



Body site matters: an evaluation and application of a novel histological methodology on the quantification of mucous cells in the skin of Atlantic salmon, *Salmo salar* L.

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Abstract

Mucous cell size and distribution were investigated in the skin of five salmon using a novel stereology-based methodology: one (48 cm) fish to test 15 tissue treatment combinations on measures of cell area and density on the dorsolateral region and, using the most suitable treatment, we mapped mucous cell differences between body regions on four (52 cm) salmon, comprising a male and a female on each of two diets. The section site, decalcification, embedding medium and plane of sectioning all impacted significantly on mucous cell size, whereas mucous cell density is more robust. There were highly significant differences in both mucosal density and mean mucous cell size depending on body site: the dorsolateral skin of the four salmon had significantly denser (about 8% of skin area) and larger (mean about 160 μm^2) mucous cells, whereas the lowest mean density (about 4%) and smallest mean area (115 μm^2) were found on the head. We found that 100 random measurements may be sufficient to distinguish differences $>7 \mu\text{m}^2$ in mean mucous cell areas. The results further suggest that salmon exhibit a dynamic repeatable pattern of mucous cell development influenced by sex, diet and possibly strain and season.

Keywords: innate immunity, mucous cells, salmon skin.

Introduction

A reliable methodology for determining mucocyte measures in fish has been lacking, even though these have been proposed as good indicators of stress, water quality, infections, diet and some life stages. Some authors suggest that the size and number of mucous cells in the epidermis is a good indication of the impact of stress because the physiological changes seem not to be mediated by cortisol (Vatsos *et al.* 2011). It has been shown that hyperplasia and hypertrophy of these mucous cells can occur in trout exposed to waterborne toxins, and mucocyte morphology has been proposed as a useful tool for biomonitoring (Ledy, Giamberini & Pihan 2003). Others state that the protection of the skin surface is because of a physical washing effect of renewed mucus rather than chemical functions of the mucus [Japanese flounder, *Paralichthys olivaceus* (Temminck and Schlegel) (Yamamoto, Kawai & Oshima 2011)]. However, recent work has shown that mucus secretion, in the gut and on the skin of fish, may be augmented by nutrition and this can affect bioactive components and natural barrier defence mechanisms associated with *Vibrio* infections in sea bass and sea lice infections in juvenile salmon *Salmo salar* L. (Sweetman *et al.* 2010; Torrecillas *et al.* 2011).

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The number and size of mucocytes in any given area is also important for interpreting the increasing number of results at the molecular level: recent work on cod, *Gadus morhua* L., shows differential immune expression and stress gene expression in the ventral and caudal regions of the skin, but practical interpretation of data rests partly on correlation with the physical attributes of the fish skin at these sites (Caipang *et al.* 2011). Fish mucus can contain lysozymes, immunoglobins, 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.

Salmon skin layers and mucous cells

Fish skin consists of the outer and inner epidermis, 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 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 and release their contents to the surface, exhibiting a large capacity for recombination and reaction to external influences (Easy & Ross 2010; Kruse *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 1975).

Mucus measurements

Measuring mucous cell number, size or density and quantitative content is fraught by the differences in size and density of cells across the body as well as subjective choices about where and what to measure. 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 previously. This gives a clear view of the structure of the selected small skin area but relatively few mucous cells for analysis in a single section. Measurements often do not consider the epidermal areas folded under and around the scales where mucous cells have been shown to aggregate in Japanese flounder (Yamamoto *et al.* 2011). The underlying assumptions may be that the largest diameter on the section corresponds to the middle of an average mucous cell and that epidermal thickness is even. Subjective choices and assumptions may bias a study.

The principles of unbiased stereology and the basic methodology of Pittman *et al.* (2011) are applied here to test the effects of body area, tissue treatment, embedding medium and section orientation on measures of mucous cell size and mucous cell density using the skin from one market size salmon. The method is further validated by application to four market size (52 cm SL) salmon at four key body sites: the head, the dorsolateral, the ventral caudal peduncle and the dorsal caudal peduncle.

Materials and methods

Fish source

A single specimen of Atlantic salmon was used for the methodology evaluation (male, length 48 cm). It was obtained from Averøy (Nofima Marin Forskningsstasjon Averøy, Norway), a commercial fish farm, under natural environmental conditions (winter 2011).

A further four Atlantic salmon of a single strain were used to implement and validate the method. These were all 52–52.9 cm long, with a male and female from each of two experimental dietary treatments in a field trial at Gildeskål Research

Station (GIFAS) under natural summer conditions in 2011.

Sampling

The single fish was caught by net from the cage and placed in a tank with anaesthesia. At Averøy, the anesthetized fish was placed in a plastic bag to preserve mucus and skin condition and killed with a blow to the head after transfer to the sampling room (transport time <2 min). A dorsolateral excision of a 7 × 7 cm skin area was made adjacent to the dorsal fin ('dorsal') and another from the lateral torso ('lateral'), including some muscle to anchor skin during fixation in 4% phosphate-buffered formalin.

