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# Ectopic epithelial cell clusters in salmonid intestine are associated with inflammation

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## Abstract

An epizootic incidence of intestinal adenocarcinomas was reported in brood fish of Atlantic salmon (*Salmo salar* L.) in 2009. The condition was associated with a specific diet inducing enteritis and morphological changes. Here, two field trials of fish up to slaughter size were initiated. In Trial 1, two different feed recipes were used. Feed I was predominantly based on marine ingredients, whereas plant ingredients were limited to soy protein concentrate and wheat. Feed II was lower in fishmeal and without soya protein, which was substituted with plant proteins from other sources. In Trial 2, a commercial feed (Feed III) was included. No macroscopic tumours were observed in 300 fish (Trial 1). At the end of both trials, samples from five different segments of the gastrointestinal tract of a total of 39 fish were investigated with morphological methods. Here, we show the presence of ectopic proliferating epithelial cells only occurring in inflamed intestine and predominantly in the second segment of the mid-intestine. Presence of ectopic epithelial cells in submucosal inflammatory foci may indicate early stages in tumorigenesis, but other possibilities such as proliferative enteric disorders cannot be excluded. Together with inflammation, carcinogenesis should be a focus of investigation in future feed trials.

**KEYWORDS**
Atlantic salmon, carcinogenesis, enteritis, inflammation, intestine, neoplasia

## 1 | INTRODUCTION

The advance of carnivorous fish species in aquaculture has been hindered by the limited availability of fishmeal and fish oil (FAO, 2014), and contemporary commercial feeds have instead used equivalent plant-derived ingredients (Powell, 2003). For the Atlantic salmon (*Salmo salar* L.), which in its natural habitat is strictly carnivorous, the increasing use of plant products in feed has been associated with morphological changes in the intestine (Bæverfjord & Krogdahl, 1996; Moldal et al., 2014). For the sustainability of the salmon industry, these disadvantages for fish health and welfare must be addressed (Olesen, Myhr, & Rosendal, 2011).

We have previously studied adenocarcinomas in farmed populations of Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) brood fish (Dale, Tørud, Kvellestad, Koppang, & Koppang, 2009). Chronic inflammation and intestinal adenocarcinoma were found in many brood fish fed a particular commercial feed containing several plant ingredients, but without soya protein. A follow-up survey of 10 brood fish farms found the same pathological changes in four farms, all using this same feed (Lyngstad et al., 2007). To our knowledge, there are no reports of intestinal adenocarcinoma in wild salmon. Further, epithelial changes were not detected in a recent characterization of the intestinal morphology in wild salmon (Løkka, Austbø, Falk, Bjerkås, & Koppang, 2013). It thus appears that the neoplasia

was associated with the novel plant feeds, but the nature of this link has not been resolved. Acute enteritis caused by diets with soya bean or pea protein has been described (Bæverfjord & Krogdahl, 1996; Penn, Bendiksen, Campbell, & Krogdahl, 2011) and has been linked to the content of saponins (Knudsen, Urán, Arnous, Koppe, & Frøkiær, 2007; Knudsen et al., 2006, 2008; Krogdahl et al., 2015). The saponin-induced enteritis was found in the hindgut (Bakke-McKellep et al., 2007). Modern commercial feeds use alcohol-extracted soya bean protein concentrates, which are virtually free from saponins.

As most feed trials in Atlantic salmon have been of relatively short experimental periods, chronic inflammation and its consequences are less well studied. Chronic inflammation and carcinogenesis are linked conditions, as in inflammatory bowel disease (IBD)-related carcinogenesis in humans (Mantovani, Allavena, Sica, & Balkwill, 2008; Odze, 2006). The sequence appears to be enteritis, epithelial dysplasia and then neoplasia. In salmon, there appears to be no studies addressing enteritis in connection with early carcinogenesis.

Here, we report the results of two long-term feed trials with respect to intestinal health under realistic sea-farming conditions, using fish up to slaughter size. The effect of replacing some of the marine fishmeal with plant protein was evaluated in both trials. In the second trial, an additional diet containing plant oil was included to ensure results relevant to current feeding practices. We show the occurrence of inflammation and ectopic, proliferating epithelial cells located predominantly, but not exclusively, in the second segment of the mid-intestine.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethical statement

The research reported in this study was approved by The Norwegian Directorate of Fisheries, allowance SF-B-48, and was carried out in accordance with national guidelines for field experiments on aquaculture fish.

### 2.2 | Feed trials

Two feed trials at two different seawater locations on the west coast of Norway were performed. Atlantic salmon in freshwater were routinely intraperitoneally injected with an oil-based vaccine and transferred to sea cages and randomly distributed in two parallel net pens with 50,000 fish per feed. Feed I was traditionally composed, containing predominantly fishmeal and fish oil, and the plant ingredients were limited to soy protein concentrate (alcohol extracted) and wheat. Feed II was lower in fishmeal and without soya protein, but with a combination of plant proteins from peas, faba beans and sunflower (Table 1), ingredients that have more recently been introduced into salmon feeds (Aslaksen et al., 2007). In Trial 2, a third, commercially available feed was included (Feed III). Feed III contained all the plant proteins in Feed I and Feed II, while two-thirds of the fish oil was replaced by canola oil (Table 1). Trial 1 ended after 24 months of

**TABLE 1** Differences in protein and oil sources between feeds. Feed I and II were used in Trial 1, whereas all three feed types were used in Trial 2

| Ingredients | Trial 1 and Trial 2 Feed I, % | Trial 1 and Trial 2 Feed II, % | Trial 2 Feed III, % |
|---|---|---|---|
| Fishmeal | 24.7 | 18.5 | 10.0 |
| Canola oil | | | 20.0 |
| Fish oil | 29.1 | 29.1 | 10.0 |
| Sunflower meal | | 4.0 | 7.5 |
| Faba bean meal | | 6.7 | 3.5 |
| Pea protein concentrate | | 17.0 | 5.0 |
| Soy protein concentrate | 20.0 | | 12.5 |
| Corn | | | 5.0 |
| Wheat gluten | 9.3 | 10.9 | 9.0 |
| Wheat whole | 11.3 | 10.0 | 5.0 |
| Other | | | 9.0 |
| Mineral/vitamin premix | 0.5 | 0.5 | 0.5 |
| Monocalcium phosphate | 1.4 | 1.3 | |
| Binders | 3.8 | 2.0 | 3.1 |
| Sum | 100 | 100 | 100 |

feeding, while Trial 2 terminated after 13 months due to an outbreak of viral pancreas disease (PD) that gives appetite loss and poor growth. PD was also suspected in Trial 1; however, this diagnosis was not confirmed. PD virus is endemic in this region. Importantly, the experiments were field trials conducted under normal industrial production settings and the possible presence of various infectious agents in these circumstances cannot be ruled out.

