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Short Communication

Novel method for quantifying salmonid mucous cells

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The primary defence mechanism of fish exposed to an infectious or damaging agent is the physical barrier of skin, gills and gastrointestinal tract and their protection mechanisms associated with the innate immune defence system, the mucosal epithelium. Mucus production helps to protect these physical barriers by acting as a diffusion barrier and lubricant whose function is to protect the epithelial cells from infection, dehydration and physical or chemical injury. Mucus also contains several bioactive components such as immunoglobulin, complement C-reactive protein, lectins, lysozyme, proteolytic enzymes, alkaline phosphatase and esterase, antimicrobial peptides and haemolysin, which exhibit biostatic or biocidal activities (Alverez-Pellitero 2008). Recent work has shown that mucus secretion, in the gut and on the skin of fish, may be augmented by nutritional components and this can affect bioactive components and barrier defence mechanisms associated with *Vibrio* infections in sea bass and sea lice infections in juvenile salmon (Sweetman, Torrecilla, Dimitroglou, Rider, Davies & Izquierdo 2010; Torrecillas, Makol, Benitez-Santana, Caballero, Montero, Sweetman & Izquierdo 2011).

The determination, therefore, of a quantitative method to assess the distribution, abundance and volume of goblet cells in various strategic locations on the Atlantic salmon, *Salmo salar* L., would provide a new tool for the further evaluation of mucosal interactions and fish health.

This paper describes a novel stereology-based method for quantification of the skin components and mucous cells of salmonids.

Fish skin is highly metabolically active and acts as a multipurpose primary defence mechanism against aquatic pathogens and parasites (Rakers, Gebert, Uppalapati, Meyer, Maderson, Sell, Kruse & Paus 2010). Fish skin contains the outer epidermis and inner dermis, transdermal scales and mucous cells. The epidermis can be further divided into the *stratum superficiale* where the squamous epithelial cells mix with a population of mucous cells; the *stratum spinosum* with some differentiated cells; and the *stratum basale* with basal cells and a basement membrane. Undifferentiated cells migrate from the *stratum basale* to the *stratum spinosum* and then recruit when necessary to the *stratum superficiale*. The epidermis is separated from the dermis by the basement membrane with filamentous proteins. The dermis has a *stratum laxum* and a *stratum*

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compactum of loose connective tissue, fibroblasts and chromatophores. Scale pockets contain scleroblasts for building scales of collagenous tissue with superficial mineralization, and the scales are anchored in place by bundles of collagen fibres.

Mucus is produced in the *stratum spinosum* by cells, which resemble mammalian goblet cells. Mucous cells are clearly visible in the *stratum spinosum* because most other epithelial cells in this layer are undifferentiated [by contrast, coho salmon, *Oncorhynchus kisutch* (Walbaum), are reported to have immature mucous cells in the basal layer (Hawkes 1974)]. From here, the mucous cells are continuously produced in the cycle to release their contents to the surface, exhibiting a large capacity for re-composition and reaction to external influences (Easy & Ross 2010; Rakers *et al.* 2010). When approaching the epidermal periphery, mucous cells of salmonids develop distinct vesicles, which enlarge the cell and maintain their integrity until release (Harris & Hunt 1975a). Fish mucus can contain lysozymes, immunoglobulins, lectins, crinotoxins and antibacterial peptides (Shephard 1994). In this way, the high content of mucus proteins and the high rate of turnover in fish skin provide a platform for potential modification of mucosal content and number.

Measuring mucous cell number, size or density and quantitative content is fraught by the differences in size and density of cells across the body. Previous studies have indicated that mucous cell density is lowest on the caudal fin and highest on the dorsal area or dorsal fin (Pickering 1974; Buchmann & Bresciani 1998). Only four studies have investigated salmonid mucus production in sea water (Pickering 1974; Fast, Ross, Mustafa, Sims, Johnson, Conboy, Speare, Johnson & Burka 2002a; Fast, Sims, Burka, Mustafa & Ross 2002b; Easy & Ross 2010), while seven have looked at salmonid skin in fresh water (Roberts, Shearer, Elson & Munro 1970; Hawkes 1974; Harris & Hunt 1975a; Pickering & Macey 1977; Pottinger, Pickering & Blackstock 1984; Buchmann & Bresciani 1998; O'Byrne-Ring, Dowling, Cotters, Whelan & MacEvilly 2003) (Table 1). Mucous cell numbers have been reported to decrease by 50% at smoltification in Atlantic salmon (O'Byrne-Ring *et al.* 2003), underscoring the importance of life stage in analysis of skin dynamics. Authors have noted the non-uniform distribution of mucous cells even within a small region (Pickering 1974) and species-specific differences in distribution patterns, as well as individual variation.

