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Variations in mucous cell numbers in gills of Atlantic salmon (*Salmo salar*) presmolt in commercial freshwater farms in Norway

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Abstract

Fish gills are heavily exposed to the external milieu and may react against irritants with different cellular responses. We describe variations in mucous cell counts in gills from healthy Atlantic salmon (*Salmo salar*) presmolts in five recirculating aquaculture system (RAS) farms and one flow-through farm. Based on certain criteria, mucous cells were histologically quantified in a defined lamellar region of the gills and the counts were analysed. Immunohistochemistry (IHC) was used to investigate epithelial responses. The median number of total mucous cells in the defined region was 59 per fish. Between the farms, the medians varied from 31 to 101 with the lowest in the flow-through farm. A regression model was fitted with "total mucous cells" as the dependent variable and with "fish length" and "fish farm" as independent variables. The proportion of variation in mucous cell counts explained by the model was twice as high when "fish farm" was included compared to only "fish length." IHC revealed proliferative responses in coherence with high mucous cell numbers. Conclusively, the variation in mucous cell counts depends on combined farm-related factors. Establishing a baseline for mucous cell counts is fundamental in the development of high-throughput monitoring programmes of gill health in farmed fish.

KEYWORDS

gill health, mucous cell, RAS, salmon

1 | INTRODUCTION

Traditionally, salmon (*Salmo salar*) smolt have been reared in land-based facilities using water from nearby rivers or lakes which flow through the production site. Flow-through farms (FT farms) depend on high water quality from the source, as means of regulating different water parameters are limited (Kristensen, Åtland, Rosten, Urke, & Rosseland, 2009). Recirculating aquaculture system (RAS) technology has developed rapidly over the last years and has been preferred in new production facilities for rearing of salmon in the freshwater stage. Because RAS technology allows close to 100% recirculation of water, it is possible to produce more fish with restricted resources (Dalsgaard et al., 2013). However, reuse of water demands efficient handling of accumulating waste products, such as particles, which

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must be removed. Additionally, the maintenance of pH levels and dissolved gases is imperative to ensure a stable environment for the fish (Dalsgaard et al., 2013; Hjeltnes et al., 2012; Kristensen et al., 2009).

Breaching of the biosecurity in RAS facilities is often critical. Accumulation of waste products can cause physical stress, which could result in reduced function of the gills (Awata, Tsuruta, Yada, Iguchi, & i., 2011; Bruton, 1985; Dahle et al., 2020). Because of their delicate structure and close contact with water, the gills are particularly vulnerable for mechanical damage and injuries caused by environmental factors (Rodger, 2007). The gills are vital not only for respiratory purposes, but also for osmoregulation, nitrogenous waste excretion, pH regulation and production of hormones (Evans, Piermarini, & Choe, 2005). In addition, the gill epithelium is an essential part of the immune system, acting as a physical and functional barrier against the outer environment (Gomez, Sunyer, & Salinas, 2013; Koppang, Kvellestad, & Fischer, 2015).

Studies of the human respiratory tract have shown that proliferation of mucous cells (or goblet cells) is one of the initial reactions towards exogenous irritants in the airways, with a resulting increase in mucus production (Rogers, 1994; Whitsett & Alenghat, 2015). Similar mechanisms are also present in the gills of fish (Gomez et al., 2013). Thus, mucous cell counts have been used to evaluate gill health in several experimental studies in salmonids (H. W. Ferguson, Morrison, Ostland, Lumsden, & Byrne, 1992; Roberts & Powell, 2003; Speare, Arsenault, MacNair, & Powell, 1997). A recent study on the histopathological responses involved in complex gill disease in farmed Atlantic salmon concluded that mucous cell hyperplasia was one of the most common pathological features (Gjessing et al., 2019). This corresponds well with the extensive mucus covering of diseased gills, a typical clinical sign of gill inflammation. Despite the well-known importance of mucous cell reactions in gills, there is scarce information on the variation of mucous cells between individual fish or possibly the variation between different fish populations. Thus, investigations addressing these questions are warranted. A baseline for mucous cell counts is fundamental in the development of a future high-throughput monitoring programme of gill health in farmed fish. This would be of special interest in RAS facilities, where gill health has been pinpointed as one of the critical concerns (Becke, Schumann, Steinhagen, Geist, & Brinker, 2018; Dahle et al., 2020; Hjeltnes et al., 2012).

