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RESEARCH ARTICLE

Evaluation of histological post-mortem changes in farmed Atlantic salmon (*Salmo salar* L.) at different time intervals and storage temperatures

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Abstract

The aim of this study was to evaluate histologic post-mortem autolytic changes in farmed Atlantic salmon. The fish were either stored at room temperature (RT, 21°C), refrigerated (4°C) or frozen (−20°C), while fish necropsy was performed at 0, 1, 4, 24 and 48 h post-storage (hps). In addition, gills were sampled at 0, 5, 10, 15, 30 and 45 min post-storage (mps) at room temperature (RT). The haematoxylin and eosin-stained tissue slides were evaluated and scored by using a semi-quantitative scoring system. Our findings demonstrated gills and pyloric caeca/pancreas as the most severely autolysed organs while heart and skeletal musculature were least affected. Generally, moderate to severe autolysis appeared first at 4 hps, while severe changes were seen at 24 hps. Gills demonstrated autolytic changes as early as 10 mps and pyloric caeca/pancreas at 1 hps. Freezing did not prevent the autolysis and even contributed to freezing artefacts, which may lead to misdiagnosis. Keeping organs refrigerated slowed the autolytic progress within the first 4 hps marginally. This study recommends gills and pyloric caeca/pancreas should be sampled as early as possible, at least within 10 min post-necropsy.

KEYWORDS
Atlantic salmon, autolysis, gills, histology, temperature, time

1 | INTRODUCTION

Immediate fixation is required for best preservation of biological cells and tissues for preparation of histological sections (Ferguson, 2006). Fixation preserves cellular and tissue morphology, minimizes the loss of molecular components and prevents decomposition, autolysis (self-digestion) and microbial growth (Bancroft & Gamble, 2008). Biological tissue is often fixated in media consisting of aldehydes, alcohol and acid-based solutions. The standard tissue fixative is 10% neutral buffered formalin (NBF) (Bancroft & Gamble, 2008; Slaoui & Fiette, 2011). In literature, there is a little information available regarding preservation of fish carcasses to minimize post-mortem autolytic changes, when necropsy is delayed and cannot be immediately performed.

Degradation of tissues after death results from three basic mechanisms: enzymatic autolysis, oxidation and microbial growth. Chemical and biological changes occur shortly after sacrifice when endogenous enzymes are released, due to loss of cellular integrity and breakdown of cell membranes (Huss, 1995). When considering fish tissue, several factors are involved in the formation of post-mortem

changes such as bacterial flora, handling/stress, seawater/ freshwater, life stage, health status, type of storage, temperature and pH (George et al., 2016; Heil, 2009; Mukundan et al., 1986). A few specific organs such as fish gills and the intestinal tract are known to be particularly vulnerable to autolysis (Mallat, 1985; Speare & Ferguson, 1989), while other organs, for example heart and skeletal muscle, show slower rates of degradation. Fish organs display faster degradation rates than mammalian organs (Ferguson, 2006). A possible explanation is the fish cells utilize glucose and glycogen from stress during handling, which in turn increases the levels of lactic acid and decreases pH. This will then promote autolysis (self-digestion) at the time of death (Gatica et al., 2008). The complex tissue structures of vertebrate organisms such as the fish body consists of different bodily components (i.e. proteins, fats [lipids] and nucleic acids etc.) that during autolysis are broken down or hydrolysed into their simple building blocks. The end products of such hydrolytic reactions become a nutrient-rich medium for the growth of microbes. Depending on whether the organism is uniformly endothermic ('warm-blooded') or poikilothermic ('cold-blooded') in addition to surrounding temperature, different rates of autolyzing processes may occur (Khuntia, 2009).

One major difference between mammals and cold-water fish such as Atlantic salmon is the body temperature. Mammals maintain constant internal temperatures and are often informally classified as endothermic. Most fish, however, are classified as poikilothermic and change their internal temperature according to the temperature of their surrounding environment (Walton & Cowey, 1982). Following the death of an endothermic animal, the body's natural processes of heat generation is 'turned off' and the internal temperature will most often decrease in a timely manner to the surrounding temperature. The surrounding temperature is usually lower than the body's core temperature who often vary between 36 and 40°C. This process will naturally preserve the tissues by reducing the rate of post-mortem degradation (Brooks, 2016). For cold-water fish, however, the opposite situation occurs. During sampling, fish are brought out of cold water, killed and left in an environment where the temperature may be higher than the original water temperature. This could increase the rate of post-mortem changes and may be one of the main reasons for why organs from fish are known to autolyze more quickly than mammalian organs (Ferguson, 2006; Heil, 2009; Mukundan et al., 1986; Roberts, 2001).

