



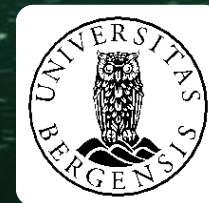
Barrier status in skin, gills and guts: mapping the dynamics of the innate immune system throughout the salmon production cycle with statistically robust results.

Karin Pittman<sup>1,2</sup>, Mearge Okubamichael<sup>2</sup>, Grigory Merkin<sup>2</sup>,  
Natalie Brennan<sup>1</sup>, Mark Powell<sup>3</sup>, Linda Andersen<sup>4</sup>, Nini  
Sissener<sup>3</sup>, Arthur Lyngøy DVM<sup>5</sup>, and Ole Jacob Myre<sup>2</sup>



Fiskr AS

INSTITUTE OF MARINE RESEARCH  
HAVFORSKNINGSINSTITUTTET



Know how to build a solid basis  
for a multinational new industry

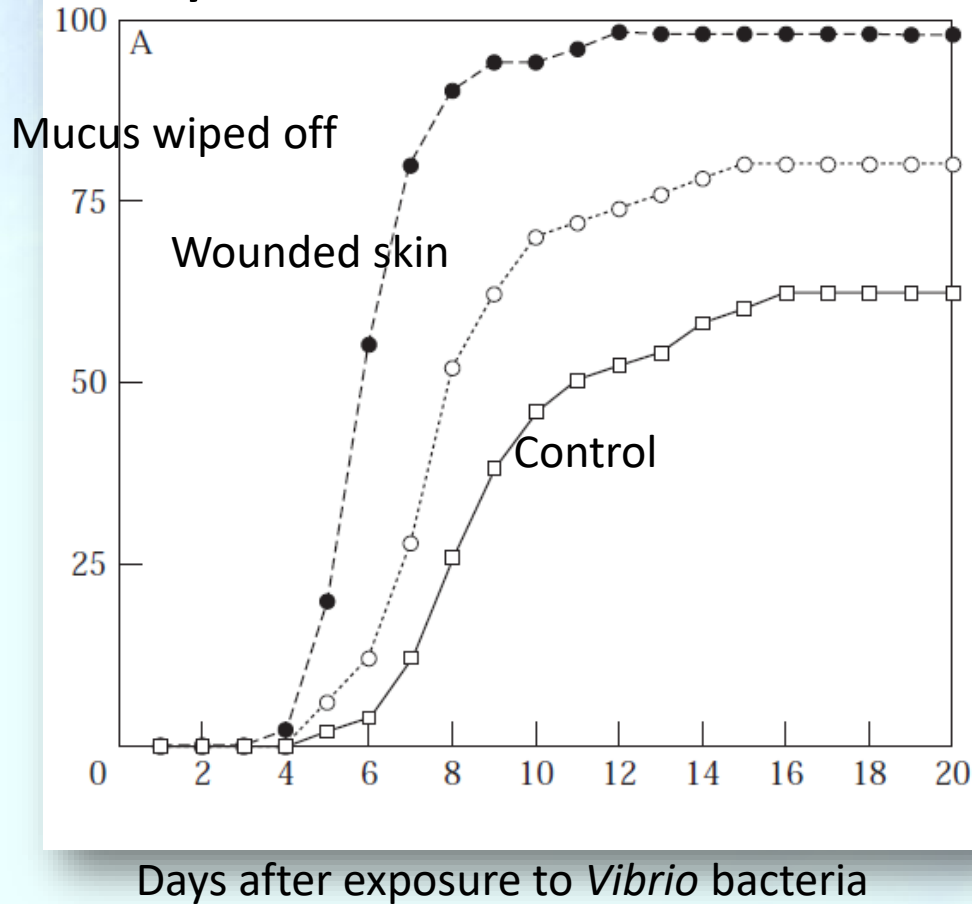


Victor Øiestad, Leif Berg and Karin Pittman  
with some of the world's first reared halibut larvae  
Hyltropolitan, Austevoll, ca 1986



# It is painfully simple: fish health is better with a good mucous layer

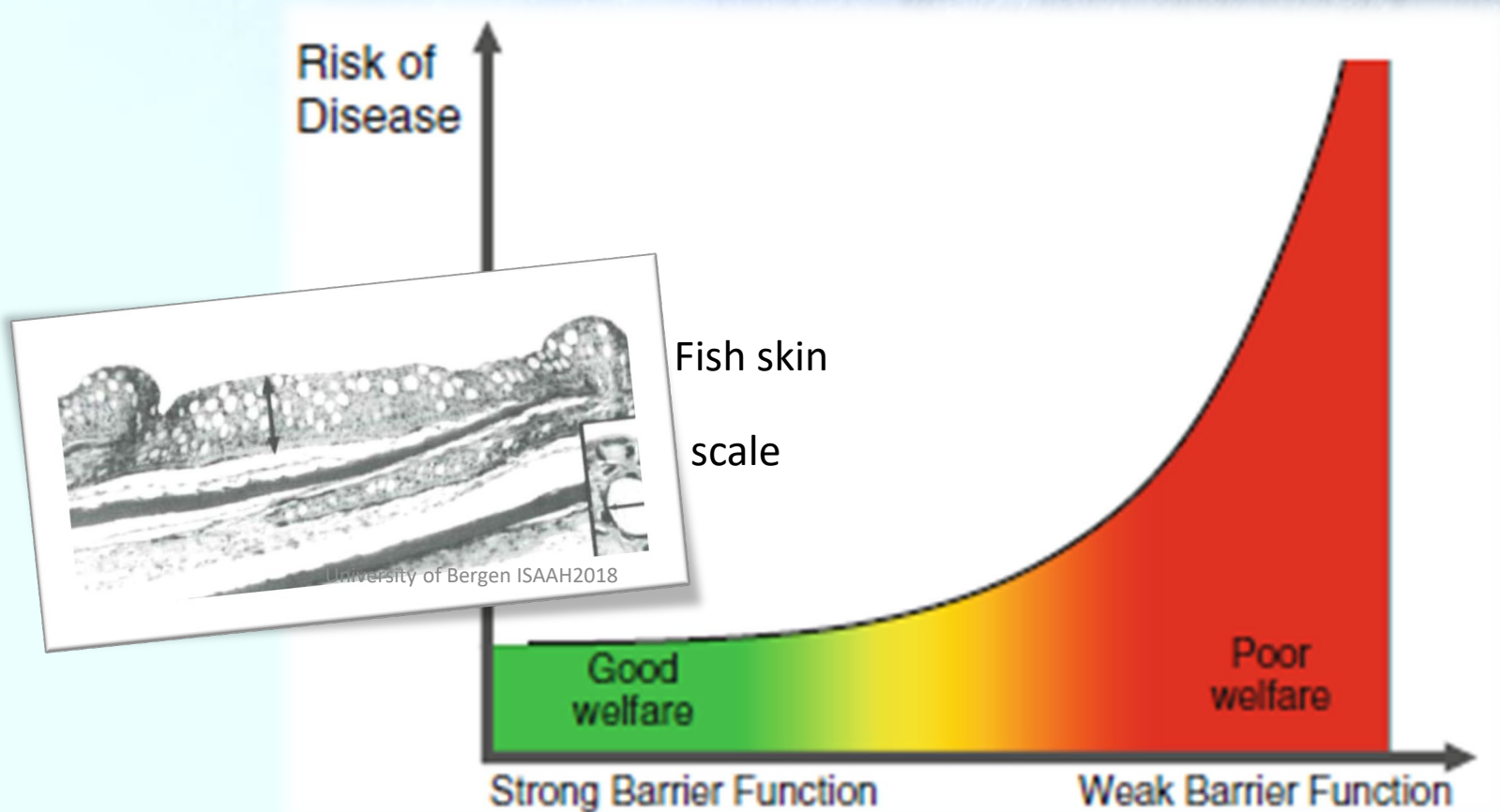
## Mortality in Atlantic salmon



From: Svendsen and Bøgwald 1997





# SKIN, GILLS, GUTS = BARRIERS

**First line of defense against parasites and disease**  
**<0.007 mm thick mucosal epithelium**  
**separates the salmon from its environment**

