Melanin-problematikken hos oppdrettslaks -

Piscine orthoreovirus (PRV) detektert i røde og svarte foci i hvit musklatur

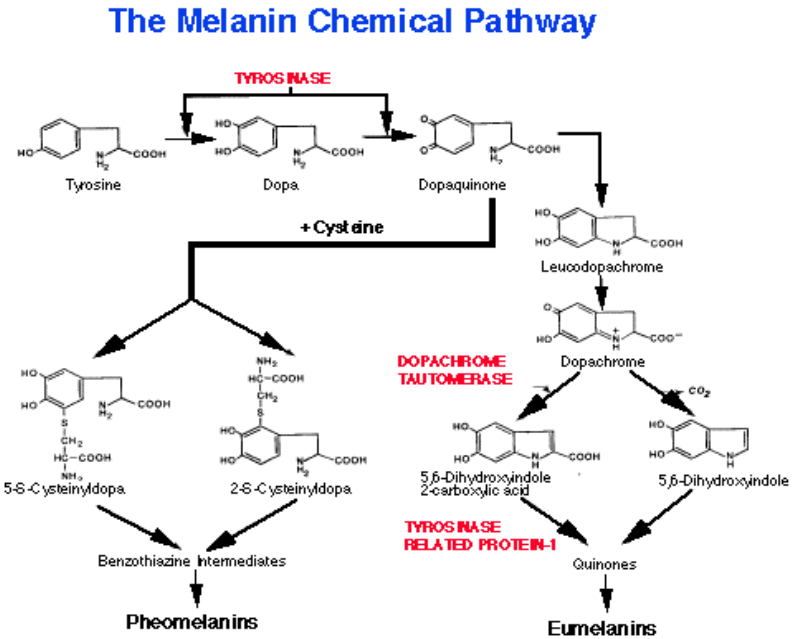
Stud. med. vet / forskerlinjestudent
Håvard Bjørgen

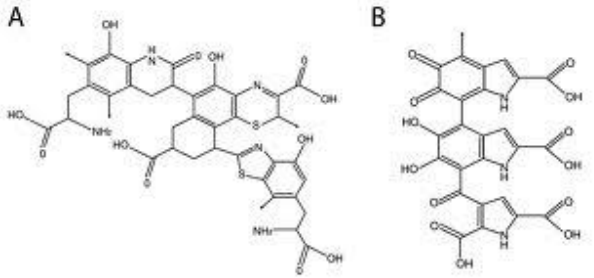
FHFs Havbrukssamling
14. oktober 2015

Disposisjon

- Melanin
- PRV
- Nye resultater
- Nye spørsmål

Hva er melanin?

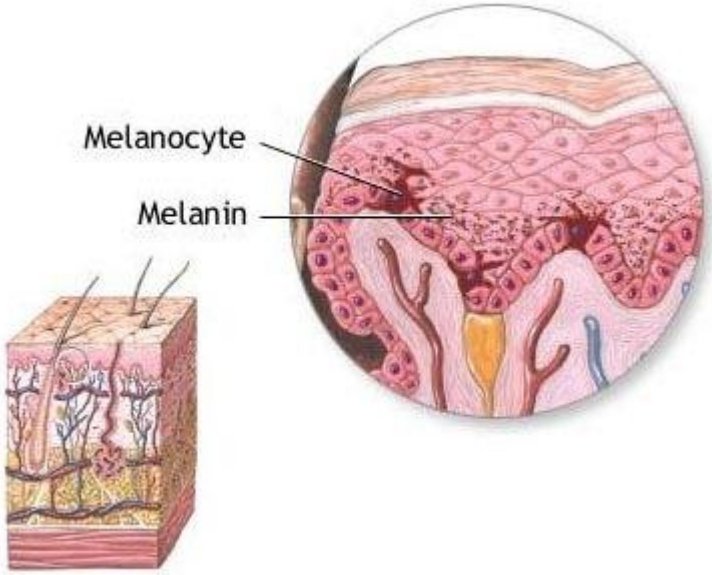


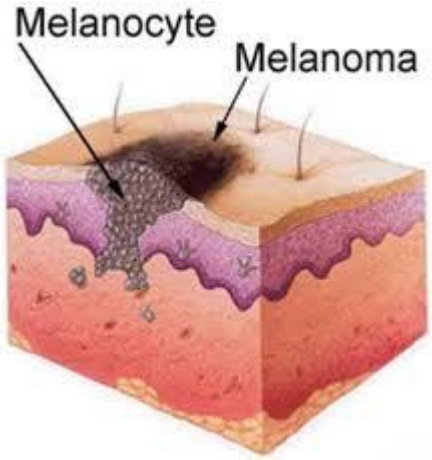


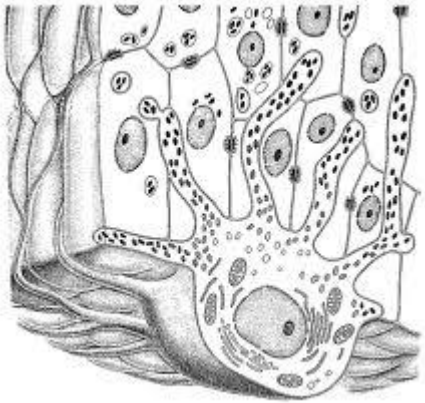
Hva er funksjonen?

- Antioksidanter – anoxi! Melanin binder frie radikaler
- Binder kobber, jern og andre tungmetaller
- Binder en rekke toksiske komponenter

Hvor dannes melanin?







Betennelse og melanin

Christensen et al.
Trends Parasitol. 2005:21;192

doi: 10.1111/j.1600-0749.2006.00319.x

Isolation of the Atlantic salmon tyrosinase gene family reveals heterogenous transcripts in a leukocyte cell line

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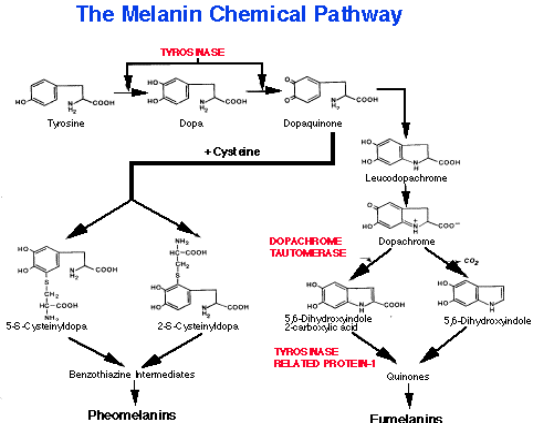
Summary

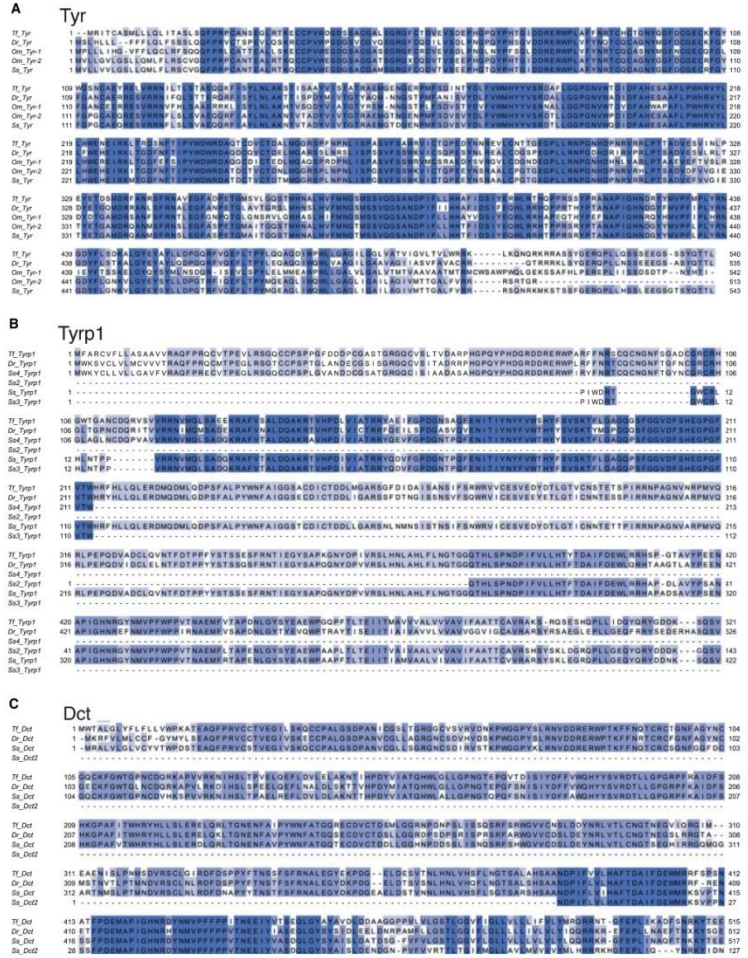
In ectothermic vertebrates, visceral organs harbor melanin-containing cells. Their ability as pigment producers is nevertheless disputed. To address expression of the key genes for melanogenesis in Atlantic salmon (*Salmo salar*), a tyrosinase-positive leukocyte cell line (SHK-1) and skin were used to obtain full-length tyrosinase (Tyr), tyrosinase-like protein-1 (Tyrp1), and dopachrome tautomerase (Dct) mRNA transcripts. In the SHK-1 cells, two different Tyrp1 transcripts were identified, one lacking exon 1. However, only the full-length version of Tyrp1 was identified in the skin. Sequen-