In this cross-sectional study, the overall aim was to investigate the prevalence of mucous cells in presmolts from five different RAS facilities and one FT farm. This was approached through two objectives: first, to implement a counting method of mucous cells in salmon gills, and second, to describe the variations in mucous cell count in salmon and between different salmon production sites.

2 | MATERIALS AND METHODS

2.1 | Material

Gill samples from a total of 220 fish were collected from six different commercial land-based salmon farms on the western and northern coast of Norway from October 2018 to January 2019. Five of the farms were RAS-based (RAS I-V), and one was a traditional flow-through facility (FT I). Two samplings of 20 fish from two different tanks were conducted at each RAS farm (a total of 40 fish from each farm) with 14 days between the samplings. From the FT farm, only one sampling of 20 fish was carried out. All fish were reported to be healthy and without signs of clinical disease. The samplings were performed in the time period between vaccination and sea transfer.

2.2 | Sampling procedure

The fish were gently netted out from the tanks and killed by an overdose of sedation (Finquel© vet, Scan Aqua), in line with regulations of the Norwegian Directorate of Fisheries (Akvakultudriftsforskriften, 2008). Weight and length were recorded. The entire second gill arch on the left side was sampled and placed in 10% buffered formalin.

2.3 | Weight and length

Weight and length of the fish were recorded from all but one fish (from RAS III). In further calculations, the fish with the missing values was given the calculated mean weight and length from the nine other individuals from the same tank at the same sampling day.

2.4 | Water transparency

Water transparency was assessed by measuring the sight depth with a modified white Secchi disc of 15 cm in diameter in each tank at each sampling time point. The measured sight depth was divided by the tank depth to get the relative sight depth expressed in percentage of the tank depth to allow comparison of results across farms. If the bottom of the tank were visible, no measurement was performed, and the water transparency was put to 100%.

2.5 | Histological investigations

After fixation for minimum 48 hr, the gill arches were processed for histology and embedded in paraffin. All samples were embedded with identical tissue orientation. Sections (2 μm) were cut and stained with haematoxylin and eosin (HE) for histological investigation and periodic acid–Schiff (PAS) according to standard procedures for the detection of mucins (Bancroft & Gamble, 2008).

Immunohistochemical investigations were applied to investigate proliferation of cells (proliferative cell nuclear antigen [PCNA], dilution 1:5,000, Dako, DK-2600 Glostrup, Denmark) and the distribution of epithelial cells (pan-cytokeratin, clone AE1/AE3, dilution 1:50, Invitrogen, Thermo Fisher, MA, USA). Gills from the fish with the lowest ($n = 3$) and highest ($n = 3$) mucous cell counts, respectively, were chosen for IHC analysis (gills from six fish in total). The method has been described

in detail elsewhere (Bjørgen et al., 2018). Briefly, sections (4 μm) were cut and mounted on Superfrost+ slides (Mentzel, Braunschweig, Germany). The sections were de-paraffinized and autoclaved at 121 degrees Celsius for 10 min. The slides were treated with phenylhydrazine (0.05%; Sigma-Aldrich, St. Louis, MO, USA) at 37 degrees Celsius for 40 min to inhibit endogenous peroxidase. Non-specific binding in reactive sites was blocked with a solution of normal goat serum diluted in 5% bovine serum albumin/Tris-buffered saline (BSA/TBS). The slides were incubated with primary antibodies for 30 min in room temperature, washed three times in phosphate-buffered saline (PBS) and further incubated with the secondary antibody (Envision© System Kit; Dako, Glostrup, Denmark) for 30 min in room temperature. Red colour was evoked with 3-amino-9-ethylcarbazole (AEC) substrate incubated for 14 min. The slides were washed in distilled water and mounted using Aquatex® (Sigma-Aldrich) mounting medium. Negative controls were prepared with PBS instead of primary antibody.