Both skin samples were rinsed in phosphate-buffered saline (PBS) and cut in eight subsamples. Four of these subsamples were decalcified in formic acid (Riedel-de Haën) (250 mL formic acid, 19.8 g NaOH in 1 L water) for 24 h at room temperature. All subsamples were dehydrated in ethanol (50%, 70% and 80%). This was followed by embedding in either Technovit 7100 (Heraeus Kulzer GmbH & Co, KG) for at least 4 h according to size and sectioning at 2 µm, or embedding in paraffin (Histowax; Leica) and sectioning at 5 µm, and mounting with Mountex® (Histolab Products AB). Slicing was performed either in traditional cross-section or in tangential sections running almost parallel to the epidermal stratifications, which are not planar. This gave the following groups (location, pretreatment, embedding and section orientation): (1) lateral, normal, Technovit, cross-section; (2) lateral, normal, Technovit, tangential; (3) lateral, normal, paraffin, cross-section; (4) lateral, normal, paraffin, tangential; (5) lateral, decalcified, Technovit, cross-section; (6) lateral, decalcified, Technovit, tangential; (7) lateral, decalcified, paraffin, tangential; (8) dorsal, normal, Technovit, cross-section; (9) dorsal, normal, Technovit, tangential; (10) dorsal, normal, paraffin, cross-section; (11) dorsal, normal, paraffin, tangential; (12) dorsal, decalcified, Technovit, cross-section; (13) dorsal, decalcified, Technovit, tangential; (14) dorsal, decalcified, paraffin, cross-section; (15) dorsal, decalcified, paraffin, tangential.

The quantification method was applied to four 52 cm SL Atlantic salmon, a male and female given Diet 1 and a male and female given Diet 2 from Gildeskål Research Station, Norway. These were captured by dipnet, anaesthetized and killed

with a blow to the head. Skin samples of about 2 × 2 cm were taken as described previously from the dorsolateral, the head, the dorsal caudal peduncle and the ventral caudal peduncle and subsequently fixed in 4% phosphate-buffered formalin. These were then dehydrated in ethanol, embedded in Technovit and tangentially sliced.

Histological analysis

Sections were stained with Periodic Acid Schiff (PAS)-Alcian Blue and processed according to Pittman *et al.* (2011). Using VIS (Version 3.6.5.0; Visiopharm Integrator System), each of the sections was outlined for relevant regions of interest (ROI) and that area was randomly subdivided into 5–100% of the total region of interest. Mucous cell number and size and epithelial area were measured. Up to 400 mucous cells were measured from each section.

Statistical analysis

Using Statistica (version 10; StatSoft, Inc.) means and standard deviations were compared by performing a paired *t*-test with a significance level of 0.05. Pearson's correlations were used to distinguish relationships between epithelial coverage and mucous cell size and mucus: epithelium ratio. For the application of the method on four fish, using R the data for cell size were Box–Cox transformed before testing with a linear mixed effects model where the individual fish was the random factor. The data for cell density were tested using a one-way ANOVA. The Tukey's HSD multiple comparison test was used to detect differences in cell size and density between body sites.

Results

Method evaluation: one fish, two adjacent body sites, 15 tissue treatment combinations

Mucous cell size. Dorsolateral and lateral samples of salmon skin displayed mucous cell sizes ranging from 10 to 666 µm², with both extremes being found in lateral sections. Maximum cell sizes in dorsolateral sections ranged from 322 to 658 µm², while in lateral sections they were slightly larger at 364–666 µm². Non-decalcified sections contained maximum cell sizes ranging from 343 to 666 µm², whereas decalcified sections had

maximum cell sizes ranging from 322 to 532 μm^2 . Technovit embedding gave maximum cell sizes ranging from 432 to 666 μm^2 , while paraffin reduced maximum cell sizes to 322–604 μm^2 . Tangential sectioning produced maximum cell sizes ranging from 342 to 666 μm^2 and cross-sections gave maximums ranging from 322 to 508 μm^2 (see Table 1). The smallest cell sizes measured (10 and 15 μm^2) were found in all groups and ranged up to 56 μm^2 (Table 1).

Mean mucous cell area. The average area of mucous cells for lateral samples was $192.573 \pm 103.201 \mu\text{m}^2$, significantly larger than for dorso-lateral samples ($155.389 \pm 96.404 \mu\text{m}^2$, *t*-test, $P < 0.001$, Fig. 1a). Tissue decalcification gave significantly smaller mean mucous cell area (decalcified mean = $162.935 \pm 96.758 \mu\text{m}^2$, non-decalcified mean = $182.322 \pm 104.980 \mu\text{m}^2$, *t*-test, $P < 0.001$, Fig. 1b). Embedding in Technovit gave significantly larger mean mucous cell areas (Technovit mean = $177.933 \pm 105.113 \mu\text{m}^2$, Paraffin mean = $164.505 \pm 95.281 \mu\text{m}^2$, *t*-test, $P < 0.001$, Fig. 1c) and tangential sectioning gave significantly larger mucous cell areas than did traditional cross-sections (tangential mean = $184.127 \pm 106.390 \mu\text{m}^2$, cross-section mean = $151.578 \pm 87.892 \mu\text{m}^2$, *t*-test, $P < 0.001$, Fig. 1d).

When determining average mucous cell area, increasing the number of mucous cell measurements per slide from 50 to 400 gave sporadically significantly different means (Fig. 2a). However, standard deviation varied between 56 and 132 μm^2 and was lowest overall with 100 mucous cell measurements per slide (84.47, Fig. 2b).