### 2.3 | Autopsy and sampling for histology

All samplings were performed in accordance with national Norwegian regulations for animal welfare (Forskrift om drift av akvakultur-anlegg §34. Avlivning av fisk). After seawater acclimatization and prior to use of the test feeds, samples were collected from 40 fish in trial 1 and from 13 fish in trial 2 (53 individuals in total). At the end of both trials, a sampling was conducted shortly before slaughter, but while the fish were still being fed. At slaughter, the first and second segments of the mid-intestine from 300 fish of each feed (I and II) in trial 1 lasting 24 months were opened and examined visually and by palpation for the presence of tumours. Such screening for macroscopic tumours was not conducted in trial 2 lasting 13 months. In total, 39 individuals were sampled from both trials (see Figure 1 and Table 2 for details). Fish were randomly collected from the two parallel net pens per feed, anesthetized and autopsied.

Samples were obtained from five different locations in the gastrointestinal tract (Figure 2). The cardiac stomach (CS), the pyloric caeca (PC) with collecting duct, the first segment of the mid-intestine posterior to the PC (FSMI), the second segment of mid-intestine (SSMI) with primary and secondary folds and the posterior intestinal segment (PS), according to anatomical nomenclature (Løkka et al.,
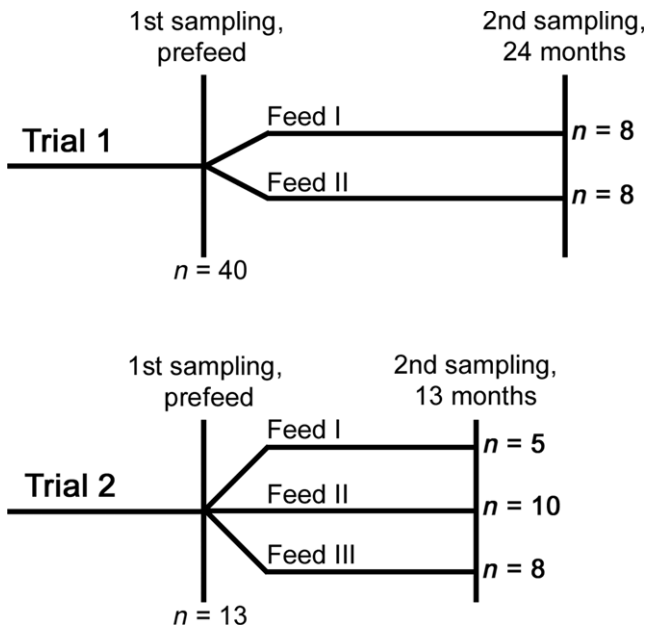
FIGURE 1 The study design. The first sampling was conducted prior to subjecting the fish to different feeds. The terminal sampling was conducted after 24 months in Trial 1 and after 13 months in Trial 2. The number of individuals sampled in different groups at different time points is indicated in the illustration

TABLE 2 Overview of sampling for histology from different feed groups: n = number of fish sampled; I = traditional salmon feed with marine fishmeal and oil and low levels of soya protein and wheat; II = as I but some fishmeal and all soya protein replaced by plant proteins from peas, faba beans and sunflower; III = commercial feed with all plant proteins used in I + II and some fish oil replaced by canola oil; n/a = not applicable

| | Trial 1 | Trial 2 |
|---|---|---|
| Feed | n | n |
| Prefeed | 40 | 13 |
| I | 8 | 5 |
| II | 8 | 10 |
| III | n/a | 8 |

2013). Tissue segments were fixed in 10% phosphate-buffered formalin for at least 24 hr, routinely dehydrated and embedded in paraffin.

## 2.4 | Histology

Histological sections were cut (2 μm) and stained according to standard procedures with haematoxylin and eosin (HE) for histological investigation and with periodic acid–Schiff (PAS) for detection of mucins. To investigate the extent of ectopic epithelial cells, serial sections (31 consecutive slides of 3 μm thickness) were made from one fish. Every fifth slide was stained with H&E. Additionally, every neighbouring slide was stained with PAS. Consequently, 14 sections were stained in total (seven with H&E and seven with PAS), with a distance of 12 μm between the stained slides.
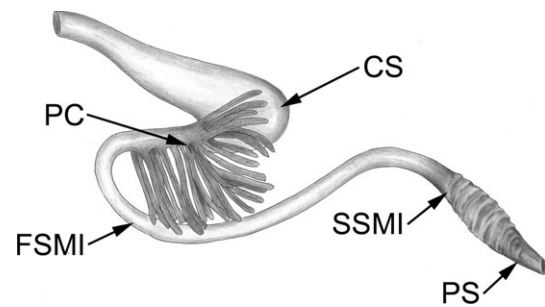


FIGURE 2 Drawing of the salmon gastrointestinal tract. Samples were obtained from five different locations (indicated in the figure): CS, cardiac stomach; PC, pyloric caeca with collecting duct; FSMI, first segment of the mid-intestine posterior to the PC; SSMI, second segment of mid-intestine with primary and secondary folds; PS, posterior intestinal segment

The paraffin-embedded material was further used for immunohistochemical investigations. Inflammation was assigned as a pathological description if infiltrating leucocytes were present in the lamina propria. A threshold between unaffected, normal tissue and affected, inflamed tissue was made solely on the presence of infiltrating leucocytes in the lamina propria. Individuals with focal, multifocal or diffuse inflammatory changes were all regarded as affected.