Introduction

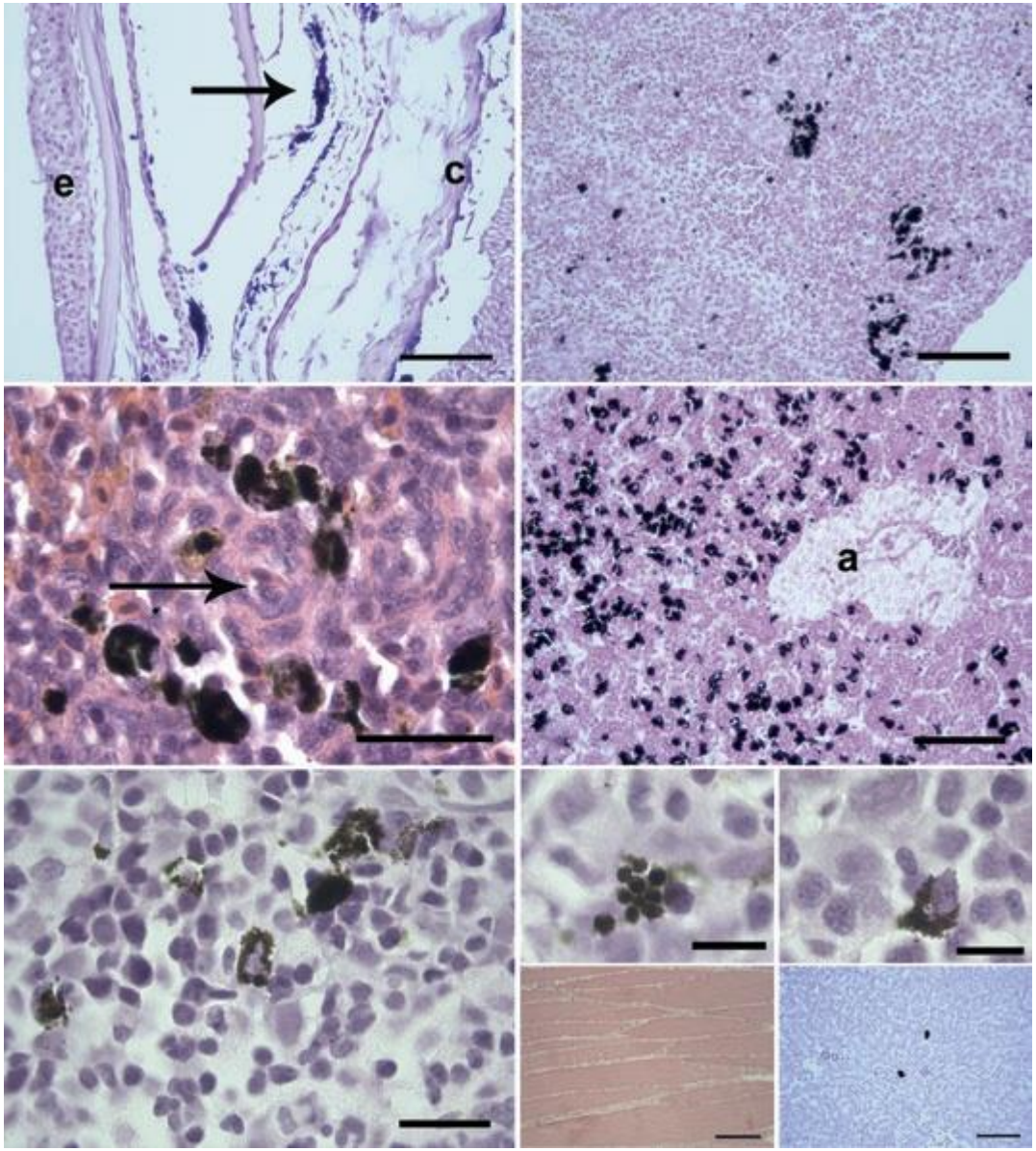
The pigmentary system of vertebrates has predominantly been addressed in cells derived from the embryonic neural tube ectoderm (Boissy and Nordlund, 1997). Pigmented cells or melanocytes in the normal adult mammal occur in the skin, uvea, retina, meninges, the inner ear, and the Harderian gland (Boissy, 1998). Such cells are characterized by their ability to synthesize melanin, a process confined within discrete organelles termed melanosomes. Melanosomes share several properties with lysosomes (Orlow, 1995; Raposo et al., 2002), and any melanocyte precursor is defined as a melanoblast (Fitzpatrick et al., 1966). The functions of melanin are not only restricted to absorb, scatter and reflect light, but also include binding of metal ions and organic cations, and acting as antioxidants and scavengers of free reactive radicals (Margalith, 1992; Sarna and Swartz, 1998). Interestingly, increasing information link the functions of the pigmentary and immune systems (Mackintosh, 2001; Raposo et al., 2002).

The principal genes encoding the enzymes involved in





PRV i røde og svarte flekker



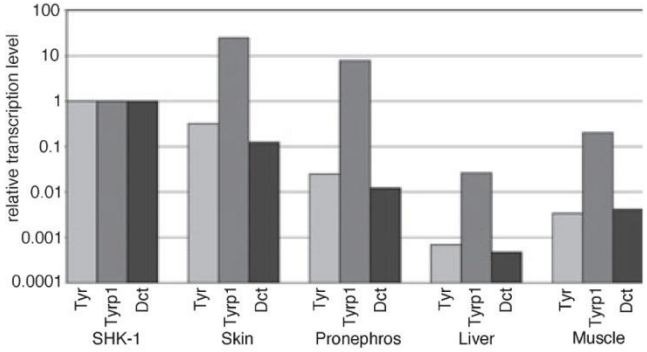
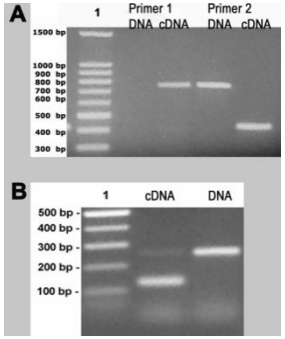


Figure 3. Histograms showing real-time quantitative polymerase chain reaction data of the tyrosinase (Tyr), tyrosinase-like protein-1 (Tyrp1) and dopachrome tautomerase (Dct). Tissues investigated are indicated. The data are presented exponentially and relative to the SHK-1 values (set as 1). The values presented are the average of triplicate runs for each gene and tissue.

PRV i røde og svarte flekker

- Primer pairs recognising a fish CD83 homolog



- Primer pair detecting Dct/TRP-2

doi: 10.1111/j.1600-0749.2006.00297.x

Melanogenesis and evidence for melanosome transport to the plasma membrane in a CD83^{+} teleost leukocyte cell line

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Key words: CD83/dendritic cell/endosomal pathway/macrophage/melanogenesis/melanomacrophage/teleost

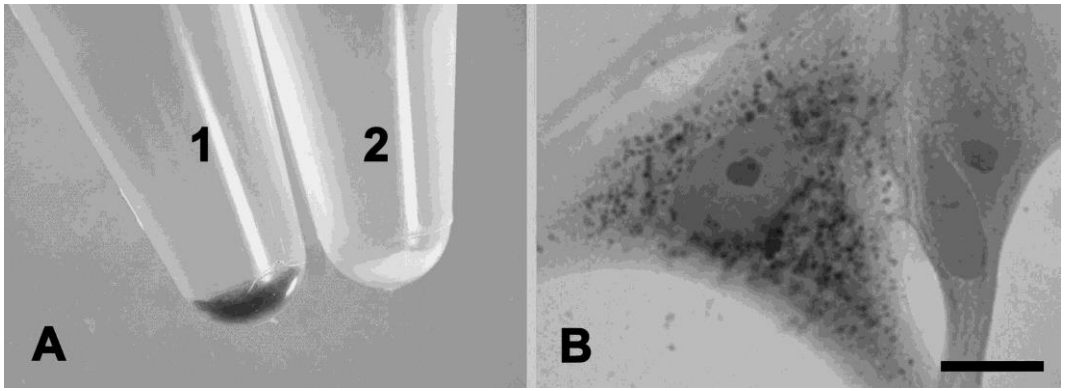
Received 1 April 2005, revised and accepted for publication 14 December 2005

