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

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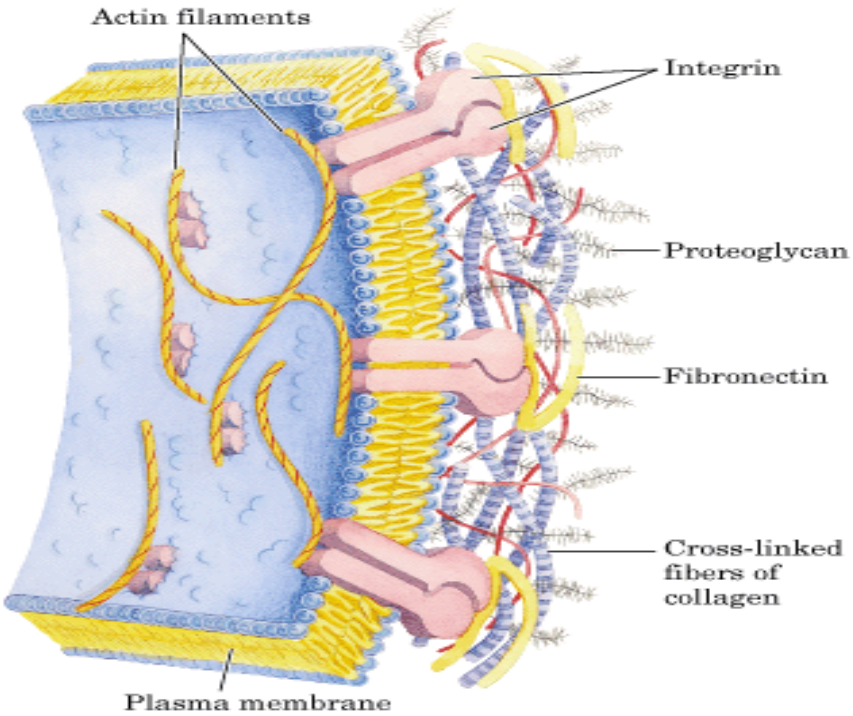
Connective tissue= a complex structural network

Extracellular matrix (proteoglycans, collagens and glycoproteins)

Adhesion proteins (syndecans, glypicans, integrins)

Cells (fibroblasts, fat cells, immune cells)

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



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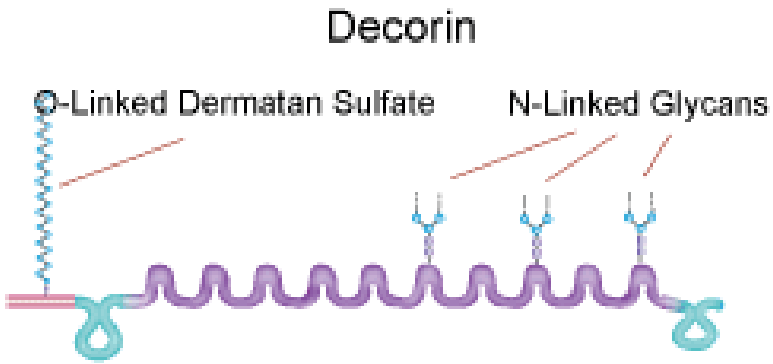
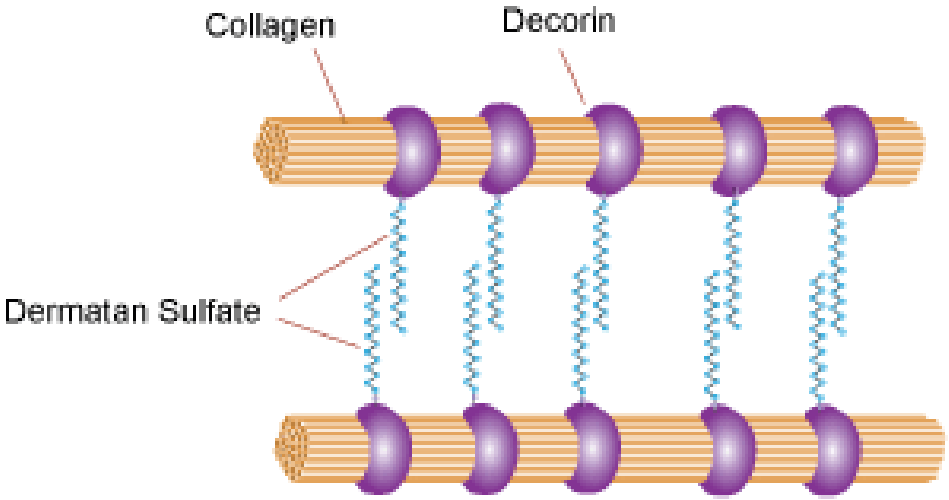
Strong interaction of carbohydrates and proteins





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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*

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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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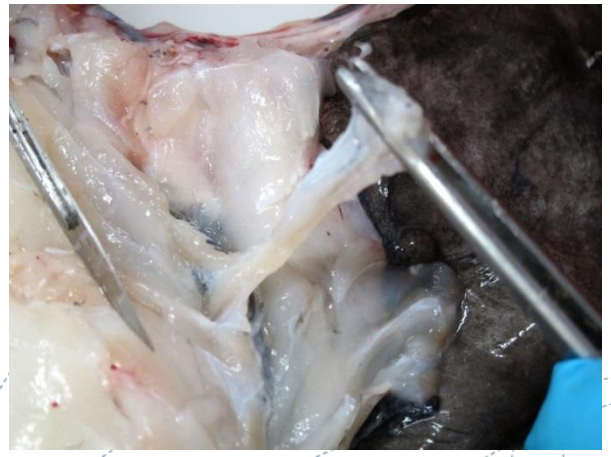
Methods used in the study