In Norway, the salmon farming industry may experience a wide seasonal change in temperature both in air and water. In addition, the farms are located geographically along a long coast line. This creates variance in storage conditions such as temperature and storage time that may affect tissue preservation. In 2020, the Norwegian Veterinary Institute section of fish diagnostic received a total of approximately 815 diagnostic cases for fish histology, of which 142 cases (17.4%) had registered autolytic or post-mortem changes within the sampled fish tissue (Geir Bornø, personal communication, April 09, 2020). Such changes decrease the sensitivity to detect microscopic lesions and can make the histologic interpretation difficult and may lead to misinterpretation of pathological changes in the healthy tissue (Dettmeyer, 2018) or missed diagnoses (Wolf et al., 2015). The microscopic diagnosis of autolytic cells and tissue requires a particularly high level of experience in microscopy (Dettmeyer, 2018), especially to differentiate autolysis from the necrotic lesions.

The aim of this work was to study autolytic changes in various organs from farmed Atlantic salmon (*Salmo salar*) stored at different temperatures over different time intervals. This was done in order to document early post-mortem changes, which may develop when sampling fish tissues in the field.

2 | MATERIALS AND METHODS

2.1 | Fish

The fish used in this study were clinically healthy farmed pre smolt Atlantic salmon (~70 g) in a fresh water flow through system (7.9°C) fed twice a day at ad libitum (Nutra Olympic), and kept under 12:12 (light: dark) light regime. Fish were fed up until the start of sampling (day 0). Different sampling regimes were adapted as described in details below.

2.2 | Sampling of gills kept at room temperature

A total of 30 fish were killed by means of benzocaine anaesthetic overdose (120 mg/L; Benzoak vet. ACD Pharmaceuticals AS). Death was confirmed by cessation of movement and respiration. The fish carcass was kept at room temperature (21°C) during organ sampling. The second gill arch were immediately sampled from five fish at the start of the study (0 min) and fixed in formalin, which served as unaffected control tissue. Five minutes later, gills were sampled from another five fish and fixed. This procedure was repeated at 10, 15, 30 and 45 min. All samples were fixed in sealed plastic containers with 10% neutral buffered formalin (Sigma-Aldrich).

2.3 | Sampling of organs stored at room temperature, refrigerator or in a freezer

A total of 39 fish were killed by means of benzocaine anaesthetic overdose as previously described. The fish were divided into three groups of 12 fish per group. Each fish was kept in a separate plastic bag and stored either at room temperature (21°C), refrigerated at 4°C or frozen at −20°C. Fish necropsy was performed at 0, 1, 4, 24 and 48 h post-storage. Organs (gills, pyloric caeca/pancreas, stomach, liver, posterior kidney, spleen, skeletal musculature and heart musculature) were immediately sampled from three fish at the start of the study (0 h time point), which served as unaffected control fish. Nine fish were sampled 1 h later, of which three fish were kept at room temperature, three fish refrigerated and three fish removed from the freezer and then thawed in cool water as described in

George et al. (2016). The coelom wall was cut open and the operculum removed from one side prior to fixation. Care was taken to ensure the whole fish, including internal organs, was immersed in formalin. This procedure was repeated with nine fish each at 4, 24 and 48 h post-storage. After a minimum of 48 h fixation, all fish were harvested for the following organs; gills, pyloric caeca/pancreas, stomach, liver, posterior kidney, spleen, skeletal musculature and heart musculature. All samples were kept in sealed plastic containers with 10% NBF (Sigma-Aldrich).