2.6 | Mucous cell count

A counting method was established to investigate the prevalence of mucous cells in the lamellae of the gills in all groups (RAS I-V and FT I). Details on method development are described in Appendix 1. The resulting method was as follows: mucous cells were counted on 20 consecutive lamellae on both afferent and efferent sides (40 lamellae on each gill filament) on three filaments, that is 120 lamellae on each fish. The counts were performed blinded to sampling date and location with sections from all samplings mixed and counted by one person. Only cells with a distinct PAS-positive cytoplasm were counted using 63X magnification. Mucous cells in the interlamellar region were not included. The counts were performed in a proximal-to-distal direction from the basis of the filament. The filament situated approximately in the middle of the angle of the gill arch was counted first, followed by the next filament according to the sequence shown in Figure 1. The counted regions had to have an intact filament with a symmetrical distribution of at least 20 lamellae; otherwise, the next filament (in dorsal or ventral direction, respectively) was selected for counting (Figure 1). Oblique sections were re-orientated and re-processed for examination. Nine samples were not suited for counting due to sample irregularities and were discarded.

2.7 | Statistical investigations

The final data set (n = 211) is described in Table 1. The sum of all counted mucous cells from each fish ("total mucous cells") was used for all calculations if not stated otherwise. A linear regression model was built to investigate the variation in mucous cell counts across sites. The dependent variable was "total mucous cells," which was log-transformed to reach the assumption of normal distribution. The independent variables tested in the model were "fish weight," "fish length," "water transparency" and "fish farm." Variables were retained in the model if $p < .05$. The residuals from the final model were checked for normality and homoscedasticity. All data were first plotted in Microsoft Excel (Microsoft Corporation), and all statistical work was performed in StataSE 15 (Stata Corporation).

3 | RESULTS

3.1 | Weight and length

The median fish weight between the different farms varied between 74 and 246 g. The median weight of all fish was 124 g, and the mean was 144 g. The median length varied between 18.5 and 27 cm across

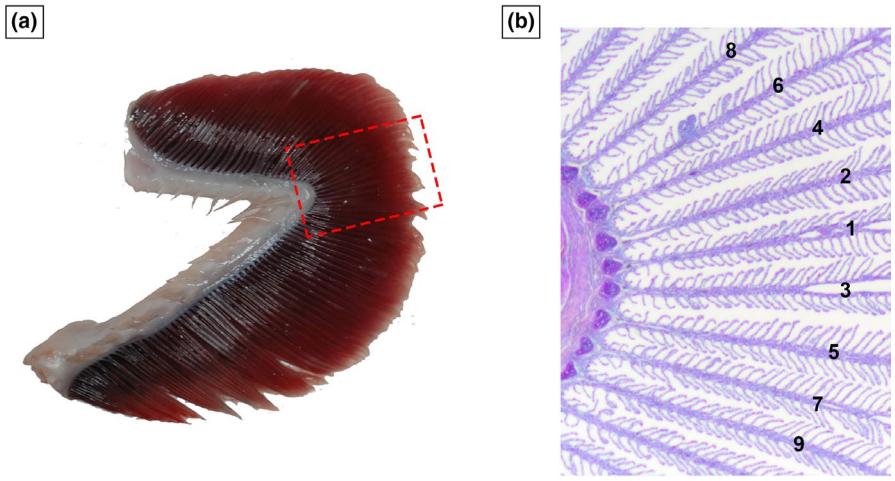

FIGURE 1 Mucous cell counting method. The starting point for the counting of each gill was determined in the middle of the angle of the gill arch (A—indicated by red box). The middle filament was termed 1 (B—indicated by 1). If the lamellae on this filament fulfilled the counting criteria (see Materials and Methods), mucous cells on this filament were counted. If not, counting of the next filament fulfilling the criteria was conducted. The selection of filament was performed in the order indicated in figure B until a total of three filaments had been counted. Mucous cells on 20 consecutive lamellae (see counting criteria in Materials and Methods) on both afferent and efferent sides were counted on each of the three filaments, that is 120 lamellae in total on each gill arch. Mucous cells in the interlamellar region were not counted