- **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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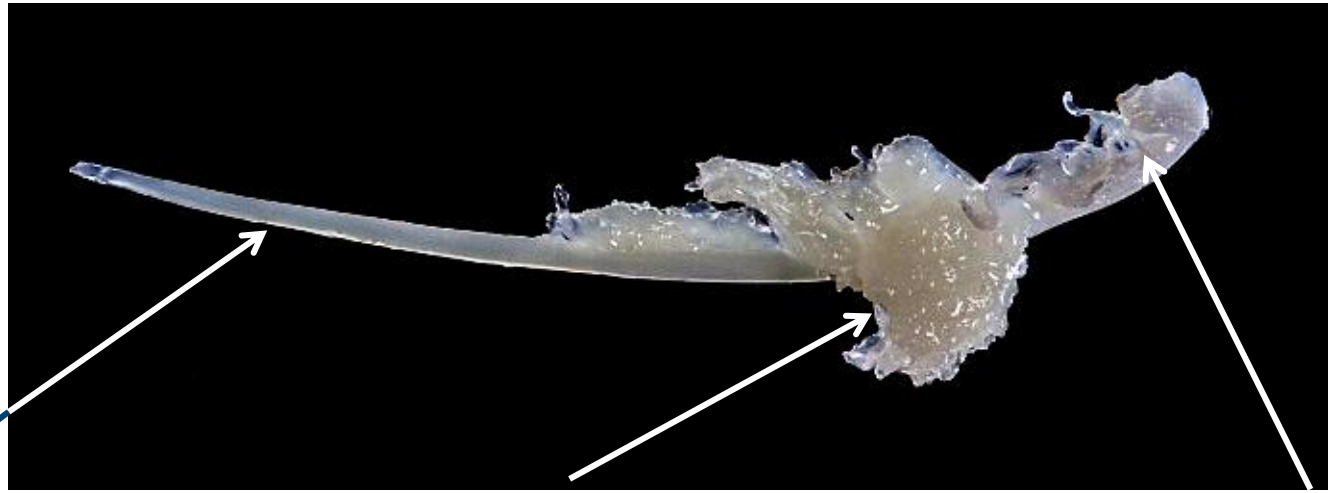
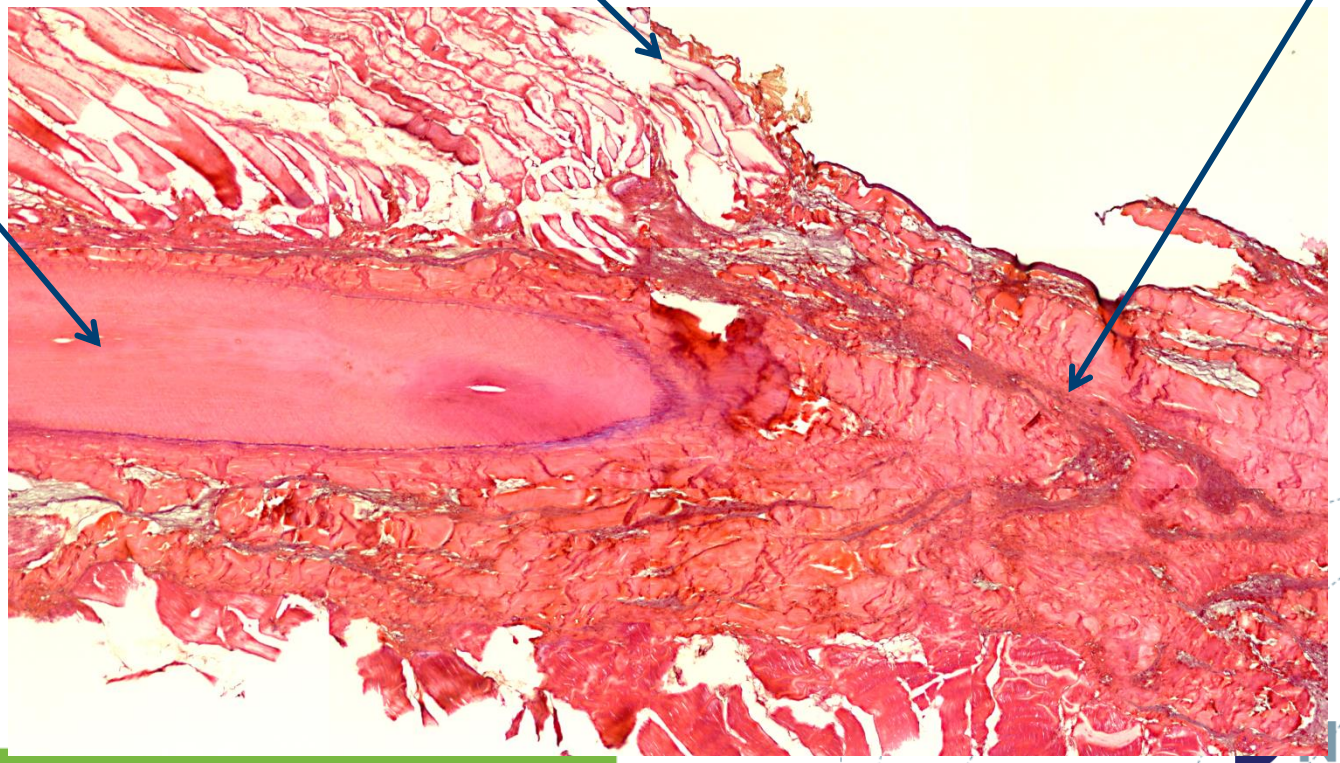


Muscle tissue

Connective tissue

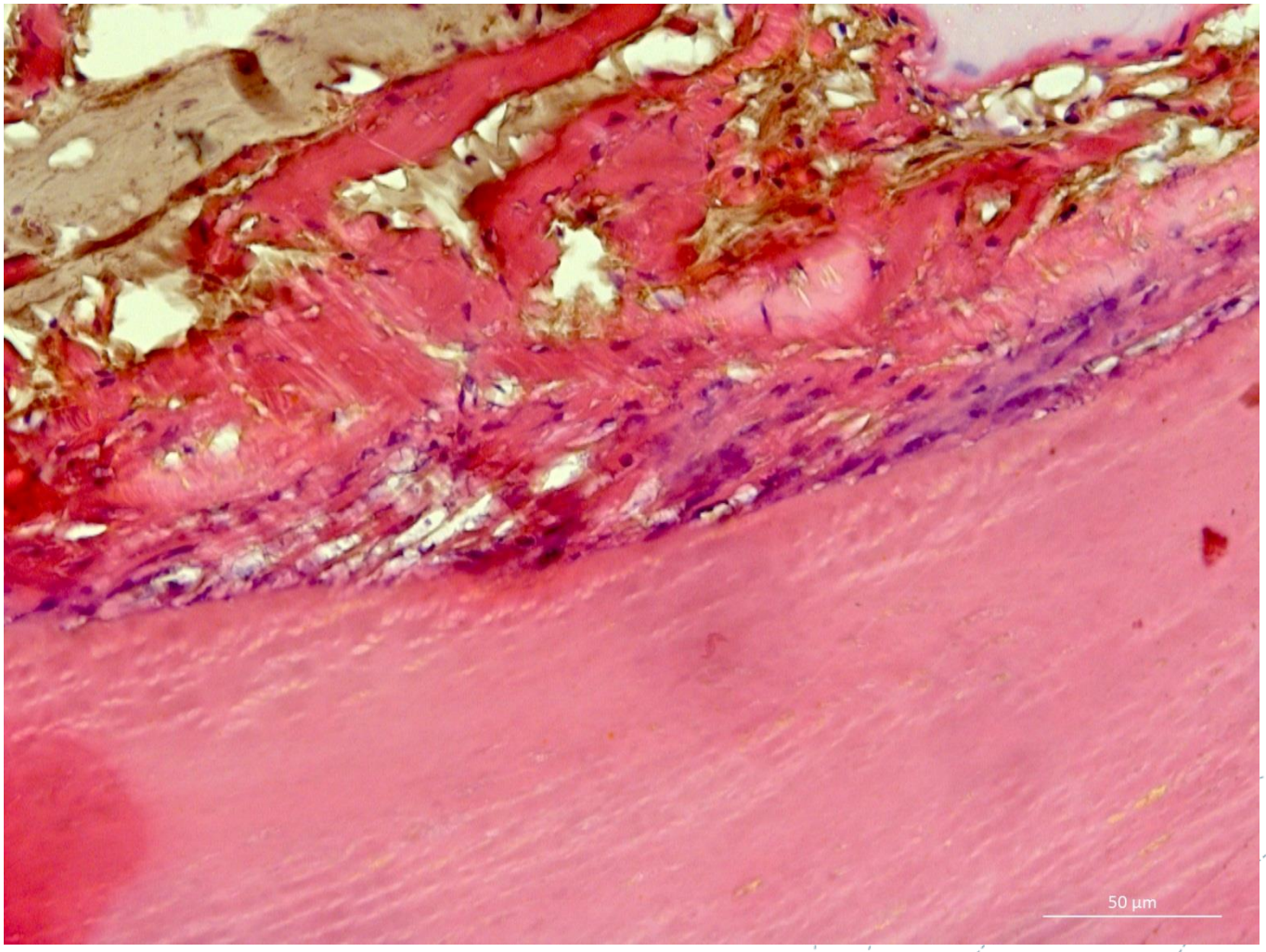
Bone



Interphase connective tissue -bone

Connective tissue

Bone



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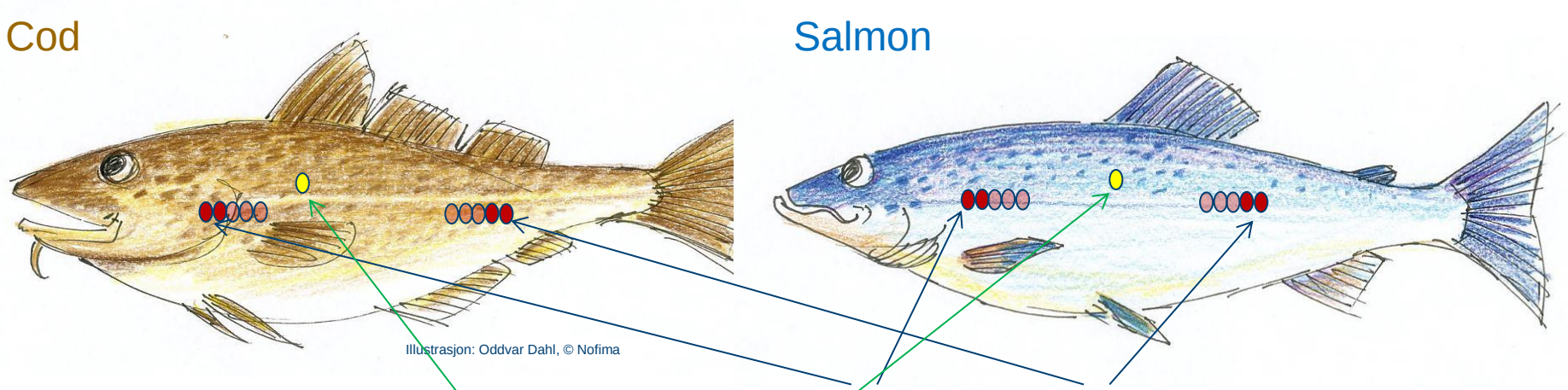
Gene expression analysis