The most common method of sampling skin for histology involves excising and embedding pieces and then slicing transverse sections to give a layered view of the skin, as described earlier. This does give a clear view of the structure of the selected small skin area but relatively few mucous cells for analysis in a single section, and measurements often do not consider the mucous cells in epidermal areas folded around and under the scales. The underlying assumptions may be that the largest diameter on the slice corresponds to the middle of an average mucous cell and that epidermal thickness is even. If primary interest is in the number and size of mucous cells per unit of epidermis, the actual orientation of the sections is less relevant.

We tested the quantitative effects of tangential vs. transverse slicing on measures of mucous cell size and ratio of mucosal to epidermal tissue and of two types of common stain for identification and enumeration of mucous cells.

In the first test, skin samples > 1–3 cm^2 were taken from an adult farmed salmon terminally anaesthetized with MS222. Sections were taken from the mid-dorsal epaxial body, the cranium, the ventral pelvis, the ventral caudal peduncle and the dorsal caudal peduncle and fixed in 4% phosphate-buffered formalin in flat-bottomed flasks. For transverse sections, small pieces were cut from the main samples and laid horizontal prior to embedding in standard paraffin blocks. For tangential sections, the sample was trimmed to fit the block laid vertically. The entire piece of fixed tissue was dehydrated (Reichert-Jung Histokinette 2000) and embedded in paraffin moulds (Kunz Instrument, WD-4). Slices of 4 μm thickness were taken with a microtome (Leica RM 2255) and stained in standard haematoxylin and erythrosin saffron stain (HES) for visualization.

In the second test, skin samples of *ca.* 2 cm^2 were taken from an anaesthetized adult farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), from the mid-dorsal epaxial body, the cranium, the ventral caudal peduncle and the dorsal caudal peduncle and fixed in the same manner as previously. These were stained with either HES or periodic acid/Schiff and alcian blue (Cook 1977) to mark the neutral and acid mucins.

Two to five non-sequential sections per location were analysed using a Leica Axioskop microscope

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Table 1 Overview of previous studies on salmonid skin and areas of sampling

Source	Species	Section location	Stain	Thickness	Units of report
Buchmann & Bresciani (1998)	*Oncorhynchus mykiss* (freshwater stage)	Epithelia of different fins, cornea and dorsal body surface	Alcian blue	–	Number per mm^2
Buchmann *et al.* (2004)	*Oncorhynchus mykiss* (freshwater stage)	Caudal fin	Alcian blue	–	Number per mm^2
Fast *et al.* (2002a,b)	*Oncorhynchus mykiss* *Salmo salar* *Oncorhynchus kisutch*	Mid-body	Hematoxylin & Eosin	5 µm	Number per 100 µm length Width of largest
Harris & Hunt (1975a,b)	*Salmo salar* *Salmo trutta*	Head, dorsal, ventral and lateral regions	Lead citrate Uranyl acetate Toluidine blue	500–600 Å 1–2 µ	Size (µm) Did not report density.
O'Byrne-Ring *et al.* (2003)	*Salmo salar*	Front shoulder flank directly behind the gill	Periodic acid/Schiff/alcian blue	5 µm	Number per mm^2
Roberts & Powell (2003)	*Salmo salar*	Second gill arch	Periodic acid/Schiff/alcian blue	5 µm	Number per inter-lamellar unit
Roberts *et al.* (1970)	*Salmo salar* *Salmo trutta*	Top of snout, top of head	Multiple stains (LM), Reynolds' citrate stain (EM)	8 µm 600 Å	Observations
Van der Marel *et al.* (2010)	*Cyprinus carpio*	Dorsolateral body	Periodic acid/Schiff/alcian blue	4 µm	Number of goblet cells per sample
Pickering (1974)	*Salmo trutta* Salvelinus alpinus	Fins, body, tail and head	Alcian blue	–	Number of mucous cells per mm^2
Pickering & Macey (1977)	*Salvelinus alpinus*	Shoulder region	Haematoxylin & eosin, periodic acid/Schiff/alcian blue	6 µm	Mean count per mm^2
Pottinger *et al.* (1984)	*Salmo trutta*	Shoulder region.	Haematoxylin/alcian blue	6 µm	Number of mucous cells per mm^2

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combined with NewCast software (Visiopharm AS) and a Prior Proscan digital stage. Epidermal area and mucous cell area were measured using stereological probes, and mucous cells were counted in systematic random sections.