2.4 | Preparation of samples for histology

Tissue samples were processed by a standard paraffin wax protocol (dehydrated, embedded in paraffin, 2 and 5 μm thick sectioned and haematoxylin and eosin [H&E] stained). Slides were evaluated by light microscopy using an Axio Lab.A1 microscope (Carl Zeiss Microscopy GmbH). Slides were also scanned by using a Hamamatsu NanoZoomer S360, and further visualized by using the software program NDP.view.2.7.25.0 (Hamamatsu Photonics K.K.).

2.5 | Histologic scoring of autolytic changes

Slides were scored by using a semi-quantitative scoring system (George et al., 2016) as follows: score 0 – None to minimal autolytic changes (<5% of the investigated tissue is affected), score 1 – mild autolytic changes (5%–10% of the investigated tissue is affected), score 2 – moderate autolytic changes (10%–50% of the investigated tissue is affected) and score 3 – severe autolytic changes (>50% of the investigated tissue is affected). Criteria for autolysis comprised one or several of the following factors (George et al., 2016) such as cellular oedema/swelling, failure to take up stain, pyknosis, karyorrhexis and karyolysis, absence of a cell nucleus due to complete dissolution or lysis, intracytoplasmic vacuolization and altered architecture of tissue unrelated to a pathological process. Specific for gills (adapted from Speare & Ferguson, 1989) were epithelial lifting of the lamellar epithelium from the pillar cells leading to development of dilated extracellular spaces in the filaments and loss of cells within the gill filaments, and epithelial cell hypertrophy. Blinded histological evaluation and visual scoring was performed by three different pathologists. The final score was defined as the median of the three scored values.

3 | RESULTS

3.1 | Changes within control group samples

In general, there were no significant cellular and architectural histological changes, linked to post-mortem processes, observed in the control group. Uptake of histologic stain was optimal for all organs. However, a few organ samples displayed mild pathologic or artifactual changes. In gills, mild multifocal lamellar fusion and mild focal inflammation of the lamellas were present in a few fish. In samples from skin and skeletal musculature, there were artifactual separation of the cuticle and epidermis. In a few samples, the epidermis was not present in the tissue biopsy. Focal areas of artifactual lifting of lamina propria from the overlying intestinal epithelium occurred within the pyloric caeca and stomach. For the other organs (liver, posterior kidney, spleen and heart musculature) there were artefacts, including tissue folding and debris under the coverslip.

3.2 | Post-mortem changes in gills stored at room temperature for 0 to 45 min

In gills, the earliest identified post-mortem changes occurred at 10 min post-storage (mps) (Figure 1). Mild changes were seen, in the form of epithelial lifting of the lamellar epithelium from the pillar cells leading to development of extracellular spaces, dilated extracellular spaces in the filaments and loss of cells within the gill filaments. Mild post-mortem changes also occurred at 15 mps. Above-mentioned changes, in addition to epithelial cell hypertrophy, cytoplasmic and nuclear swelling of epithelial cells, loss of tissue architecture and loss of stain uptake of a severe degree, occurred at 30 and 45 mps (Table 1).

3.3 | Post-mortem changes in organs stored at room temperature (21°C)

At 1 hps, signs of post-mortem changes within the gills, liver and the pyloric caeca was observed. The gills had moderate changes in the form of epithelial lifting of lamellar epithelium from the pillar cells leading to development of extracellular spaces, dilated extracellular spaces in the filaments, epithelial cell hypertrophy, cytoplasmic and nuclear swelling of epithelial cells, loss of tissue architecture, loss of cells within the gill filaments, loss of stain uptake and loss of cellular details (Figure 2). Mild changes occurred in the pyloric caeca, in the form of separation of lamina propria from the basal membrane and disintegration of the mucosa. The liver had mild autolytic changes, in the form of pyknotic cell nucleuses. No changes occurred in posterior kidney and spleen.

At 4 hps, the gills had severe stain loss and post-mortem changes similar to those observed at 1 hps. The liver and pyloric caeca/pancreas had moderate autolytic changes in the form of pyknosis, stain loss and loss of cellular architecture (Table 2). The intestinal mucosa were separated from underlying tissue and cellular structures were mixed within the intraluminal centre of the pyloric caeca. By comparison to the pyloric caeca, the stomach did not show any observable autolytic changes at 4 hps. Posterior kidney and spleen had mild autolytic changes after 4 hps.

