SKIN, GILLS AND GUTS OF FISH: THE SLIMY BARRIERS RESPOND TO IMMUNE CHALLENGES BY CHANGING THE PATTERNS OF MUCOSAL EPITHELIA. WHAT WE CAN DO WITH MUCOSAL MAPPING

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- Salmobreed
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- Alltech Japan
- Cooke Aquaculture, Canada

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- Industry
- Regional Research Fund
- Innovation Norway
- Norwegian Research Council
SKIN, GILLS, GUTS = BARRIERS

First line of defense against parasites and disease
All these barriers have mucous cells

Fish skin
scale
# Mucous Cells

<table>
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<tr>
<th>substance</th>
<th>antibacterial</th>
<th>antifungal</th>
<th>antiviral</th>
<th>antiparasitic</th>
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<td>H2A peptider</td>
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<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1 oncorhyncin2</td>
<td>✓</td>
<td>✓</td>
<td></td>
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<tr>
<td>H6 oncorhyncin3</td>
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<td>pleurocidin</td>
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<td>✓</td>
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<tr>
<td>Sal-2</td>
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<td>✓</td>
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<td>complement factors</td>
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<td>Antigen-antibody</td>
<td>Antigen-antibody</td>
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<td>(proteases etc)</td>
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<td>lectins</td>
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<tr>
<td>interferon</td>
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</tbody>
</table>
Skin turns blue at «landing sites»
(experimental work)
Anectodal evidence from fish farms said that some feeds were giving more mucous and less lice but all attempts to measure this were failing.

Could we make a method to measure mucous cells?
Q: how do you take one (1) slice of the sky and find out how big the balloons are and how tightly packed they are?

A: Design-based stereology

*YES: Mucosal mapping*

*Mucosal mapping is complementary to all other existing technologies*
• Salmon lice  
  **challenges**  
• Salmon welfare  

Weekly lice counts  

Sampling for skin response
Similar structures between mammals and fish, except fish have live cells at the surface
Goblet cell = mucous cell
Nice pictures but not good statistics....

From Gomez et al., 2013
Where to measure health outside the blood?

Whole fish
Growth, mortality

Organ level (skin, gills, guts)
Composition and cell types
**Barrier health (skin, gills, guts)**
Regulates growth potential, disease etc.
**Summarizes effect of several hundred genes**

Gene level
Up- and down regulation

«... technology is outpacing our ability to interpret the DNA that's decoded. How much does a particular variant change risk for an individual?»
«Science» journal, 5 December 2014
Design-based stereology (Pittman Protocol)

Examples of salmon skin sections giving statistically robust objective measures of:

i) mucous cell area
ii) mucous cell density
iii) area/density (barrier strength)

Control

Aquate SPMP

mucus cells
pink – neutral mucins
blue – acid mucins

scale
pigment
What we do is difficult AND validated*

1. Number of mucous cells alone means little
   < 5 is «less» than 1

2. Direct area of cell can mislead
   Same cell but profile changes

3. Numerical density is not volumetric density
   -5 cells per mm2 is not eg. 5% of tissue filled with mucous
   -numerical density is not directly comparable with much

*Pittman et al. 2011; Pittman et al. 2013; Torrecillas et al. 2015
The Repeatable Basics: Significantly larger mucous cells on dorsal (p<0.01)

Pittman et al. 2012
The Repeatable Basics: Significantly denser mucous cells on the dorsal (p<0.05) i.e. how much of the epithelia is mucus.
1. Sulefisk - field trial on commercial farm, 4 cages with 2 control 2 Aquate-fed groups - 280 000 salmon (about 50 m tons), 4 sampling dates, 7 months duration

2. Gifas1 - field trial on research station, 60 000 salmon, 12 cages with 3 cages for each of 4 diets (control, plus three other diets), 5 sampling dates over 4 months

3. Gifas2 - field trial on research station, 60 000 salmon, 12 cages dose-response to 2 levels of trial diet plus control diet, 3 sampling dates over 2 months

4. Averøy - controlled trial at Salmobreed, two specially bred families - 1 sampling date

5. Gill test - gills from GIFAS2 first date control fish

6. Seabass (Spain) – guts, skin and gills at research station Las Palmas; 720 fish, 4 diets in triplicate, 8 weeks

7. Delousing salmon (Norway) – 1 day before, then 2, 8 and 15 days after, samples of skin, gills, esophagus following exposure to hydrogen peroxide

8. Yellowtail (Japan) – diet and parasite control, (Kochi Univ and Alltech)

9. Salmon (Canada) – diets, skin, intestine (Cooke Aqua)
Findings:
1. Diet makes a **significant** difference to innate immunity
2. The Happy accident
3. What is happening when skin reacts

4. Gills – salmon wild and farmed
5. Guts – seabass and diets
6. Yellowtail skin – diets and parasites
7. The big picture
4 diets, 4 months, 60000 salmon
-Diet really does make a difference

Cell area

Date

Cell area

Aquate SG

The Happy Accident
B (Aquate SG) increases cell density most

B (Aquate SG) has fastest response in mucosal tissue (barrier strength)

Cage-wise parasite counts - B (Aquate SG) has least
Parasites go where mucosal cells are small and few
Family does **not** explain lice counts, but mucosal measures do (so far)

RDA statistical tests on quantified data)

N=29 salmon
Pittman et al., in prep
Different states of skin

Resting state  Responding state  Going toward sick

Size, density and barrier strength of mucous cells and skin changes.
Illustration of how we think mucosal barrier behaves when parasites attack

Parasite arrives
- large mucous cells are replaced by more small cells
- smaller cells move quicker through skin
- many smaller cells wash off parasites

Skin returns to resting state if parasites gone
Mucosal Mapping in Salmonids

Complementary to all other methodologies

Inventor Prize 2013
Hordaland Fylkeskommune
(most fish farms in Norway)
Mucosal Mapping on salmon gills and skin
- highly significantly different mucous cell populations
- different control mechanisms

N= 10 salmon
* p<0.05
** p<0.001
*** p<0.0001

Barrier strength
Mucosal Mapping of wild salmon gills:

- Wild had shorter lamellae
- No mucous cells in lamellae
- Healthier?