- 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

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

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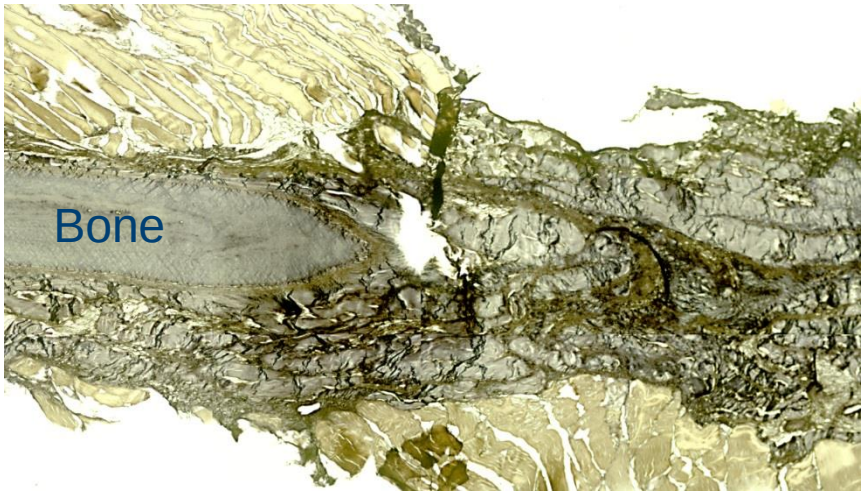
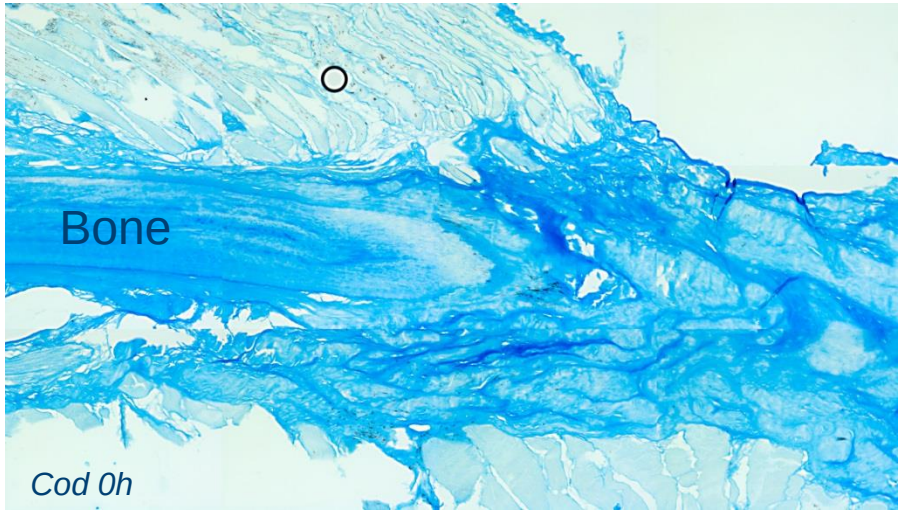
The connective tissue is rich of elastin, proteoglycans and collagens



Dark colour: Elastin
Brown: Muscle



Dark colour: Proteoglycans
Light blue: Muscle

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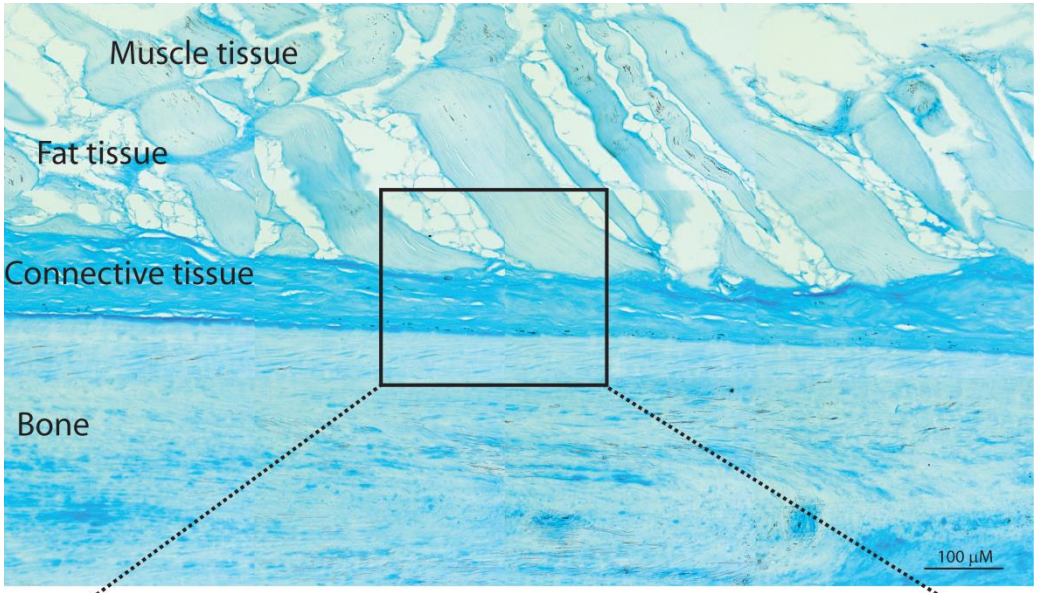

Tight connection between bone, connective tissue and muscle in cod!!

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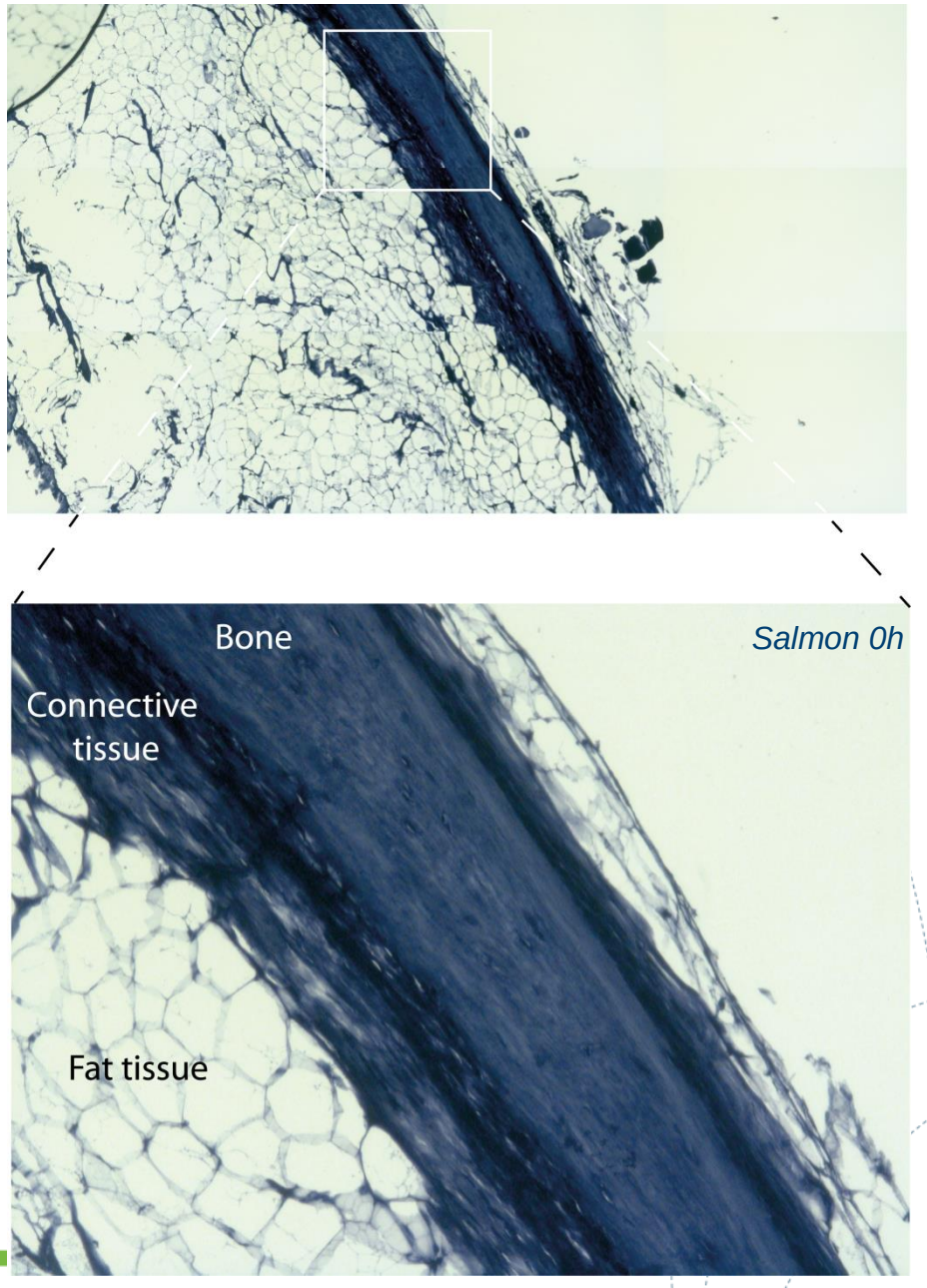
Tight connection between bone, connective tissue and fat in salmon!!