Measurements based on tangential slicing resulted in larger areas of mucosal and epidermal tissue, whereas the average cell area and the ratio of epidermal to mucosal tissue were similar to those generated from transverse sections (Fig. 1). It is significant that although 'flat', the fixed sections were not planar and all layers of epidermal stratification could be exposed repeatedly in a single tangential slice, allowing counts not only of superficial mucous cells but also deeper cells. Furthermore, as slicing of the epidermal tissue may expose cells at varying levels of their structure, identification of mucous cells cut near their periphery was easier when PAS/alcian blue was applied. This stains specifically for mucopolysaccharides, whereas HES is a common but more general stain of cell nuclei and eosinophilic structures. Ease of specific cell identification may lead to less variation in the results (Fig. 2). Average cell size, mucous cell density in a measured epithelial area or mucosal cell volume relative to epithelial volume, all of which can be generated by our method, may be more useful for further research than largest cell size because of the high reactivity of mucous cells and their variable spatial distribution (Fast *et al.* 2002b).

Importantly, the amount of work necessary to generate a measurable tangential section was about one-third to one-quarter of that for transverse sections. Up to embedding of tissue, the preparation time is equal. Thereafter, time needed is determined by the reduction in number of necessary slices for tangential sections compared with transverse sections and consequently, the reduction in the handling time of each block and the number of slices that must be stained. If we calculate that each transverse section would be 4 μm thick whereas the tangential section could cover a square centimetre or more of epidermis, then 250 transverse sections are needed to cover the same epidermal area as one tangential section. Furthermore, the larger amount of relevant tissue available reduced time needed for handling and analysis of each sampled fish. Identification and enumeration of the mucous cells was easier using the PAS/alcian blue staining method than using the HES stain.