Significant differences were found in mean mucous cell area between all treatments at all quantities measured, except 50 cell measurements in the decalcified vs. normal treatment (*t*-test, $P > 0.05$, Table 2). When individual mucous cell sizes were plotted against the % ROI the regression showed a very slight negative slope (-0.151), little correlation to the individual data points (Pearson's correlation, $r = -0.043$) and a very narrow confidence interval (Fig. 3a). However, when mean mucous cell areas were plotted against % ROI, the slope was more negative (-0.357) and the correlation was higher (Pearson's correlation, $r = -0.323$), although the confidence bands were broad (Fig. 3b).

Mucous cell area: epithelial area. The grand mean mucous cell area: epithelial area (cell density) was

Table 1 The range of minimum and maximum mucous cell areas (μm^2) in processed skin tissue from a single male salmon. Row headings indicate body area (lateral or dorsolateral), decalcification or non-decalcified skin, embedding medium (Technovit or paraffin) and angle of section (tangential or cross-section). Column entries indicate smallest and (largest) minimum cell areas within each category and (smallest) maximum cell area within each category

	Lateral	Dorsolateral	Decalcified	Normal	Technovit	Paraffin	Tangential	Cross-section
Minimum (μm^2)	10 (55.69)	14.92 (32.30)	10 (40.74)	14.92 (55.69)	14.92 (46.45)	10 (55.69)	10 (55.69)	14.92 (35.99)
Maximum (μm^2)	666.413 (364.812)	658.806 (322.315)	532.804 (322.315)	666.413 (324.834)	666.413 (432.492)	604.06 (322.315)	666.413 (342.834)	508.938 (322.315)

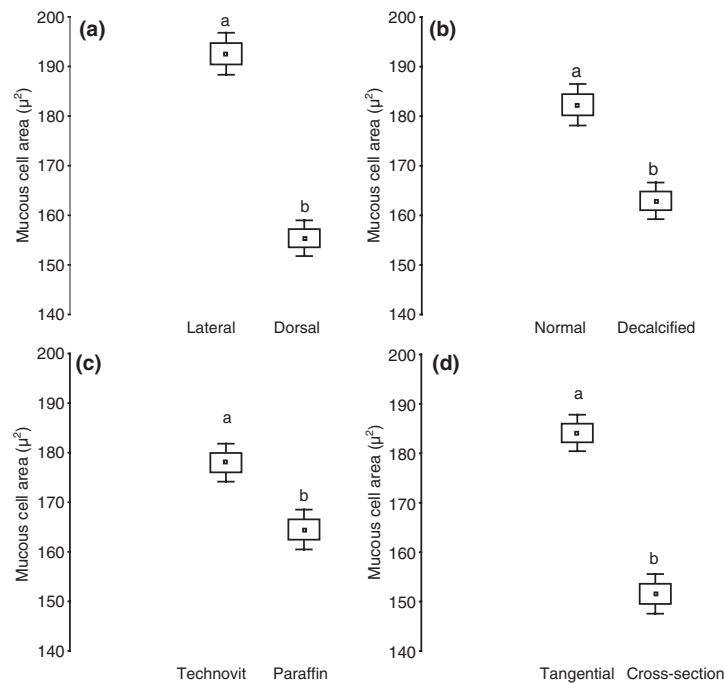


Figure 1 (a) Mean mucous cell area in μm^2 (\pm standard error) of sections taken from the dorsal and the lateral skin of a single 48 cm (standard length) male salmon. (b) Mean mucous cell area of the salmon skin which has been decalcified (decalcified) for scales or has been untreated (normal). (c) Mean mucous cell area in sections of the salmon skin embedded either in Technovit or paraffin. (d) Mean mucous cell area of the salmon skin sectioned in traditional cross-section or tangentially. Different letters indicate significant differences at $P < 0.05$.

0.137 ± 0.067 . There were no significant differences on this ratio caused by body area, tissue treatment, embedding medium or section orientation (Fig. 4). The ratio was not significantly affected by % ROI covered (Pearson's correlation, $r = -0.059$, slope $\ll 0.001$, Fig. 5). However, six of the eight points representing the dorsolateral were below the regression line, indicating lower mucous cell densities from this location on the fish body.

Method application: four fish, four body sites, one tissue treatment

Both mucous cell size and mucous cell density in the four salmon are significantly affected by body site ($F_{3,12} = 10.543$, $P = 0.001$ and $F_{3,12} = 4.526$, $P = 0.024$, respectively, ANOVA). The following paragraphs will examine the results for size and density in detail.

Mean mucous cell area on head, dorsolateral and caudal peduncle. There are consistent and significant regional differences in the mean mucous cell

size and density of the four salmon. The mean cell sizes ranged from $160 \mu\text{m}^2$ on the dorsolateral skin (largest) to $115 \mu\text{m}^2$ on the head (smallest; Fig. 6a). Mucous cell sizes were larger, and the differences were highly significant, in skin from the dorsolateral vs. the head region ($P < 0.001$, Tukey's HSD; Table 3), the dorsolateral vs. the dorsal caudal peduncle ($P = 0.001$, Tukey's HSD) and the ventral caudal peduncle vs. the head region ($P < 0.001$, Tukey's HSD). Significantly larger mucous cell sizes were found on the ventral caudal peduncle vs. the dorsal caudal peduncle ($P < 0.01$, Tukey's HSD).