## 2.5 | Immunohistochemistry

For immunohistochemistry (IHC), 4 μm thick sections were mounted on glass slides (Superfrost©; Mentzel, Braunsweig, Germany), incubated for 24 hr at 37°C, dewaxed in xylene and rehydrated in graded alcohol baths (absolute alcohol, 96% alcohol and 70% alcohol) before transferring to distilled water. Antigens were next demasked by heat treatment (autoclave) in 0.01 M citrate buffer, pH 6.0 at 120°C for 10 min, followed by treatment with phenylhydrazine (0.05%; Sigma-Aldrich, St. Louis, MO, USA) for 40 min at 37°C to inhibit endogenous peroxidase. The slides were rinsed three times in phosphate-buffered saline (PBS). Non-specific binding was blocked by goat normal serum diluted (1:50) in 5% bovine serum albumin (BSA) in PBS. The primary antibody in 1% BSA/Tris-buffered saline (TBS) was added and incubated for 30 min in room temperature. The sections were then rinsed three times in TBS and further incubated with a secondary antibody (from EnVision© System kit; Dako, Glostrup, Denmark) for 30 min at room temperature. The slides were again washed three times in TBS, and the sections were incubated with AEC or DAB (from EnVision© System kit) for, respectively, 14 or 7 min to develop the colour (red or brown). All sections were washed with distilled water and counterstained with Mayer's haematoxylin for 1 min and mounted with polyvinyl alcohol media pH 8.0.

For identification of epithelial cells, cytokeratins were recognized using a monoclonal mouse pan-cytokeratin antibody, clone AE1/AE3 (Invitrogen, dilution 1:50). A monoclonal antiproliferating cell nuclear antigen (PCNA) antibody, clone PC10 (Dako), was used to detect proliferating cells (dilution 1:10,000). Polyclonal antisera recognizing MHC class II β chain (dilution 1:1,400) (Koppang et al., 2003) was

used to mark antigen-presenting cells. A monoclonal antisera recognizing trout CD3ε (dilution 1:400) (Boardman, Warner, Ramirez-Gomez, Matrisciano, & Bromage, 2012) was used to mark T-lymphocytes. Negative controls were performed using 1% BSA instead of the primary antibody. The SSMI of all individuals was analysed with the above-mentioned antibodies.

## 2.6 | Immunofluorescence—double-labelling

From each feed group, five individuals were randomly selected for IF analysis of the SSMI. Sections were dewaxed and demasked as described in the immunohistochemical protocol. Unspecific binding was prevented with 10% normal goat serum in PBS/0.5% Tween®80 (Sigma-Aldrich) for 30 min. A mix of two primary antibodies (recognizing MHC class II β chain and CD3ε) were added and incubated at 4°C overnight (Boardman et al., 2012; Koppang et al., 2003). Sections were then incubated with a mix of secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG) (dilution 1:400; Molecular Probes, Inc., OR, USA) for 60 min and mounted with polyvinyl alcohol media pH 8.2. The sections were investigated with a Zeiss Axio Imager M2 microscope (Carl Zeiss, Oberkochen, Germany), and images were obtained with a Zeiss Axiocam 506 mono using software Zen Pro 2012.

## 2.7 | Statistical analysis

Associations between intestinal segments and frequency of fish with inflammation (Table 4) and inflammation and ectopic epithelial cells (Table 5) were assessed by Fishers' exact test (Stata® software).

## 3 | RESULTS

None of the altogether 53 fish sampled prior to the two trials displayed any macroscopic or histological intestinal changes.

## 3.1 | Screening for intestinal tumours on slaughter, Trial 1

On slaughter after 24 months of feeding, none of the 300 fish fed Feed I or II displayed macroscopically observable or palpable intestinal changes in the gastrointestinal tract.

## 3.2 | Histological characterization of inflammation and ectopic epithelial cells in both trial 1 and trial 2

Although no macroscopic changes were observed, alterations of the tissue architecture were detected by histology. The five intestinal segments of all fish were evaluated for the occurrence of inflammation and ectopic epithelial cells in the subepithelial tissues. No changes were detected in the stomach cardia or in the PS ("rectum"). The PC were negative for both inflammation and ectopic epithelial cells except in one individual with such changes in the ceca as well as in the SSMI.

The SSMI with primary and secondary folds was most frequently affected by inflammation or ectopic epithelial cells among the four proximal segments (Table 3). One fish diverged from this pattern as both inflammation and ectopic epithelial cells were detected in the FSMI while no affection of the SSMI was found. Typical changes in affected gut included clustered epithelial cells in the connective tissue of the lamina propria (Figure 3a), in the space between the basal lamina of the folds and *stratum compactum*, that is, outside the epithelium and thus termed "ectopic" (Figure 3b). Serial sections of ectopic clusters confirmed that the cells truly were ectopic and without connection to the epithelium (data not shown). Single ectopic cells were occasionally seen and also epithelial cells penetrating the basal membrane of the intestinal folds were detected, indicating that these cells are in the process of becoming ectopic (Figure 3c). No ectopic epithelial cells were found on the serosal side of the solid, collagenous *stratum compactum*. The amount of mucin in the ectopic cells varied, but was often present in large quantities (Figure 3d). In H&E sections, ectopic cells were most easily detected when present in distinct clusters. Most often, there were multiple clusters together (Figure 4a), but some single clusters were also observed (Figure 4b). The cells showed a moderate-to-severe pleomorphism, exhibiting a high nucleus/cytoplasm (N/C) ratio (Figure 4c) and intense proliferative activity as demonstrated by PCNA staining (Figure 5a). The staining also revealed dysplastic changes with cell nuclei present from the basement membrane and up to the luminal border, demonstrating loss of epithelial intracellular organization (Figure 5a). Cytokeratin immunostain was strong in most ectopic cells (Figure 5b), in keeping with cells of epithelial origin. Fish devoid of both inflammation and ectopic epithelial cells showed few PCNA-positive cells in the lamina propria; however, epithelial changes could still be encountered (Figure 5c). Cytokeratin-positive cells were not detected in the lamina propria of fish without inflammatory changes and ectopic epithelial cells (Figure 5d). Serial sections stained for PAS, cytokeratin and PCNA showed colocalized reaction in ectopic cells for the characteristics of epithelium, mucus and proliferation (Figure 6).

