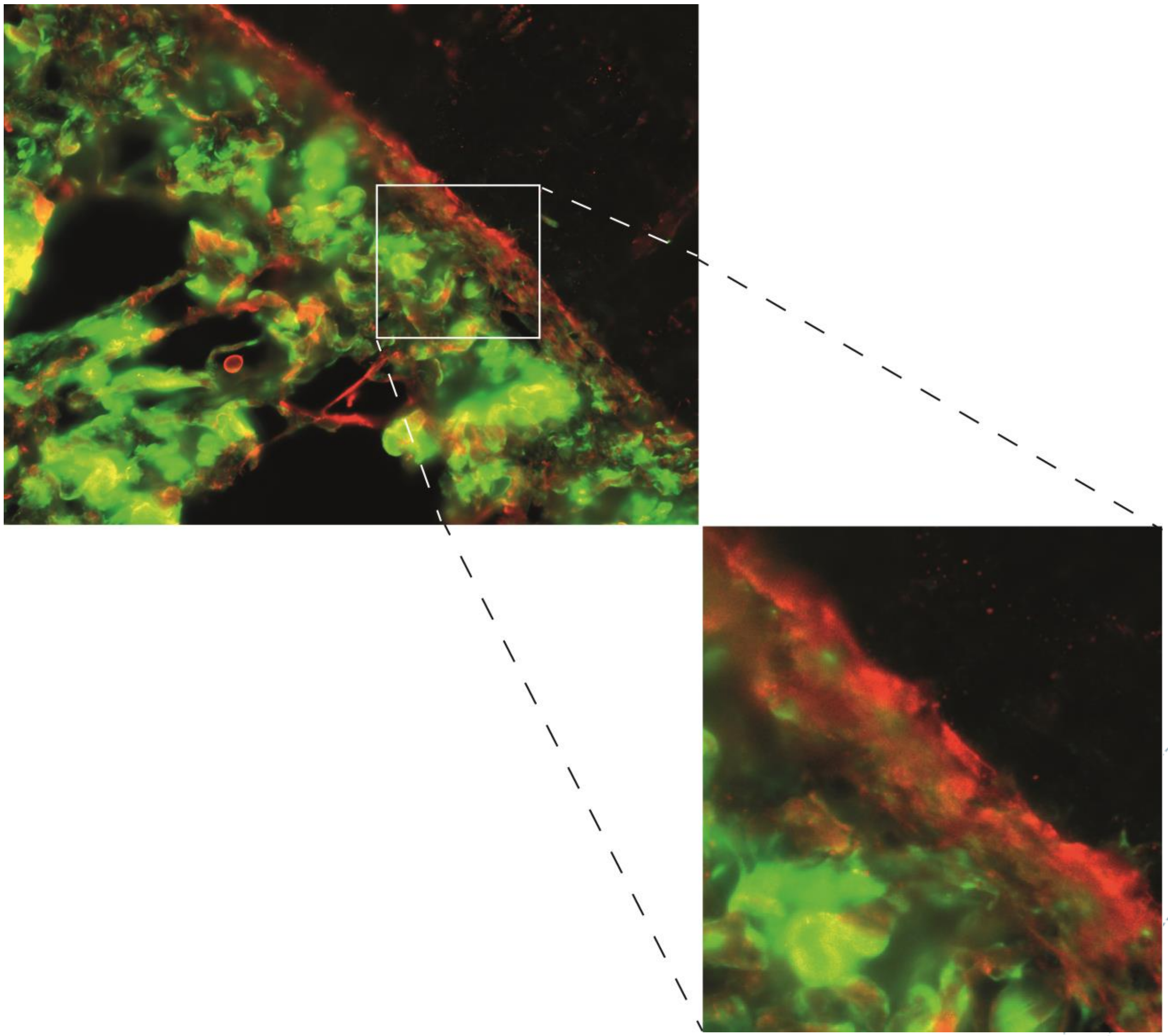
The network of carbohydrate binding protein and collagen in the splitting area





