Fish Wear Their Immune System on the Outside – What This Means for Aquaculture and Ecology

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Abstract: After 40 years of intensive aquaculture, we know the fish as an *animal* better than we ever did from millenia of fishing. The fundamental differences between aquaculture animals and agriculture animals include the time spent in a controlled stable environment for juvenile organogenesis (upto 70% of total lifespan for terrestrials vs 0-5% in aquatics), the number of evolutionary whole genome duplication events contributing to the current genome (2R for terrestrials and 3R or 4R for aquatics) and the body location of the mucosal immune barrier (inside for terrestrials and both inside and outside for aquatics). The external slimy layers (mucosa of skin, gills, and intestines which are the outside of the inside) make up this mucosal immune barrier. They contain antiviral, antifungal, antiparasitic and antibacterial substances in constant dialogue with the environment for over half a billion years. The mucous cells exhibit an organ-wide repeatable response to stimuli such as therapeutics, stress, diet and environment. The application of an unbiased standard method, mucosal mapping or Veribarr™, over 12 years and over 100 trials in ecology and aquaculture has contributed to unprecedented understanding of how teleost mucosa function. Gills, which comprise about 50% of the surface area of fish, have proven to be the most sensitive early warning of systemic dysregulation. Some results from commercial-scale productions, from "detective work" and from controlled lab studies will be highlighted in the talk along with a plea for development of standards for fish health. The scope for growth in aquaculture as an industry resides in establishing and *maintaining* good stock health.

Keywords: Digital fish health, mucosal immunology, One Health, Mucosal Mapping

Abstrak: Selepas 40 tahun akuakultur secara intensif, kita lebih mengenali ikan sebagai haiwan daripada yang pernah kita lakukan selama beribu tahun dalam perikanan. Perbezaan asas antara haiwan akuakultur dan haiwan pertanian termasuk masa yang dihabiskan dalam persekitaran terkawal yang stabil untuk organogenesis juvana (sehingga 70% daripada jumlah jangka hayat untuk terestrial dan 0-5% dalam akuatik), bilangan peristiwa evolusi penduaan genom keseluruhan yang menyumbang kepada genom semasa (2R untuk daratan dan 3R atau 4R untuk akuatik) dan lokasi mukosa penghalang imun (di dalam untuk daratan dan kedua-dua di dalam dan di luar untuk akuatik). Lapisan berlendir luaran (mukosa kulit, insang, dan usus yang berada di luar dan bahagian dalam) membentuk penghalang imun mukosa ini. Ia mengandungi bahan antiviral, antikulat, antiparasit dan antibakteria dalam dialog berterusan dengan alam sekitar selama lebih setengah bilion tahun. Sel-sel mukosa mempamerkan tindak balas berulang seluruh organ kepada rangsangan seperti terapeutik, tekanan, diet dan persekitaran. Penggunaan kaedah piawai yang tidak berat sebelah, pemetaan mukosa

atau Veribarr™, selama 12 tahun dan dengan lebih 100 percubaan dalam ekologi dan akuakultur telah menyumbang kepada pemahaman yang belum pernah berlaku sebelum ini tentang bagaimana mukosa teleost berfungsi. Insang, yang merangkumi kira-kira 50% daripada luas permukaan ikan, telah terbukti sebagai amaran awal yang paling sensitif terhadap disregulasi sistemik. Beberapa hasil daripada pengeluaran berskala komersial, daripada "kerja detektif" dan daripada kajian makmal terkawal akan diserlahkan dalam kertas ini bersama-sama dengan rayuan untuk pembangunan piawaian untuk kesihatan ikan. Skop untuk pertumbuhan dalam akuakultur sebagai industri terletak dalam mewujudkan dan mengekalkan kesihatan stok yang baik.