But what happens during storage?

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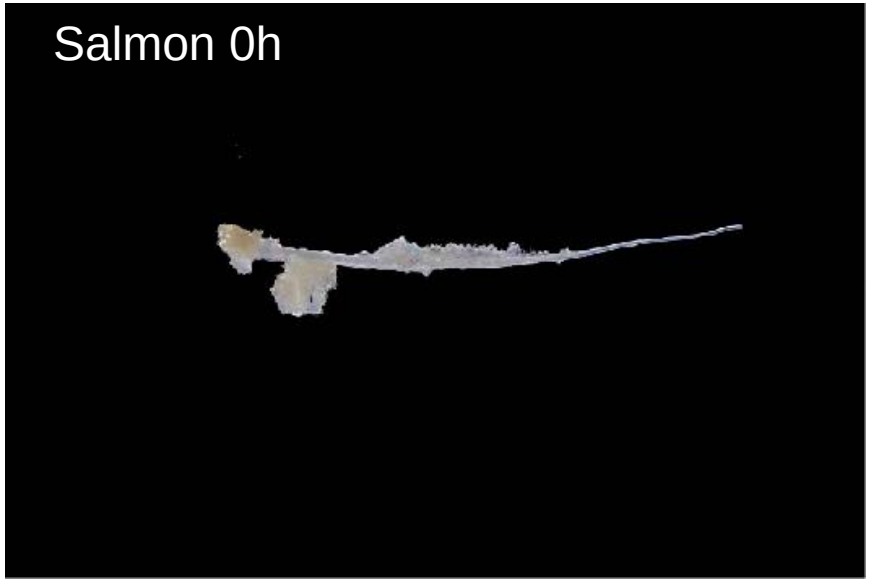

Salmon 0h

Cod 0h

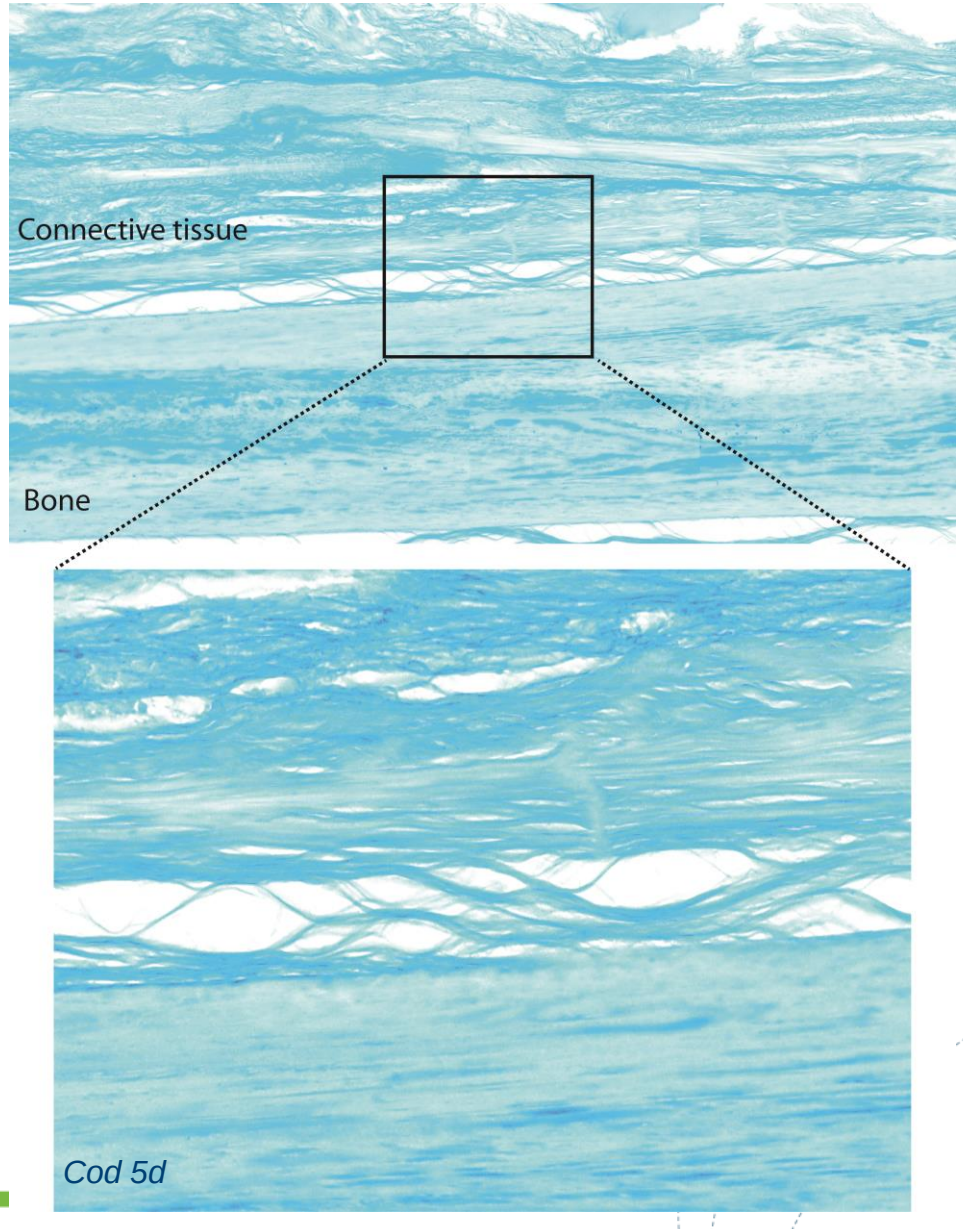
Salmon 5 days

Cod 5 days

Elastin fibres are broken in cod

Cod 5 day.