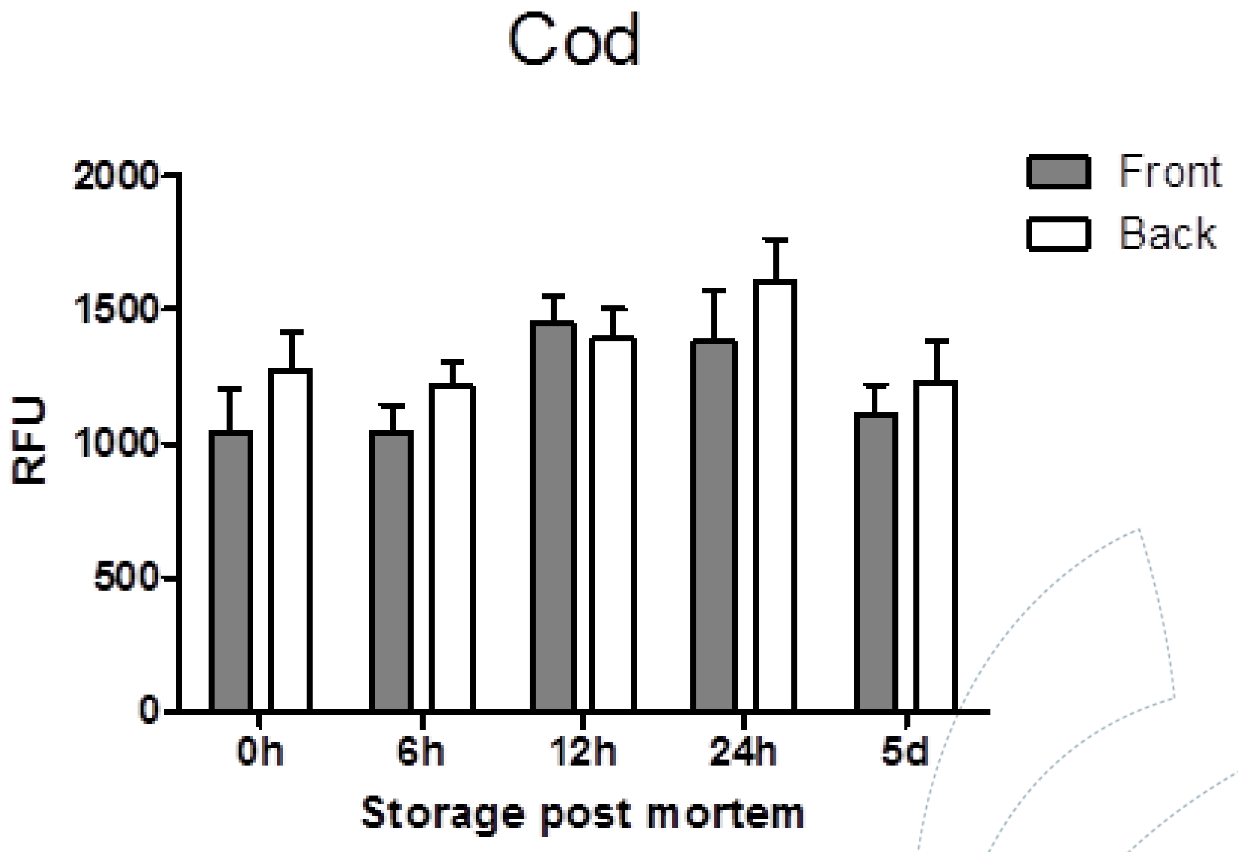




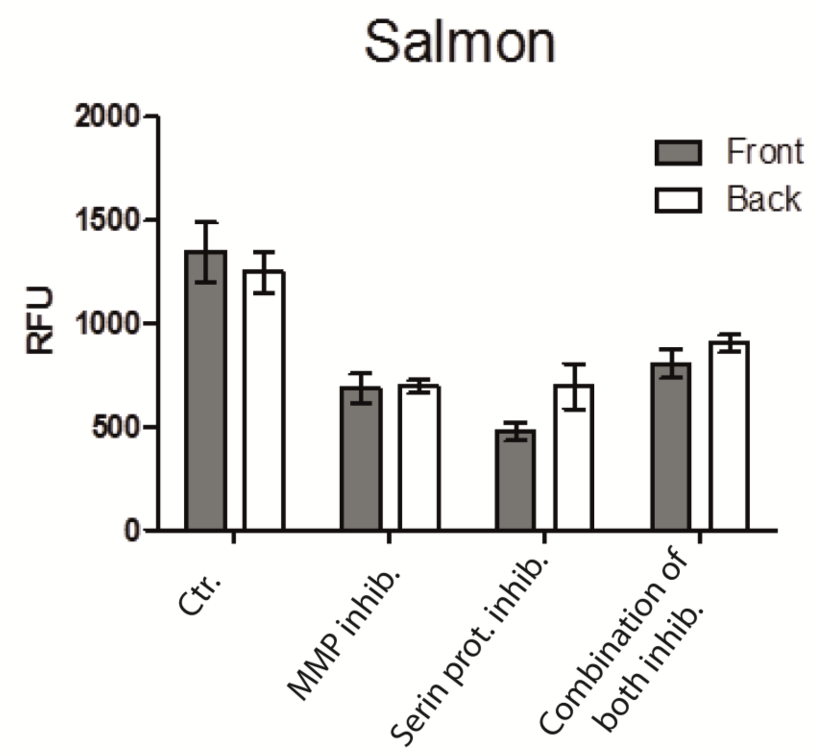


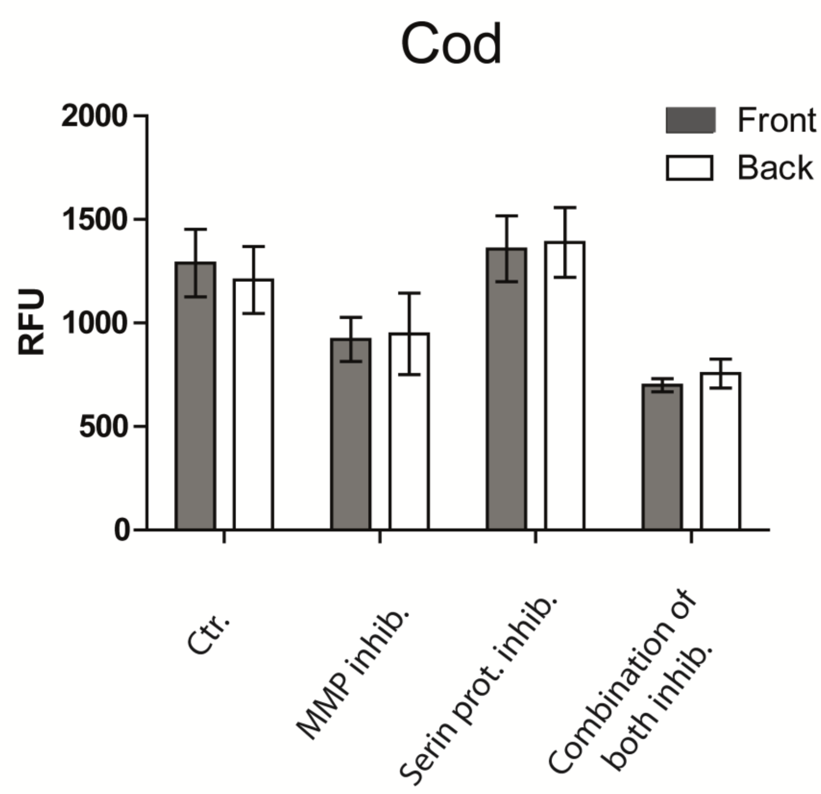
Metalloproteases are active right after slaughter in salmon

Metalloproteases activity is high during the storage period in cod



Different types of degrading enzymes are active





Conclusions

- The connective tissue is important!
- Differences between muscle and pin bone area
- Differences between salmon and cod
- Differences in enzymatic profile between salmon and cod
- The connective tissue is broken during storage into threadlike structures
- It is the attachment between bone and connective tissue that is degraded *post mortem*

Further work

- Histology of salmon and relevant candidate proteins identified by microarray analysis
- Study changes in proteins by proteomics during storage (0h and 5d)
- Study changes of relevant single proteins by western blotting during the storage period
- Identification of MMP types and localization

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How can we use this information?

- When we know the pin bone biology we can:
  - Develop better methods for pin bone removal
  - Predict and examine which external factors that can be important
  - Optimize the pulling force

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