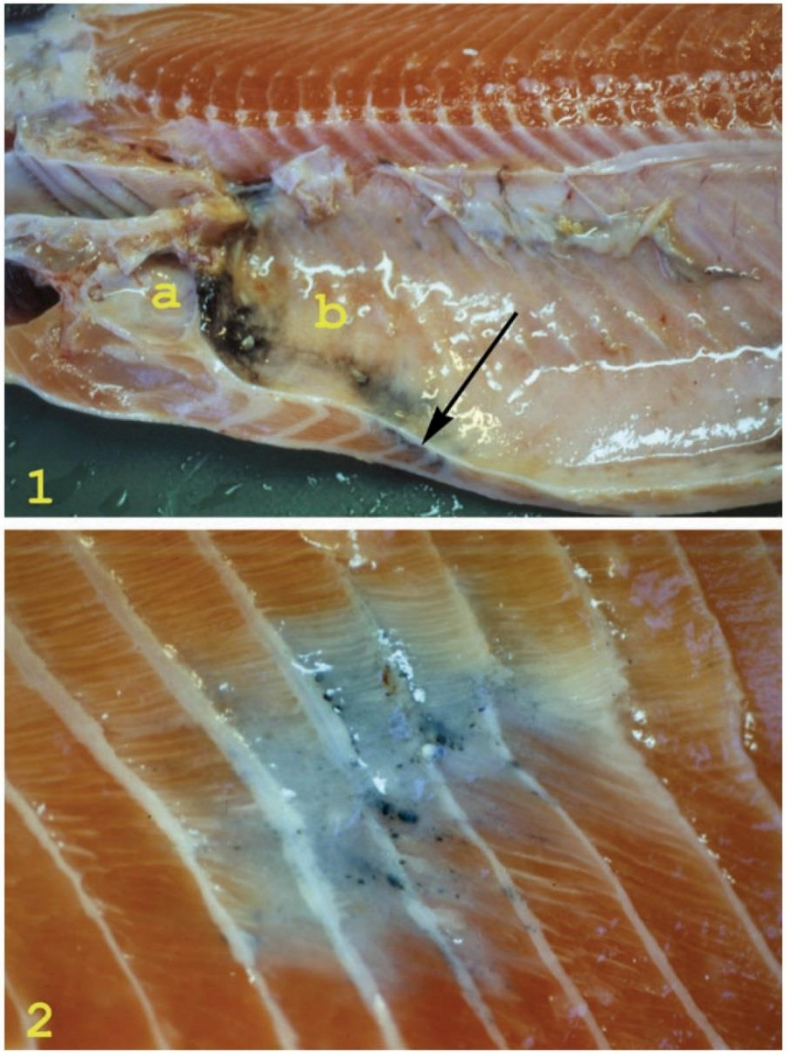
Summary

Introduction

Melanins are complex polymeric pigments, which are formed by a wide variety of living organisms ranging from fungi and bacteria to higher vertebrates (Margalith, 1992; Orlow, 1995; Raposo et al., 2002). Common for

- A; 0,1 mM PTU inhibit tyrosinase dopachrome production from L-DOPA
- B; a few long cultured cells showed melanin reduction potential





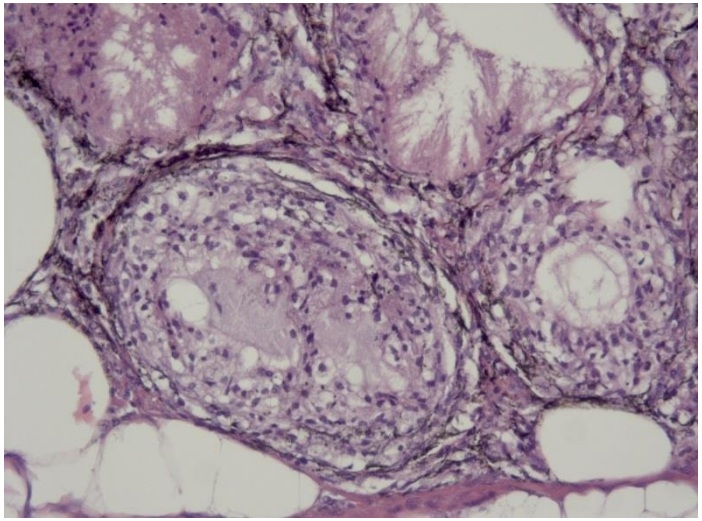
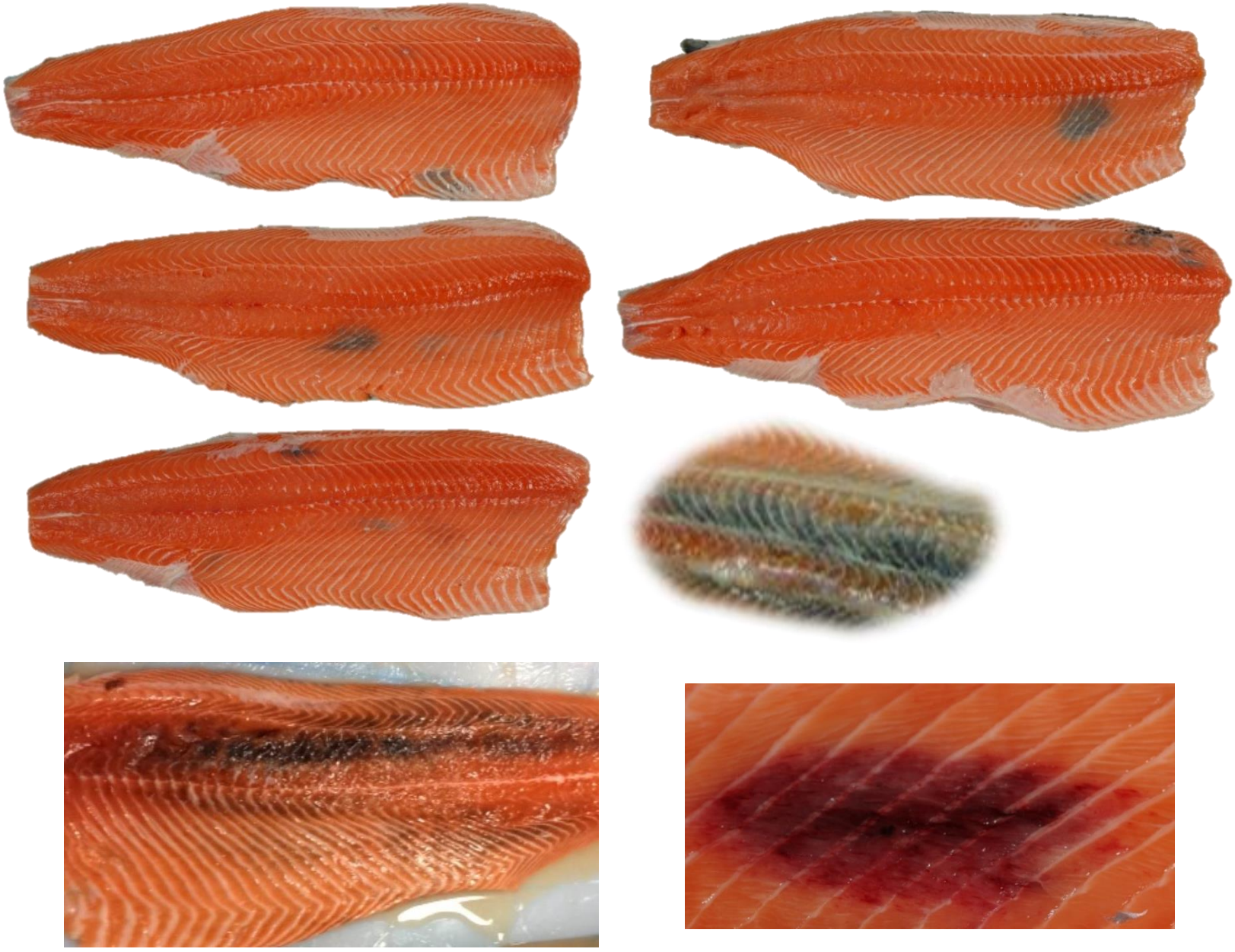


Figure 1 Gross pathological changes in the carcass of an Atlantic salmon. The pericardial cavity (a) is normal, but severe melanization is apparent in the abdominal cavity (b). Melanized musculature subjacent to the peritoneum is seen on the cut surface (arrow).
Figure 2 A melanized area in the musculature of an Atlantic salmon. The peritoneum is removed and darker foci are seen in a dark to grey area involving five myosepta. The lesion is situated laterally in the fish, covering the area of the lateral organ. Note the contraction in the musculature, disrupting the curves of the intramuscular septa.

PRV i røde og svarte flekker



PRV i røde og svarte flekker

Journal of Fish Diseases 2014, 37, 327–340 doi:10.1111/jfd.12106

The effect of vaccination, ploidy and smolt production regime on pathological melanin depositions in muscle tissue of Atlantic salmon, *Salmo salar* L.

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Abstract

The presence of melanin in muscle fillets of farmed salmon represents a considerable quality problem for the salmon industry with major economic concerns. In this study, we have examined the presence of abnormal pigmentation in vaccinated versus unvaccinated Atlantic salmon, *Salmo salar* L., and evaluated possible differences between diploid and triploid fish. Furthermore, the impact of the smolt production regime at ambient (4.5 °C) versus elevated temperature (16 °C) was investigated. Pigmented muscle spots were analysed for the expression of genes involved in melanization (tyrosinase gene family) and immune-related response in addition to morphological investigations. The proportion of fish with intramuscular melanin deposits was not significantly different between vaccinated and unvaccinated fish, regardless of ploidy. However, an interaction between vaccination and smolt regime was shown, where smoltification at elevated temperature after vaccination increased the number of affected individuals compared with vaccination followed by simulated natural smoltification. Furthermore, there were overall more fish with melanin spots amongst the triploids compared with their diploid counterparts. Transcription of the tyrosinase gene family confirmed an onsite melanogenesis in all pigment spots. The histological examination and the expression of the immune-related genes revealed a chronic polyphasic myopathy that was not affected by vaccination, ploidy or smolt production regime.