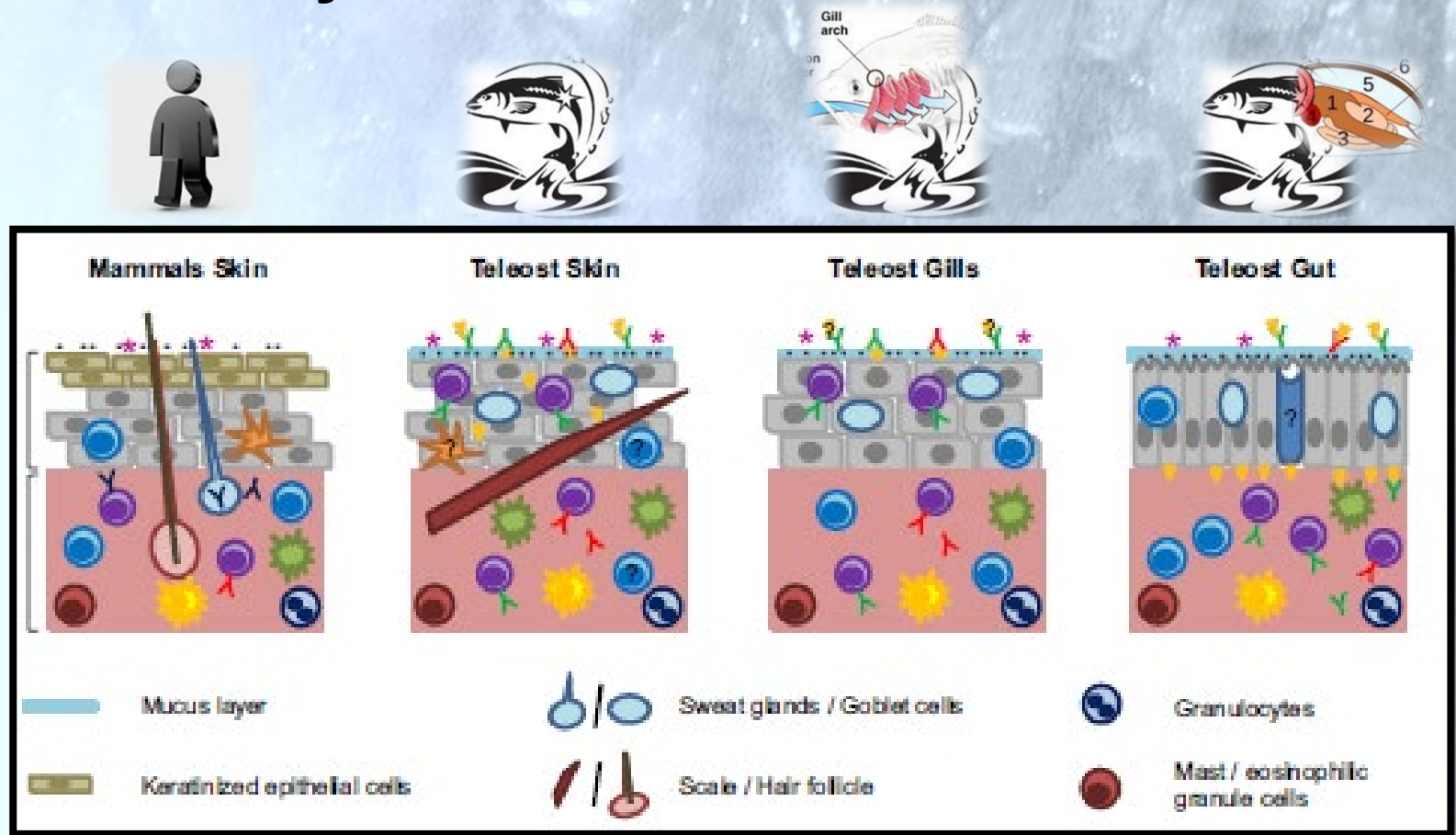


# Slime is Pro-Active

from Rakers 2013 Nature

substance	antibacterial 	antifungal 	antiviral 	antiparasitic 
H2A peptider	✓	✓		
H1 oncorhyncin2	✓	✓		
H6 oncorhyncin3	✓	✓		
pleurocidin	✓	✓		
Sal-2	✓	✓		
complement factors	Antigen-antibody	Antigen-antibody	Antigen-antibody	Antigen-antibody
hydrolytic enzymes (proteases etc)	degrade	degrade	degrade	degrade
IgM, IgT	basic antibodies	basic antibodies	basic antibodies	basic antibodies
lectins	pathogen recognition	pathogen recognition	pathogen recognition	pathogen recognition
mucus extract			✓	✓
interferon			✓	

# Brief anatomy of barriers



Similar structures between mammals and fish, except fish have live surface cells

Goblet cell = mucous cell

Nice pictures but not good statistics....



# Where to measure health outside the blood?

**Whole fish**

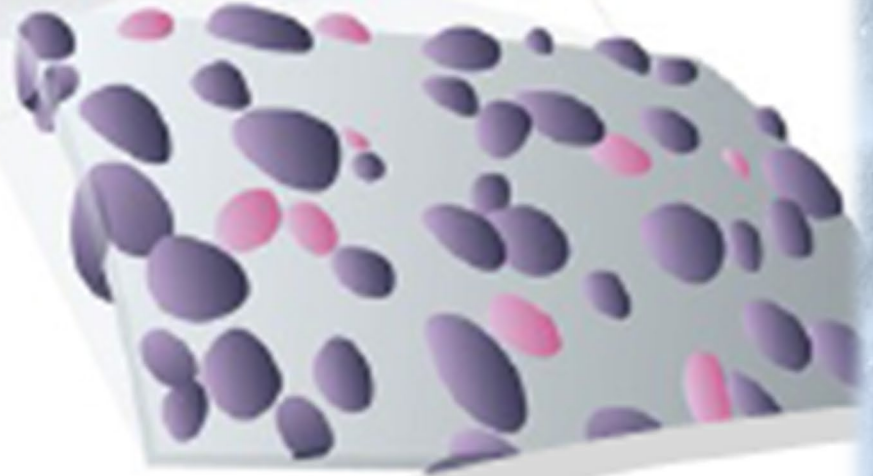
Growth, mortality



**Organ level (skin, gills, guts)**

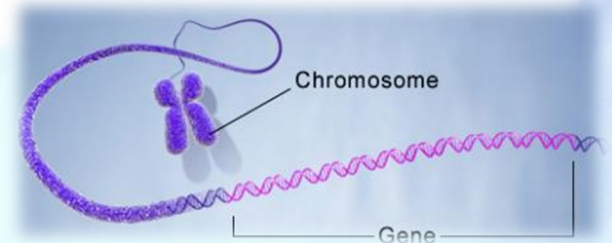
Regulates growth potential, disease etc.

Summarizes effect of several hundred genes



**Gene level**

«... technology is outpacing our ability to interpret the DNA that's decoded. How much does a particular variant change risk for an individual?»

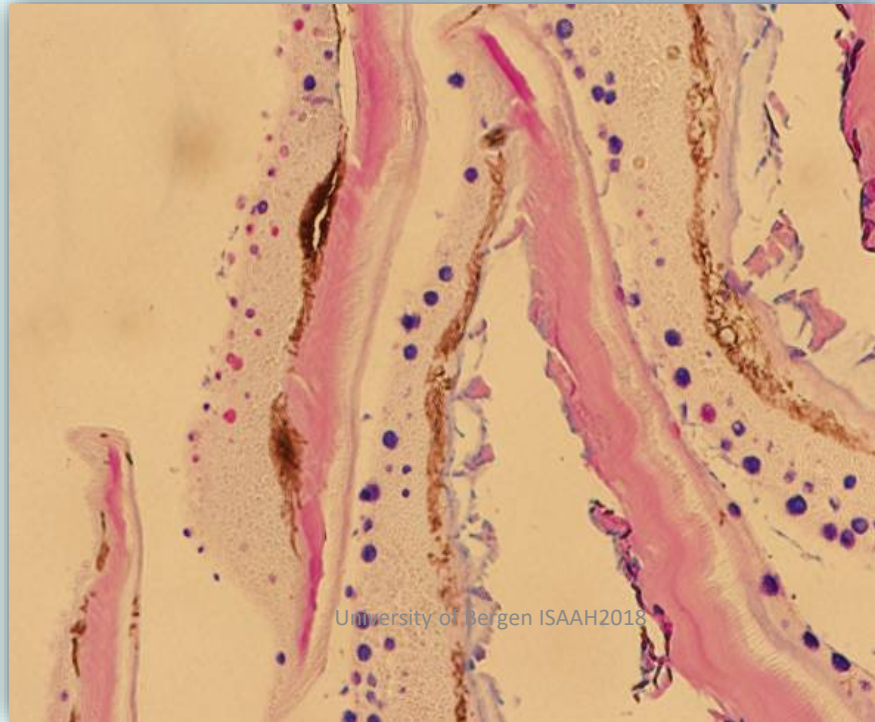


# Method: Design-based stereology, Industrially applicable

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

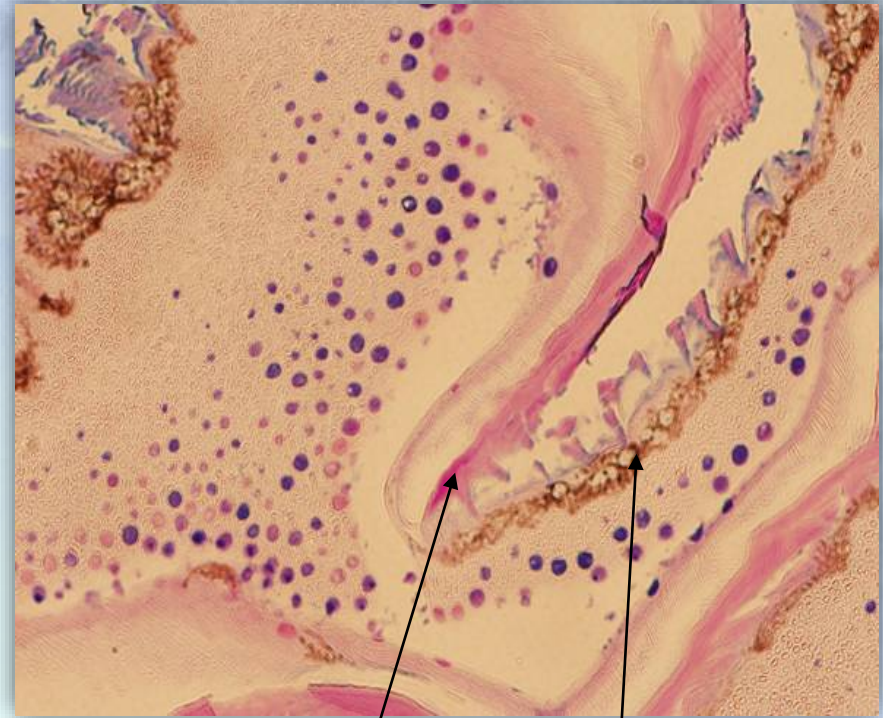
- i) mucous cell area A    ii) mucous cell density D    iii) barrier status ( $1/A:D$ )