**TABLE 3** Numbers of individuals with presence of inflammation/ectopic epithelial cells, respectively, in the five gastrointestinal segments in 39 examined salmons from both trials

| Feed | Trial | Cardia | PC | FSMI | SSMI | PS |
|---|---|---|---|---|---|---|
| I *n* = 13 | 1 and 2 | 0/0 | 0/0 | 2/1 | 6/3 | 0/0 |
| II *n* = 18 | 1 and 2 | 0/0 | 2/1 | 1/0 | 13/8 | 0/0 |
| III *n* = 8 | 2 | 0/0 | 1/0 | 3/0 | 6/4 | 0/0 |

FSMI, first segment of the mid-intestine; PC, pyloric caeca; PS, posterior intestinal segment; SSMI, second segment of mid-intestine.
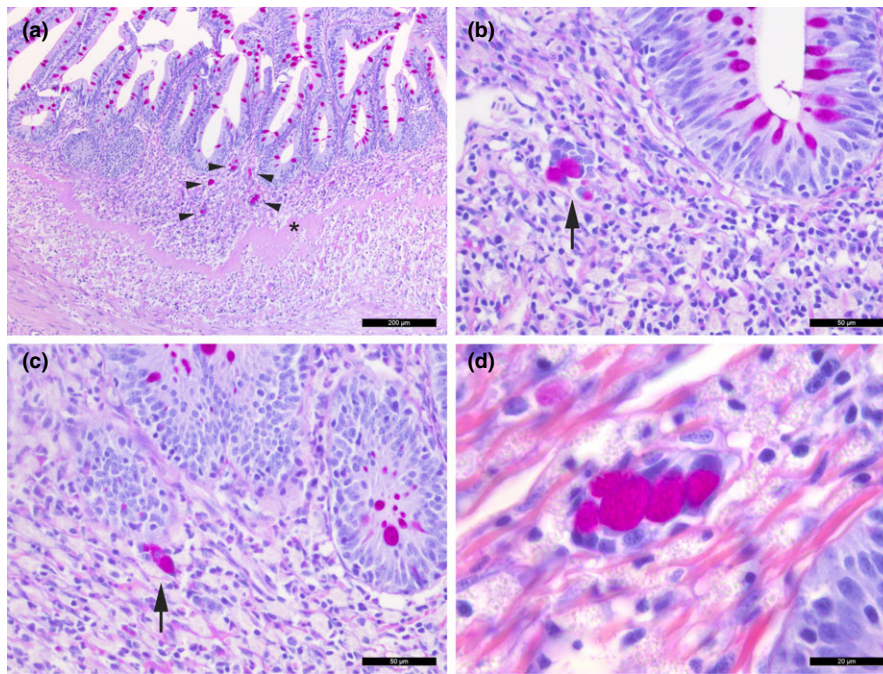
**FIGURE 3** Histological investigations of periodic acid–Schiff (PAS)-positive cells in the lamina propria of the second segment of mid-intestine. PAS stain. (a) A focal thickening containing multiple clusters of PAS-positive cells (arrowheads). The *stratum compactum* is indicated with asterisk. Note the irregular appearance of the epithelium in the nearby fold bottom. Fish from Trial 1, Feed 2. (b) A cluster of PAS-positive cells (arrow) located in close proximity to the basal end of a primary fold. Fish from Trial 2, Feed 2. (c) PAS-positive cells (arrow) penetrating the basal lamina of a primary fold. Fish from Trial 2, Feed 3. (d) High-resolution image of a cluster of PAS-positive cells. Fish from Trial 2, Feed 3. (a) scale bar = 200 μm; (b, c) scale bar = 50 μm; (d) scale bar = 20 μm

## 3.3 | Immunohistochemistry and IF for immune cells

Sections stained for MHC class II β chain and CD3ε chain displayed immuno-positive cells in the epithelium and in the lamina propria, either as focal or multifocal infiltrates (Figure 7). Severe inflammation was occasionally present, characterized by a general thickening of the lamina propria and infiltrates of both antigen-presenting cells and T-lymphocytes (Figure 7c and f). In addition, scattered immunopositive cells were detected beneath the *stratum compactum* (Figure 7c and f). Immunofluorescence (IF) double-labelling displayed a similar staining pattern as the IHC, as CD3ε$^+$ and MHC class II$^+$ cells were detected adjacent to each other in the epithelium and in the lamina propria (Figure 8). Double immune-positive cells for both MHC class II and CD3ε were not observed.

## 3.4 | Statistical analysis of colocalization of ectopic epithelial cells and inflammation

The difference in frequency of inflammation and ectopic epithelial cells between the SSMI and the three more proximal segments was highly significant (Fisher exact test $p = .001$) (Table 4). The presence of leucocyte infiltrates in the lamina propria was evaluated and compared to the presence of ectopic epithelial cells. There was a significant association between inflammation and the presence of ectopic

epithelial cells ($p < .001$), as no ectopic cells were detected in fish devoid of inflammation (Table 5).