Introduction

It is tempting to manage aquaculture as though it is agriculture under water, but the aquatic animals we are rearing are very fundamentally different in 3 aspects: firstly, farmed terrestrial animals may spend upto 60% of their total lifespan from sperm-meets-egg to harvest in the protected stable environment of a maternal womb or large egg, relying on this to develop into a functional small or juvenile animal. By contrast farmed aquatic animals exert little or no maternal stability to make a juvenile and instead may broadcast or lay fertilized eggs externally and let the new organism develop in response to the variable environmental signals such as temperature, light, nutrition and physical enclosure. Secondly, the broad genetic vocabulary needed for aquatic juvenile development in a variable environment exists because of a teleost-specific whole genome duplication about 350 million years ago, or about 200 million years before men got a Y-chromosome, and contrasts to the more limited 2R genome duplication of terrestrial animals (Sato and Nishida 2010). This magnanimity regarding gene function in aquatics means that mammalian correlates for syntenic genes or para- and orthologs of teleosts may be nonexistent or misidentified. Thirdly, the external slimy surfaces of the skin and gills reflect and respond to the higher microbial diversity of eg. inland water vs air (Walters and Martiny 2020) and are the fish's immune system on the outside composed of *living* cells. It has already been shown that wiping off the mucus from the skin leads to higher mortalities than does physically wounding the skin of fish (Svendsen and Bogwald 1997). The external mucosal barriers of skin and gills have thus been protecting aquatic animals for over 500 million years (Xu, Takizawa et al., 2016). These mucosa plus that of the intestines are the interactive user-interface between the organism and the aquatic environment (Minich, Poore et al., 2020, Salinas, Fernandez-Montero et al., 2021) (Fig. 1).

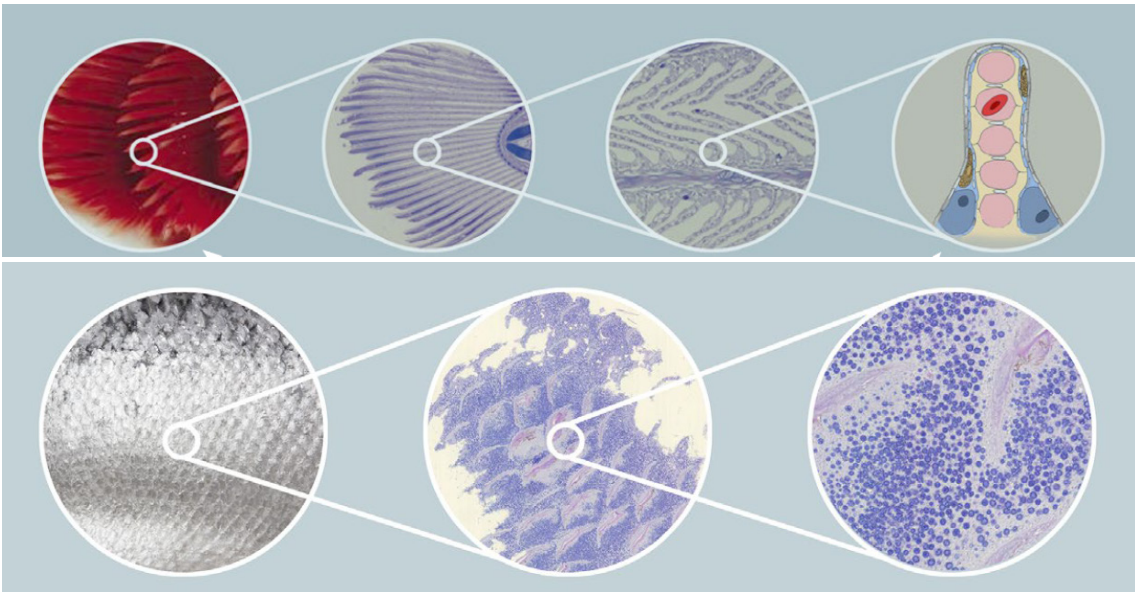

Figure 1: Top: Location of protective mucous cells in the gill lamellae (respiration). Left: macroscopic image of healthy gills; Midleft: histological preparation of gill tissue with Periodic Acid Schiff – Alcian Blue making mucous cells blue dots; Midright: closeup of prepared gill lamellae with a few blue mucous cells on narrow lamellae; Right: illustration of placement of mucous cells (brown) in the double layered membrane between water and blood cell (red). Illustration by K. Moe.

Bottom: Location of protective mucous cells on the skin, over the scales. Left: macroscopic image of healthy skin; Middle: histological preparation of skin tissue with Periodic Acid Schiff – Alcian Blue making mucous cells blue dots and scales pink crescents; Right: closeup of multiple blue mucous cells and 3 pink crescents of scales. All images from "The Robust Fish" (2019) with permission.

A mucosal membrane depends by definition on the presence of mucous cells which form an integral part of the dialogue between fish and environment and show distinct differences according to body site (Pittman, Pittman et al. 2013). The dynamic changes in the size and amount of mucous cells (hyperplasia and hypertrophy) are often cited as key characteristics of treatments and responses to pathogens, and Gjessing *et al*. (2019) state that gill disease complexes are characterized by 3 frequent findings: subepithelial leukocytes, epithelial cell hyperplasia and mucus cell hyperplasia. Foyle *et al*. (2020) highlight the essential role of healthy gills, noting that indicators of gill disease include hyperplasia and hypertrophy of epithelial and mucus cells.