When the data were grouped according to sex, both had equally small cells on the head but females had larger dorsolateral mucous cells and males had larger cells on the caudal peduncle (Fig. 6b). The largest sex-associated difference in mean mucous cell area was on the ventral caudal peduncle. Diet gave large differences in the mean cell area at all body parts except the dorsolateral, where both treatments had mean cell areas of about $160 \mu\text{m}^2$ (Fig. 6c). One of the diets seemed to exacerbate the difference between the ventral

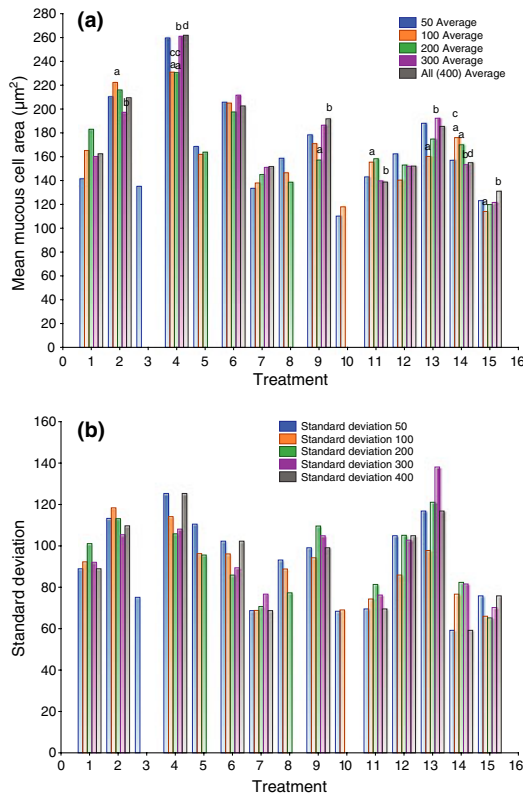


Figure 2 (a) Comparison of mean mucous cell area in skin of a single male salmon across all treatment groups as determined by measuring 50, 100, 200, 300 and 400 randomly selected mucous cells. Different letters indicate significant differences within a group. Groups: (1) lateral, normal, Technovit, cross-section; (2) lateral, normal, Technovit, tangential; (3) lateral, normal, paraffin, cross-section; (4) lateral, normal, paraffin, tangential; (5) lateral, decalcified, Technovit, cross-section; (6) lateral, decalcified, Technovit, tangential; (7) lateral, decalcified, paraffin, tangential; (8) dorsal, normal, Technovit, cross-section; (9) dorsal, normal, Technovit, tangential; (10) dorsal, normal, paraffin, cross-section; (11) dorsal, normal, paraffin, tangential; (12) dorsal, decalcified, Technovit, cross-section; (13) dorsal, decalcified, Technovit, tangential; (14) dorsal, decalcified, paraffin, cross-section; (15) dorsal, decalcified, paraffin, tangential. Some groups did not have enough cells for all counts. (b) The standard deviation for the above mean mucous cell areas. Groups as in a.

and dorsal mucus development, with about 60 µm² larger cells on the ventral portion of the tail than on the dorsal from Diet 1, whereas salmon fed Diet 2 had a more consistent cell size on both sides of the tail. In these cases, there were only two fish beneath each point for comparison thus rendering moot the question of significant differences.

Table 2 Tabular overview of significant differences of mean mucous cell size (µm²) when 50, 100, 200, 300 and 400 cells were compared for treatment effects. Paired *t*-tests for each category. Mean Gr 1 is the first of the pair of categories being compared, Mean Gr 2 is the mean of the second of the pair

	50		100		200		300		400	
	Mean Gr 1 (µm ²)	Mean Gr 2 (µm ²)	Mean Gr 1 (µm ²)	Mean Gr 2 (µm ²)	Mean Gr 1 (µm ²)	Mean Gr 2 (µm ²)	Mean Gr 1 (µm ²)	Mean Gr 2 (µm ²)	Mean Gr 1 (µm ²)	Mean Gr 2 (µm ²)
Lateral vs. dorsal	181.65	152.87	184.02	147.84	186.10	151.64	190.42	153.16	192.57	155.39
Decalcified vs. normal	162.64	169.51	156.51	171.20	161.01	173.86	163.47	176.93	162.93	182.32
Technovit vs. paraffin	175.94	155.28	171.20	155.53	172.39	160.36	175.71	162.15	177.99	164.50
Tangential vs. cross-section	180.24	150.37	174.62	151.36	176.56	154.58	182.63	150.41	184.13	151.58
			<i>P</i> -value	<i>P</i> -value	<i>P</i> -value	<i>P</i> -value	<i>P</i> -value	<i>P</i> -value	<i>P</i> -value	<i>P</i> -value
			≤0.001	0.003	0.002	≤0.001	0.001	0.001	≤0.001	≤0.001
			0.340	0.004	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001

P-values > 0.05 indicate no significant difference and are shown in bold.