## 4 | DISCUSSION

We have in this paper described an inflammation in the SSMI in sea-farmed Atlantic salmon fed plant protein and shown an association between inflammation and ectopic epithelial cells. Ectopic epithelial cells were never detected if inflammation was not present. The phenomenon was observed with three different diets. In wild-caught fish, ectopic epithelial cells have not been observed (Løkka et al., 2013). In our previous study (Dale et al., 2009), adenocarcinomas were often large and macroscopically visible, while smaller tumours had desmoplasia—a hard, fibrous stroma that made them easily palpable in the soft intestinal tissue. Screening for macroscopic changes revealed that four of ten brood fish populations were affected (Lyngstad et al., 2007). In the present study, no such changes were detected when screening for tumours at slaughter. Further, we performed histological investigations on a smaller number of fish for in-depth qualitative studies addressing several intestinal segments. While histological examinations showed no pathological changes before the start of the feed trials, we found inflammation and ectopic epithelial cells in all groups at slaughter. Such changes, previously not reported in slaughter-sized fish, may have serious implications for both fish health and ethical considerations.
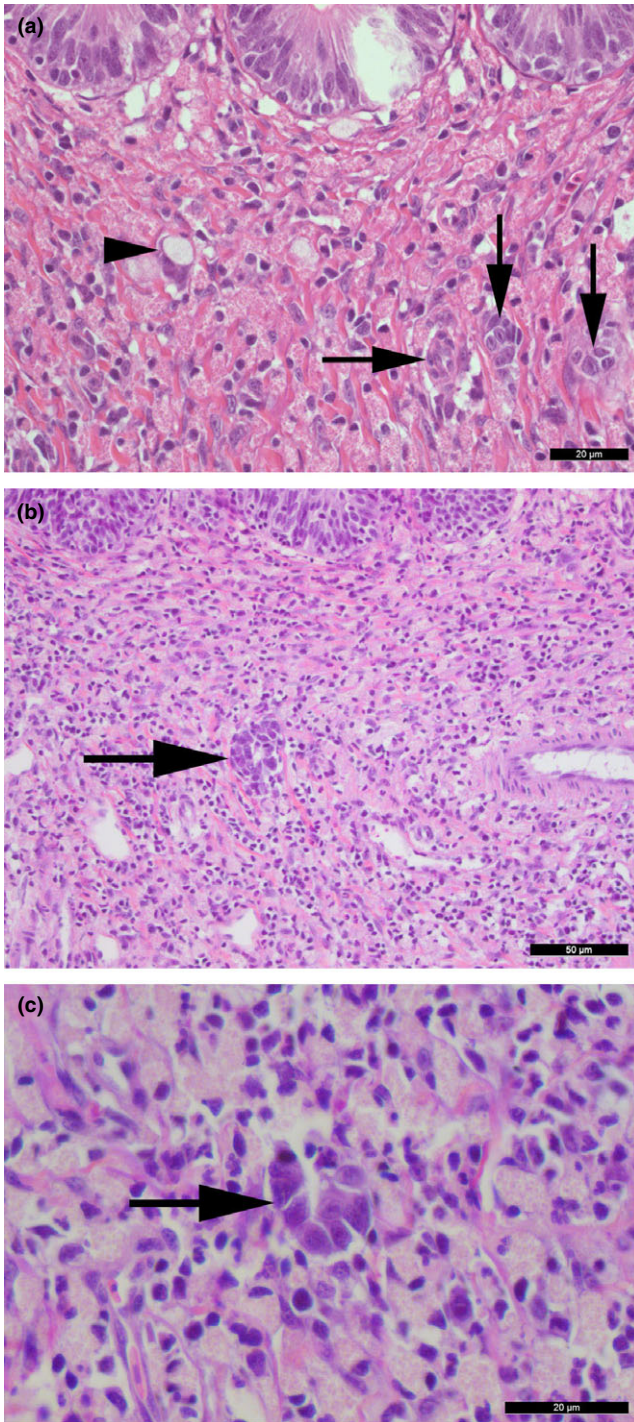
**FIGURE 4** H&E images of ectopic epithelial cells from three different individuals. (a) Multiple clusters of ectopic, hyperchromatic epithelial cells (arrows) are present in the lamina propria. A possible mucus-containing cell is indicated (arrow head). Fish from Trial 2, Feed 1. (b) A cluster of ectopic epithelial cells (arrow) in the depth of a heavily inflamed lamina propria. Fish from Trial 2, Feed 3. (c) A single cluster of ectopic epithelial cells surrounded by inflammatory cells. Note the hyperchromatic phenotype and high N/C ratio. Fish from Trial 2, Feed 2. (a, c) scale bar = 20 μm, (b) scale bar = 50 μm

Ectopic epithelial cells in the SSMI were detected in 15 of 25 individuals with inflammatory changes. We deliberately use the term ectopic to state only that epithelial cells beneath the epithelial basement membrane, that is, outside the epithelium, are located in an abnormal position. Serial sections showed the ectopic cells to be clustered and not strings of cells connected to the regular epithelium. The presence of cytokeratin and mucus was used as indicators of their epithelial nature, and no MHC class II expression was observed in such cells. Beneath the epithelial basement membrane, the presence of mucus that cannot be secreted to the intestinal lumen is clearly pathological and also made the cells easily recognizable. In IBD, epithelial dysplasia precedes extra-epithelial changes. In our study, we observed epithelial dysplasia with cell nuclei present from the basement membrane and up to the luminal border. However, registering the presence of dysplasia in all individuals proved more difficult and inaccurate than registering ectopic cells, primarily due to the highly branched intestinal folds, often resulting in skewed sections through the epithelium. Proliferation and invasiveness are important hallmarks for malignancy, and intramucosal adenocarcinomas are most often characterized by the presence of single cells or small glandlike structures that infiltrate the submucosa (Odze, 2006). The ectopic epithelial cells appeared motile, unpolarized and strongly positive for PCNA, indicating high proliferation activity. They often displayed cell atypia in the form of a high nucleus/cytoplasm ratio. Taken together, the ectopic epithelial cells could represent the first, easily recognizable sign of tumourigenesis.