Loss of stain started appearing in all other organs at 24 hps. The samples from all organs were at 24 hps more eosinophilic in colour and the contrasts within the tissue were not as clear, as in samples from earlier time points (Figure 3). At 24 and 48 hps,

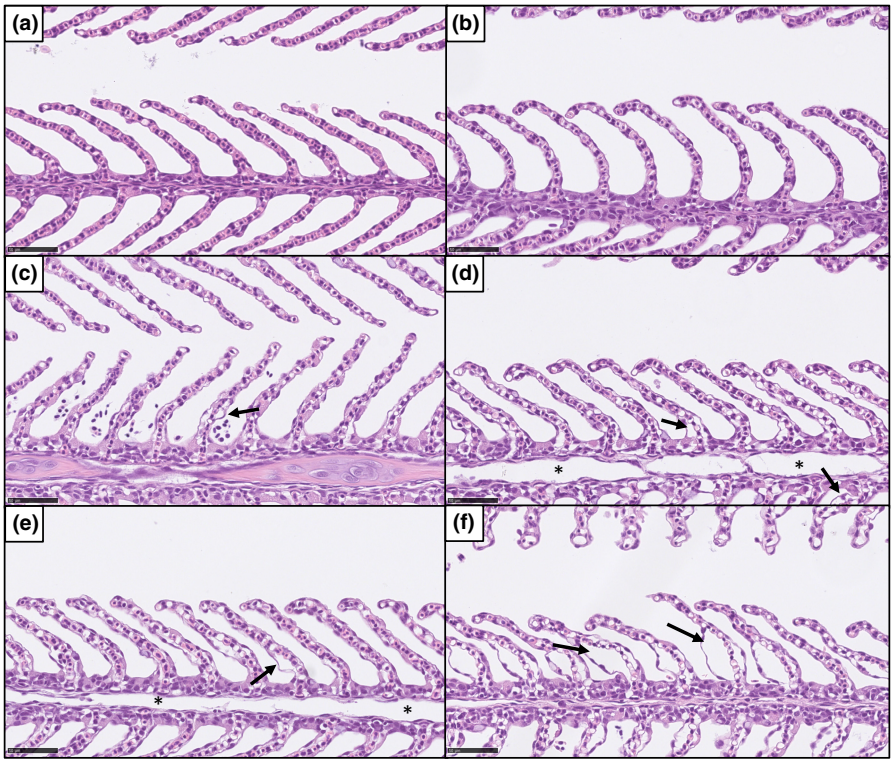

FIGURE 1 Histologic images of gills kept at room temperature (21°C) at 0 (a), 5 (b), 10 (c), 15 (d), 30 (e) and 45 (f) minutes post-storage. Fresh samples (a) included for reference. Asterisk (*) shows separation of the gill filament and the black arrows show epithelial lifting leading to development of extracellular spaces. Scale bar 50 μm

Organs/tissue room temperature (RT) (21°C)	Histological score over different time intervals					
	0 min	5 min	10 min	15 min	30 min	45 min
Gills	0	0	1	1	3	3

TABLE 1 Median score for gill autolysis over time at 21°C (0 = minimal, 1 = mild, 2 = moderate and 3 = severe) $N = 5$ for all groups

there was generally loss of cellular details in all internal organs, while the skeletal musculature had the least amount of changes (Table 2 and Figure 2). At 48 hps, all organs had severe post-mortem changes, except for the skeletal musculature (Figure 2) that had mild changes, including an increase of cellular spaces, loss of stain uptake and tissue architecture. The epidermis, dermis and subcutaneous adipose tissue were in general difficult to grade, due to severe loss of tissue during preparation of autolytic samples. As such, the histological examination focused on changes within the skeletal musculature.

At 24 hps, all organs had moderate to severe post-mortem changes, except for the skeletal and heart musculature. Finally, at 48 hps, all organs had severe post-mortem changes, except for mild and moderate changes within the skeletal and heart musculature, respectively (Table 3). In general, samples stored over longer time periods (at both 4°C and 21 °C) had more changes in the form of cell and tissue separation, loss of tissue architecture and loss of stain uptake. When comparing the scores of organs stored at 4 and 21°C, there is generally a small delay in the onset and development of post-mortem changes at 4°C, especially for liver, spleen, skeletal and heart musculature.