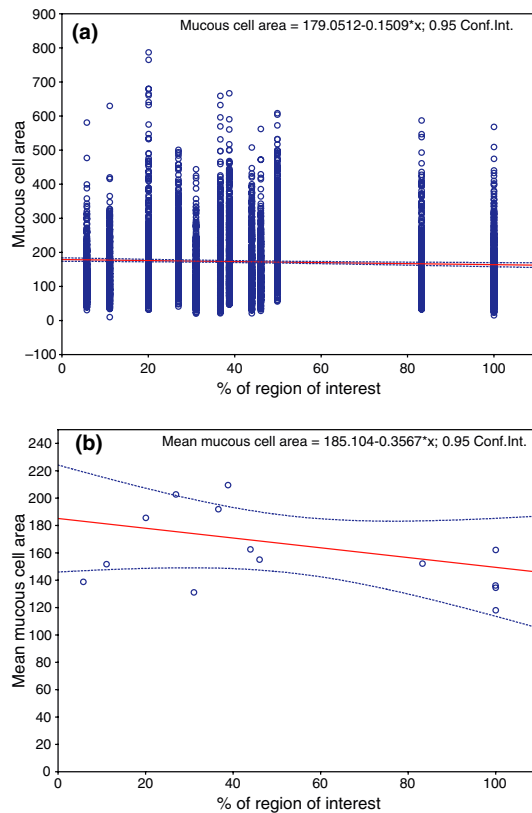


Figure 3 (a) The relationship between individual mucous cell measurements and percentage of the region of interest covered by the counting frames. The solid line is the regression ($179.0512-0.1509x$) and the dashed lines indicate 95% confidence interval. Four slides had 100% coverage and are grouped together. (b) The relationship between the mean mucous cell areas and the percentage of the region of interest covered by the counting frame. The solid line is the regression ($185.104-0.3567x$) and the dashed lines are the 95% confidence interval.

Mean mucous cell density on the head, dorsolateral and caudal peduncle. Significant differences in mucous cell density are apparent between body parts where the pattern found for cell sizes repeats itself. The lowest densities are found on the heads of the four fish (about 0.042 or about 4% of the epidermal area) and density is more than doubled at the dorsolateral (Fig. 6d). Mucous cell densities were significantly higher in the skin from the dorsolateral vs. the dorsal caudal peduncle ($P < 0.05$, Tukey's HSD; Table 3) and from the dorsolateral vs. the head ($P < 0.05$, Tukey's HSD).

There were no significant differences in cell size or density of samples from the head vs. the dorsal caudal peduncle nor between samples from the

ventral caudal peduncle and the dorsolateral. The mucous cell densities alone were not significantly different between the ventral and the dorsal caudal peduncle nor between the ventral caudal peduncle and the head, despite significant differences in cell sizes.

Differences in density are further associated with sex and diet, with females having higher densities on the head and dorsolateral and males having higher densities on the tail (Fig. 6e). Although there are too few individuals sampled to make any meaningful comparisons for significant differences, the most consistent difference in mean mucous cell densities was due to diet, with Diet 1 giving higher densities at all body parts (Fig. 6f). The tail area of fish fed Diet 2 showed more equal cell densities on the ventral and dorsal relative to Diet 1. Diet 1 is associated with a near doubling of the density on the head and on the ventral caudal peduncle relative to Diet 2 and about 15% higher densities on the dorsolateral skin.

Discussion

Mucous cell measurements were significantly affected by even small changes in body site, by the angle of sectioning within body sites, by decalcification and by embedding medium. The largest mucous cell measurements within a body site were obtained on non-decalcified, Technovit-embedded tangential sections. Body site gave highly significant differences on mucous cell density, but there was no effect of tissue treatment on this parameter. Mucous cell size and density varied significantly across the body, exhibiting repeatable patterns with some interesting differences between groups. In the following, we discuss the nuances of the method evaluation and the consequences of results from the application of the method.

Method evaluation: one salmon, two adjacent body sites, 15 tissue treatment combinations

Mucous cell size. The minimum cell size was around $10 \mu\text{m}^2$ across all treatments. However, paraffin-embedded slides may have shrunk any smaller mucous cells to the point where they are indistinguishable from random epithelial discoloration. Others have also found Technovit to be superior to paraffin in maintaining cellular detail (Yeung 1999). Mucous cells were identified through the use of PAS-Alcian Blue staining and

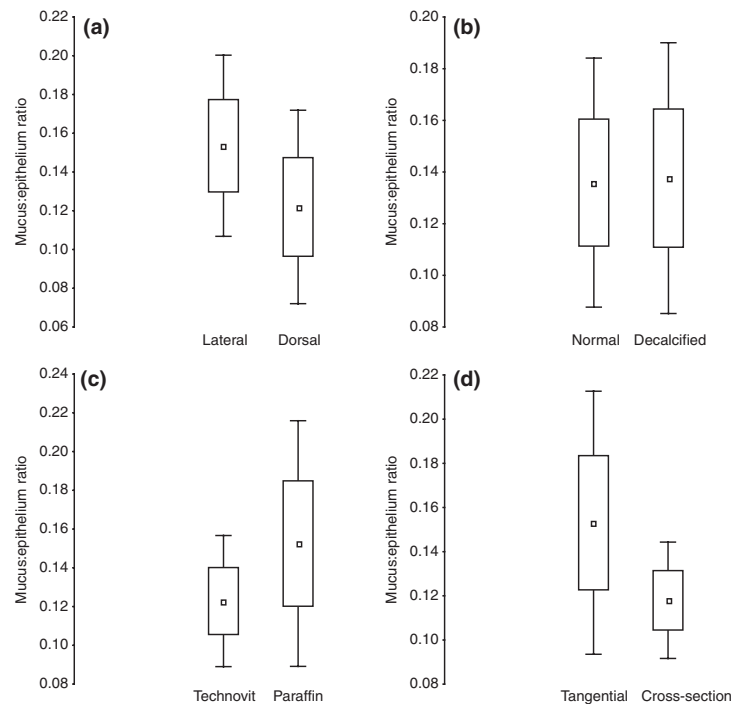


Figure 4 (a) Mean ratio of mucous cell area: epithelium area (\pm standard error) of sections taken from the dorsal and the lateral skin of a single 48 cm (standard length) male salmon. (b) Mean ratio of mucous cell area: epithelium area of the salmon skin which has been decalcified (decalcified) for scales or has been untreated (normal). (c) Mean ratio of mucous cell area/epithelium area in sections of the salmon skin embedded either in Technovit or paraffin. (d) Mean ratio of mucous cell area/epithelium area of the salmon skin sectioned in traditional cross-section or tangentially.