## Control



mucous cells  
pink – neutral mucins  
blue – acid mucins

## Treatment



scale

pigment

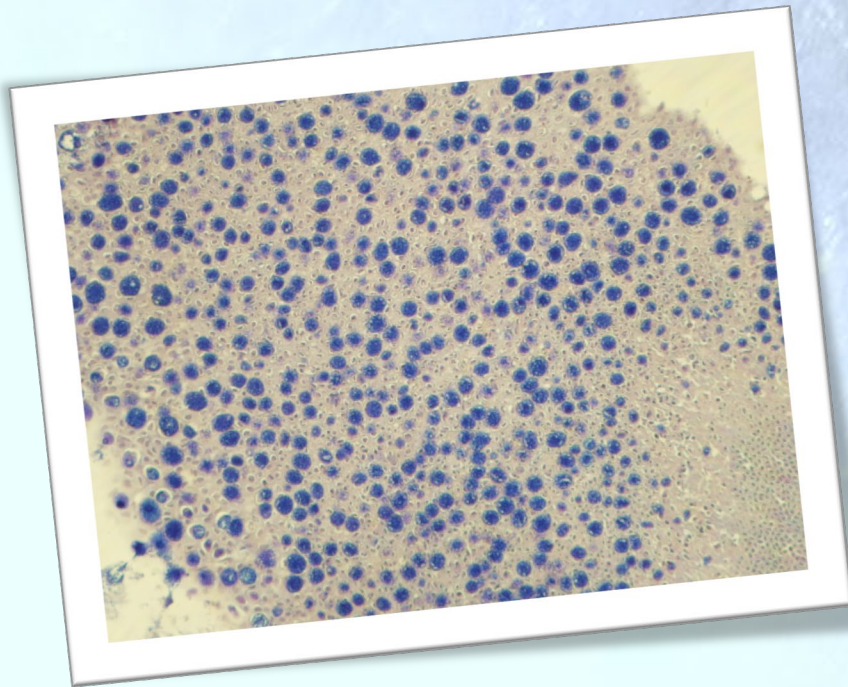
Stain: Periodic Acid Schiff-Alcian Blue



UNIVERSITY OF BERGEN

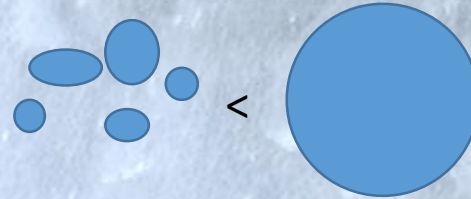


# What we do is difficult AND validated



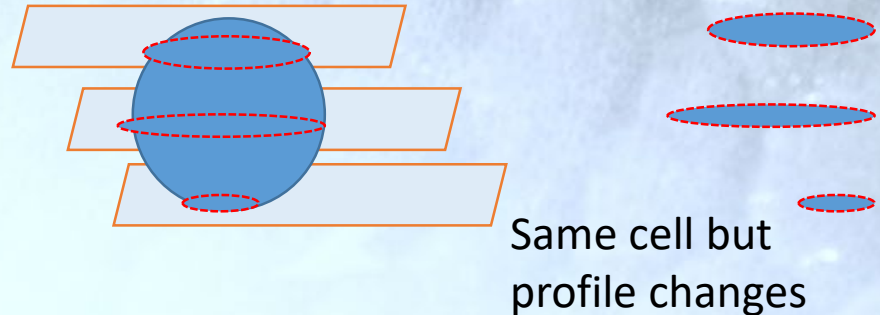
*Dorsal skin of trout  
PAS-AB stained*

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

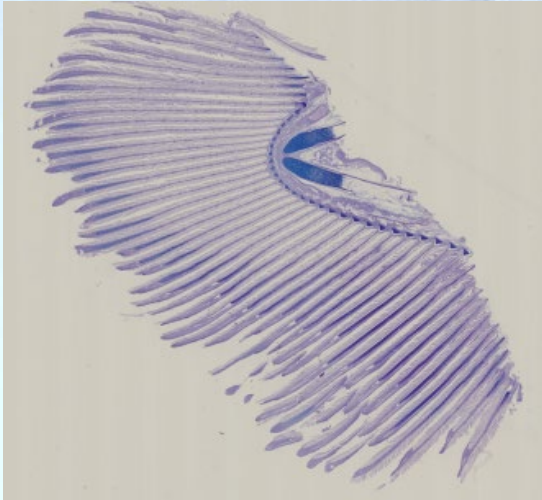
3. Numerical density is not **volumetric density**

-5 cells per mm<sup>2</sup> is not eg. **5% of tissue filled with mucous**

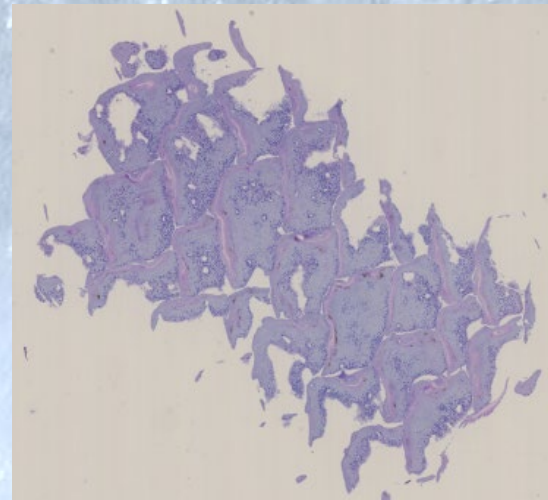
-numerical density is not directly comparable with much

Examples of salmon skin sections giving statistically robust objective measures of:  
i) mucous cell area **A**    ii) mucous cell density **D**    iii) **barrier status** ( $1/A:D$ )

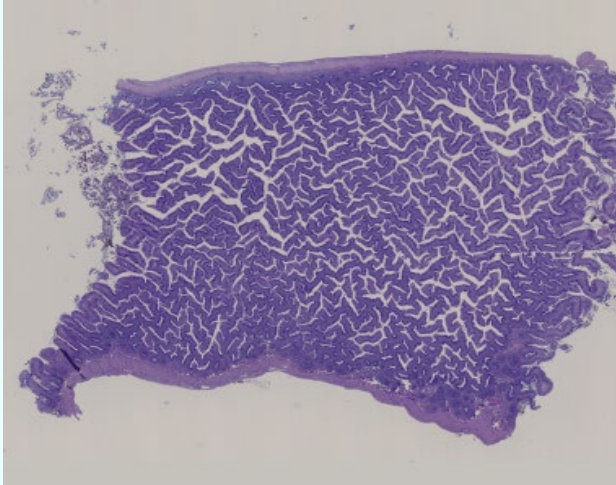
GILLS



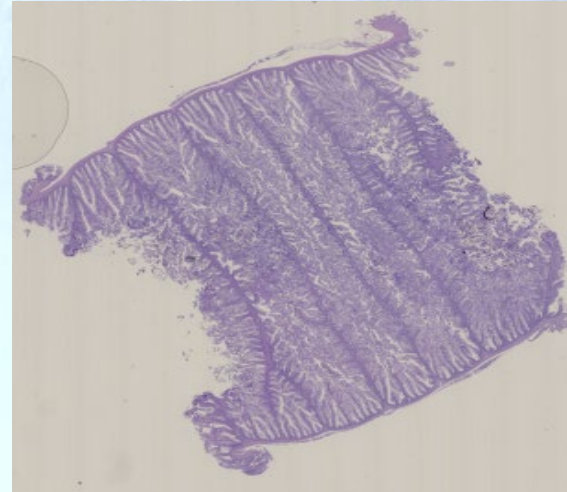
DORSAL SKIN



FOREGUT



HINDGUT



Mucosal epithelia are ancient forms of self protection  
- Possible to plot all tissue results in same figure



# HYPOTHESIS Pathogenesis

Each tissue has an optimum size & density of mucous cells  
(Homeostasis)

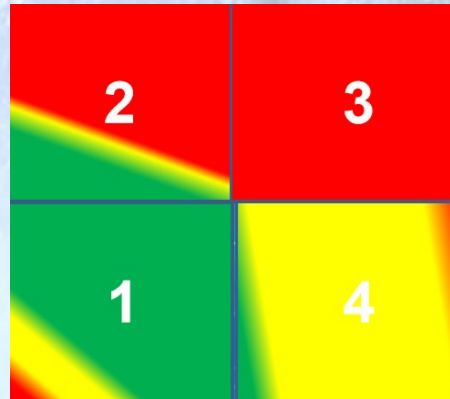
Response to immune-challenges is first in cell size then cell density

Clinical conditions occur with both too much and too little

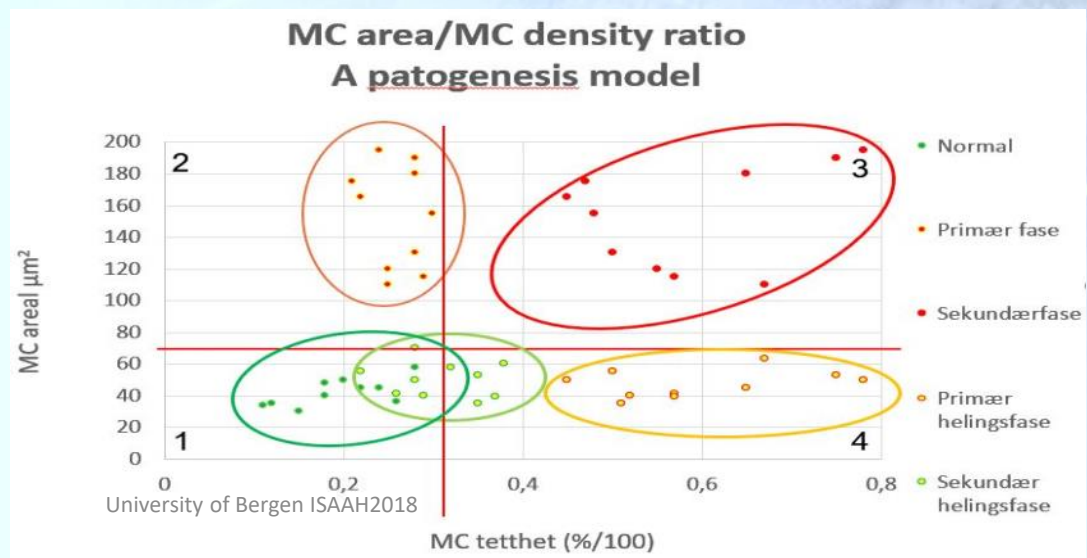
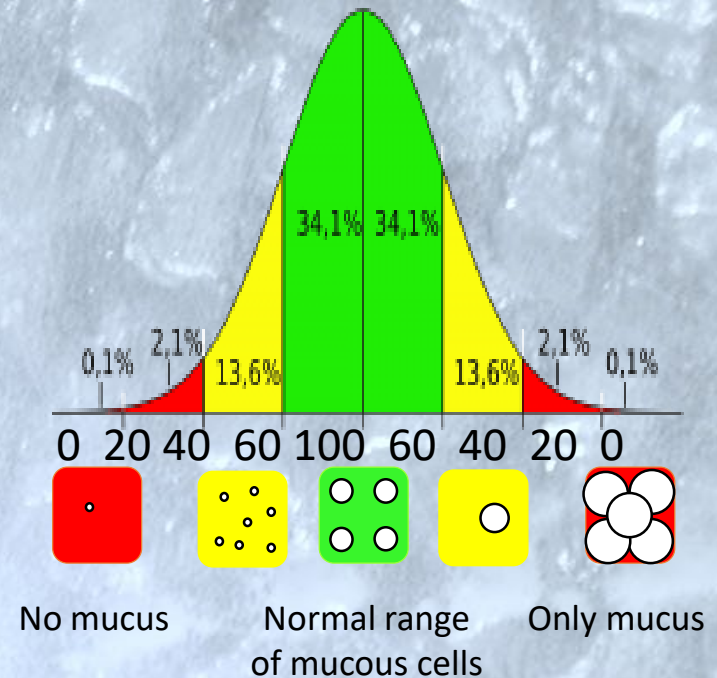
The Immune system is always responding.