Since 2011, the dynamic morphometrics of the key characteristic of any mucosal epithelia, its mucous or goblet cells (MCs), have been made measurable and statistically reliable through the application of mucosal mapping (Pittman, Sourd et al., 2011, Pittman, Pittman et al., 2013). Key work has looked at the microbia populating various mucous layers on the fish body and the immunoglobulins produced (Salinas, Zhang et al. 2011, Minich, Härer et al. 2022) current thinking suggests that mucosal epithelia produce a wide variety of substances and secretory immunoglobulins "on demand" as a function of the dynamic mucosa (Dang, Pittman et al., 2020, Salinas, Fernandez-Montero et al., 2021). Therefore, rather than focusing on the particular substances released at a chemical or organelle level, we have focused on the level of biological organization above this, the cellular and tissue responses of external mucosal barriers.

The dynamic function of the mucosa is both physical and biochemical and is exquisitely sensitive to changes in the environment. For the sensitive gills, this has been demonstrated both in the wild and in controlled or farmed situations: in thirty wild sculpins sampled at 3 stations along a pollution gradient of heavy metals from an abandoned mine in a Greenland fjord, Dang et al. (2019) found that gill lamellar mucous cells were in significantly higher density when there was a high environmental lead (Pb) load, that the size of the mucous cells in the gill filament were positively correlated with the lead level in the liver and that smaller skin mucous cells were associated with higher parasite loads. The study demonstrated that environmental characteristics induced significantly different morphodynamics of three mucosal tissues (skin, gill lamellae and gill filament) in the same species, agreeing with the concept that body site matters to the mucosal response parameters (Pittman, Pittman et al., 2013). The study also demonstrated nuanced protective abilities of the mucosal epithelia and potentially diagnostic properties.

In a series of controlled trials, salmon were exposed to various doses of peracetic acid (PAA) and repeat exposure after 2-3 weeks (Lazado, Haddeland et al., 2020, Haddeland, Lazado et al., 2021, Lazado, Timmerhaus et al., 2021, Lazado, Strand et al., 2022). Salmon not only showed that lamellar mucous cells were always significantly smaller (less than 70 square microns) and less than 2% of the volume of the epithelium while those in the filament were larger and upto 4 times more abundant, confirming the distinct nature of these two populations. The gill cells also showed an initial transient subacute hypertrophy which was linked to a small, generalized stress response. In the skin, mucous cell sizes marginally increased with increasing dose of PAA during the first exposure whereas the second exposure gave no significant changes with dose (Lazado Haddeland et al., 2020), suggesting not only adaptation or learning by this living layer but also showing the strength of the skin barrier compared to the sensitivity of the gill barrier. Rantty found that salmon gills, esophagus, and skin can take about 2 weeks to recover from one treatment of H_2O_2 and postulated that low post-treatment feeding was also due to irritated esophagus (Rantty 2016).

An application of this therapy to controlled infection with Amoebic Gill Disease made clear that the properties of mucous cell sizes and volumetric densities are a sensitive indicator of health, recovery or vulnerability (Lazado Strand et al., 2022). Four treatments were applied: one Control group was uninfected and untreated while three groups were infected with AGD and one was given no treatment, one treated with 5 ppm PAA for 30 minutes and one with 10 ppm PAA for 15 minutes. Samples collected after 24 hrs revealed that the infected groups had begun their cellular hyperplasia relative to the Control group and oxidative stress was measured in the initial phase. By 4 weeks the treatment differences in cell sizes and abundance were significant – untreated AGD induced the largest cells at the highest density and surprisingly the 10 ppm dose gave significantly larger denser mucous cells than the Control group whereas 5 ppm was closer to the Control group measures, suggesting that twice the dose of PAA was half as effective against AGD. The Control group itself maintained its gill mucosal values near those of healthy wild salmon. The authors found that longterm infections corresponded with dysregulation of systemic reactive oxygen species (ROS), a concomitant elevated antioxidant production and altering inosine and guanosine (Lazado, Timmerhaus et al. 2021) as well as inducing lower cortisol responses. The affected tissues responded differently, with the liver and gill being more sensitive than the skin, and the gills displaying mucous cell hypertrophy after the second and third exposure. Metabolomics showed that in the gills, genes for immunity and for ribosomal functions were significantly affected by oxidants, whereas the liver was the site of genes involved in targets of oxidation-reduction. In the skin, whose mucosal morphodynamics remained relatively unchanged, changes were dependent on the duration of exposure and some ribosomal functions were impacted (Lazado Timmerhaus et al., 2021).