The histological results alone do not prove that the ectopic cells are neoplastic. Epithelial cells respond efficiently to damage, becoming migratory and proliferating to cover denuded surfaces before settling as ordinary, surface epithelium again. This change back and forth from an epithelial to a mesenchymal phenotype, often-designated epithelial–mesenchymal transition (EMT), is normal during embryogenesis and in response to tissue damage (Hay, 1995; Kalluri & Weinberg, 2009). On the other hand, EMT has been linked to the development of epithelial cancer. The mesenchymal features of motility coupled with the enormous proliferative capacity of epithelium could be important to the often-malignant metastasis of many epithelial cancers (Kölbl, Jeschke, & Andergassen, 2016). Indeed, the brood fish adenocarcinomas did metastasize (Dale et al., 2009). In the younger slaughter fish in the present study, the ectopic epithelial cells were present in an inflamed lamina propria. This inflammation could compromise the integrity of the stem cell niche of the fold bottoms leading to EMT and abnormal location of epithelial cells and provide a carcinogenic environment.

Not all enteric inflammatory conditions with changes of the types seen in this study develop into malignancy. In porcine proliferative enteropathy (PPE), a common disease in swine production, epithelial changes in the intestine can resemble a neoplasm. Histologically, epithelial dysplasia is evident and infiltration into the underlying tissue is occasionally observed (Roberts, Rowland, & Lawson, 1980). Typically, an immature epithelium extends into the lamina propria and to the organized lymphoid tissue (Peyer's patches) of the submucosa. Intestinal epithelial cells have occasionally been found in mesenteric lymph nodes, thus ectopic. In PPE, the protrusion of adenomatous epithelial cells into the lamina propria and the related
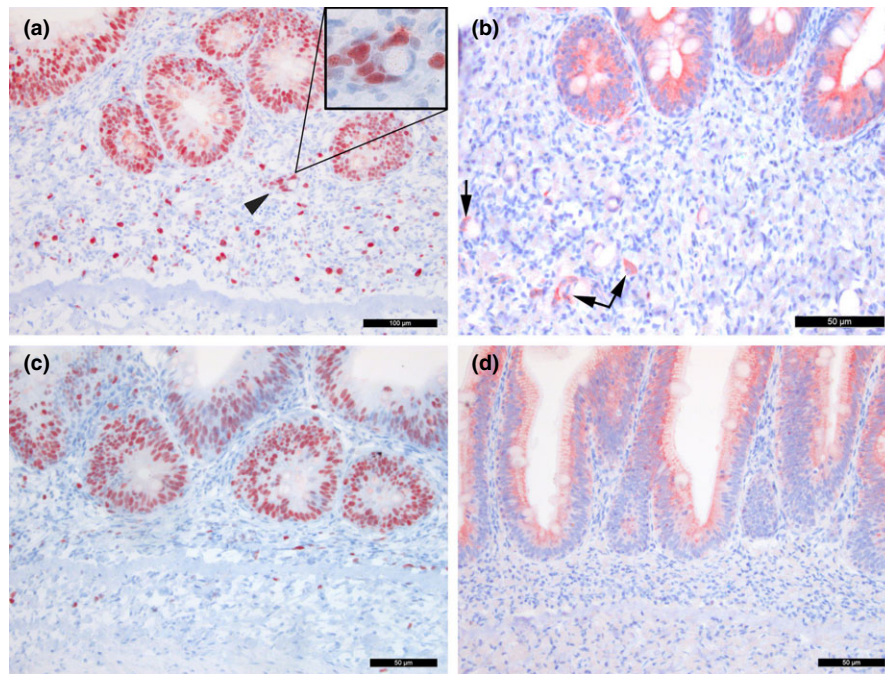
**FIGURE 5** Immunohistochemical investigations of ectopic epithelial cells in the second segment of mid-intestine. (a) A cluster of proliferating cell nuclear antigen (PCNA)-positive cells (arrowhead). The lamina propria is generally thickened and contains several single PCNA-positive cells. Note that the irregular epithelium in the fold bottoms is highly positive. High-resolution image in the upper right corner. Fish from Trial 2, Feed 2. (b) Several cytokeratin-positive cells in the lamina propria. The epithelium displays strong cytokeratin reactivity. Fish from Trial 2, Feed 2. (c) Few PCNA-positive cells are present in the lamina propria. The epithelium in the fold bottoms is PCNA-positive and appears irregular. Fish from Trial 1, Feed 1. (d) Cytokeratin staining is evident in the epithelium of the folds. No positive cells are seen in the lamina propria. Fish from Trial 2, Feed 1. (a) scale bar = 100 μm, (b–d) scale bar = 50 μm
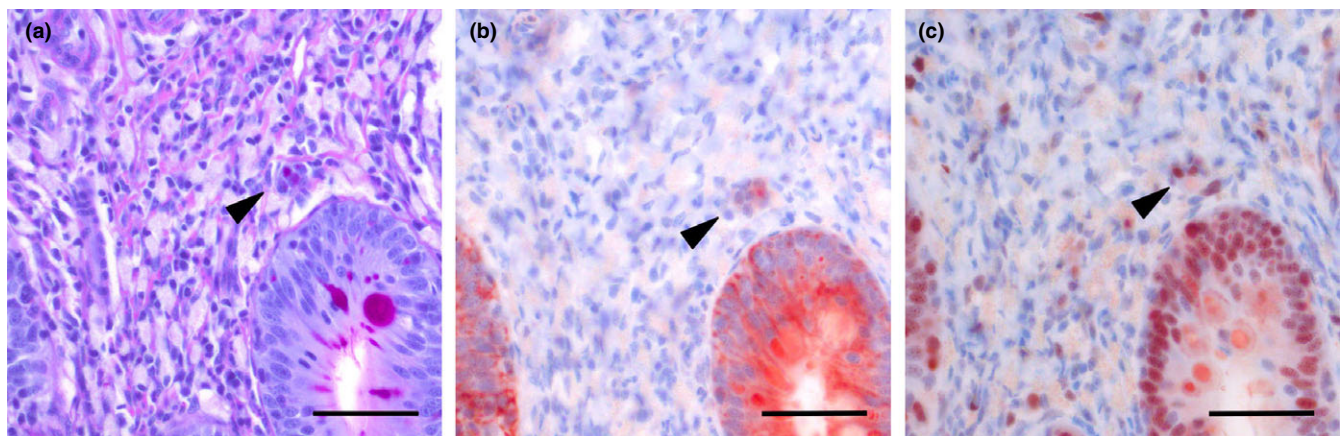