TABLE 1 Descriptive statistics for main variables, grouped by farm

Farm	Number of fish	Tank depth (cm)	Fish weight [g] *median (min–max)*	Fish length [cm] *median (min–max)*	Relative sight depth [%] *median (min–max)*	Mucous cell count *median (min–max)*
FT I	20	350	112 (56–218)	20 (17–24.8)	100	31 (21–73)
RAS I	38	400	74 (50–124)	18.5 (15.5–22)	58 (53–84)	44 (21–71)
RAS II	39	300	152 (52–284)	22 (16–29)	60 (54–63)	44 (16–257)
RAS III	39	400	246 (146–432)	27 (24–33)	100	89 (42–170)
RAS IV	39	410	95.5 (66.5–127)	20 (18–22)	100	68 (19–156)
RAS V	36	450	162 (111–218)	24 (21–27)	84 (57–84)	101 (35–216)
Total	**211**	—	**124 (50–432)**	**22 (15.5–33)**	**53–100**	**59 (16–257)**

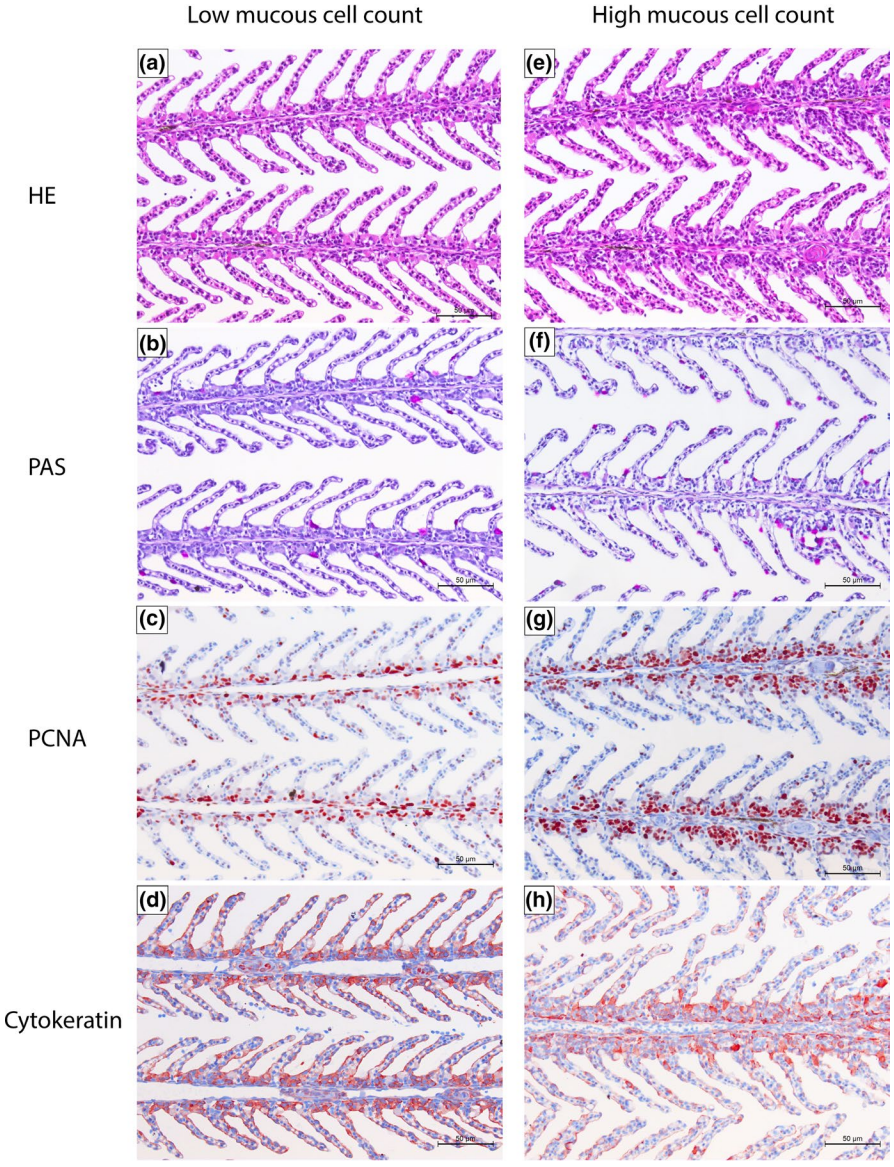

FIGURE 2 Histological investigations of gills with low (A-D) and high (E-H) mucous cell counts. A) HE stain showing gill filaments and lamellae of normal character. B) PAS stain showing sparse amounts of scattered PAS-positive cells on the lamellae. C) PCNA stain revealing proliferating cells at the base between the lamella (red colour). Scattered PCNA-positive cells are evident in the lamellae. D) Cytokeratin stain showing a dense epithelial network (red) in the interlamellar region. Cytokeratin reactivity is also evident in the respiratory epithelium of the lamellae. E) HE stain showing gills with a thickened and cell-rich filament. F) PAS stain showing multiple PAS-positive cells on the lamellae. A focal cluster of hyperplasia with many PAS-positive cells is evident in the lower right corner. G) PCNA stain revealing a proliferative response in the interlamellar region which appear thickened. H) Cytokeratin stain showing epithelial hyperplasia of basally located cells in the interlamellar epithelium. The stain appears less dense and organized than in image D. Pockets of non-epithelial cells are found within the epithelium

the six farms, and the median length of all fish was 22 cm (mean 22 cm). The median values of weight and length are described in Table 1.

3.2 | Water transparency

Water transparency varied between 54% and 100% sight depth in relation to the tank depth. The lowest and the highest records of sight depth in each farm are shown in Table 1. In three of the farms (FT I and RAS III-IV), the bottom of the tank was visible at both sampling points. The tank depth varied between 300 and 450 cm in the different farms (Table 1).