Gills: The best early warning & indicator general health

Foregut: Earliest response to diet



Primary in green sector (1)  
→ Normal condition in the tissue  
Primary in red sector (2)  
→ Primary inflammation  
Red sector (3)  
→ Secondary inflammation  
Primary yellow sector (4)  
→ Recovery

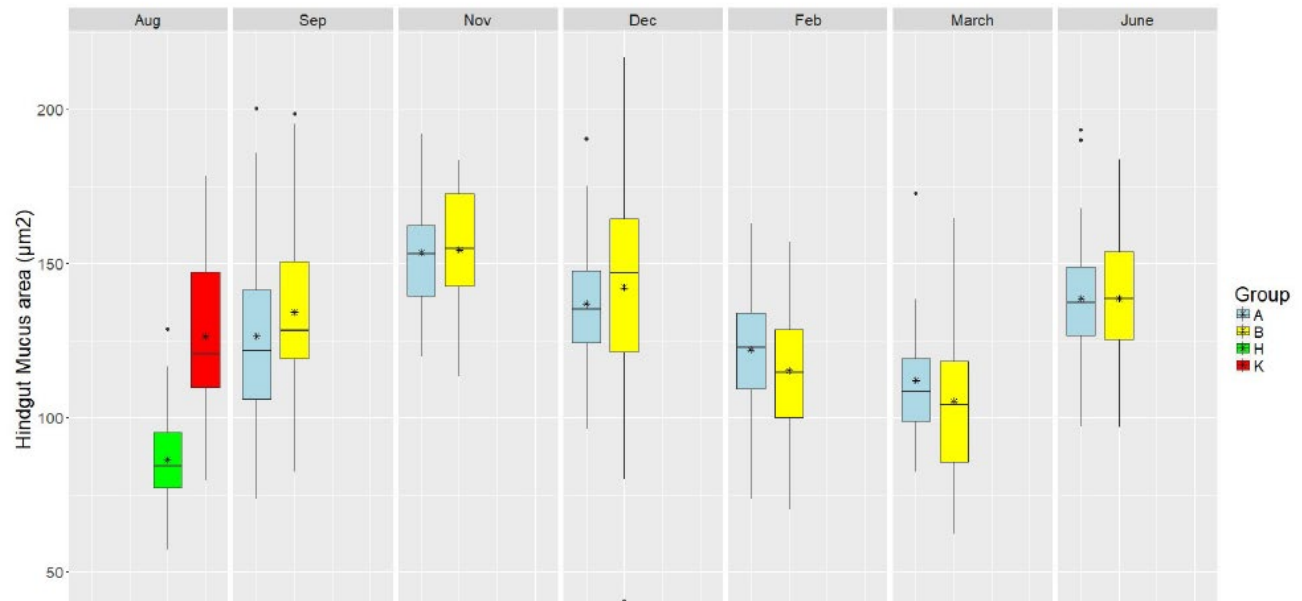




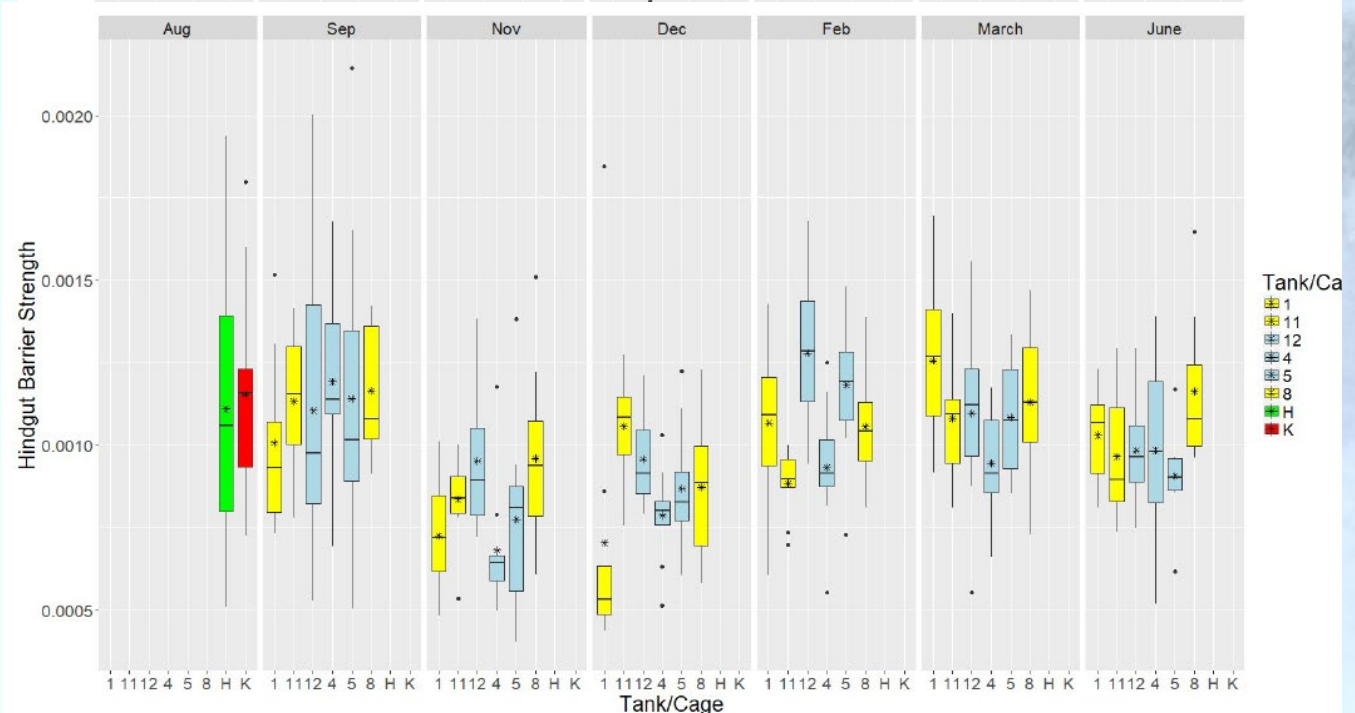
# **The guts are the foundational organ**

- building blocks for immunity
- diet can clearly affect  
immune responses
- easily distressed in salmon

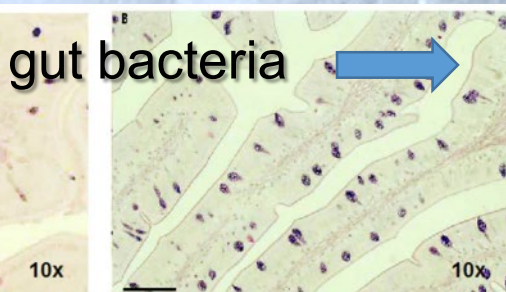
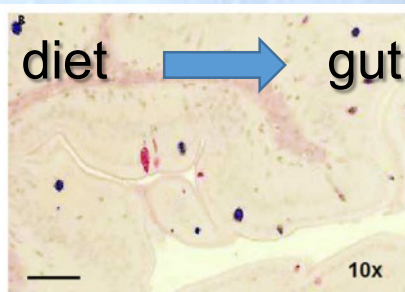
Salmon  
generation study  
**Hindgut area**  
group means  
- 6 cages and  
2 diets  
n=30-45  
Production cycle



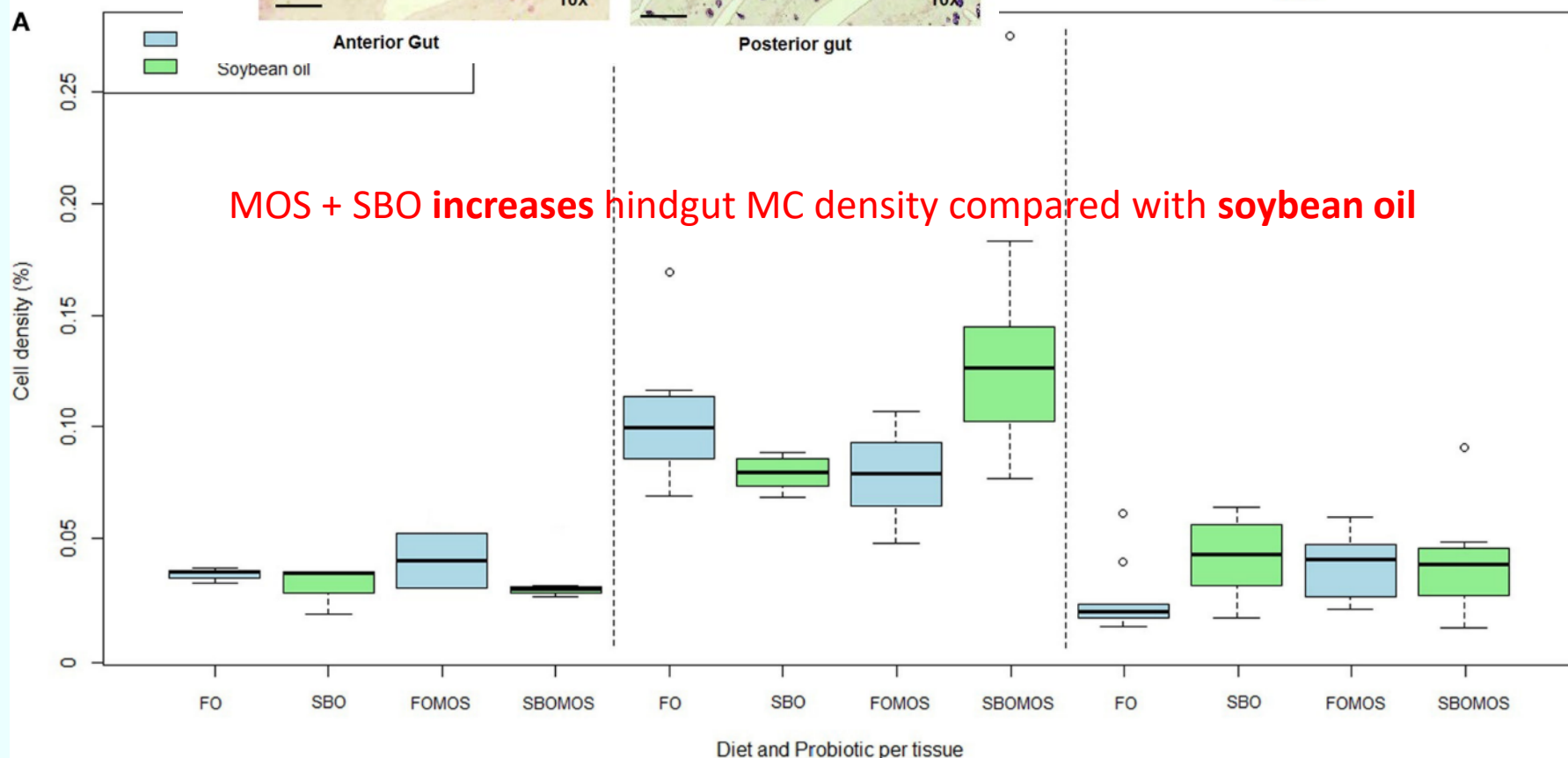
**Hindgut  
Barrier Status**  
(1/(A:D)  
in cages,  
Responding to  
net changes in  
Nov-Dec