Work continues…!

Wild had shorter lamellae
No mucous cells in lamellae
Healthier?

Work continues…!
Can gill measures indicate general health?
-Trygve Sigholt, BioMar, 27 June 2014
Mucosal Mapping in Seabass guts and skin

- **Diet** → **Gut bacteria** → **Mucosal tissue response**

- **Anterior Gut**
- **Posterior gut**
- **Dorsolateral skin**

**Fish oil** vs **Soybean oil**

- **MOS + fish oil** decreases hindgut MC size
- **MOS +SB Oil** increases hindgut MC size

**Diffusion rates up with decreased cell size**
Mucosal Mapping in Seabass guts and skin

MOS+ fish oil decreases hindgut mucous cell density compared with fish oil
MOS + SBO increases hindgut MC density compared with soybean oil

Dietary mannan oligosaccharides: counteracting the side effects of soybean meal oil inclusion on European sea bass (Dicentrarchus labrax) gut health and skin mucosa mucus production? Frontiers in Immunology 6:397. doi: 10.3389/fimmu.2015.00397
Patterns of mucous cell distribution may be correlated with differential gene expression and be a vital clue to innate immune responses to a variety of pathogens and inflammatory agents.

- Good epithelial turnover combined with differentiation of cells
- Stronger immune reactivity
- Slightly increased ability for immune substances to diffuse
- Lowest cell migration & diffusion rates, epithelial hyperplasia, more cell differentiation and turnover, and more anti-inflammatory agents

Downregulated IL-6, IL-10, and TGFβ (functioning of mucous barrier and immune homeostasis)

From: Torrecillas et al., 2015
Highly significantly larger mucous cell size on dorsal than on head ($p < 2.2e^{-16}$)

Higher densities on dorsal than head ($p < 0.006$)

Diet 1

Sex significantly affects density ($p = 0.046$)

Diet 2

Diet 4 group has nearly significantly lower mucous cells densities ($p = 0.056$)

Diet 7

N=24 on Day 81

From Maxwell H 2015 «Quantification of epidermal mucous cells in Canadian Atlantic salmon». Masters thesis. BIO UiBergen
Mucosal Mapping vs salmon lice infestation

Adult attached lice repress mucous cell production at site and repress general mucosal immunity (Thorsen, in prep, N= 45 fish).

Attached adult lice clear the way for higher levels of new copepodites (Mo, 2015).

13.2 % density
AVG M 176 μm²

12.3 % density
AVG MC 182 μm²

11 % density
AVG MC 157 μm²

0.4% density
AVG MC 109 μm²

Control

Controlled infestation with salmon lice copepodites

2nd infestation
No adult lice

2nd infestation
With adult lice

Attachment site

From: Thorsen, in prep
Skin response to salmon lice is stage specific

proper animation first draft by Egil Paulsen
Gills are still recovering more than 2 weeks after treatment.

Esophagus responds with highest density of mucous cells which persists for 3 weeks.

N=22 fish (88 samples)

From: Rantty I, 2015, in prep.
Mucosal Mapping and Cell Shape Analysis

Gut mucous cells may be elliptical

Segmented image of salmon Esophagus mucous cells
(from Merkin based on Rantty, in prep)

Hard to make correction factors for elliptical cells but cells here have minor:major axis ratio between 1:5 and 1:1

Courtesy: Grigory Merkin
Mucosal Mapping and Japanese Yellowtail

Control 対照区

Day 0 Control, n= 5 females and 5 males

Female:
- 75μ² (1.4%)
- 56μ² (0.7%)

Male:
- 68μ² (1.5%)
- 62μ² (1%)  
- 81μ² (2.8%)  
- 15μ² (0.1%)  

Pectoral Side:
- 79μ² (1.7%)
- 72μ² (1.5%)

Compare scale size with parasite size

Courtesy: Alltech Japan/Quantidoc
The big picture summary

Mucosal Mapping shows:

1. how fish skin reacts to parasites, diseases and treatments;
2. how fish guts react to dietary components
3. how salmon skin, gills and guts react to delousing
4. THAT DIET MAKES A SIGNIFICANT DIFFERENCE

*If we can measure it, we can improve it*

tailor diets, select broodstock, select sites

Strengthen the barriers!
Healthy barriers = healthy fish

Mucosal Mapping

Health
- Good welfare
- Few challenges to homeostasis

Disease Pathology
- Many challenges to homeostasis
- Poor welfare
VISION

A health assessment strategy with a code for quantifiable health and welfare
-the presence of health not the absence of disease
Quantidoc owns the IP for this diagnostic method of quantitatively assessing mucous cells and is commercializing the product for industrial application.

- Diagnostic – health status (skin, gills and guts)
- Quantitative, objective & comparable
- Statistically robust
- Links diet & immunity
- Summarises the effects of >200 genes
- Important tool for monitoring and improving fish health and welfare

Contact: quantidoc@gmail.com