3.3 | Histopathological assessment

Histopathological changes were observed in only one fish with areas of subepithelial leucocyte infiltrates, epithelial cell hyperplasia and mucous cell hyperplasia.

3.4 | Immunohistochemistry—PCNA and cytokeratin

PCNA staining of gills with low mucous cell counts (Figure 2a and b) revealed proliferative cells at the base between lamellae, that is the interlamellar region of the filament (Figure 2c). Scattered positive cells were evident in the lamellae. Cytokeratin staining coincided with the PCNA stain, showing a dense red stain in the interlamellar region (Figure 2d). Additionally, the pavement cells of the lamellae were cytokeratin-positive. Gills with high mucous cell counts (Figure 2e and f) had a strong PCNA reaction in the interlamellar region, which also appeared thickened (Figure 2g). Cytokeratin staining of such gills was paler and more loosely organized than in gills with low mucous cell counts (Figure 2h). Infiltrates of other, non-epithelial cells were also evident in the interlamellar region.

3.5 | Mucous cell counts

The total number of mucous cells for each fish varied between 16 and 257, with a median of 59 (mean: 70.5). The median (min–max) values of mucous cell count from fish in each farm varied between 31 (21–73) as the lowest median and 101 (35–216) from the farm with the highest median value (Table 1 and Figure 3). The distribution of mucous cells in relation to fish weights is displayed in Figure 4. Further, the scatter plot in Figure 5 shows the relationship between mucous cell counts and fish length in each farm.

3.6 | Statistical analysis

The final regression model included "fish length" and "fish farm" as independent variables. "Fish length" and "fish weight" were highly correlated (0.96), and length was chosen because of higher explanatory power in the model. "Water transparency" was not statistically significant as a predictor of mucous cell count in the model. The final model had an adjusted R-square value of 0.44. If "fish farm" was removed, the adjusted R-square for the model was reduced to 0.23. Fitted values versus residuals indicated that the assumption of homoscedasticity was met, and the model residuals showed a normal distribution.