Threadlike structures containing proteoglycans in cod



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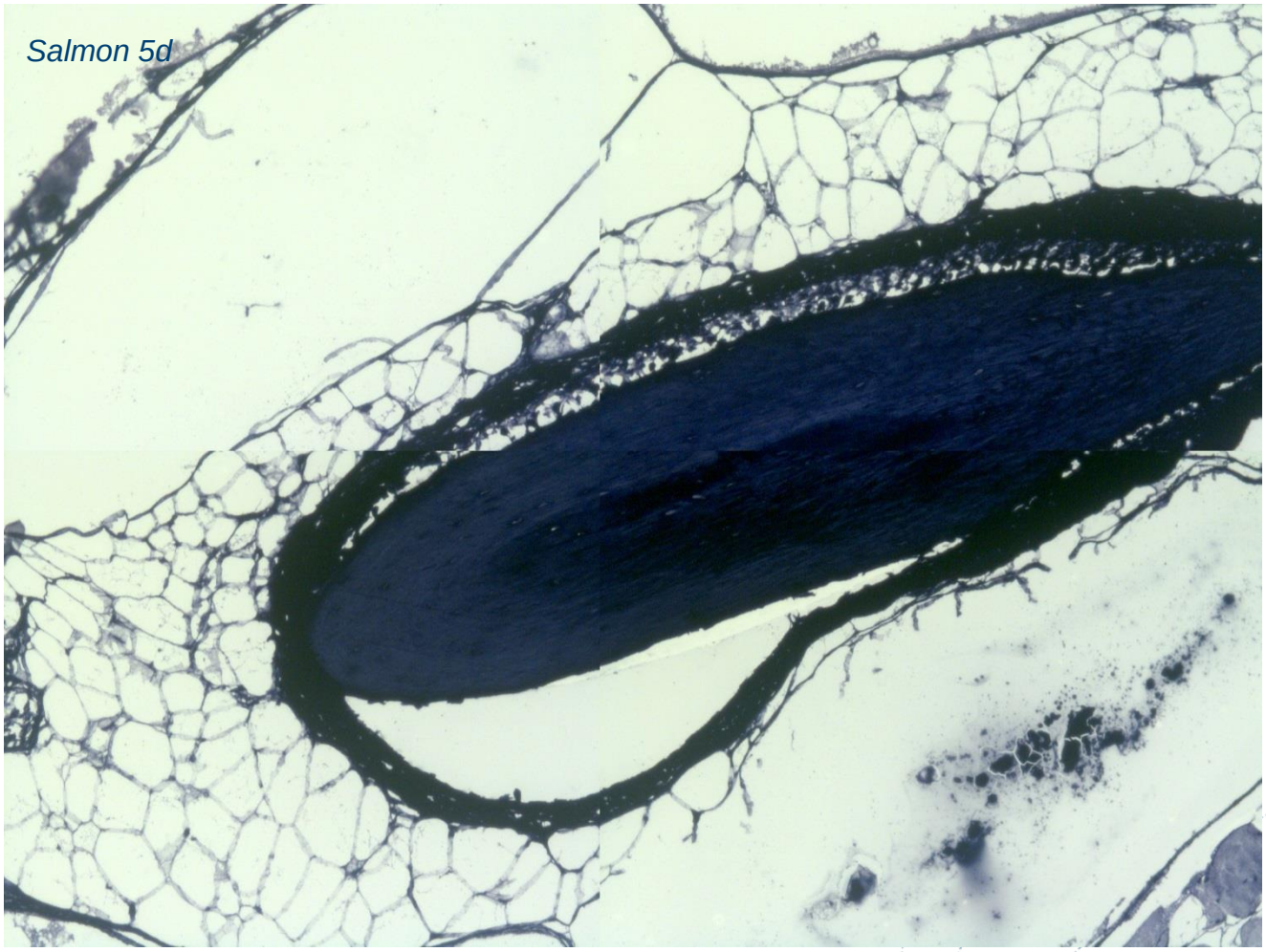
The connective tissue is intact in salmon, but it is loosen from the bone



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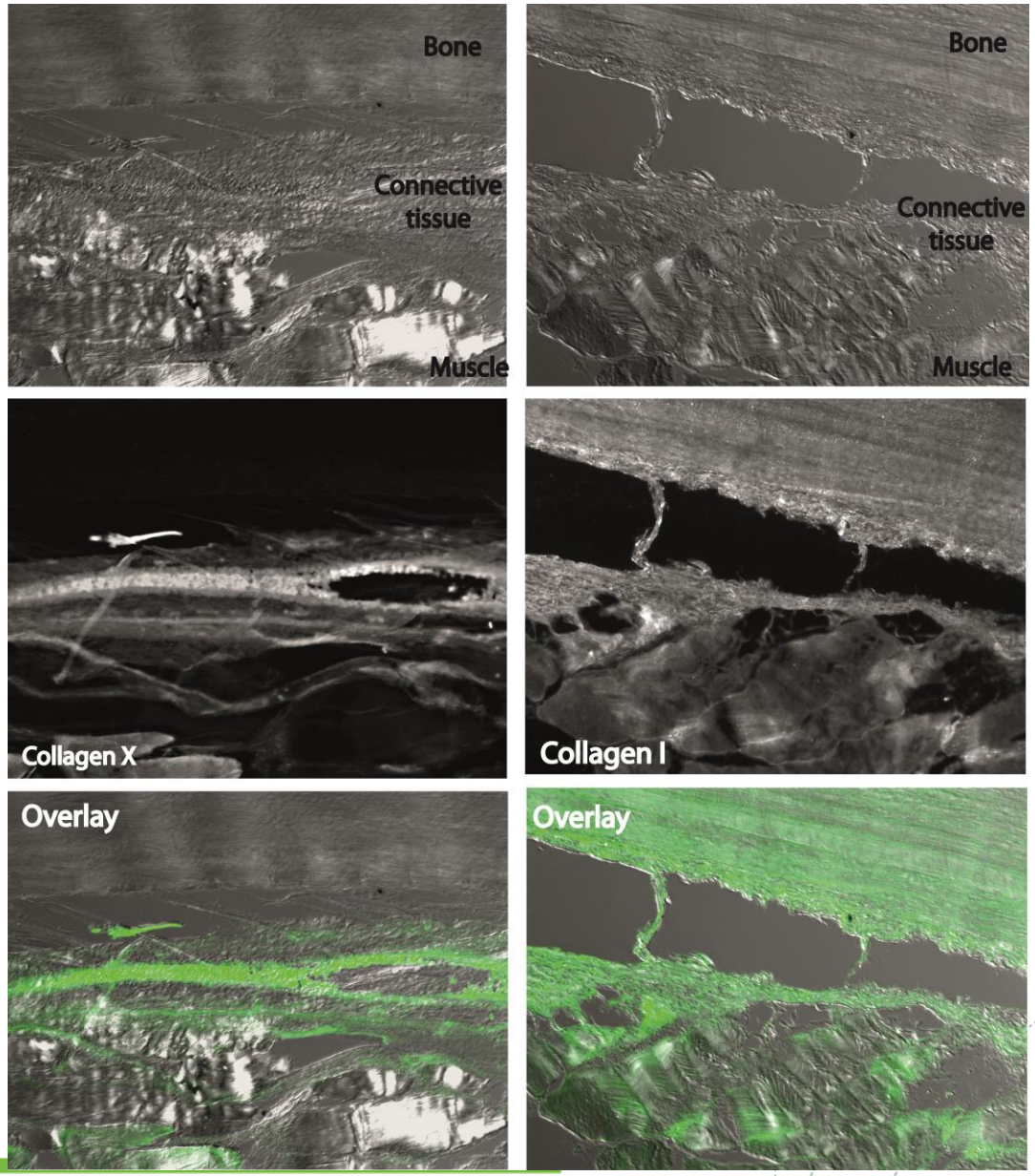
Localization of collagen X and I in the splitting area of cod