Keywords: diploid, melanin, melanogenesis, melanomacrophage, triploid, vaccination.

Introduction

Pigmented muscle changes, often called 'black spots', represent a quality problem of considerable size for the Atlantic salmon, *Salmo salar* L., industry (Mørkøre & Heia 2012), and the aetiology and pathogenesis of this condition have so far remained an enigma (Berg *et al.* 2006; Larsen *et al.* 2012; Mørkøre 2012). Histological investigations of pigmented muscle lesions show that they are dominated by inflammation and pigmented cells (Koppang *et al.* 2005; Larsen *et al.* 2012), frequently termed 'melanomacrophages' in piscine morphological characterization (Agius & Roberts 2003).

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Journal of Fish Diseases

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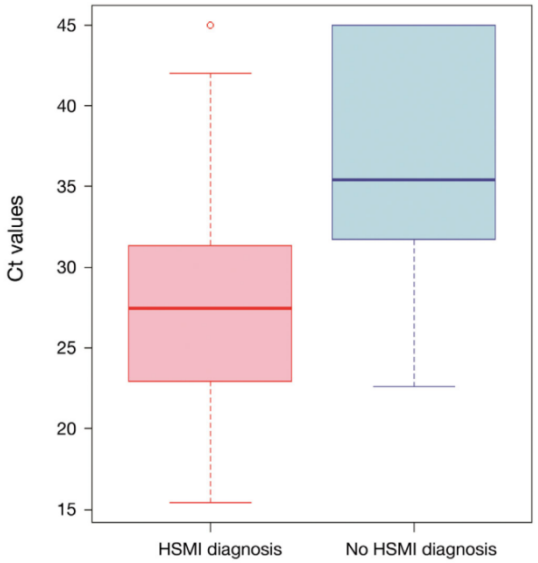
PRV- causalagens HSMI?

- **Assosiasjon: PRV & HSMB**
  Utbrudd i felt og smitteforsøk:
- Signifikant økt PRV load

- Hver 4. diagnose!
- Første månedene i sjøfasen
- Forandringer i rød muskulatur og myocardium



Vol. 99: 7–12, 2012
doi: 10.3354/dao02451

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published May 15

Quantification of piscine reovirus (PRV) at different stages of Atlantic salmon *Salmo salar* production

Marie Løvoll, Marta Alarcón, Britt Bang Jensen, Torunn Taksdal, Anja B. Kristoffersen, Torstein Tengs*

Norwegian Veterinary Institute, Ullevaalsveien 68, 0454 Oslo, Norway

ABSTRACT: The newly described piscine reovirus (PRV) appears to be associated with the development of heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon *Salmo salar* L. PRV seems to be ubiquitous among fish in Norwegian salmon farms, but high viral loads and tissue distribution support a causal relationship between virus and disease. In order to improve understanding of the distribution of PRV in the salmon production line, we quantified PRV by using real-time PCR on heart samples collected at different points in the life cycle from pre-smolts to fish ready for slaughter. PRV positive pre-smolts were found in about 36% of the freshwater cohorts and a general increase in viral load was observed after their transfer to seawater. A reduction in viral loads was recorded when fish approached slaughter (18 mo in sea cages). Sequencing of positive samples did not support the hypothesis that outbreaks are caused by the spreading of a particular (virulent) strain of PRV.

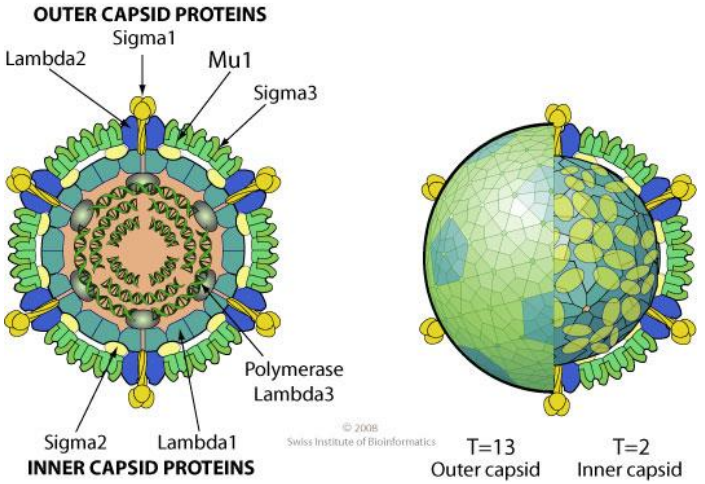
KEY WORDS: Piscine reovirus · PCR · Salmon · Aquaculture · HSMI

HSMB og *Piscine orthoreovirus*

	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
HSMI	83	94	162	144	135	131	162	142	134	181

Mortalitet varierer fra 0-20%

.
Photo: Trygve Poppe

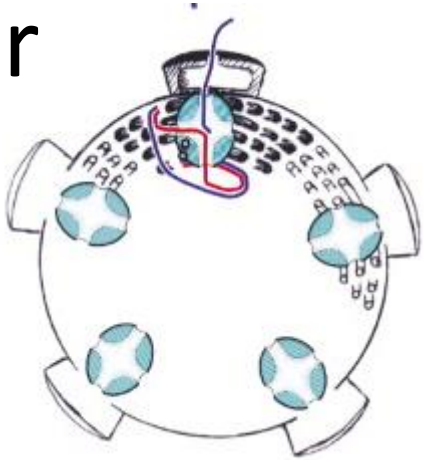


	Prevalence of PRV
SW salmon farms	Very high (close to 100%?)
FW salmon farms	<50%
Wild salmon	13% (Garseth 2013)

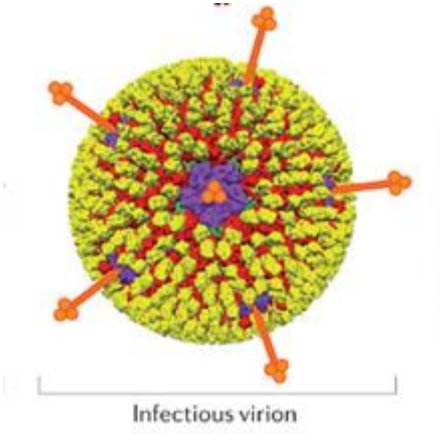
HSMI - an emerging disease

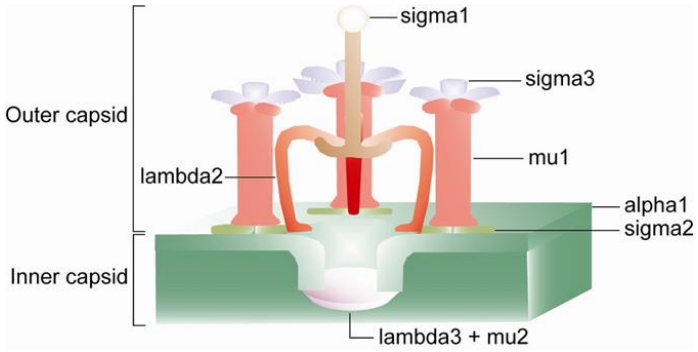
- PRV is ubiquitous in Atlantic salmon SW farms
- Transmission routes are not well described
  - Horizontally
  - Vertically?
- Virulent and less virulent strains.
- Vaccination
  - Difficult to cultivate
- Selective breeding?