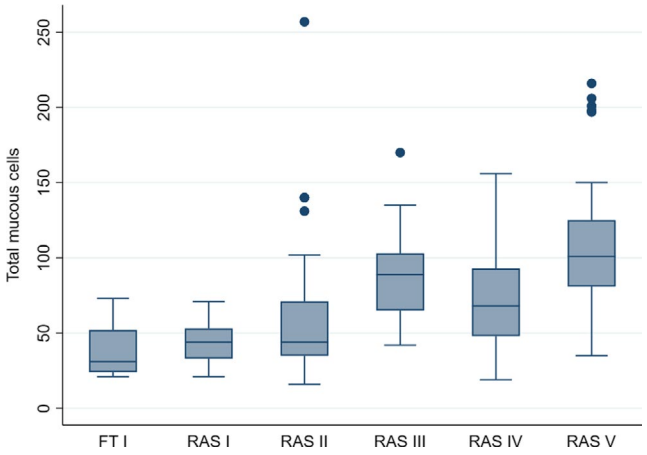

FIGURE 3 Distribution of total mucous cell count in each fish between the different farms

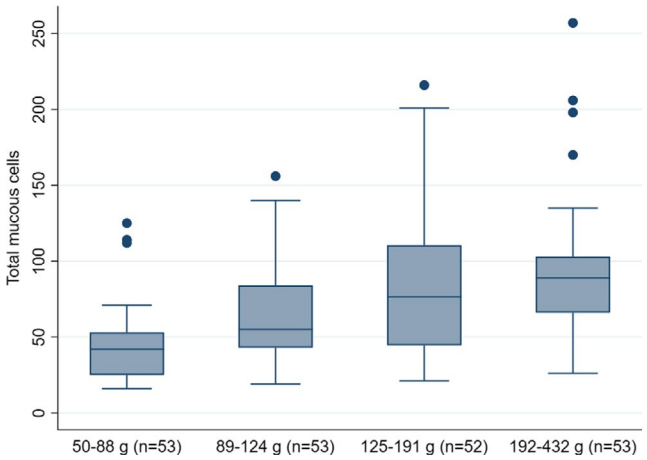

FIGURE 4 Total mucous cell count in relation to fish weight. The fish are split into equal groups by weight

4 | DISCUSSION

To our knowledge, this is the first study describing the variation in mucous cell count from gills of clinically healthy salmon reared in commercial fish farms in Norway. Based on earlier publications, we have developed a method of counting mucous cells from salmon gill histology samples. The results indicate that variations in the number of mucous cells depend on farm-related factors, when fish length is

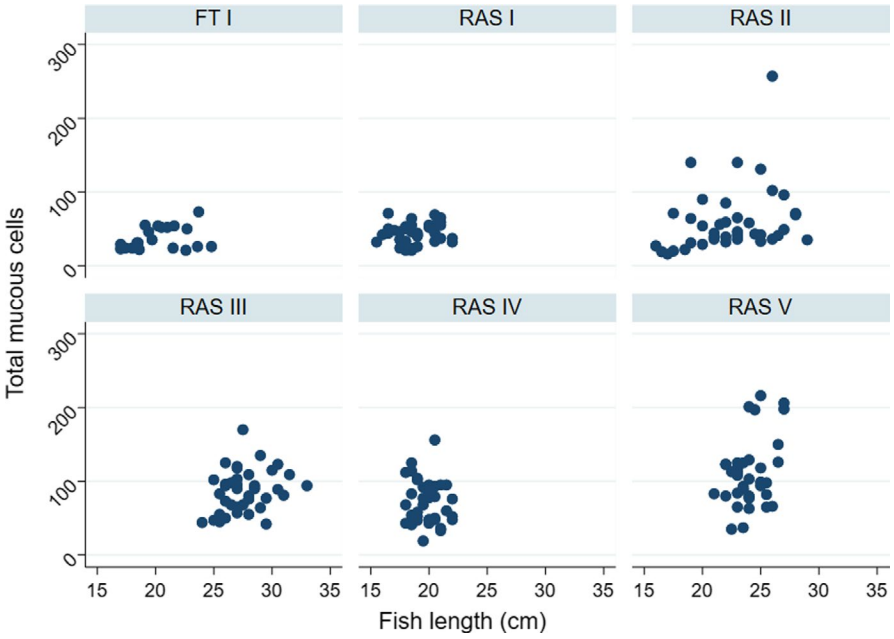

FIGURE 5 Scatter plot to show the distribution of total mucous cells counted in each fish by fish length

accounted for by the regression model. This suggests that factors related to the fish farm will have an impact on the number of mucous cells in each fish. Immunohistochemical investigations indicated an early proliferative response in the gill epithelium correlating with increased mucous cell counts.