**FIGURE 6** Serial sections of a focal cluster of ectopic cells (arrowheads) in the lamina propria of the second segment of mid-intestine. (a) Periodic acid–Schiff staining demonstrating mucinous content in ectopic cells. (b) Ectopic cells appear as cytokeratin-positive. (c) Mitotic activity in ectopic cells shown by PCNA staining. Fish from Trial 2, Feed 2. (a–c) scale bar = 50 μm

inflammatory response is associated with a massive bacterial infection. Although this condition resembles a neoplasm (histologically), its behaviour is that of an infectious disease (Roberts et al., 1980). Most cases of PPE are self-limiting due to the elimination of the bacteria. In comparison, the feed exposure in our trials was constant and thus persisted for the entire experimental period.

Both inflammation and ectopic epithelial cells were each highly prevalent and significantly associated in the SSMI. Investigations of

the normal distribution of MHC class II, most prominent in professional antigen-presenting cells, and CD3ε, which is a T-cell specific molecule (Koppang et al., 2003, 2010; Løkka et al., 2014), provided the basis for our characterization of the inflammatory response. Here, we showed that the thickening of the lamina propria was mainly due to the presence of MHC class II+ cells and T cells. In humans, T cells may express MHC class II (Kambayashi & Laufer, 2014). Their presence in fish is unknown; however, no such
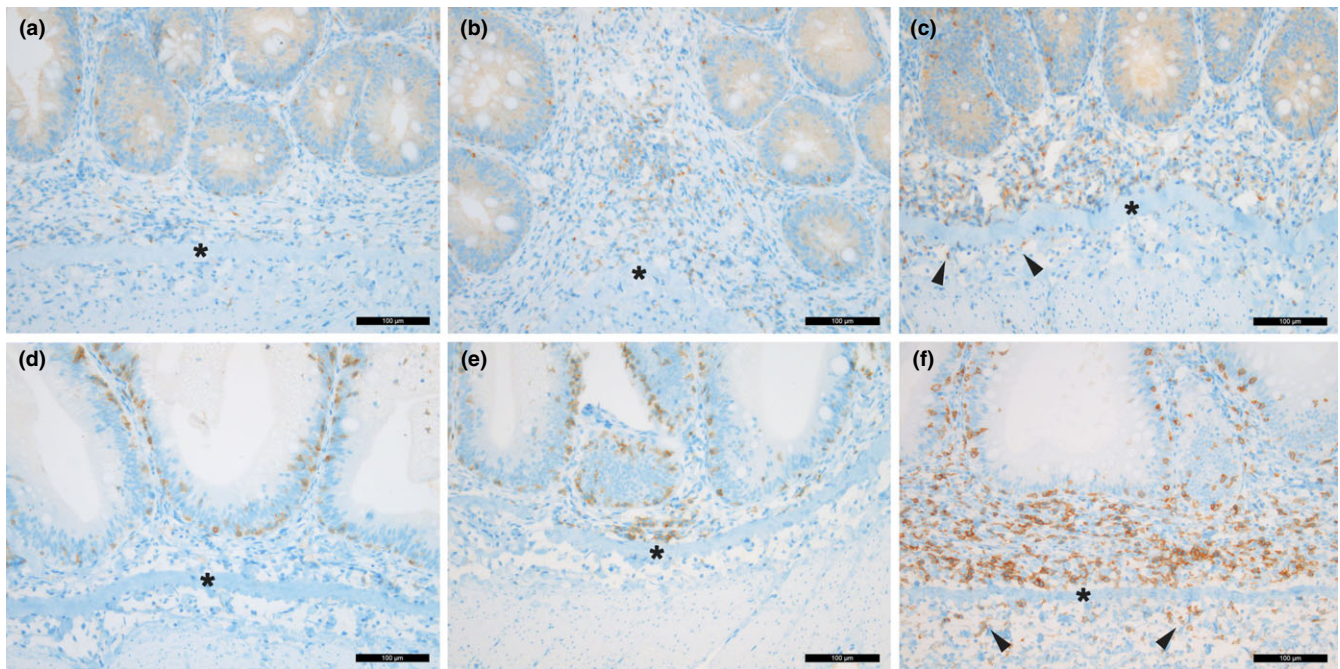
**FIGURE 7** Immunohistochemical investigations of Atlantic salmon second segment of mid-intestine. The *stratum compactum* is shown with an asterisk. (a) Normal distribution of MHC class II^{+} cells, mainly located basally in the epithelium and with negligible amounts in the lamina propria. Fish from Trial 2, Feed 1. (b) A focal infiltrate of MHC class II^{+} cells in the lamina propria. Fish from Trial 2, Feed 2. (c) Diffuse inflammation with abundant MHC class II^{+} cells. Immunopositive cells also appear basal to the *stratum compactum* (asterisk), though in a sparse amount. Fish from Trial 2, Feed 2. (d) Distribution of CD3ε^{+} cells intraepithelially and basal to the enterocyte nuclei in non-inflamed intestine. Fish from Trial 2, Feed 1. (e) A focal infiltrate of CD3ε^{+} cells beneath the epithelial basal membrane. Fish from Trial 2, Feed 3. (f) Diffuse inflammation (not restricted to the lamina propria) with vast amounts of CD3ε^{+} cells, mostly above, but also under the *stratum compactum* (asterisk). Fish from Trial 2, Feed 2. (a–f) scale bar = 100 μm
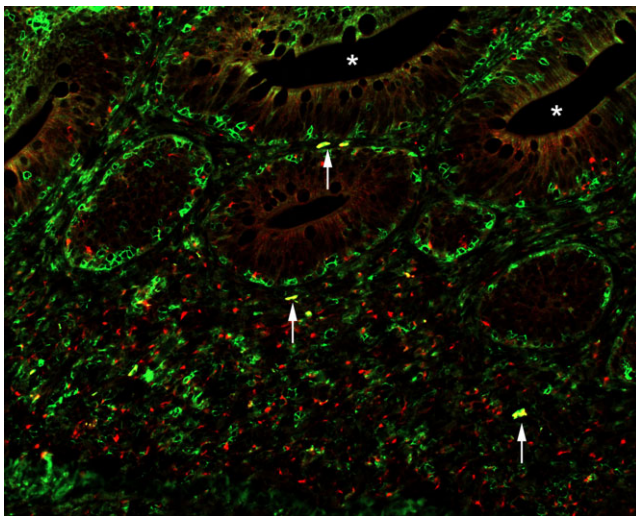