3.4 | Post-mortem changes in organs kept in refrigerator (4°C)

At 1 hps, the gills had moderate post-mortem changes, similar to those described above at 21°C. In addition, the pylorus caeca/pancreas had mild changes, similar to those described at 21°C. No other organs had observable post-mortem changes at this time point (Table 3).

At 4 hps, the posterior kidney had moderate post-mortem changes (Table 3), in the form of clear spaces within the interstitium and tubular cell separation from surrounding tissue. In addition, mild post-mortem changes appeared within the heart, in the form of vacuolization of cardiac myocytes and some endothelial separation.

3.5 | Post-mortem changes in organs kept in freezer (−20°C)

At 1 hps, the posterior kidney had mild post-mortem changes similar to those described at 4°C. The gills had severe post-mortem changes similar to those described at 1 hps at 4°C, in addition to several freezing artefacts. The heart musculature had mild changes at 1 hps, and throughout the time course of the study. For other organs, there were no observable post-mortem changes at this time point (Table 4).

At 4 hps, all organs, except for the heart musculature, had moderate to severe changes in the form of tissue autolysis and freezing artefacts. Observable freezing artefacts were generally in the form of crystallization and cell and tissue separation. At 24 hps (Figure 4)

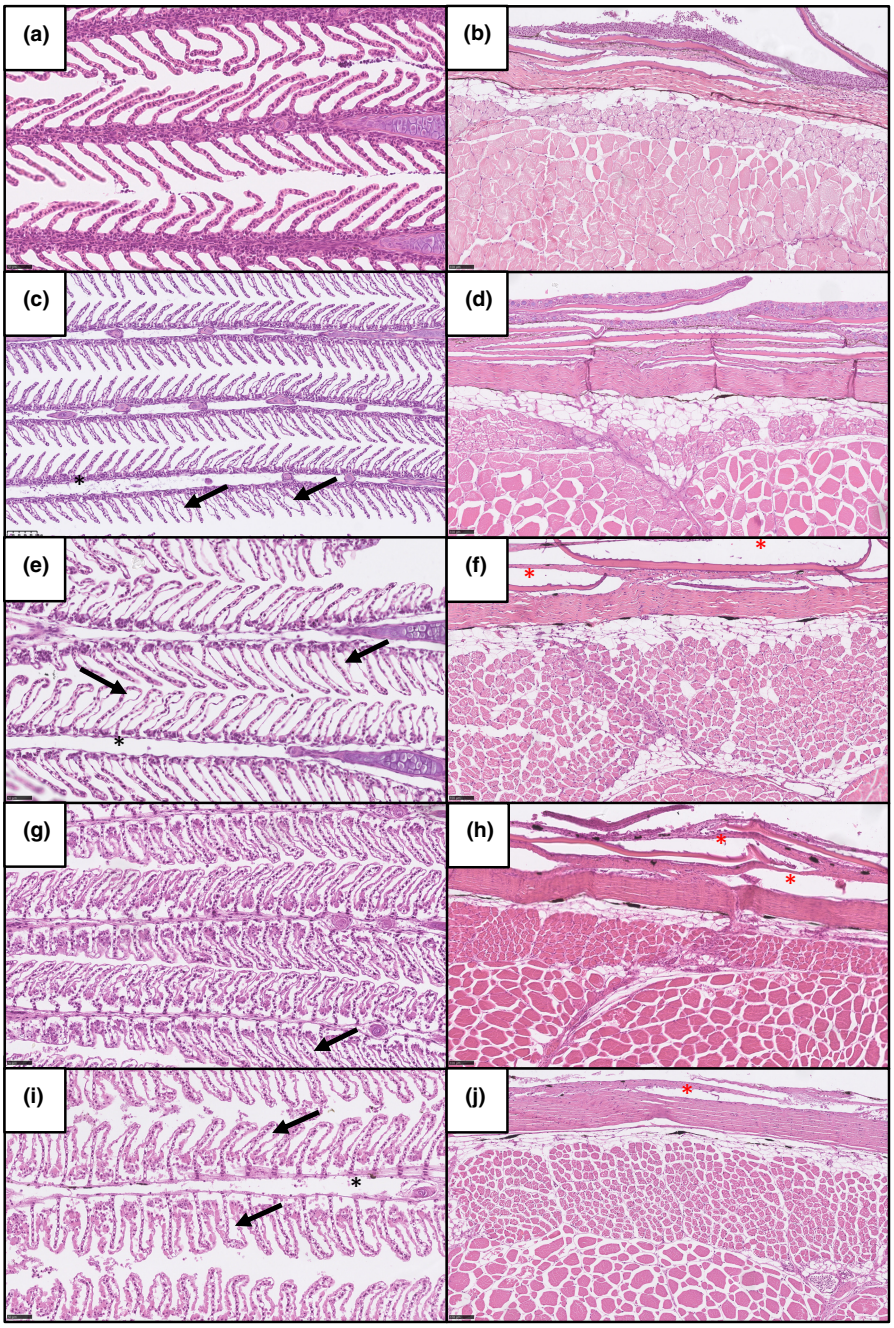

FIGURE 2 Histologic images of gills (a, c, e, g and i) and skeletal musculature and skin (b, d, f, h and j) kept at room temperature (21°C) at 1 (c and d), 4 (e and f), 24 (g and h) and 48 (i and j) hours post-storage. Fresh samples (a and b) included for reference. Dark asterisk (*) in gill shows separation of the gill filament and the black arrows show epithelial lifting leading to development of extracellular spaces. Red asterisk (*) in skin show separation between epidermal and dermal skin layers. Scale bar 50 μm (gills) and 100 μm (skeletal musculature)