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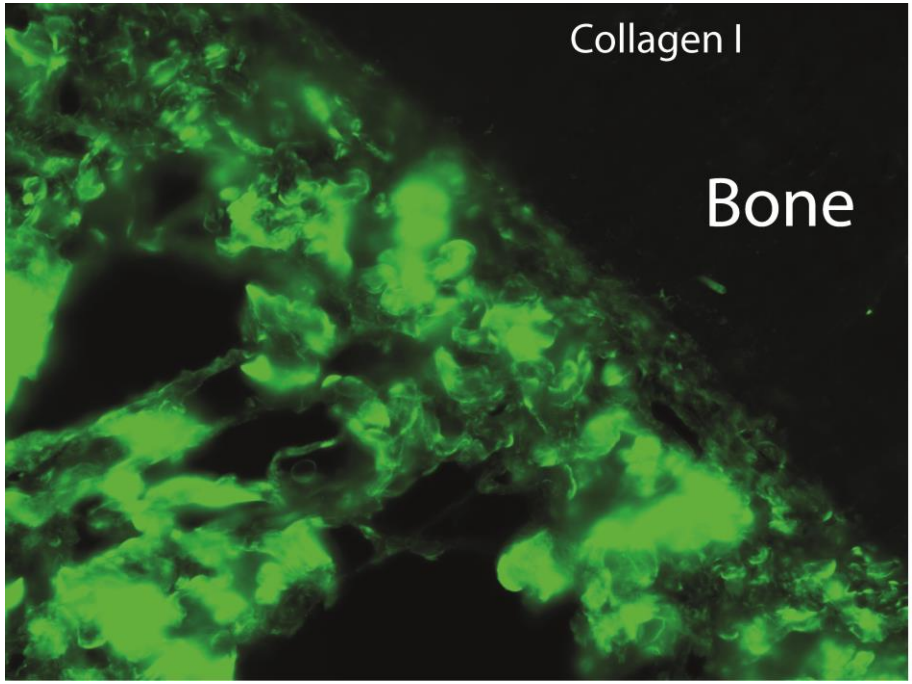
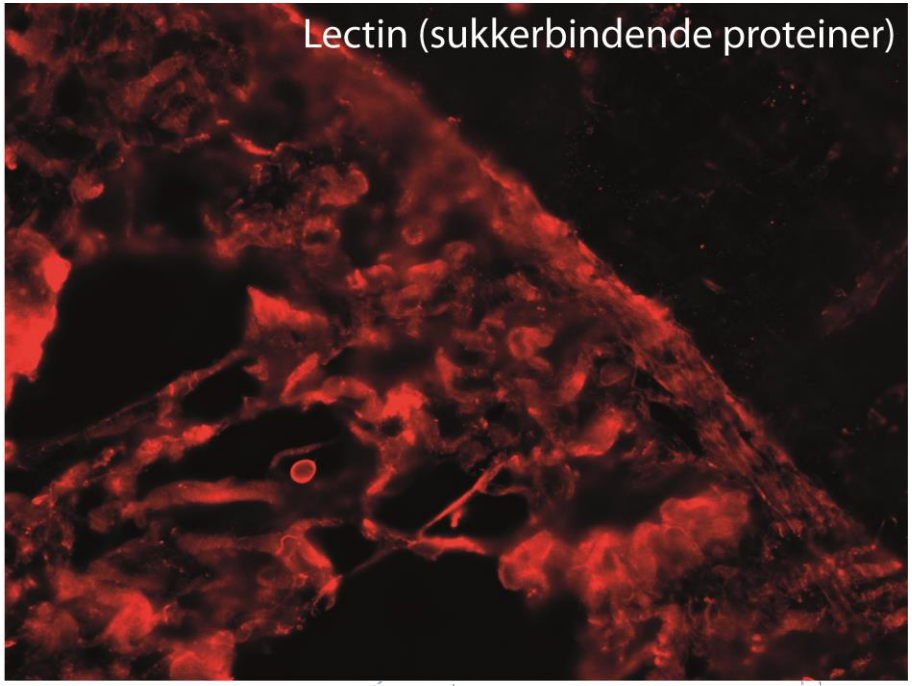
The network of carbohydrate binding protein and collagen in the splitting area in cod





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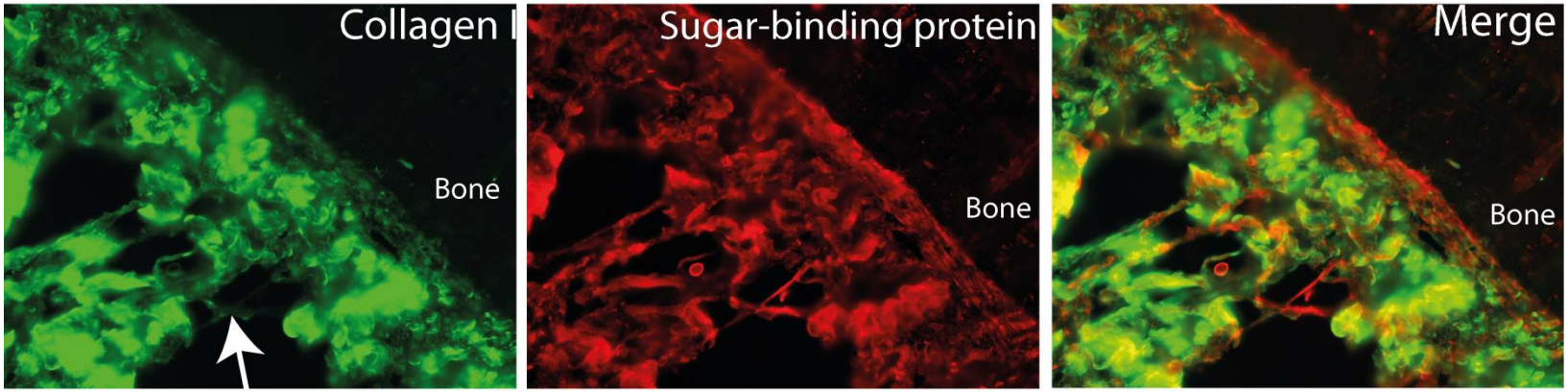
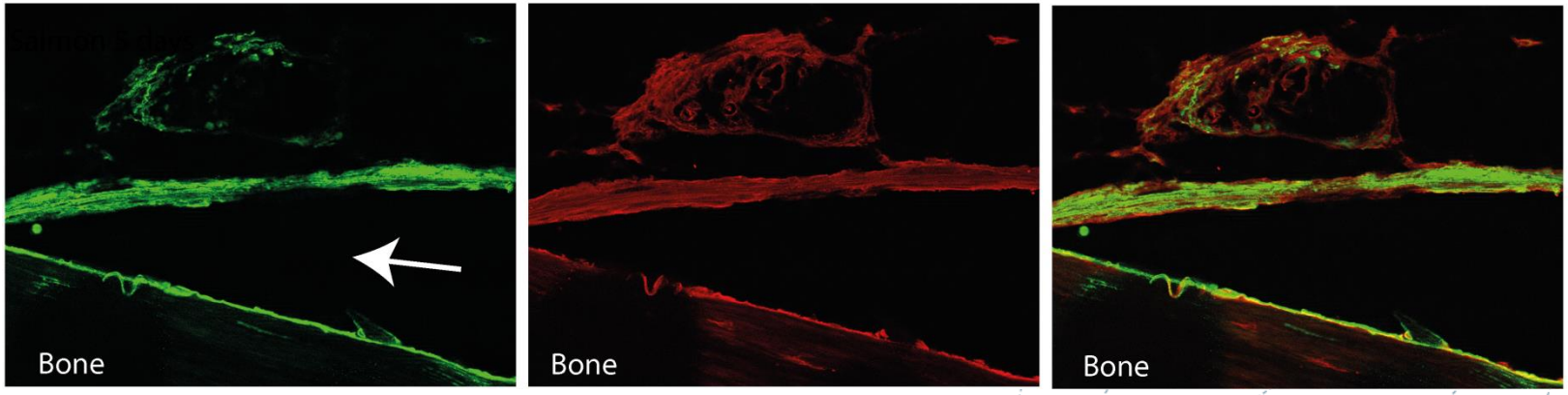
Differences in splitting area between salmon and cod