The External Immune system of Fishes and One Health

The gill mucosal barrier is exquisitely sensitive and responsive to its environment. Asking "What is Gill Health?", Foyle and colleagues argue that homeostasis or the ability to maintain physiological function in the face of stressors is key, despite no clear evidence on how far the limits can be stretched before health is compromised and little consensus on its objective measure (Foyle, Hess et al., 2020). However, as this is an external part of the immune system in constant contact with water, particles and pathogens, all the fish in a site are experiencing the same environment and will display much the same response in this evolutionarily conserved barrier. It is impossible to vaccinate against a poor environment. If the fish are healthy it should imply the site is healthy.

But health is more than just the absence of a pathogen or disease, as shown by the ecotoxicological responses of sculpins (Dang, Pittman et al. 2019). Indeed the presence of certain parasites, viruses or bacteria may be unimportant to overall health or may fail to cause the expected disease (Nylund, Roed et al. 2021). Thus we come to the interaction between farming systems and fish, where the technological demands of increasingly industrialized systems seem to exceed the biological adaptability of the fish, leading to "lifestyle" disease and mortalities (Gjessing, Steinum et al., 2019, Sommerset, Walde et al., 2021). The basic levels of stress, noise, background chemistry, feed composition, stocking density, frequency of handling and treatment method all induce changes which impact on mucosal and systemic health, leading to an enormous variety of up- and down regulated genes. However it is necessary to understand the measure of baseline health before selecting the response variables and comparing across incomparable trials: Wiik-Nielsen and colleagues found that the seawater intake for a land-based facility could provide a venue for bacterial infections of the gill which would transmit horizontally within the system which could be exacerbated by elevated temperatures, inadequate hygiene and reduce available oxygen levels (Wiik-Nielsen, Gjessing et al., 2017). Low level algal invasions, eutrophication, increased environmental temperatures also expand the wide number of inputs to which the gills and the skin must react and illustrate the multidimensional complexity of health and homeostasis.

Defining gill or skin health in a way that can be reproduced on other individuals or conditions is crucial to understanding the wide variety of responses found by an even wider variety of authors. The inherent plasticity of developing fish also means that there is as yet no standard "best" size because the organogenesis is determined by the environmental inputs combined with the flexible genetic cascades (Pittman, Yufera et al., 2013). Since the presence or absence of secreted mucus and mucous cell hypertrophy characterize many identified clinical diseases and some histocomplex disorders, the focus on this evolutionarily conserved feature offers some clarity. In general healthy gill lamellae are the site for gas exchange and oxygenation of the blood and the mucous cells are contained within a double membrane between the water and the blood cells. Healthy gill lamellae have little need for mucous cells to offset toxins or pathogens which would occlude respiratory surfaces. As such the larger and denser the lamellar mucous cells are in afflicted gills, the thicker the lamellae and the further the distance for gasses to diffuse (Haddeland, Lazado et al., 2021). That the scope of growth can be impacted by the bacterial interaction in lamellae is evidenced by the negative association between gill microbiome diversity measures and mass of the fish (Minich, Härer et al., 2022), such that faster swimming fishes have lower gill microbiome diversity (Minich, Härer et al. 2022) and healthy fast swimming tuna have few mucus cells on the lamella (Merkin et al, *in prep*).

The dynamic range of mucous cell sizes in fish species indicates vulnerability to many challenges as cell sizes increase and biotensegrity decreases (Ingber 2006, Matthews, Overby et al.

2006). Across 12 fish species the largest densest mucous cells are in the skin, followed by the gill filament and the smallest population resides in the gill lamellae (Merkin et al. *in prep*.). Each of these mucous cell barriers displays a repeatable quantifiable response to challenges and to restitution, circling around a putative homeostasis point for physiological functions. This can then be used to establish a standardized tool of gill health or skin health on which to posit molecular or other results across systems and across species to build a more uniform approach to reporting on fish health.

Practical applications

The fish is always responding to its environment and when we industrialize that environment the demands for response from the fish may exceed the biological scope for homeostasis. New locations, new technologies and even new species or dietary ingredients may pose unexpected challenges to fish production. By measuring the barrier cells rather than the molecules, we see the summary of the effects of many hundreds of genes, both known and unknown, while still being infinitely more sensitive than simple growth and mortality. This has practical application for those seeking to improve their production.