Orthoreovirusstruktur



- Det serotypespesifikke antigen for orthoreoviruses er protein σ1. Antigen gjenkjennelse av dette proteinet er basis for tre hovedserotyper av MRV og 5–11 serotyper av ARV.
- Antistoff mot σ1





PRV- in RBC

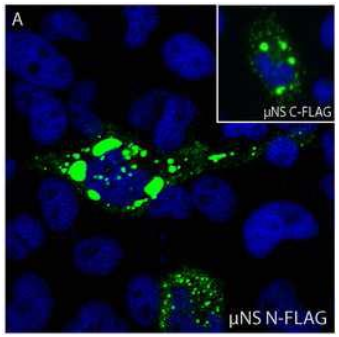
Virusinklusjoner i RBC

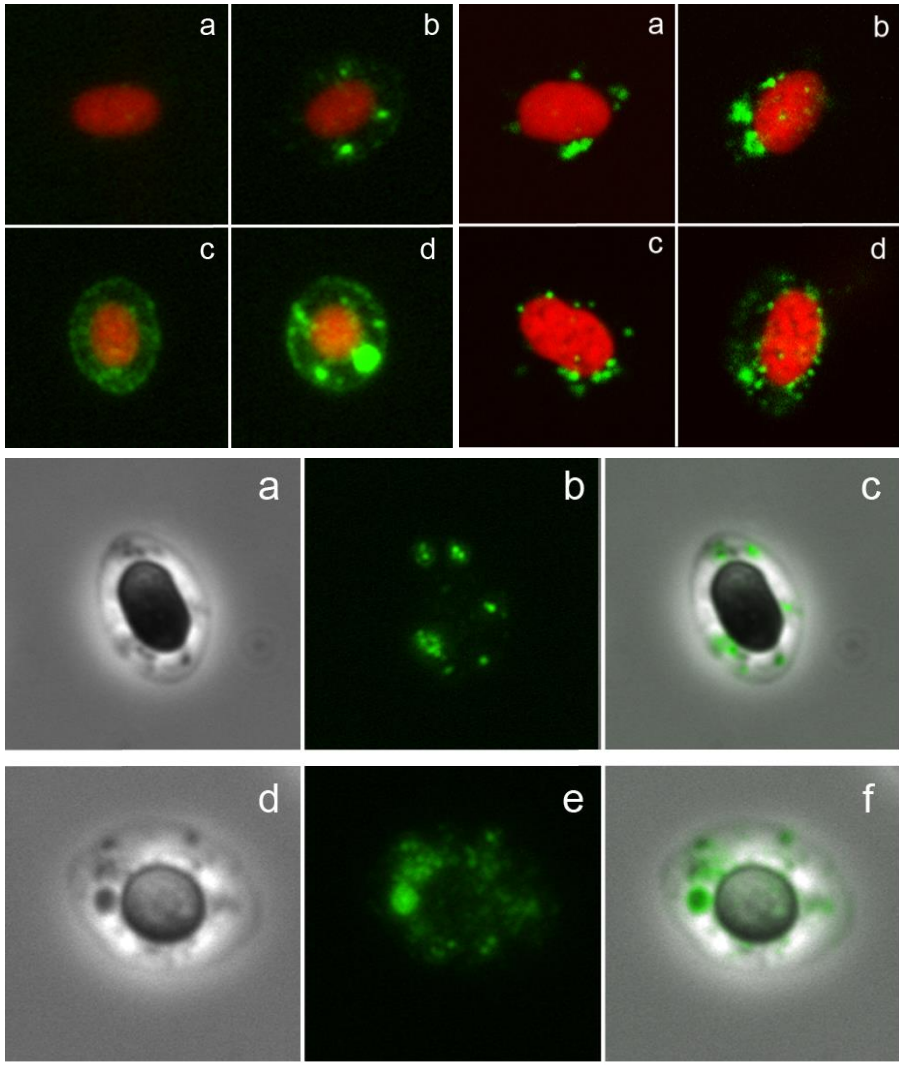
- Variabel størrelse
- cytoplasmatiske
- PRV protein
- dsRNA

Phase contrast

Co-localization w/PRV staining

Viral factories



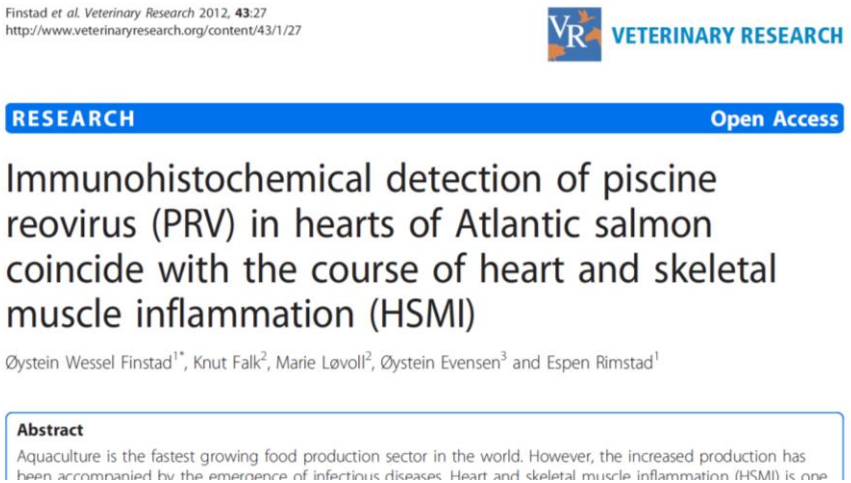


PRV i røde og svarte flekker

- Røde flekker: Blødninger og en polyfasisk muskeldegenerasjon – indikerer en kronisk, bakenforliggende faktor
- Svarte flekker viser også polyfasisk muskeldegenerasjon i tillegg til muskelreparasjon og granulomatøs betennelse – indikerer også en kronisk, bakenforliggende faktor

Bakgrunn for gjennombruddet

- Infeksjon? Måtte utelukkes!
- Fikk antistoff av virologene -> Teste på melaninflekker!



Finstad *et al. Veterinary Research* 2012, **43**:27
http://www.veterinaryresearch.org/content/43/1/27

VETERINARY RESEARCH

RESEARCH Open Access

Immunohistochemical detection of piscine reovirus (PRV) in hearts of Atlantic salmon coincide with the course of heart and skeletal muscle inflammation (HSMI)

Øystein Wessel Finstad[1*], Knut Falk[2], Marie Løvoll[2], Øystein Evensen[3] and Espen Rimstad[1]

Abstract

Aquaculture is the fastest growing food production sector in the world. However, the increased production has

Bakgrunn for gjennombruddet

- Samarbeid!
- Lerøy
- HI (Matre)

Materiale & metode

Group	Fish (n)	Age (y)	Broodstock	Water	Length (cm)	Weight (g)
A	25	2	Farmed	Sea	n/a	4144
B	35	2	Farmed	Sea	n/a	4056
C	10	2	Farmed	Sea	n/a	4696
D	26	n/a	Farmed	Sea	n/a	n/a
E	20	3	Farmed	Sea	73.5 ± 5.6	4908 ± 1009
F	42	3	Farmed	Sea	60.5 ± 5.5	2763 ± 815
G	42	3	Farmed	Sea	57.4 ± 7.7	2953 ± 747
H	10	n/a	Wild	Fresh*	81 ± 12.5	5020 ± 2210

- Immunohistokjemi (PRV, PD, IPNV)
- RT-qPCR (PRV, IPNV, ISAV, PMCV, SAV VHSV).

Resultater

Group	Fish (n)	Melanised changes (n)	Red changes (n)	No changes (n)	Heart changes* (n)
A	25	9	1	15	20
B	35	1	0	34	5
C	10	3	7	0	n/a
D	26	26	0	0	n/a
E	20	17	1	2	15
F	42	1	0	41	0
G	42	0	0	42	0
H	10	0	0	10	0

Hva med «blødninger»?