Cod 5 days

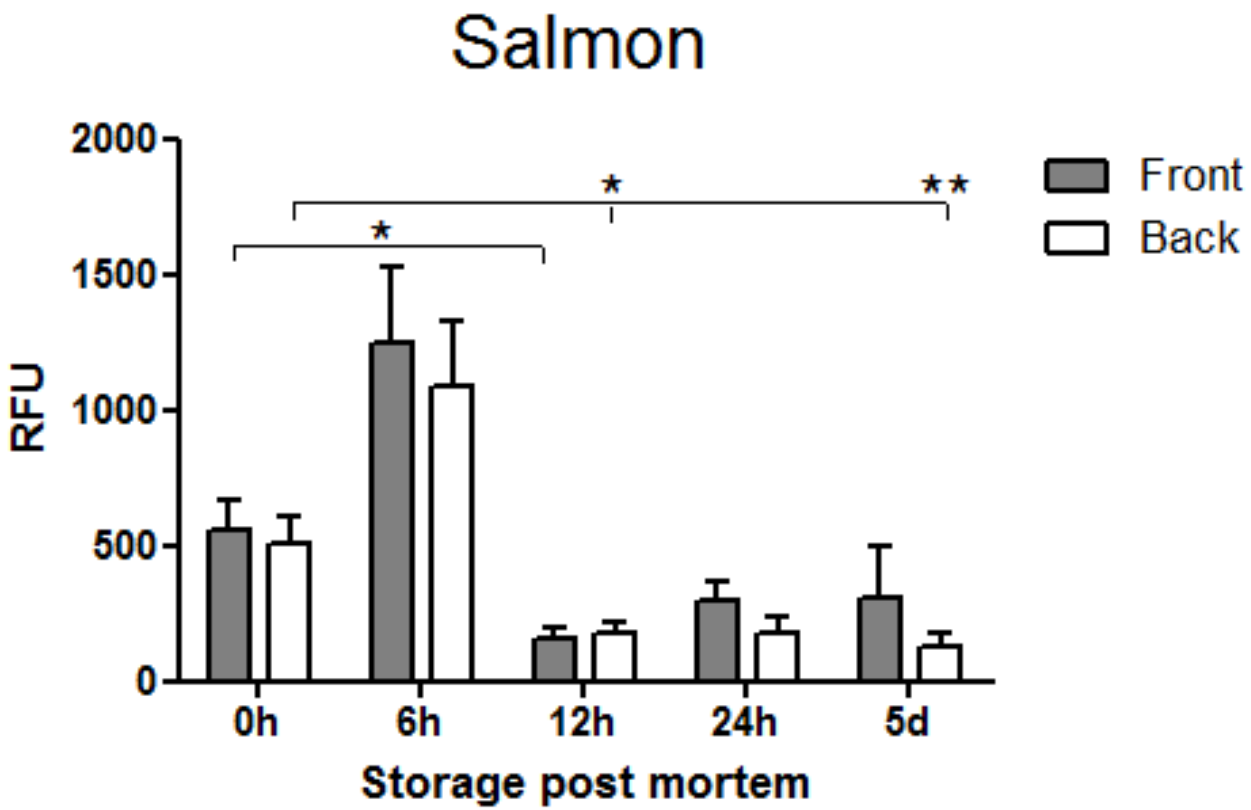


= Splitting area

Salmon 5 days



Metalloproteases are active right after slaughter in salmon



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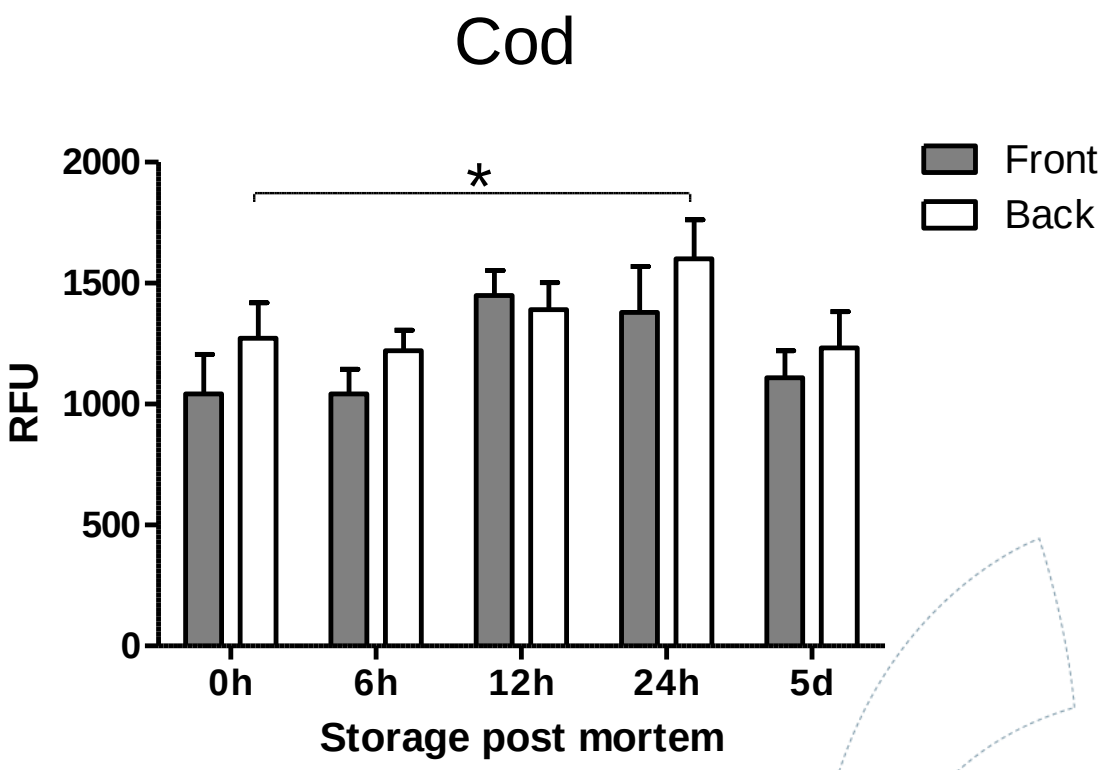
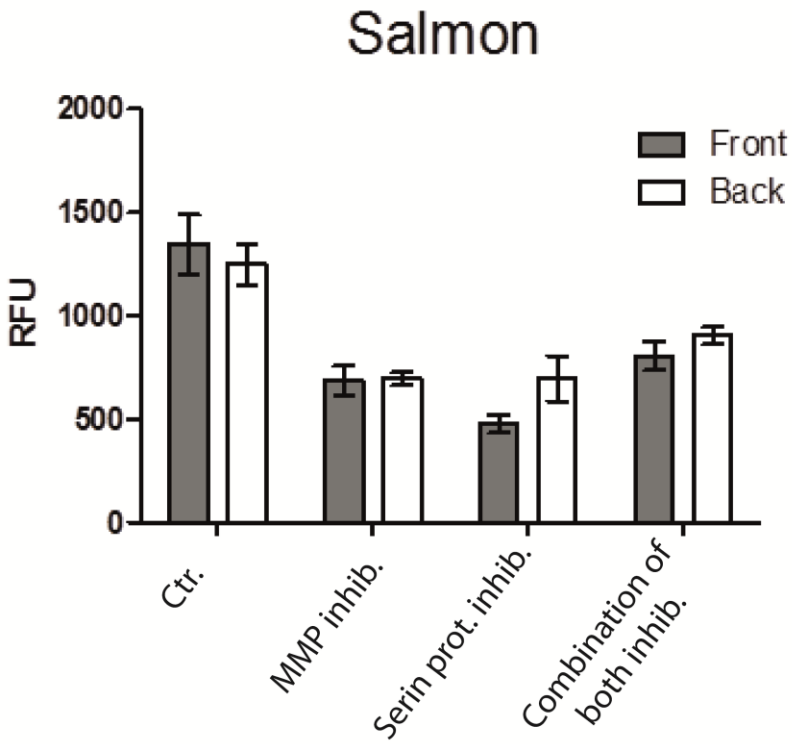
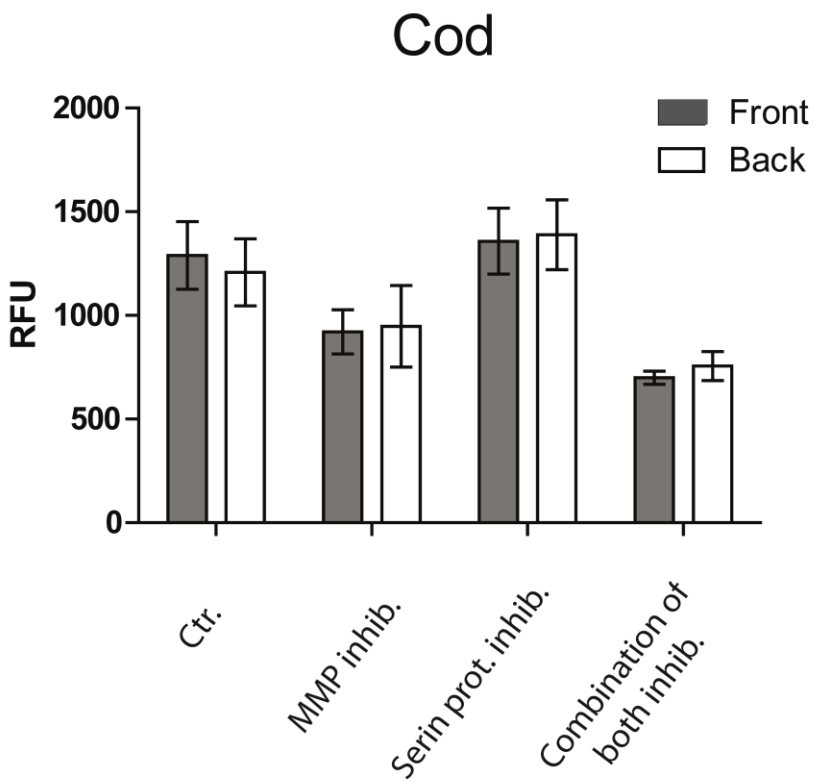
Metalloproteases activity is high during the storage period in cod