**FIGURE 8** Immunofluorescence double-labelling with antibodies recognizing MHC class II and CD3ε molecules. Diffuse inflammation in the lamina propria, with infiltrates of lymphocyte-like MHC class II^{+} cells (red) and CD3ε^{+} cells (green). The fluorescent signals do not overlap. Autofluorescence was detected in erythrocytes (arrows). Intestinal lumen is indicated (asterisks). Fish from Trial 1, Feed 2

**TABLE 4** Inflammation (A)/ectopic epithelial cells (B) and gastrointestinal segment in 39 examined salmons. Significantly more frequent inflammation and ectopic epithelial cells ($p$ = .001, Fisher's two-sided exact test) in the second segment of mid-intestine (SSMI) than in the three more proximal segments

|  | Anatomical location | |
|---|---|---|
|  | 3 more prox segments[a] | SSMI |
| **(A)** | | |
| Inflammation | | |
| Yes | 9 | 25 |
| No | 30 | 14 |
| **(B)** | | |
| Ectopic cells | | |
| Yes | 2 | 15 |
| No | 37 | 24 |

[a]Cardia, pyloric caeca and first segment of the mid-intestine.

The inflammation was not evenly distributed throughout the gastrointestinal tract. In the cardia, no inflammation was detected. In the PC, three of 39 individuals displayed inflammatory changes, while a few fish had inflammation in the PC or in the FSMI. In contrast, 25 of 39 fish had inflammation of the SSMI. These results identify the SSMI as the main location of feed-related intestinal inflammation. In another study (Van den Ingh, Krogdahl, Olli,

expression was observed in the present inflammatory changes. The diagnosis of mural enteritis is thus justified by several criteria, with changes similar to that of IBD in humans (Odze, 2006).

**TABLE 5** Inflammation and ectopic epithelial cells in the second segment of mid-intestine of 39 examined salmons. ($p < .001$)

| Inflammation | Ectopic epithelial cells | |
| --- | --- | --- |
| | Yes | No |
| Yes | 15 | 10 |
| No | 0 | 14 |

Hendriks, & Koninkx, 1991), a group of salmon fed a diet containing full-fat soya bean meal showed inflammatory changes only in the distal intestine, corresponding to the SSMI according to new anatomical nomenclature (Løkka et al., 2013). In our study, inflammation also occurred in other segments of the gastrointestinal tract. Nevertheless, we point to the SSMI as the obvious region of choice for studies addressing feed-related intestinal inflammatory responses, in keeping with previous results from our group (Løkka et al., 2014).

We can only speculate about the reasons behind the predilection of inflammation for the SSMI. Several studies have found the teleost SSMI as the most immunologically active segment, with endocytosis and antigen uptake (Løkka & Koppang, 2016). Further, in mammals, the mucus layer covering different regions of the gastrointestinal tract varies significantly according to the function of the particular location. We do not know of any similar studies in fish, but there are reasons to believe that the same phenomenon exists here, explaining different antigen access to different regions of the intestine (Løkka & Koppang, 2016). In a murine experimental model for human IBD, changes are located in the colonic segment and it has been shown that the barrier effect of the mucus is lost, allowing the rich, normal microbiota of this segment to provoke inflammation (Johansson, Larsson, & Hansson, 2011; Schütte et al., 2014).

In the present study, ectopic epithelial cells were detected in all three diet groups and in two different field trials. The common denominator for the presence of ectopic epithelial cells was thus inflammation. The influence of a possible salmonid alphavirus infection (or any other infectious agent) remains speculative. Importantly, two different research populations at separate locations shared the same pathological changes with the main conclusion being that ectopic epithelial cells do not occur in the absence of inflammation. The prevalence of histological changes varied between the different diets, with a trend to more changes as the level of plant ingredients increased. However, even with the more traditional diet based on mainly marine ingredients, some inflammation and ectopic epithelial cells did occur. This could indicate that even the soya protein or wheat in the more traditional feed is not completely safe, as ectopic epithelial cells have not been detected in the intestines of wild salmon (Løkka et al., 2013). Some background level of enteritis due to unrecognized reasons is also to be expected during realistic, long-term feed trials. Even though ectopic epithelial cells were not detected in wild salmon, enteritis did occur (Løkka et al., 2013). Experimentally, stressful husbandry conditions for Atlantic salmon have been shown to increase intestinal epithelial permeability, which may have serious consequences if the feed contains potentially harmful components (Sundh et al., 2010). Such combinatory effects

are unpredictable, and long-term realistic feed trials are necessary to investigate the safety of modern aquaculture practices.

As the number of new components in salmon feed will likely continue to increase, intestinal changes like inflammation and ectopic cells could become a growing concern in aquaculture production. Safety testing will benefit from the knowledge of the appearance, location and mechanisms of the intestinal changes that we have reported here. Our results stress the necessity of applying histological approaches for such testing.

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