# Mucosal Mapping in Seabass guts and skin



mucosal tissue response



Torrecillas S, Montero D, Caballero MJ, Pittman KA, Custódio M, Campo A, Sweetman J and Izquierdo M (2015)

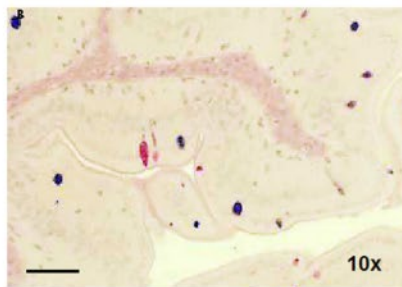
Dietary mannan oligosaccharides: counteracting the side effects of soybean meal oil inclusion on European sea bass

(*Dicentrarchus labrax*) gut health and skin mucosa mucus production? *Frontiers in Immunology* 6:397. doi: 10.3389/fimmu.2015.00397

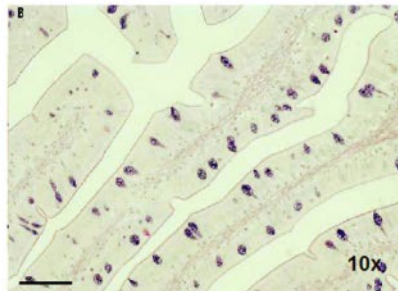


# Mucosal Mapping in Seabass guts and skin

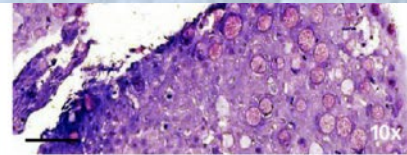
diet → gut bacteria → mucosal tissue response



Anterior Gut

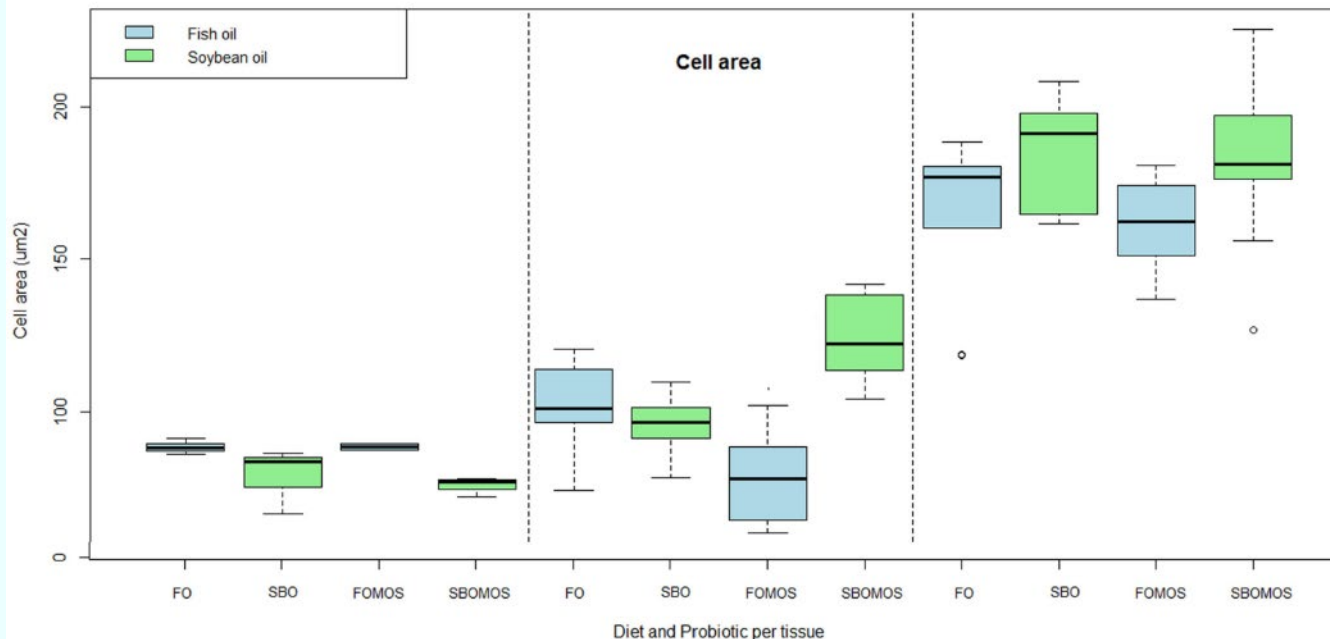


Posterior gut



Dorsolateral skin

Diffusion rates up  
With decreased cell  
size



MOS + fish oil  
decreases  
hindgut MC size  
**(better immunity)**

MOS +SB Oil  
increases  
hindgut MC size  
(normal gut folds..)

# Mucous responses in Seabass guts and skin

diet → gut bacteria → mucosal tissue response

foregut

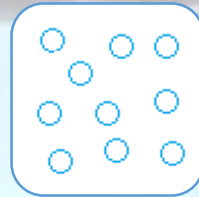
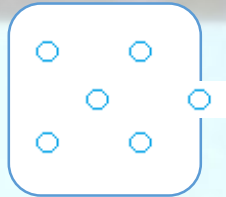
hindgut

hindgut

Dietary evaluation

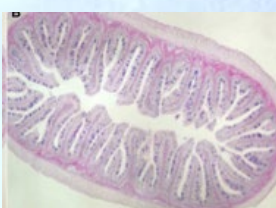
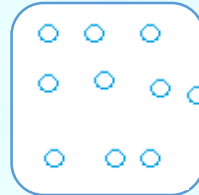
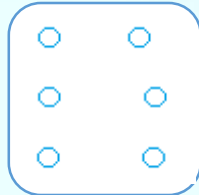
Fish oil

Seabass gut  
Mucous cell  
size and density  
(to scale)



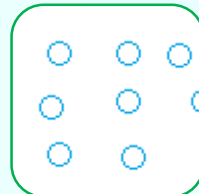
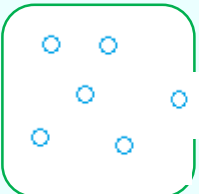
Good epithelial turnover  
combined with differentiation of cells

Fish oil + MOS



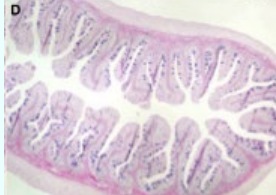
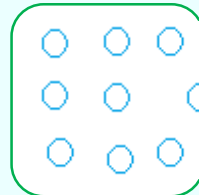
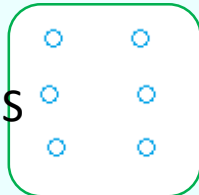
Stronger immune reactivity  
slightly increased ability for  
immune substances to diffuse

Soybean oil



Downregulated IL-6, IL-10,  
and TGF $\beta$  (functioning of  
mucous barrier and  
immune homeostasis)

Soybean Oil + MOS



Lowest cell migration & diffusion rates,  
epithelial hyperplasia,  
more cell differentiation and turnover,  
and more anti-inflammatories

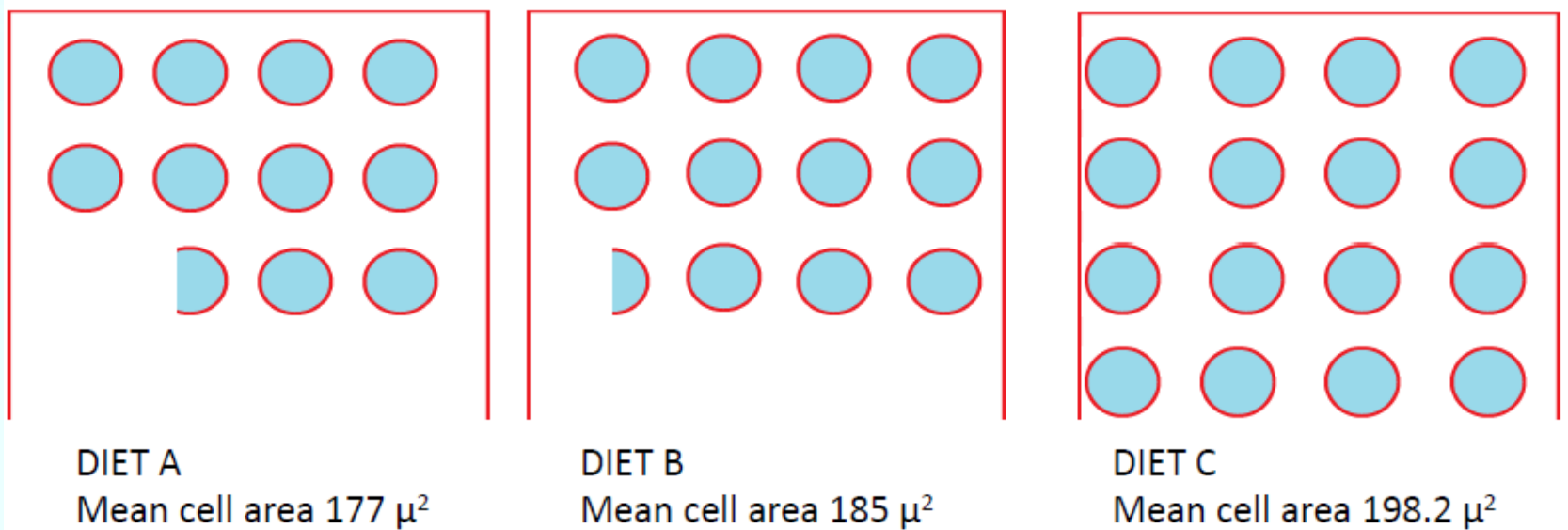
From: Torrecillas et al., 2015

# **The skin is the shield**



«Dice» reconstruction of size and density of mucous cells  
in dorsal skin from 3 diets