required sufficient mucins within the cell to be stained. Thus, there are two ways in which the minimum can be obtained, either as the smallest size of cell at which mucins are produced deep in

the epithelium or as cells cut at their periphery (i.e. smallest resolution of this method). Mucins vary greatly in size (322–13 288 residues) and many facets of the mucin family remain to be elucidated (Hill & Perez-Vilar 1999). Mucins themselves can be of two basic types, membrane bound or secreted, and both are highly glycosylated (Bansil & Turner 2006) opening the possibility of more sensitive histochemical identification along with quantification.

Maximum cell sizes were significantly affected by treatment. The large variation in maximum cell area can be due to the irregular form of the larger mucous cells (Kryvi & Totland 1997) as well as the point of intersection. The largest measured cell was found in tangentially sectioned, non-decalcified, Technovit-embedded lateral sections (group 2). This group also gave the largest standard deviation. Decalcification may be necessary to process skin of fish species with thick scales which impede histological sectioning, thereby affecting the absolute measure of cell size. However, our method will allow quantification and direct comparison

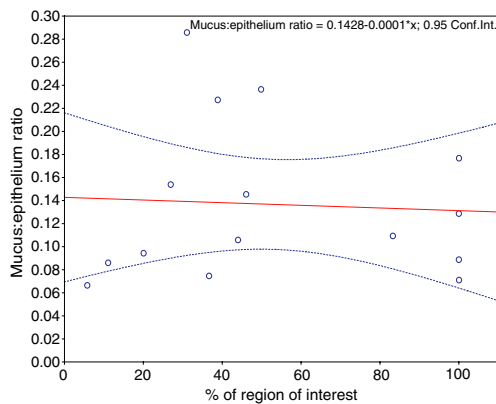


Figure 5 The relationship between the mean mucous cell areas and the percentage of the region of interest covered by the counting frame. The solid line is the regression ($0.1428 - 0.001 \times$) and the dashed lines are the 95% confidence interval.

within a treatment regime. The range of within-individual maximum mucous cell area can be more than double (322 vs. 666 μm^2) and therefore indicates the limited value of this measure in comparing treatments. The limited applicability of maximum cell size further highlights the need for

objectively determined mean cell sizes in addition to numerous measures.

Table 3 Multiple comparison test (Tukey’s HSD) of mean mucous cell area and mean mucous cell/epithelium density in four salmon (52 cm SL)

Basis for comparison	Body site 1	Body site 2	P-value
Mean mucous cell area	Head	Dorsal C.P.	0.851
	Dorsolateral	Dorsal C.P.	0.001
	Ventral C.P.	Dorsal C.P.	0.010
	Dorsolateral	Head	<0.001
	Ventral C.P.	Head	<0.001
	Ventral C.P.	Dorsolateral	0.983
Mucous cell density	Head	Dorsal C.P.	0.999
	Dorsolateral	Dorsal C.P.	0.045
	Ventral C.P.	Dorsal C.P.	0.281
	Dorsolateral	Head	0.042
	Ventral C.P.	Head	0.269
	Ventral C.P.	Dorsolateral	0.675

C.P., caudal peduncle.
Mean mucous cell area is in μm^2 . Significant differences are highlighted in bold font.

Mean mucous cell area. Mean mucous cell areas were not significantly different within a processed slide whether 50 or 400 cells were measured. Significant differences in mean mucous cell area between all 15 processing groups were found using 50, 100, 200, 300 or 400 cell measurements. The smallest difference between mean mucous cell areas that was not significantly different was approximately $7 \mu\text{m}^2$. This indicates that 100 random measurements may be sufficient to distinguish differences $>7 \mu\text{m}^2$ in mean mucous cell areas.

Sections from the lateral tissue showed significantly larger mucous cells than sections from the adjacent dorsal tissue of the same fish. This concurs with previous work such as a recent Japanese study that found that mucous cell number and size of the Japanese flounder were affected by bacterial infection with both factors differing by infection means and body region (Yamamoto *et al.* 2011). Previous studies have indicated that