Mucous cells can be identified by several different staining methods and can be quantified using different approaches. Ferguson et al. (1992) acquired the number of mucous cells per a given gill area, while Speare et al. (1997) described a mucous cell index based on total counts of all lamellae on a filament. Others have taken into account the size of each mucous cell, in addition to the distribution, in a stereology-based method as described in Pittman et al. (2011) and Dang et al. (2019). Based on the work by Ferguson et al. (1992) and Speare et al. (1997), we have developed a modified method, where mucous cells are counted on the gill lamellae according to given criteria (see Appendix 1). To ensure accurate cell counts, mucous cells in the interlamellar region were not included. The high cell proliferation rate in this region made counting of single PAS-positive cells difficult; thus, only the lamellae were counted. The method proved suitable for our study, and the output variable (mucous cell count) was owed significant results in the regression model.

In this study, the number of mucous cells in the gill lamellae of clinically healthy fish was investigated. According to our counting method, filaments with less than 20 symmetrical pairs of lamellae were rejected and thus not counted. The method therefore excluded gills with common pathological changes such as lamellar hyperplasia. However, if there were focal pathological changes in the gills, but the other areas of the filaments fulfilled the counting criteria, the sample was counted and included in the study. In total, only one out of 211 gill samples showed pathological changes, making it difficult to conclude on how focal pathological changes affected the number of mucous cells.

Our results indicate fish size is associated with the number of mucous cells found in the gills. This is not unexpected in order to keep a constant ratio of mucous cells versus epithelial cells during growth. However, regarding median values of mucous cells from each farm, fish from RAS I and RAS II had a median of mucous cells close to the FT farm (FT I), and the median of RAS V was more than twice as high as RAS I and RAS II. At the same time, the weight and length of the fish in RAS II and RAS V were similar, indicating that some other factor than the size must explain the variation in mucous cell counts observed between farms. No gill diseases were reported in any of the fish groups included in the study. In the regression model, size of the fish (fish length) and fish farm as fixed effects together accounted for 44% of the variation in mucous cell counts. Meanwhile, the size alone only accounted for 23% of the variation explained by the model. This means that in these data, when the size of the fish is accounted for, factors within the fish farm had a substantial contribution to the proportion of explained variation in the number of mucous cells counted from each fish. Noteworthy, the lowest mean of mucous cells was found in the FT farm. One could speculate that favourable environmental conditions in the flow-through environment coincided with a low mucous cell count. However, the result should be interpreted with caution, as the material from the FT farm was limited to 20 fish from one sampling. Regarding the matter of individual or farm-related factors, fish from FT I and RAS IV were equivalent in size but fish from RAS IV on average had double the mucous cell counts compared to the FT farm. However, further studies are needed to tease out which farm-level factors contribute to the variation in mucous cell count, as well as to establish causal pathways on how, for example, management and the environment in the farms affect the mucous cell numbers.

The plastic responsiveness of mucous cells makes them important first-line defenders in the epithelial lining of the gills. Mucous cells are modified, highly polarized epithelial cells that produce and secrete

mucins at their apical surface. Hyperplastic and metaplastic mucous cell responses are commonly seen in several infectious gill diseases (Ferguson, 2006) but also with other stressors such as formalin treatment or high ammonia concentrations (Ferguson et al., 1992; Speare et al., 1997). It thus seems likely that other alterations in the environment, for example variable water parameters, may affect mucous cell dynamics and epithelial cell homeostasis. The cellular response in gills with both high and low mucous cell counts, respectively, was investigated using IHC targeting PCNA, a conserved marker for proliferation. Gills with low mucous cell counts revealed PCNA-positive cells mainly restricted to the interlamellar region, consistent with the location of the stem cell niche of the lamellae (Ferguson, 2006). Cytokeratin staining confirmed that these cells were mainly epithelial cells with a prominent and dense staining pattern. Gills scored with a high mucous cell count showed marked proliferation in a thickened interlamellar region. Cytokeratin staining revealed a paler staining pattern of more loosely arranged epithelial cells, allowing the presence of, for instance, leucocytes, reflecting an inflammatory reaction. Taken together with the high mucous cell count, this indicates an early organ response and in this case possibly towards environmental factors.