Our data indicates that the skin can be relatively insensitive to things which can impact the gills greatly. Therefore, the skin is the shield and gills are the sentinel guard giving early warning of change. The gills are the "lungs" of the fish, where function can be compromised long before it is macroscopically visible. The skin itself can nonetheless be victim of many "lifestyle diseases" such as becoming dry and actually shield-like (few mucous cells but many epithelial cells) or thin and easily disrupted and much depends on the experienced life history of the fish. Since the mucosa lie above and around the scales, when a fish loses scales it also loses the mucosal barrier. This can however grow back if given sufficient pause and continue to protect the fish (Sveen, Karlsen et al. 2020) but repeated insults (or "scale loss") lead to the documented vulnerability of fish with removed mucus (Svendsen and Bogwald 1997). Therefore, we can begin to consider the skin shield as the two-way interactive user-interface of the fish. This shield responds to surface contacts which confer a higher diversity of microbial population than in the gills, secreting immunoglubulins according to need and reflecting the physical ambience (Minich, Härer et al. 2022). The gills respond to the water and its particles, pathogens and quality, as well as to stress of many kinds. The healthy gill sentinel guard has few and small mucus cells in the lamellae whereas the healthy skin shield has sufficient mucous production to fulfil its normal physiological needs. (The intestines, the foundation of health, are another story for a longer paper.) In general, we have a "Fish Detective" always responding to the farm and its routines, helping identify the sources of impact on the external immune system (Fig. 2).

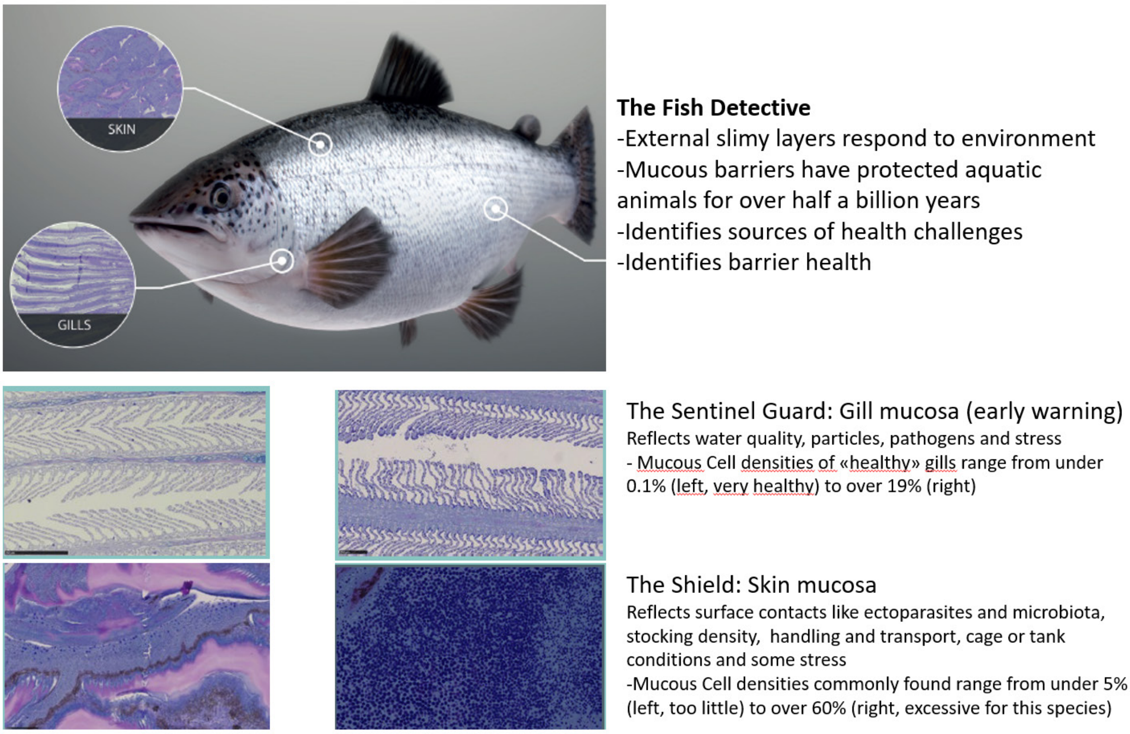

Figure: 2. The Fish Detective: external mucosal barriers respond quantitatively to the environment and can help identify healthy routines on-farm, good sites, and areas for improvement. Images from "The Robust Fish" (2019) available from QuantiDoc with permission.

Conclusion

To accompany the growing need to document One Health across species and systems it is imperative to understand the mechanisms by which mucous cell production influences and responds to immune substances and microbial signals for the maintenance of healthy mucosal barriers (Salinas, Fernandez-Montero et al., 2021). The health of the oceans is the health of our fish and ultimately of us. The interactive mucosal barrier between organism and environment has been slimy for half a billion years. It is time we literally took its measure.

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