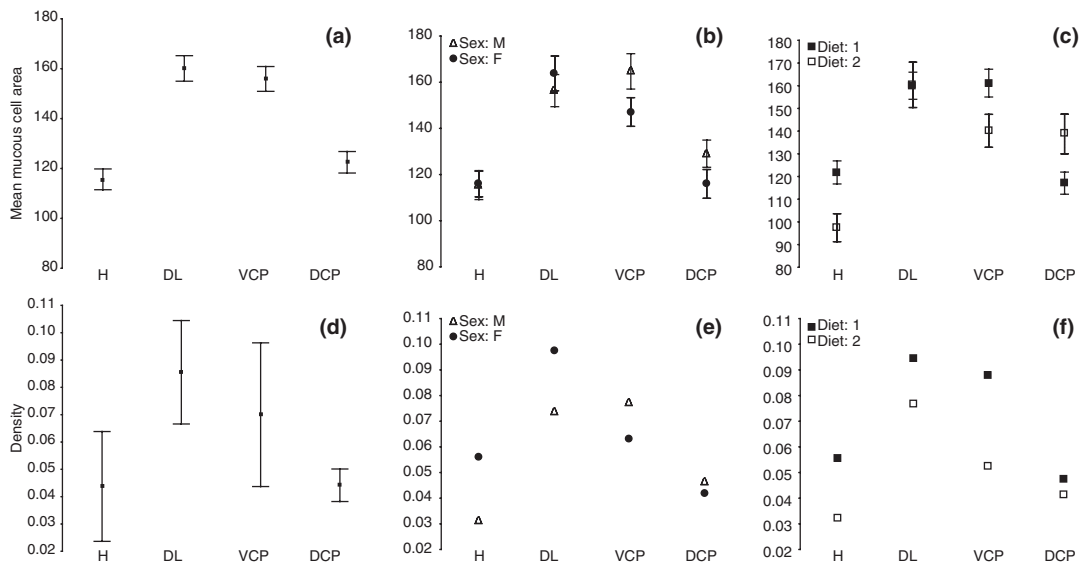


Figure 6 Overview of the mean mucous cell area ($\mu\text{m}^2 \pm 95\%$ confidence interval) and mean mucous cell/epithelium density (mean $\pm 95\%$ confidence interval) at four body sites in four Atlantic salmon of 52–52.9 cm SL. (a) Mean mucous cell area of the head (H), dorsolateral (DL), ventral caudal peduncle (VCP) and dorsal caudal peduncle (DCP) of all four salmon. (b) Mean mucous cell area of four body sites in four salmon grouped according to gender (two females, two males). (c) Mean mucous cell area of four body sites in four salmon grouped according to diet (two fish on diet 1, two fish on diet 2). (d) Mean mucous cell density at four body sites in four salmon. (e) Mean mucous cell density at four body sites in four salmon grouped according to gender. (f) Mean mucous cell density at four body sites in four salmon grouped according to diet. Please see Table 3 for an overview of significant differences.

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 *et al.* 2002a,b; Easy & Ross 2010), while seven have looked at salmonid skin in fresh water (Roberts *et al.* 1970; Hawkes 1974; Harris & Hunt 1975; Pickering & Macey 1977; Pottinger, Pickering & Blackstock 1984; Buchmann & Bresciani 1998; O'Byrne-Ring *et al.* 2003). 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 to 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. This study joins the other studies in reinforcing the need to standardize the source location of mucous tissue when comparing between treatments or between fish.

Mean mucous cell area declined slightly with greater tissue area coverage. However, it must be noted that all slides with 100% coverage were cross-sections. These gave significantly smaller cell areas than did tangential sectioning and may have contributed to the declining trend line in Fig. 3b. Removal of these points gave a positive slope of +0.315, with a very broad confidence interval (data not shown). The correlation between the mean mucous cell area and regression line is low (−0.35), indicating a stronger effect of treatment on mucous cell area than of ROI covered.

Mucous cell area: epithelial area. The mucus/epithelium ratio is the mucous cell density based on area rather than number, making it comparable between individual fish and body areas. A large number of small cells may give the same mucosal density as a few large cells. Given that mucous cells enlarge as they mature and migrate to the epithelial surface, measuring the area rather than only the number may give a better representation of the protective capacity of the mucus for the organism.

Treatment did not affect mucus/epithelium ratio. This may indicate that any shrinkage in the mucous cells owing to treatment method is accompanied by similar shrinkage in the epithelium. There was no correlation between

percentage area analysed (% ROI) and mucous cell density.

Method application: four salmon, four body sites, one tissue treatment

The skin of the head, dorsolateral, ventral caudal peduncle and dorsal caudal peduncle were examined in four salmon using tangential sectioning, no decalcification, Technovit embedding and the method of Pittman *et al.* (2011). The results revealed repeatable, reliable patterns in mucous cell size and distribution in salmon skin. The dorsolateral skin contained the highest density (about 8% of the area of volume of the epithelium) and usually the largest size of mucous cells (about $160 \mu\text{m}^2$), whereas the head exhibited the lowest density and smallest size (about 4% and $110 \mu\text{m}^2$). That even the small number of fish sampled resulted in highly significant differences in mean mucous cell size as well as significant differences in mucous cell density at different body sites indicates that the method is both sensitive and robust.

Comparisons between the fish raise a number of issues. Significantly larger and denser mucous cells on the dorsolateral body indicate that this would be the site of greatest mucosal production, concurring with previous studies (Pickering 1974; Buchmann & Bresciani 1998), whereas the significantly smaller and scarcer mucous cells on the head suggest a site of little mucosal production. The difference between body sites seems also to be affected by sex and thus raises the issue of a sex-related response to various infestations and treatments. Male–female differences abound in the animal world and mucous production may be no exception. This pattern of mucous cell distribution may be correlated to the differential gene expression in fish such as cod (Caipang *et al.* 2011) and be a vital clue to the infestation pattern of various ectoparasites such as the salmon lice (*Lepeophtheirus salmonis* and *Caligus elongatus*) (Gjerde, Ødegård & Thorland 2011).