- how it «looks» on average in the skin of these Norwegian salmon



**Interpretation:** both size and density are important.

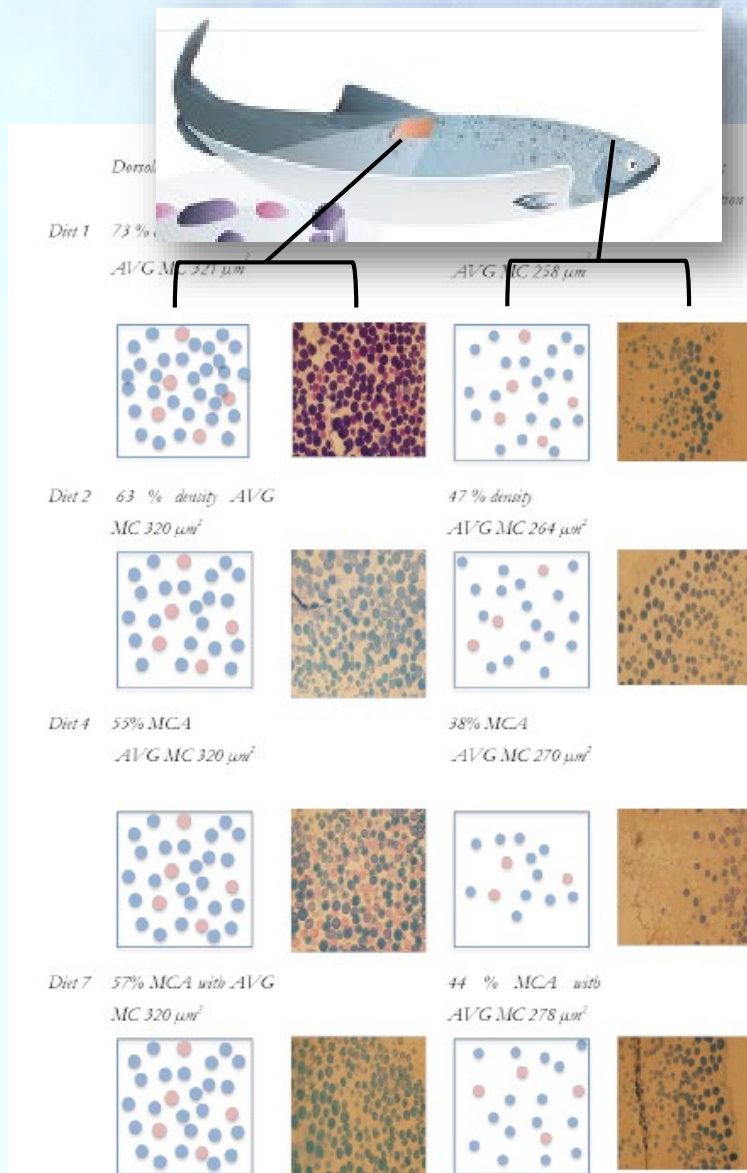
**Density** is an indication of how much mucous reaches the outside (in C 30% more)

**Size** is an indication of how quickly the cells can fill and move to the outside

(these cells are in the normal size range for ca 2 kg fish)

-in C the cells are insignificantly bigger so they move and fill insignificantly slower

# Mucosal Mapping on Canadian salmon treated with 4 diets



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 ( $p = 0.056$ )

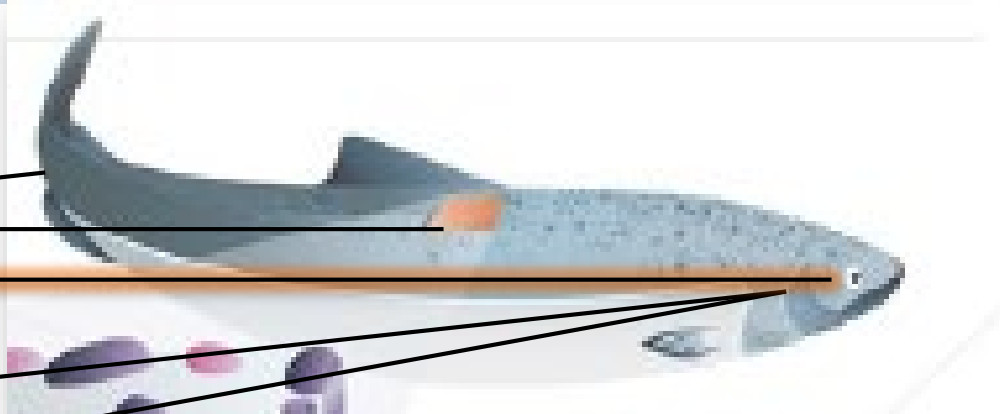
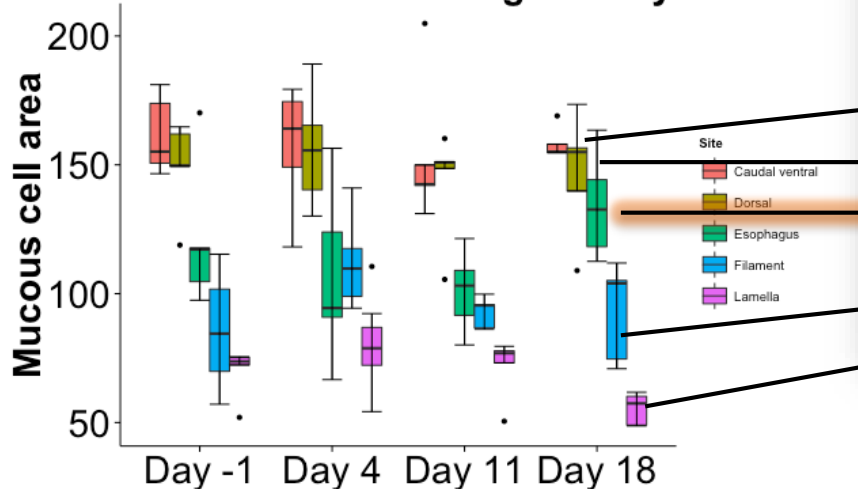
Diet 4

Diet 7

N=24 on Day 81

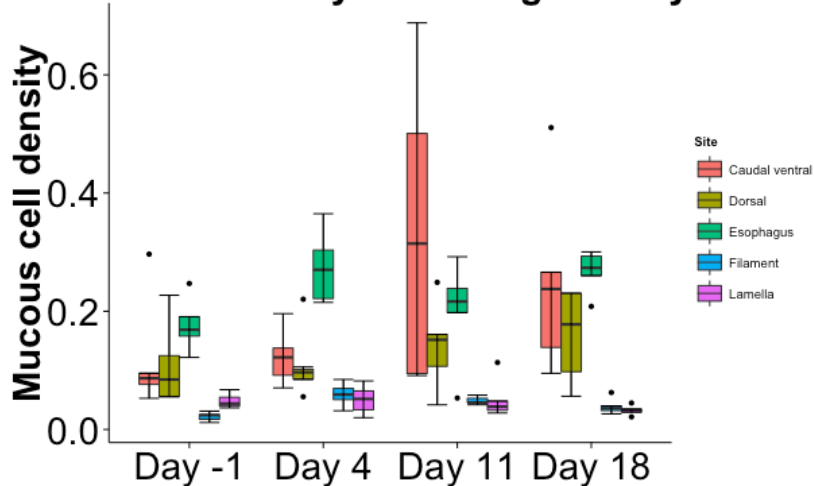
# Delousing with hydrogen peroxide

**Mucous cell area according to body sites**



Gills are still recovering more than 2 weeks after treatment  
-Filament mucosa is different than lamellar mucosa

**Mucous cell density according to body sites**



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

N=22 fish (88 samples)