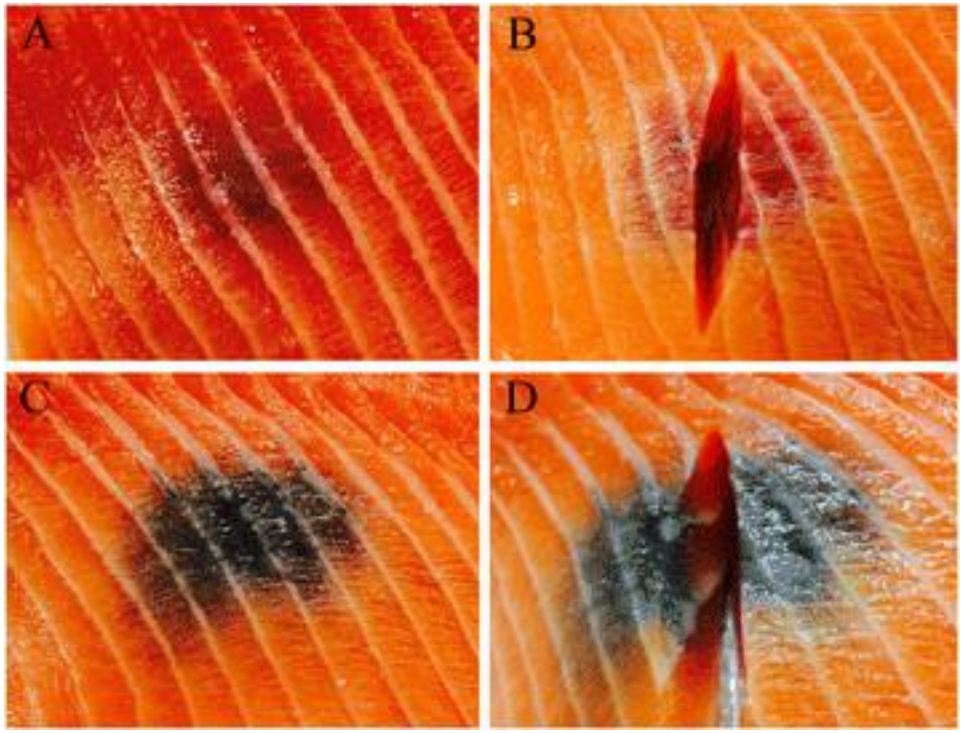
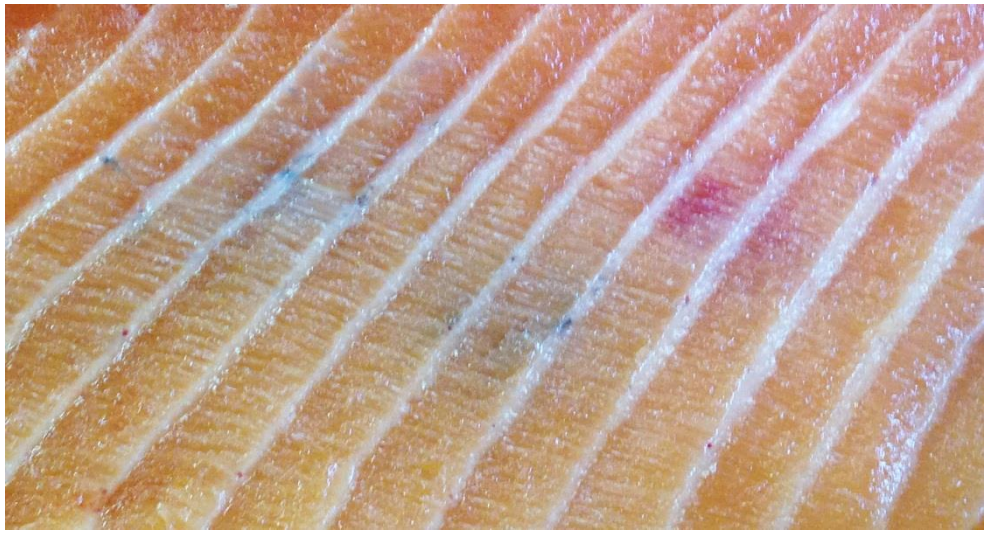
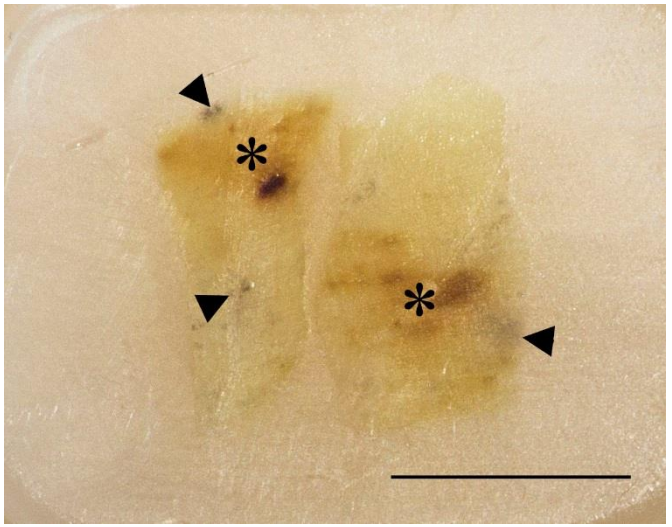
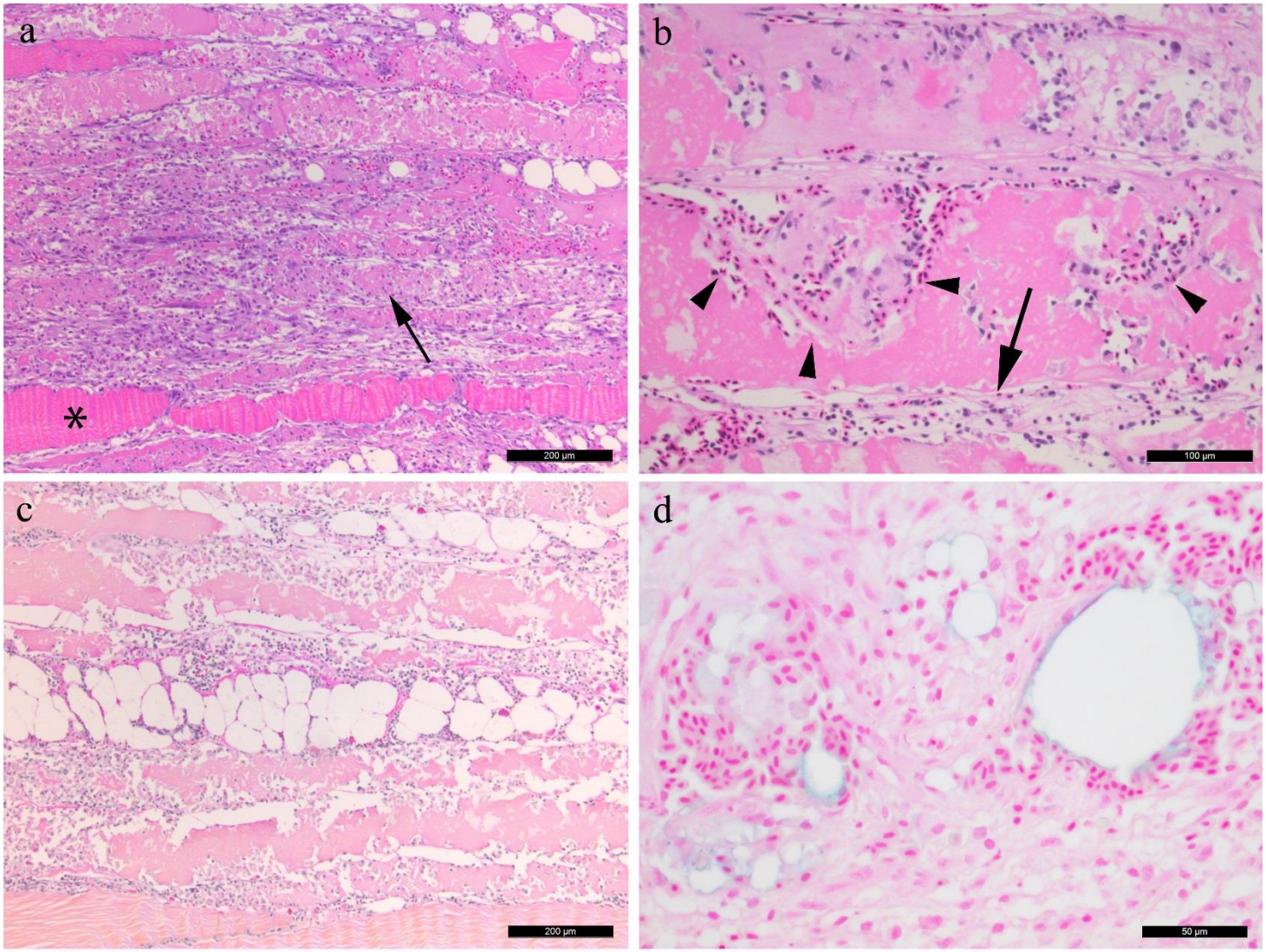


Foto: Håkon R. Sæbø

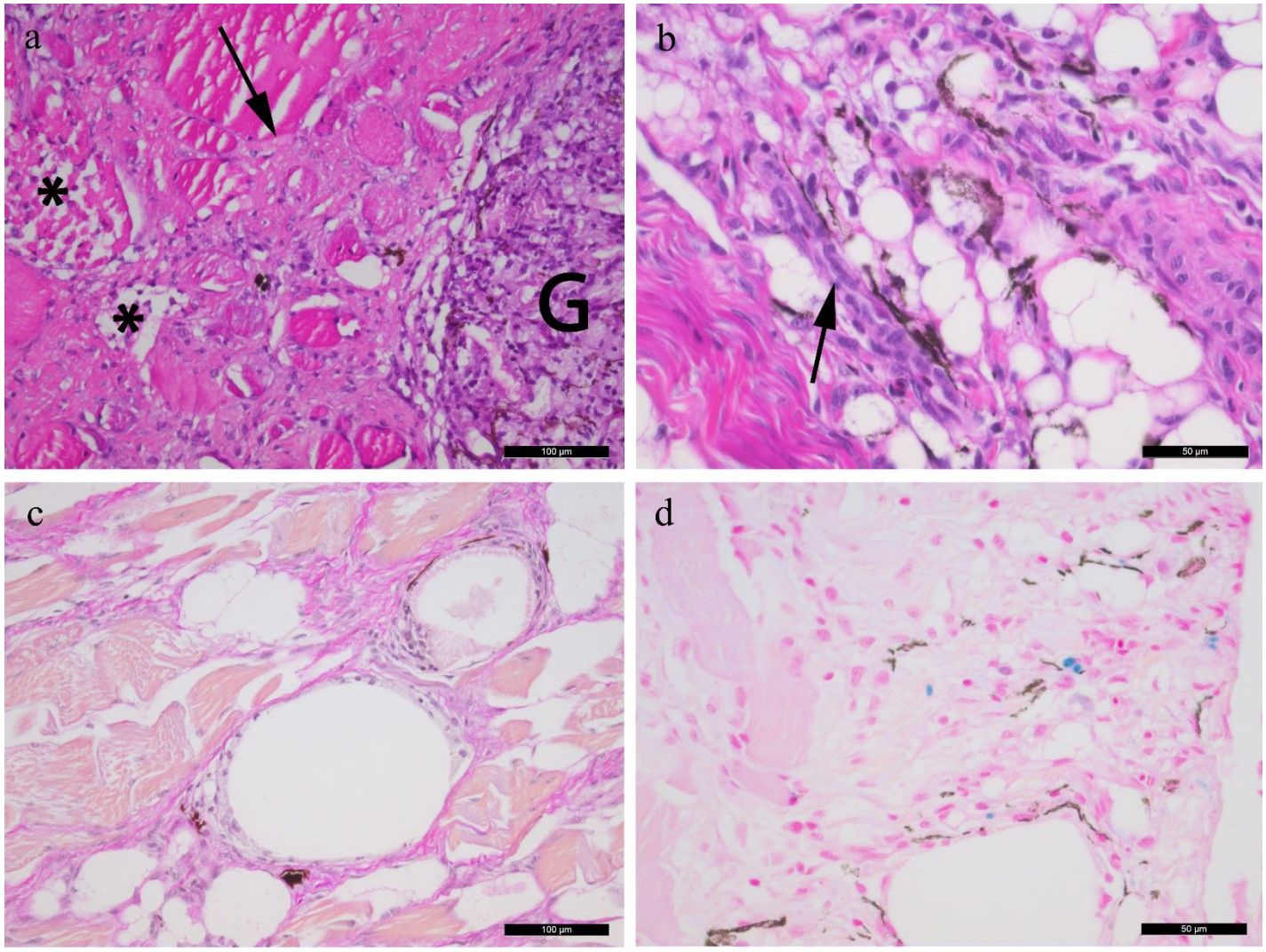
Hva med «blødninger»?