and 48 hps, all organs, except heart muscle and spleen, had severe post-mortem changes similar to those described after 24 hps at 4°C. Organs were observed both with and without freezing artefacts. The spleen started showing moderate changes at 24 hps (Figure 4), and then severe changes at 48 hps (Table 4).

4 | DISCUSSION

Immediate fixation of biological specimens is not always possible. In order to reduce the rate of post-mortem changes in fish organs, we investigated the effect of different storage temperatures and time. We compared storage at room temperature (21°C), in a refrigerator (4°C) and in a freezer (−20°C) for a period of 48 h after sacrifice, before fixation in formalin. Gills, heart, posterior kidney, spleen, liver, pyloric caeca with pancreatic tissue and skeletal musculature were selected and are routinely used for histopathological investigation in fish diagnostics. Gills were also investigated for shorter duration (0–45 mps) before fixation. The type of post-mortem changes and the effect of time and storage temperature on the development of these changes, were investigated by histological examination of HE-stained tissue sections of affected organs.

TABLE 2 Median score for organ autolysis over time at 21°C (0 = minimal, 1 = mild, 2 = moderate and 3 = severe) $N = 3$ for all groups

Organs/tissue room temperature (RT) (21°C)	Histological score over different time intervals				
	0 h	1 h	4 h	24 h	48 h
Gills	0	2	3	3	3
Pyloric caeca/pancreas	0	1	2	3	3
Stomach	0	0	0	3	3
Liver	0	1	2	3	3
Posterior kidney	0	0	1	3	3
Spleen	0	0	1	3	3
Skeletal muscle	0	0	1	1	1
Heart muscle	0	0	1	2	3

TABLE 3 Median score for organ autolysis over time at 4°C (0 = minimal, 1 = mild, 2 = moderate and 3 = severe) $N = 3$ for all groups

Organs/tissue refrigerator (RF) (4°C)	Histological score over different time intervals				
	0 h	1 h	4 h	24 h	48 h
Gills	0	2	2	3	3
Pyloric caeca/pancreas	0	1	1	3	3
Stomach	0	0	0	3	3
Liver	0	0	0	3	3
Posterior kidney	0	0	2	2	3
Spleen	0	0	0	2	3
Skeletal muscle	0	0	0	0	1
Heart muscle	0	0	1	2	2