From: Rantty I, 2015, Masters thesis UiB



# Skin mucus density and barrier status in salmon production

**Origin differences in FW**

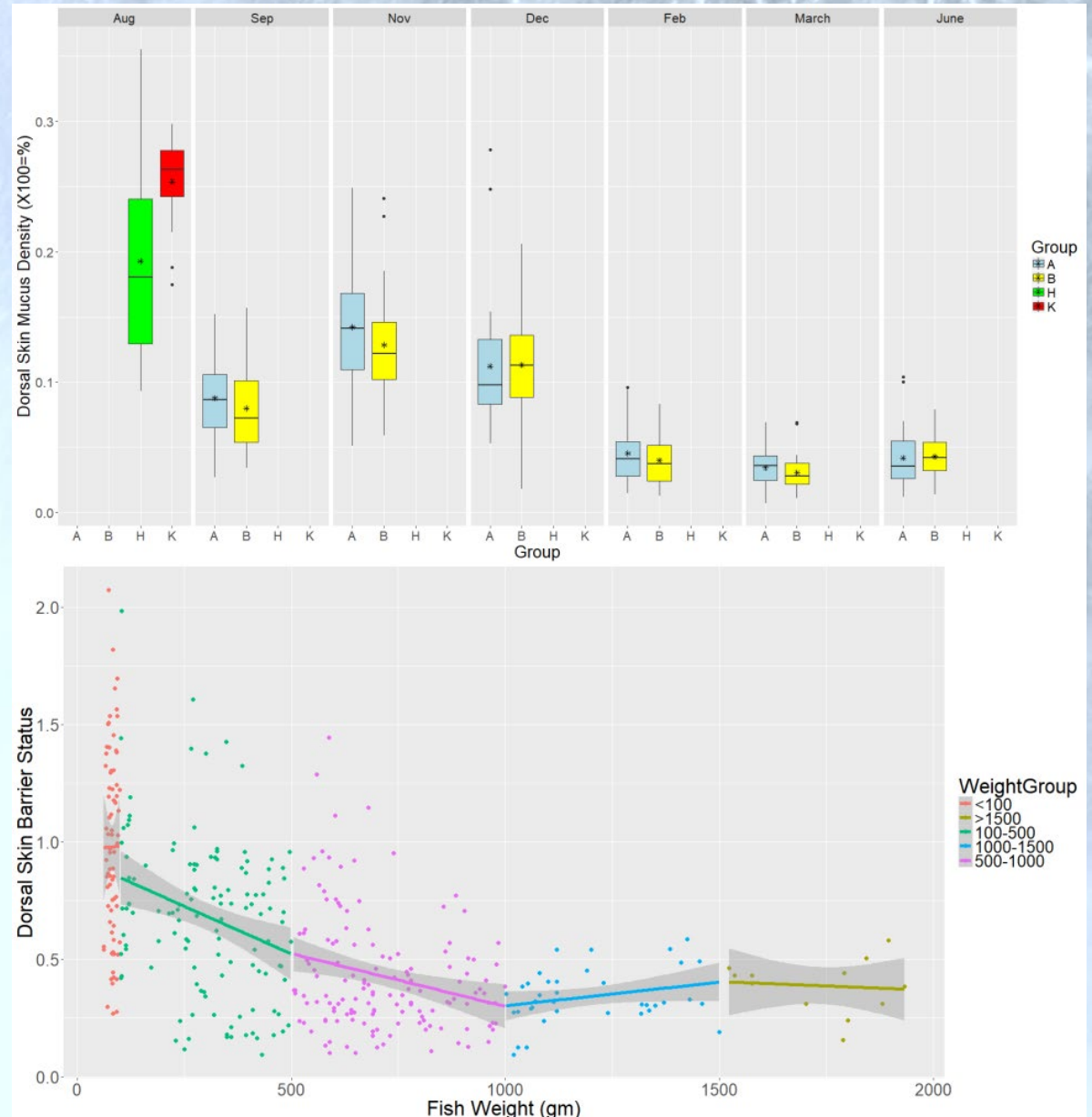
**Effect wiped out in SW**  
**Skin dries out/ tightens**

**Delousing in Feb onward**  
**reduces remaining**  
**mucous**

**SW values <5% but**  
**normal can be upto 30%**

**Slight reduction of barrier**  
**status with increasing**  
**lice load**

**Skin shield was**  
**weakened over time**  
**by treatments**



# **The gills are the sentinel organ**

- ~50% of surface area of the fish
- 2 populations of mucous cells
  - i) lamellar – small, low density when healthy
  - ii) filament – larger, denser, reflect systemic health
- early warning signal

# Gills lamellar measurements

## «Generation study»

### Freshwater

**Small cells & low density**  
**Same after transfer to SW**  
**Clear origin differences**  
**Advantage Group H**

### Nov / Dec 16

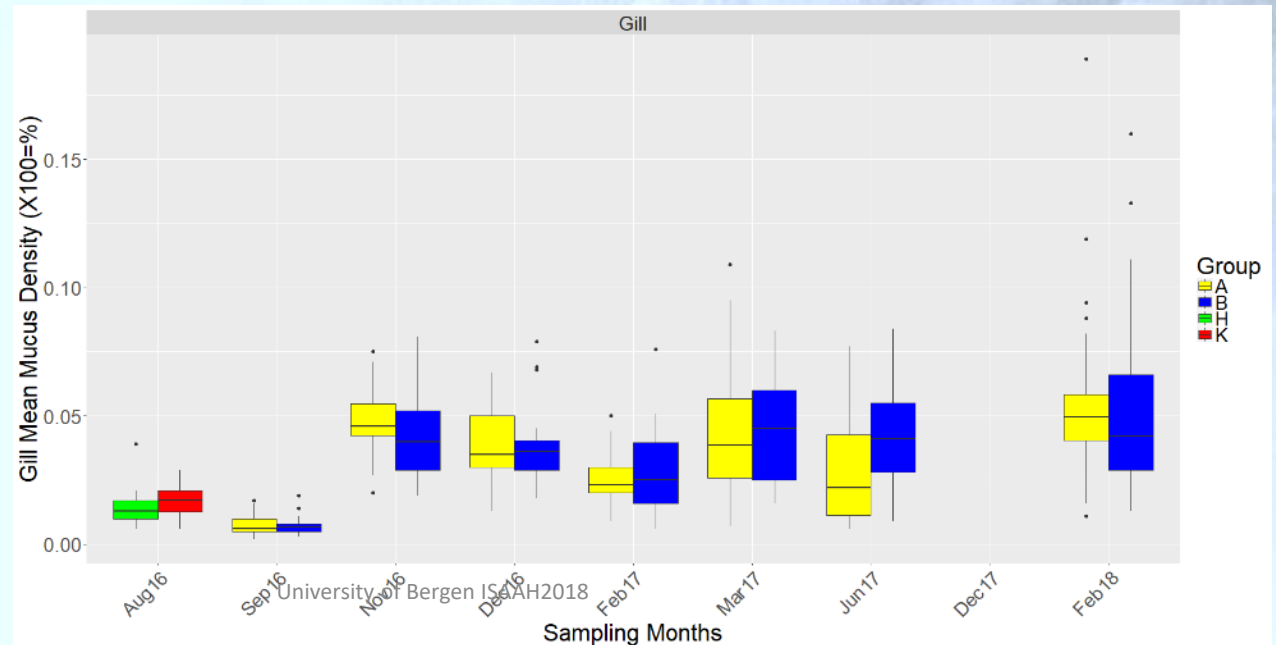
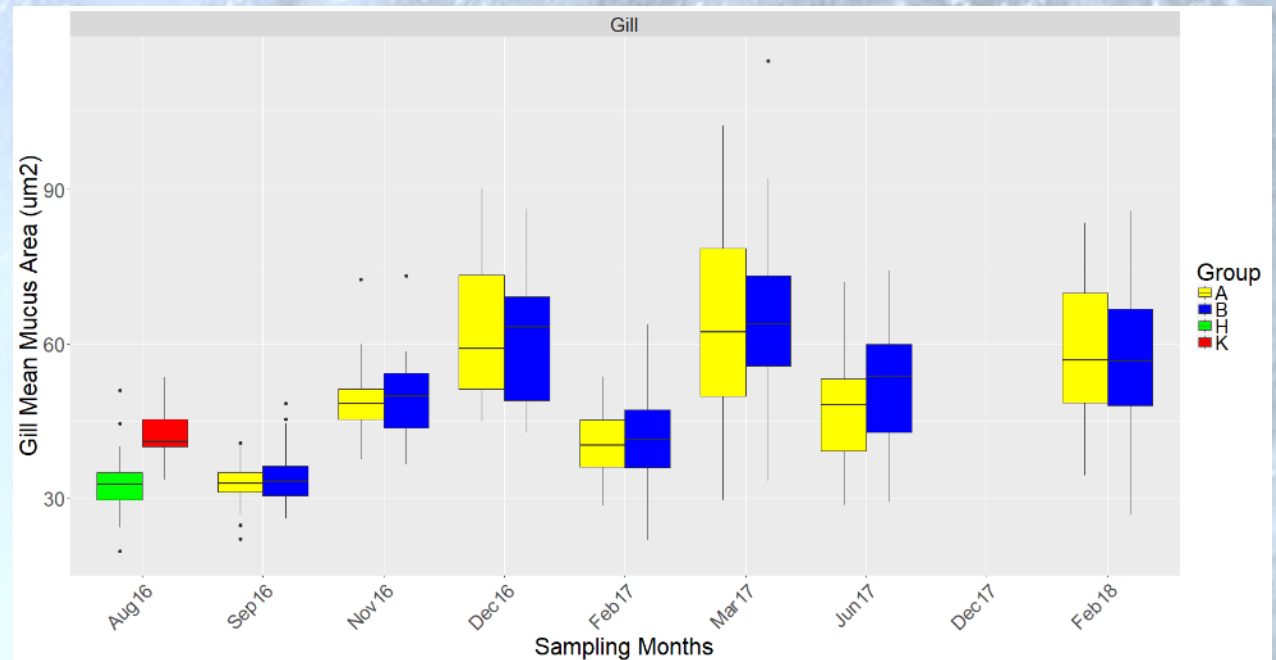
**Sizes & density increases**  
**Net change impacts all**

### Feb / Mar 17

**H2O2 impacts all**

**Gills always responding**  
**(significant differences**  
**WITHIN Gill Score 0-1)**

*n=30-45 under each box*  
*2 diets in triplicate*





# Gill lamellae = respiration and excretion

- barrier status is predictor of scope for growth?