Different types of degrading enzymes are active





Enzyme activity 6h

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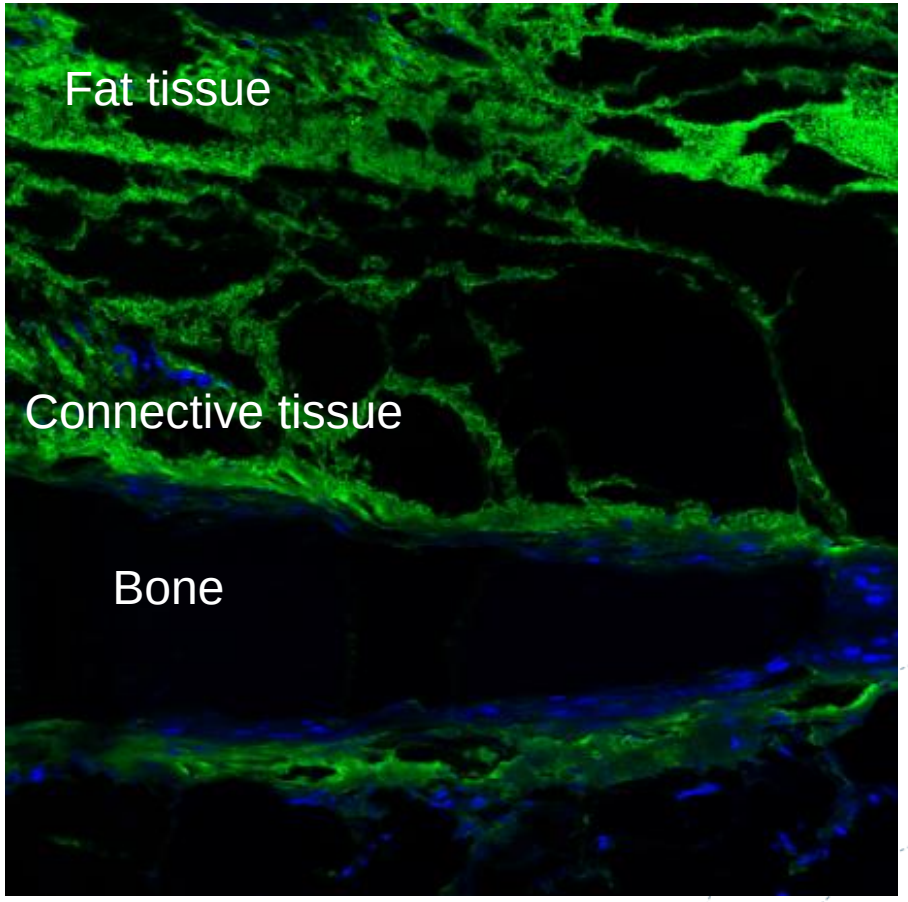
MMP activity in connective tissue and fat tissue, but not close to the bone



- Green color= enzyme activity
- Blue colour= nucleus

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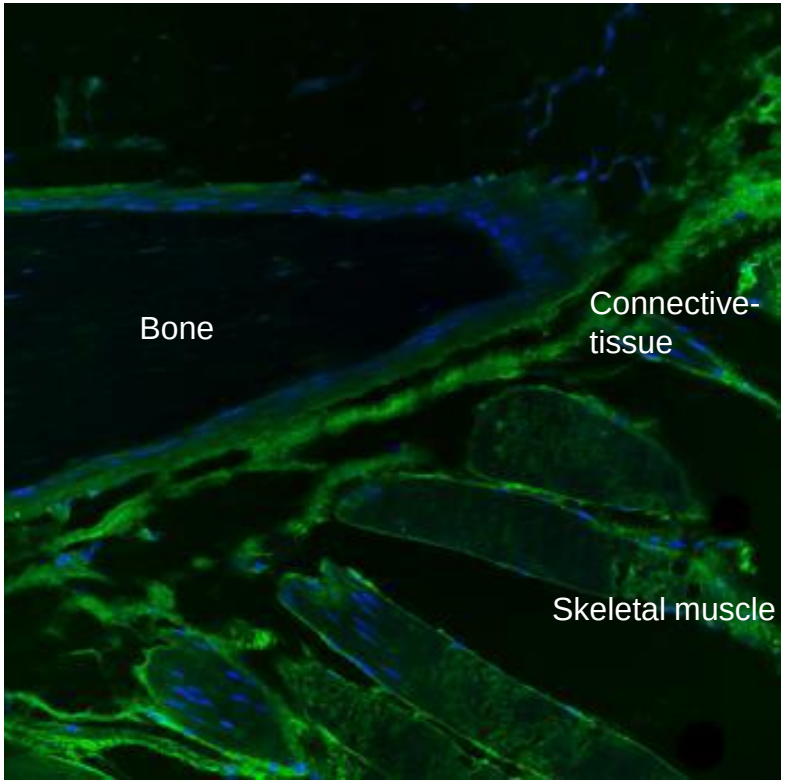
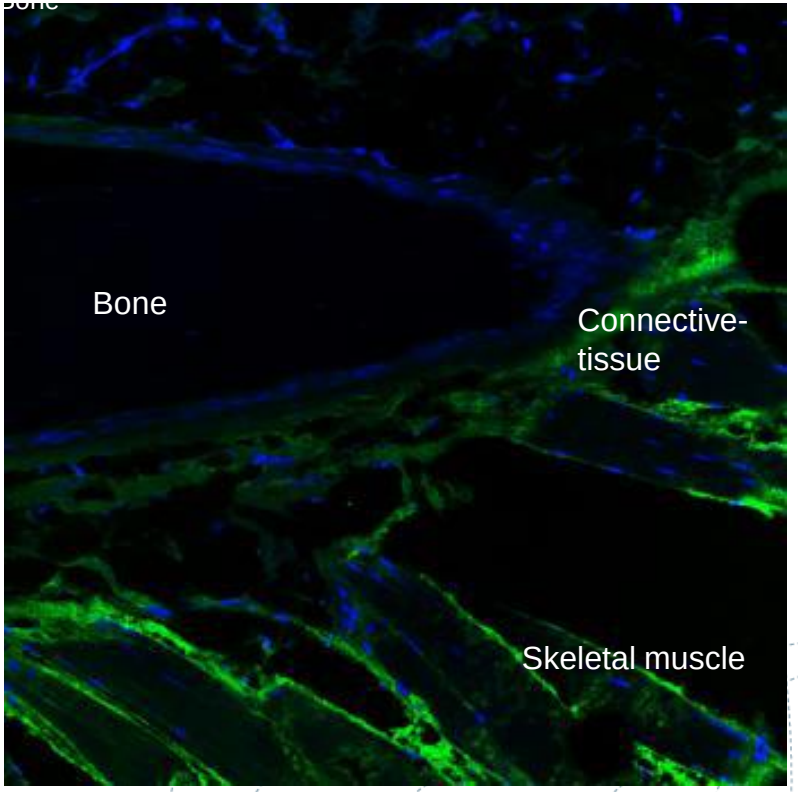
The MMP activity differ between pinbone area and in skeletal muscle

Control



GM 6001 inhibitor



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Further work

- 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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What do we know	What do we not know ?
The major structure of pinbone area is different between cod and salmon: Cod (skeletal muscle-connective tissue-bone) Salmon (fat-connective tissue-bone)	**Different type of process/mechanical solution for removal of pinbone in salmon and cod?**
Differences in the degradation profile between cod and salmon . • For both species it is the attachment between bone and connective tissue that is degraded *post mortem* • The connective tissue is broken during storage into threadlike structures in cod. A different degradation pattern is observed in salmon	**How do the degradation pattern influence on the pulling force?**
Correlation between enzyme activity and degree of degradation • Differences in enzymatic profile between salmon and cod	**Do stress or different handling, storage temperatures influence the enzyme activities?** **Are the differences between cod and salmon?** **Is it possible to increase/steer the enzyme activity?**

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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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