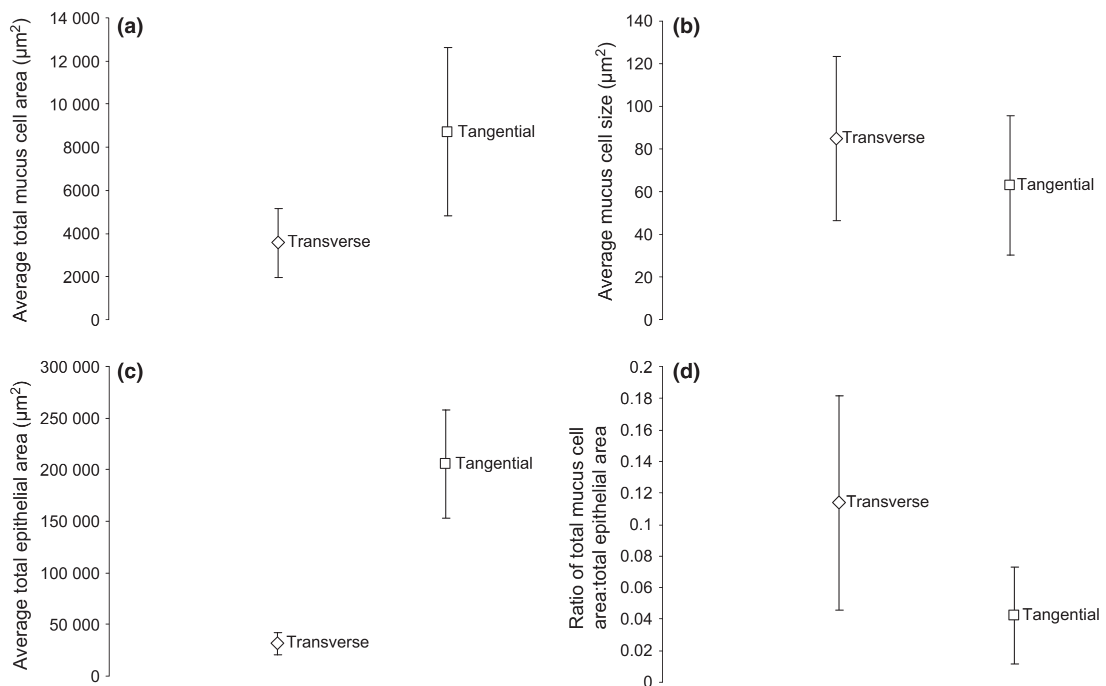

Figure 1 Comparison of (a) average total mucous cell area, (b) average mucous cell size, (c) average total epithelial area and (d) ratio of total mucous cell area to total epithelial area in non-sequential tangential vs. transverse sections of a single adult farmed salmon. All results from mid-dorsal epaxial skin. Vertical bars show SD for transverse or tangential sections.

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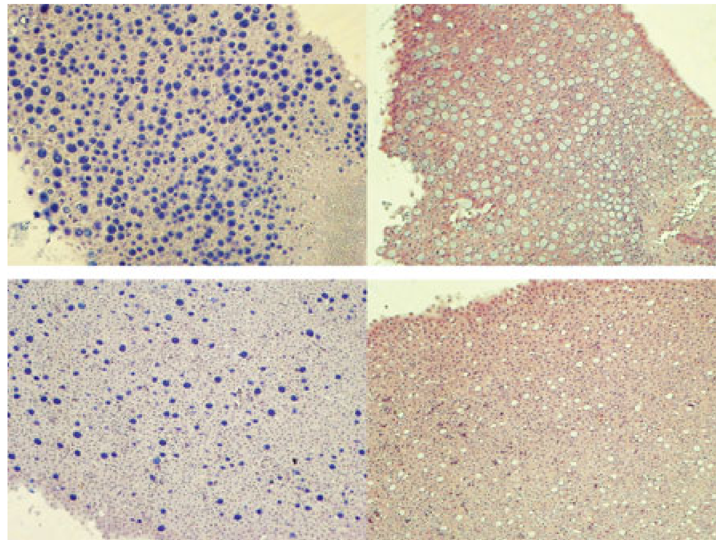

Figure 2 Comparison of mucosal tissue measures from mid-dorsal epaxial (upper row) and cranial (lower row) skin of a single adult farmed rainbow trout stained with hematoxylin–erythrosin or periodic acid/Schiff/alcian blue (mucous cells blue or pink). Two to five non-sequential slices gave mean mucous cell area and mucous cell density relative to epithelium of 161.5 ± 123.6 μm^2 and 0.20 ± 0.15/μm^2 (upper left); 197 ± 44.5 μm^2 and 0.15 ± 0.1/μm^2 (upper right); 120 ± 64.8 μm^2 and 0.06 ± 0.04/μm^2 (lower left) and 42.2 ± 14.3 μm^2 and 0.09 ± 0.04/μm^2 (lower right).

Thicker sections of tissue may further reduce handling time, through the use of e.g. whole mounts (see for example Buchmann, Bresciani & Jappe 2004). However, further work is needed to test tissue permeability when there are scales present as well as the penetration characteristics of both alcian blue and periodic acid Schiff on deep, immature mucous cells, which are picked up by the current method.

Tissue delineation by software and systematic uniform random sampling of areas of interest prevents observer bias in measurements. Within-individual variation may be larger than between-individual variation (e.g. mucous cell density on the flank is generally higher than on the cranium; own data). This stereology-based method does not count and measure all mucous cells in a slice but rather estimates the number and size using probes to yield sufficient data to achieve the desired variance (precision), in accordance with stereological principles (Howard & Reed 2005). In experimental work with treated fish groups, an efficient method would perform as few measurements as possible to achieve this precision. The main question is what level of variation in the estimates is acceptable, while a secondary question is how much work is necessary for an acceptable variance in the estimates. We continue testing to address both these questions in practical demands of reproducibility and efficiency. This method lays the foundation for quantitatively addressing questions relevant to the efficacy of modulating mucosal production, the effect of interventions against salmon lice and for investigating the quantitative ontogeny of teleost mucosal defences.

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