**Smolt origin differences  
Sept 2016-Feb 2017**

**High barrier status is  
generally correlated with  
high weight**

**Effect increases with time**

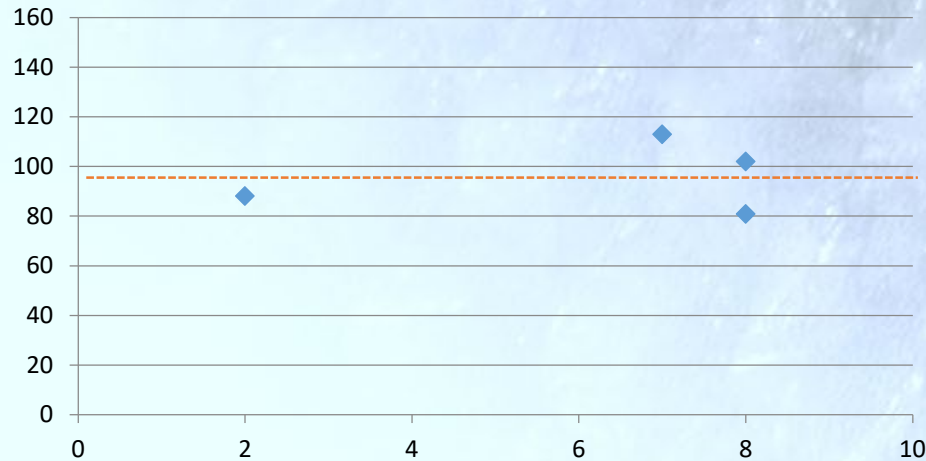


*From project CAC2016G-Integrert lusehåndtering – test av ulike kombinasjoner forebyggende tiltak mot lakselus*  
*Partners: Marine Harvest, Skretting, Inst of Marine Research, FHF, Quantidoc*  
*University of Bergen ISAAC2018*

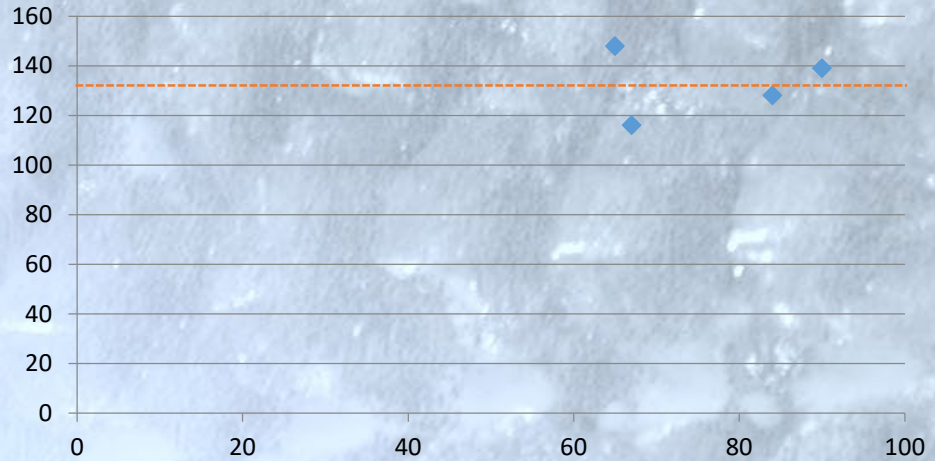
# Few mucous cells on healthy lamellae, filament has more

## Non-lethal biopsy gives reproducible results

Lamellar MC area vs cell nr



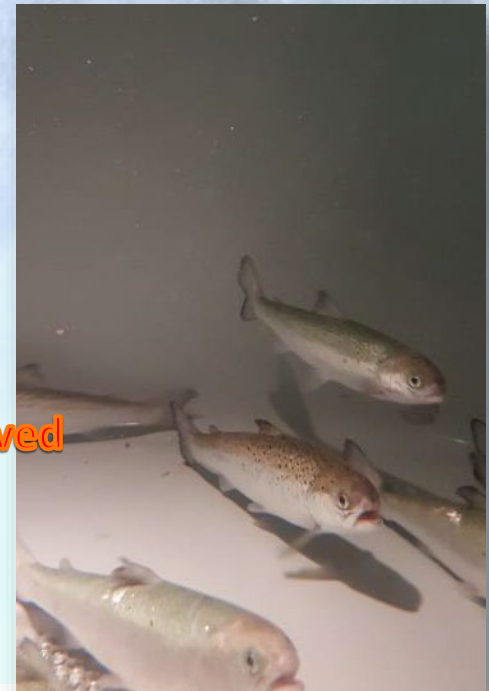
filament MC area vs nr cells



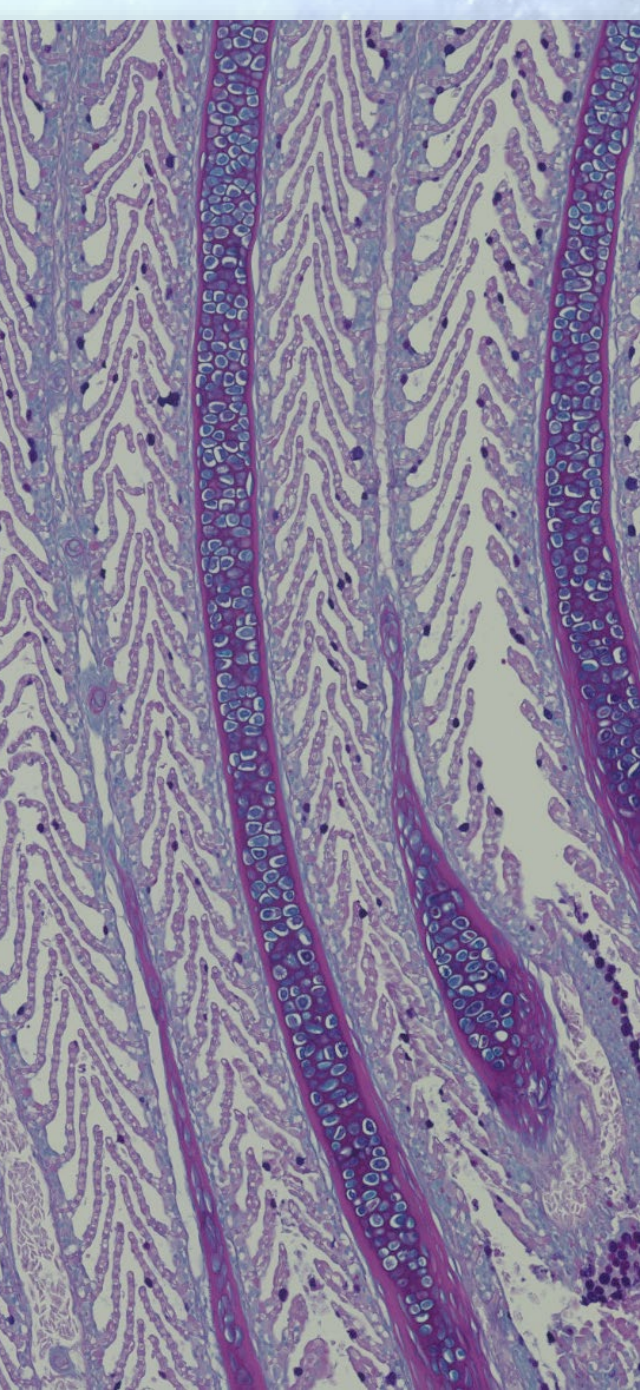
Mark clipping  
from 2nd gill arch



All fish survived







## Beta-testing the model: Gills as predictor

Data integrated into standard growth models and anonymized rawdata into an economic model:  
(R. Tveterås, 2018), verifiable with empirical field data

Simplified:

Growth rate =  $g(w, ^\circ C, GBS)$

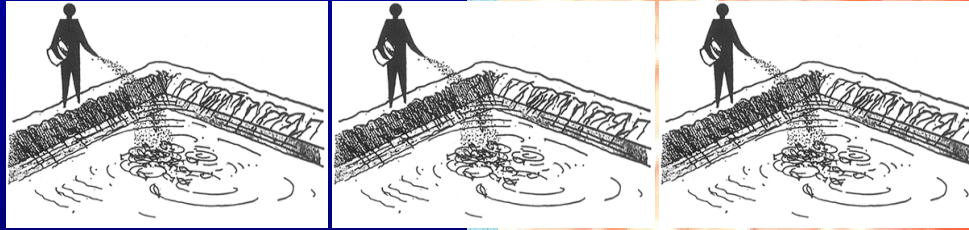
$g$  is initial weight

$^\circ C$  is temperature

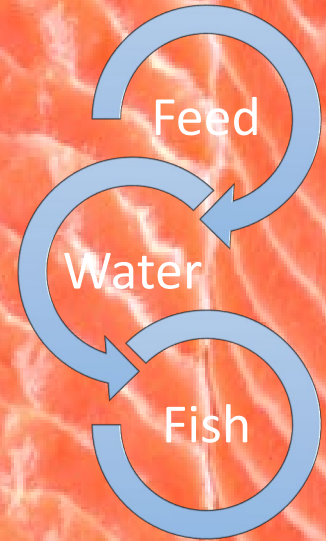
GBS is Gill Barrier status

This may improve production planning to optimize site utilization and profitability.





High Quality Feed	High Quality Feed	High Quality Feed
Bad water quality	Good water quality	Good water quality
Weak gills	Good gills	Gill disease
Impaired growth	Strong growth	Impaired growth
<b>Conclusion:</b> «The feed is bad»	<b>Conclusion:</b> « <b>Very good feed</b> »	<b>Conclusion:</b> «The feed is bad»



# Acknowledgements

- Research Council of Norway (NFR)
- Regional Research Council of Western Norway (RFF)
- Innovation Norway
- Bergen Technology Transfer
- Fisheries and Aquaculture Industry Research Fund (FHF)
- Nofima
- Alltech
- SalmonGroup
- Salmobreed
- Marine Harvest
- Skretting
- Aller Aqua
- Biomar
- Cooke Aquaculture
- DTU Aqua
- Steinsvik AS
- Universidad de Las Palmas de Gran Canaria
- Dalhousie University
- University of Tasmania
- NCE Seafood

And many many students from 1993-2018

# Mucosal Mapping to Veribarr™ Quick Timeline

- Developed 2010 at Univ of Bergen to be industrially applicable
  - First publications 2011, 2013
  - 2013 Inventor Prize; 2015 GAA Aquaculture Leadership and Innovation Prize
  - Quantidoc - **quant**ification and **doc**umentation, startup 2014 – now 4 employees
  - Quantitative histology - Stereology
  - Developed custom software – applying machine learning
  - Complementary to all other existing methods
- 
- **Veribarr™ – Verification of Barriers**
  - - Machine analysis of Goblet cells (as primary mucus producers):
  - - Average Cell Area +/- SD in  $\mu^2$
  - - Relative Density (volumetric % of Goblet cells in tissues)
  - - Barrier status calculated as  $1/(A:D)$
- 
- **Proof of concept**
  - Database with >9000 analyses tied to relevant metadata
  - Values tied to temperature, size, season, feed and sex etc. etc.
  - Salmon, salmontrout, yellowtail, lumpfish, seabass, sculpins
  - 6 countries and counting...