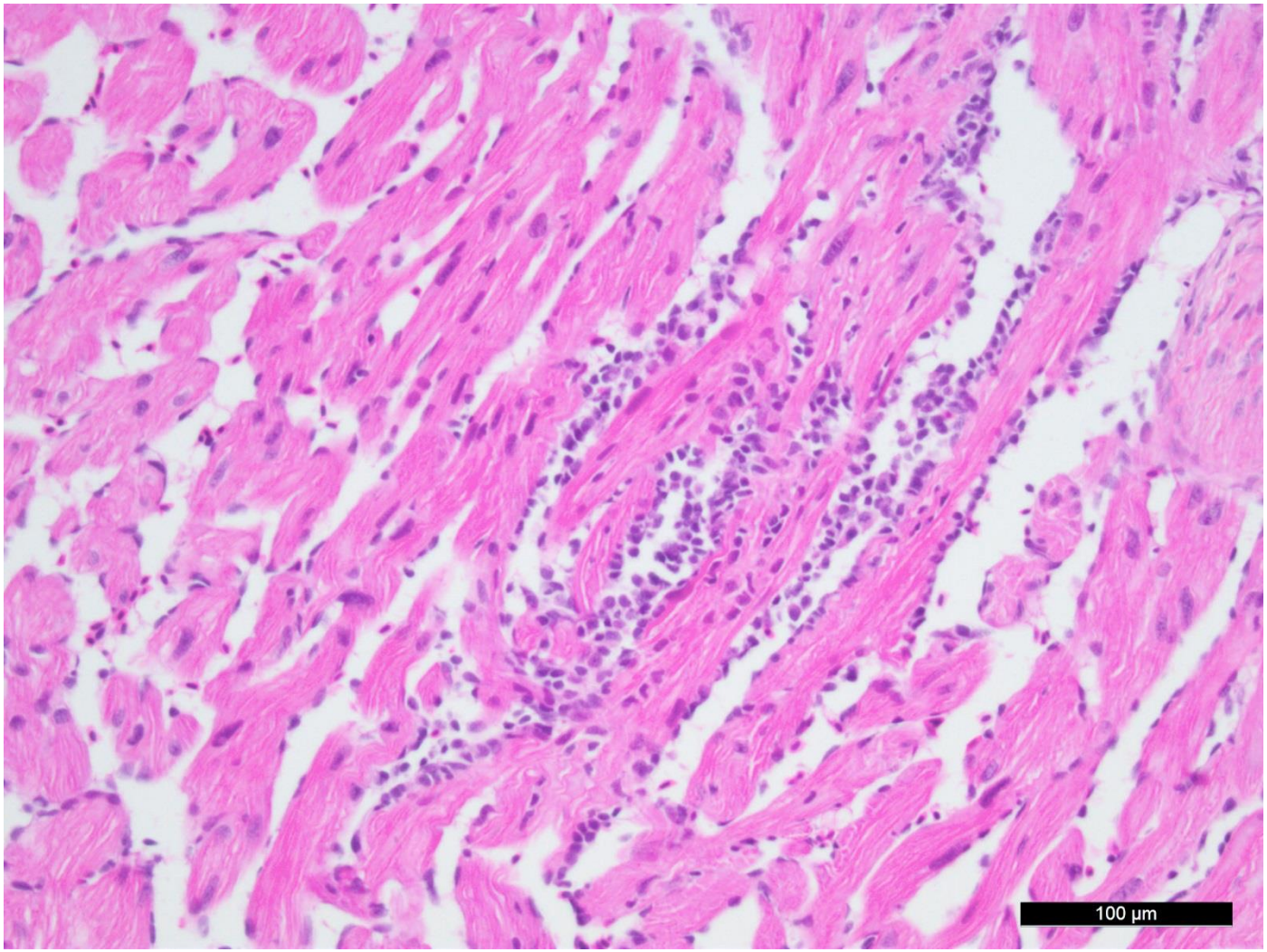





a
*
200 µm
b
100 µm
c
200 µm
d
50 µm

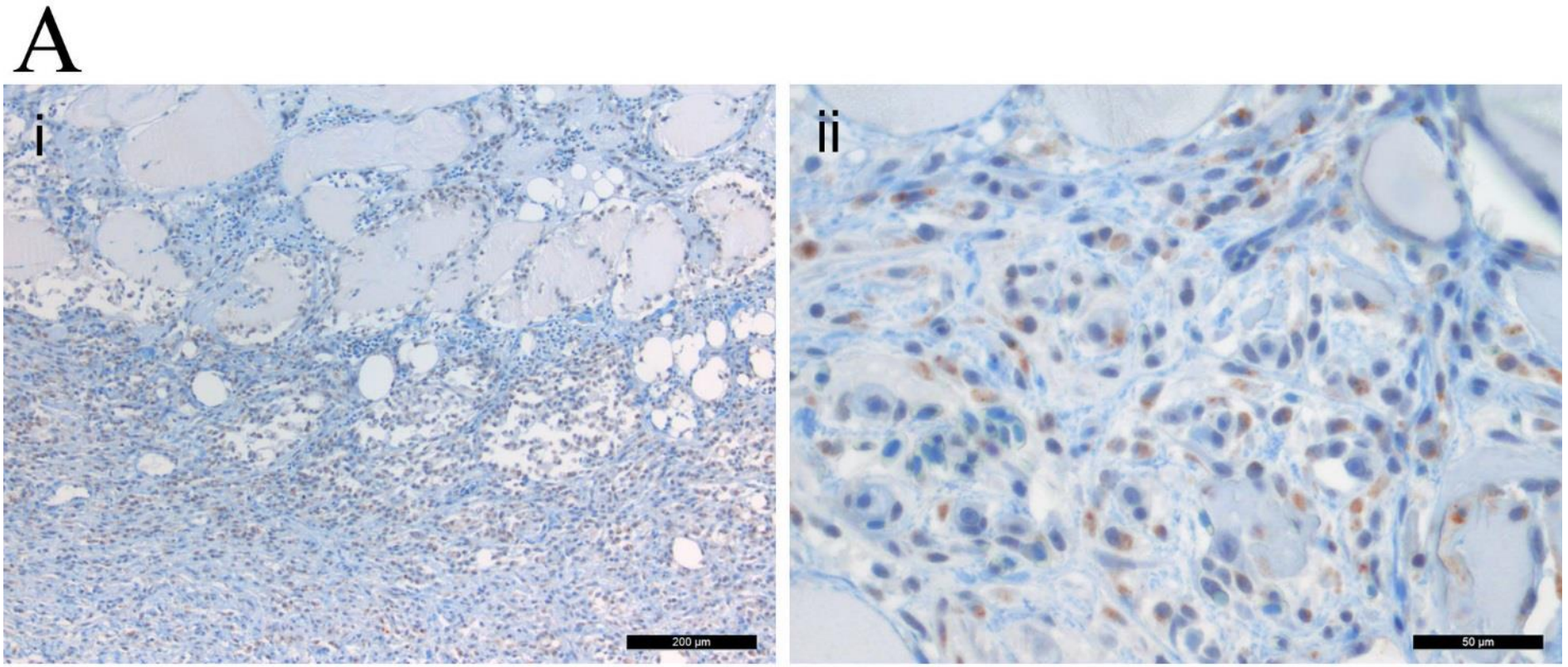
Akutt nekro-hemorrhagisk myositt


a
*
*
G
100 µm
b
50 µm
c
100 µm
d
50 µm


100 µm

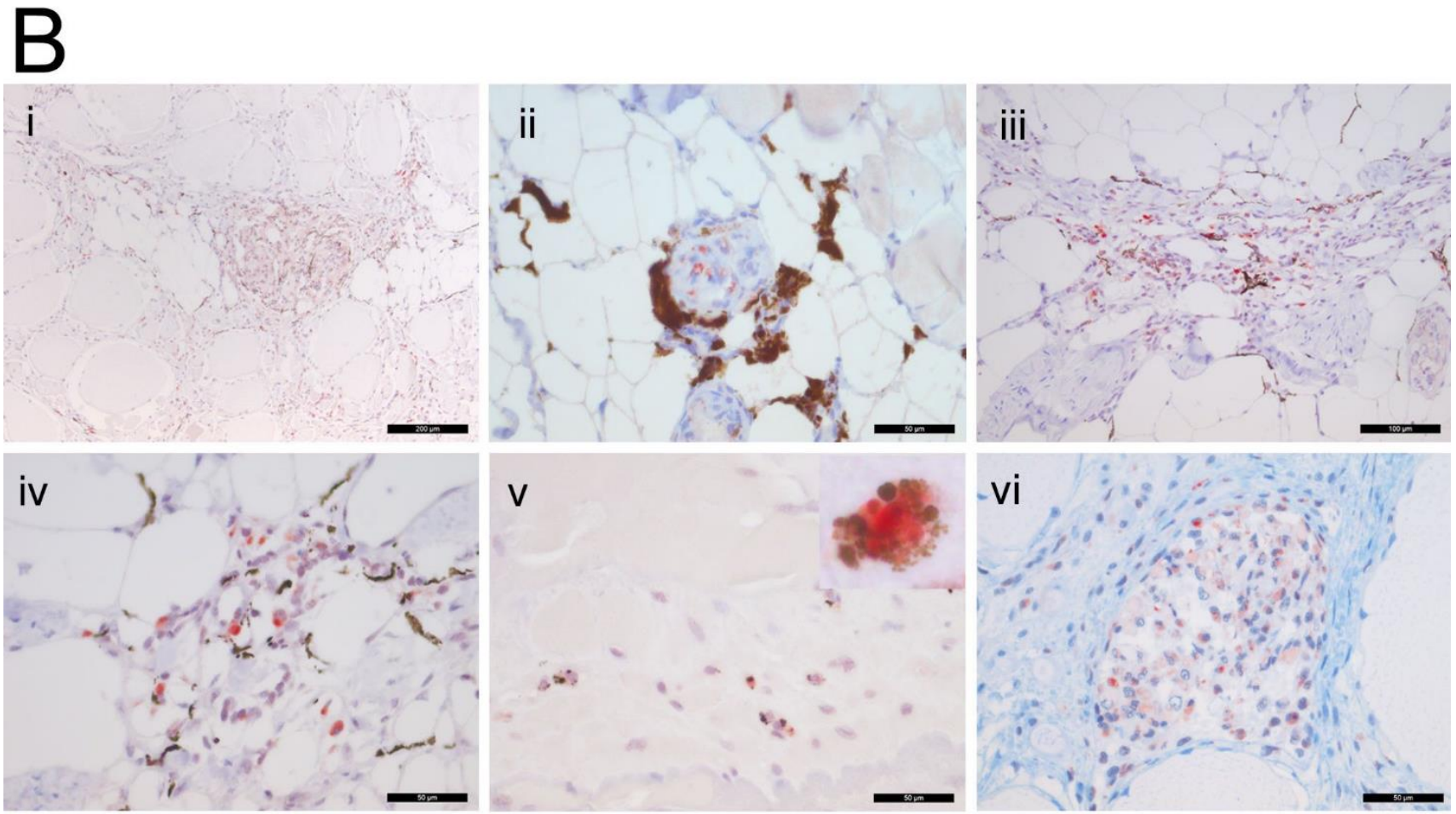
Group	Fish (n)	Melanised changes (n)	Red changes (n)	No changes (n)	Heart changes* (n)
A	25	9	1	15	20
B	35	1	0	34	5
C	10	3	7	0	n/a
D	26	26	0	0	n/a
E	20	17	1	2	15
F	42	1	0	41	0
G	42	0	0	42	0
H	10	0	0	10	0

Immunohistokjemi

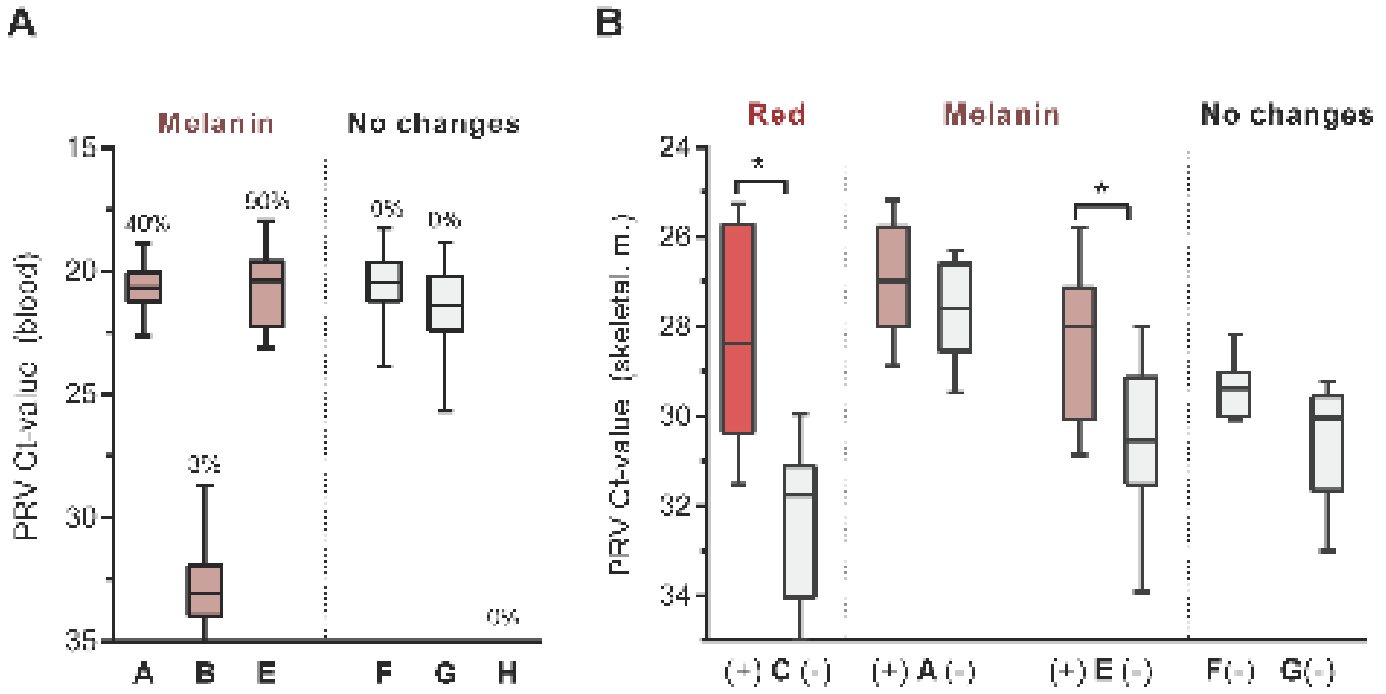


To forskjellige anti-PRV sera viste samme resultat

Immunohistokjemi



RT-qPCR


A
Melanin
No changes
PRV Ct-value (blood)
15
20
25
30
35
40%
3%
90%
0%
0%
0%
A
B
E
F
G
H
B
Red
Melanin
No changes
PRV Ct-value (skeletal. m.)
24
26
28
30
32
34
*
*
(+) C (-)
(+) A (-)
(+) E (-)
F(-)
G(-)

Spørsmål videre

- Hvilken effekt har virusstammer?
- Hvilken effekt har virusmengde?
- Hvilken effekt har alder på fisken ifht infeksjonstidspunkt?
- Hvilken effekt har ulike driftsforhold?
- Hva er sammenhengen mellom HSMI og svarte flekker?
- Hvorfor finnes ikke svarte flekker i villfisk selv om den er full av virus?
- Hvorfor fant vi ikke svarte flekker i fisken som gikk i kar på Matre selv om den var full av virus?

Patogenesen!

Virus finnes over alt! Også i klinisk frisk fisk!

Hvordan kan vi komme videre?

- Finne ut hvordan viruset induserer sykdom, herunder svarte flekker (forsøk, patologiske studier, epidemiologi)
- Finne ut om det er spesifikke virusegenskaper (stammeinformasjon) som er essensielle (feltinformasjon, virologiske studier)
  - Hvordan smitter virus, f.eks. opptak i gjeller og tarm
  - Hvordan bryter vi smitteveiene
  - Hvordan overlever virus
  - Hva er virusreservoir

Oppsummering

- Vi har funnet viruspartikler i alle undersøkte røde og svarte flekker
- Vi har redegjort for overgangen fra akutt (røde) til kroniske (svarte) forandringer
- Vi har ingen data som peker i andre retninger enn virustilstedeværelse
- Men vi vet ikke om virus initierer forandringene

Poeng: Gi sikrere råd til næringen!

Bedre data og bedre kunnskap gjør at vi kan gi sikrere råd!

Takk for oppmerksomheten!