Water transparency measurements with Secchi disc proved difficult to perform in land-based facilities. Strong water currents and variation in light conditions caused inaccuracies during measuring across the tanks and thus represent a source of error in this variable. In three of the farms, the bottom of the tank was visible at both sampling occasions, reducing the variation of this parameter in the data set. However, the results obtained indicate no relationship between water transparency in the tanks and mucous cell counts in the gills. Given the uncertainty of the water transparency measurements, these results should be interpreted with caution. Future studies on the matter should include alternative methods to assess water quality, for example turbidity.

In conclusion, this cross-sectional study shows a variation in gill mucous cell counts in between six different commercial salmon farms, and more than 200 healthy salmon. This suggests that mucous cell counts can become a monitoring tool for gill health in the future.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

DP planned the study, sampled the material, carried out statistical analysis and wrote the manuscript. HB planned the study, carried out histological investigations and wrote the manuscript. AF planned the study, sampled the material, counted the mucous cells and commented on the manuscript. LAH planned the study, sampled the material, counted the mucous cells and commented on the manuscript. EOK planned the study, evaluated immunohistochemistry and commented on the manuscript. AN contributed to drafting of the manuscript, aided in statistical analyses and commented on the manuscript. MS planned the study, carried out statistical analysis, supervised the study and commented on the manuscript.

DATA AVAILABILITY STATEMENT

The data set generated in the study is not included but is available from the corresponding author on reasonable request.

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APPENDIX 1

PRELIMINARY COUNTING METHOD

We specified the selection of filaments and lamellae by several criteria. To be suitable for counting, the criteria demanded normal morphological appearance of the 20–26 first (at the base of the filament) lamellae on both sides of the filament, including symmetry in length and width in lamellae. Thus, the basis for the mucous cell numbers in this paper was consistently obtained from proximal parts of the filaments. If the lamellae at the basis of the filament were irregular, the count could start more distal on the lamella (up to the 6th pair), given that the next 20 consecutive lamellae were acceptable for counting. Only lamellae fulfilling these criteria were counted; however, the selection of different filaments was random.

PRELIMINARY MUCOUS CELL COUNTS

To evaluate the counting method, 48 random slides were counted by two independent examiners. The mucous cell counts from each filament were summed, giving a total number of mucous cells for each fish. Examiner 1 (AF) had a median of 40.5 mucous cells (range 16–140) per fish, and examiner 2 (LAH) had a median of 44.5 mucous cells (range 13–150) per fish. The correlation between the examiners was 95.7%. The mean inter-examiner difference on each slide was 7.7 mucous cells (SE: 0.8). There was no apparent inter-relation between the inter-examiner difference and the number of mucous cells. Fourteen of the 48 slides had an inter-examiner difference of more than ten mucous cells, and among those, the mean inter-examiner difference was 14.9 (SE: 0.9).

Based on the preliminary counts, an additional criterion was added to specify the order of selection of filaments. The filament situated approximately in the middle of the angle of the gill arch was counted first, followed by the next filament according to the sequence shown in Figure 1. The counted regions had to have an intact filament with a symmetrical distribution of at least 20 lamellae; otherwise, the next filament (in dorsal or ventral direction, respectively) was selected for counting until three filaments were counted (Figure 1).

After re-evaluating the 14 slides with the additional criterion, four of the slides were rejected by both examiners. These slides were remade, resulting in three acceptable and one rejected slide. The rejected slide was discarded, leaving 13 slides in the group. The mean inter-examiner difference in this group decreased to 6.0 (SE: 0.8), and the mean inter-examiner difference among all slides in the evaluation ($n = 47$) decreased to 5.1 (SE: 0.4) mucous cells. The overall correlation between examiners improved to 98.1%. The 34 slides with an inter-examiner difference below ten mucous cells were determined to be adequate, and thus, these were not re-counted.

All remaining slides were counted by one examiner following the improved counting method, as described in Materials and Methods.