Diet appears to strongly influence mucous cell density and size, with both factors being most different on the ventral caudal peduncle. Thus, it may be postulated that nutrition can affect mucous production at particular body sites and help in directed treatments against ectoparasites. Other researchers have found an influence of diet on gut mucosal production (Sweetman *et al.* 2010; Torrecillas *et al.* 2011). Furthermore,

experiments where mucus mediates either pharmaceutical components or other innate immune factors must consider the number and density of those mediating mucous cells.

Further issues are raised by the comparison between fish sources. The dorsolateral measure of the four fish from Gildeskål Research Station (two female, two male, all 52 cm long and sampled in summer) are somewhat smaller and less dense than those from the single 48-cm long male salmon sampled in winter from Averøy. This then raises the question of seasonal as well as strain difference in mucosal production. It may therefore be postulated that detecting differences in the efficacy of treatments against for example sealice may be confounded by the effects on mucous cell production from nutrition, from seasonal effects and from fish sex.

The results here underscore the need to examine the role of mucous cells and their population dynamics across the body of teleosts. In a recent work on cod skin, Caipang *et al.* (2011) found expression of antibacterial genes (g-type lysozyme, metallothionein, BPI/LBP, galectin, hepcidin, transferrin), antiviral genes (fortilin and IRF-1, ISG-15 and methyltransferase), cytokine genes (IFN- γ , CC-chemokine and two interleukins), four glucose transport genes and four stress-related genes (Cu/ZN-SOD, catalase, GSH-Px and Hsp-70) with many of these showing a ventral/dorsal differentiation. These authors argue that both the type and number of cells per unit area of skin as well as the presence of various types of mucus secretory cells in both regions of the skin could account for the differences in gene expression.

The behaviour of mucins is complicated because of their large size and complex structure. Mucin domains involve variable hydrophilic/hydrophobic properties, hydrogen bonds and electrostatic interactions, as well as their ability to aggregate and form complexes with other polymers. These properties are also of direct relevance to the numerous diseases involving mucins and to the problems of nutrient uptake and drug delivery through the mucus barrier (Bansil & Turner 2006). Mucus contains several bioactive components such as immunoglobulin, complement C-reactive protein, lectins, lysozyme, proteolytic enzymes, alkaline phosphate and esterase, antimicrobial peptides and haemolysin which exhibit biostatic or biocidal activities (Allvarez-Pellitero 2008). About 20 mucin genes have been identified and partly

cloned in humans and homologues exist in the mouse and the rat. Different collections of genes are expressed in different tissues (Bansil & Turner 2006) and changes in histochemistry of mucous secretions have been reported in fish (e.g. Urawa 1992; van Der Marel *et al.* 2010). In his thesis, Subramanian (2009) found constitutively produced antimicrobial substances from the epidermal mucus of Arctic char, brook trout, striped bass, koi carp, haddock, cod and hagfish, not only showing differences in activity against various pathogens but also with the hagfish acidic epidermal mucus extract displaying potent antimicrobial activity at minimum bactericidal concentrations against all screened pathogens.

Skin diseases have serious economic impact for fish farms, in terms of growth loss, mortalities, treatment costs and increased susceptibility to other pathogens. The host skin reaction to infection or toxins involves shifts in mucous cell populations and density as well as shifts in mucous cell metabolism leading to changes in mucus quality. Mucous cell properties may determine host susceptibility to certain skin diseases as well as playing a role in pathogen affinity and pathogenicity – Olafsdottir & Buchmann (2004) suggest that, in freshwater salmon, Dexamethasone treatment against *Gyrodactylus derjavini* parasites has the effect of reducing mucous cell densities on caudal and pelvic fins. In each case, mucus production enhances the physical and biochemical shields for fish health.

Concluding remarks

Mean mucous cell area is sensitive to the sampled body site, as well as histological processing. Mucous cell density as a ratio of mucus to epithelium is a relatively robust measure that can be used to compare body areas as well as the effects of treatments on fish species. Our study shows that maximum mucous cell size can vary twofold between sections from an individual fish and has limited applicability. In salmon, we find a pattern of significantly larger and more dense mucous cells on the dorsolateral body and smaller less dense cells on the head. Regional differences between the head, dorsolateral and caudal peduncle are highly significant. There are also differences between male and female fish, with females having more dense mucous cells on most of the body than do males, as well as important

influences of diet on mucous cell distributions. Using our systematic random approach, measuring as few as 100 mucous cells was enough to distinguish significant differences in mean mucous cell area.

As this is the first application of the novel method, many issues are raised about mucous cell production, including the effects of gender, size, diet, season, body site and individual variation. Handling only a few samples from many individuals, as with design-based stereology (Boyce *et al.* 2010), can address questions of quantification at the population or treatment level while limiting the impact of the individual's state. The data garnered using stereology in this study suggest that salmon mucous cells have a repeatable distribution with some consistent variations between and within individuals. This method allows unbiased comparison of mucous cell dynamics in fish from different environments, strains, genders and sizes. This method furthermore lays the foundation for selected breeding and directed intervention to build up a sustainable primary immune system and bring the skin of the salmon itself back into the fight for increased health and welfare.

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