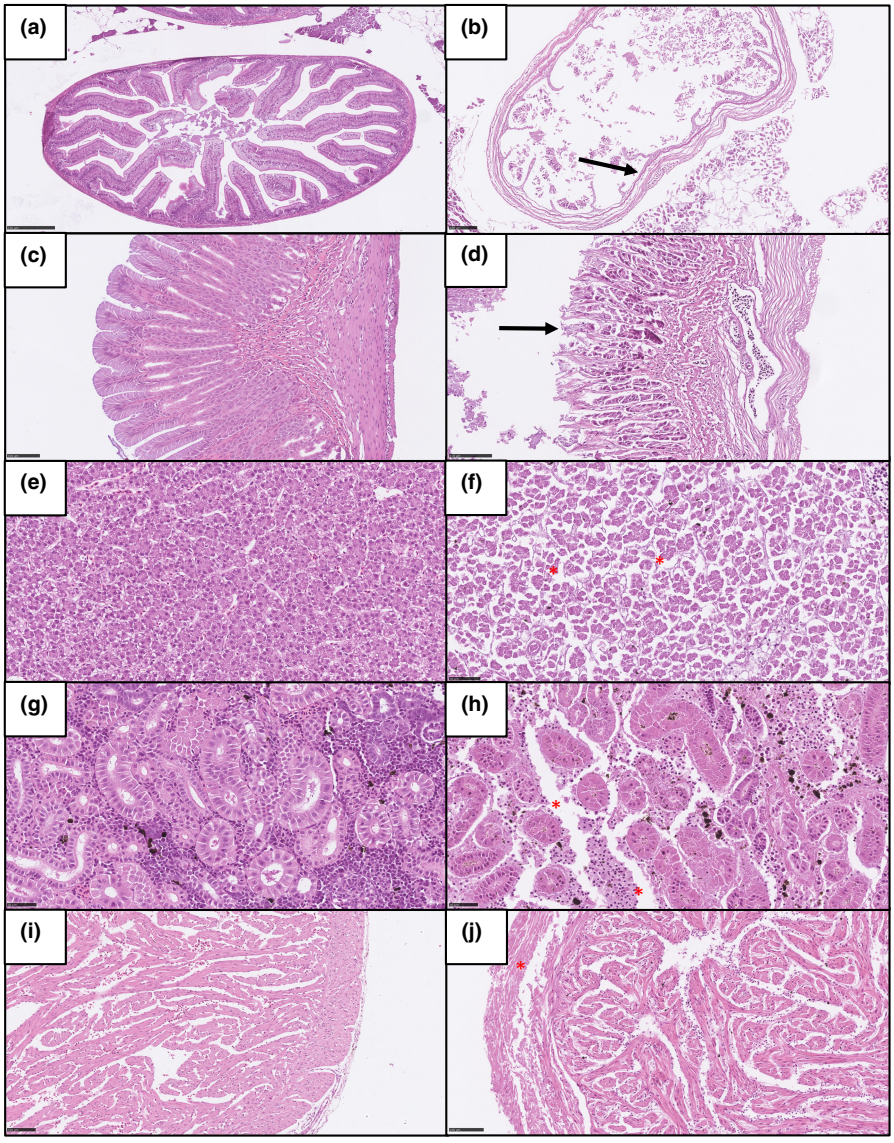

FIGURE 3 Histologic images of different tissues kept at room temperature (RT). Control samples are included for reference. (a) Pyloric caeca and pancreatic tissue, control sample (0 h). (b) Pyloric caeca and pancreatic tissue (24 h), dark arrow shows disintegration and loss of epithelial mucosa. (c) Stomach, control sample (0 h). (d) Stomach (24 h), dark arrow shows disintegration of epithelial cells in gastric mucosa. (e) Liver, control sample (0 h), (f) Liver (24 h), red asterisk (*) shows destruction of liver hepatocytes and sinusoids. (g) Posterior kidney, control sample (0 h), (h) Posterior kidney (24 h), red asterisk (*) shows destruction of kidney, especially the haematopoietic tissue. (i) Heart musculature, control sample (0 h), (j) Heart musculature (24 h), red asterisk (*) shows moderate destruction of cardiac myocytes. Scale bars 50 μm (posterior kidney, liver), 100 μm (heart musculature, stomach and pyloric caeca with pancreatic tissue (24 h)) and 250 μm (pyloric caeca with pancreatic tissue (0 h))

TABLE 4 Median score for organ autolysis over time at −20°C (0 = minimal, 1 = mild, 2 = moderate and 3 = severe) $N = 3$ for all groups

	Histological score over different time intervals				
Organs/tissue freezer (−20°C)	0 h	1 h	4 h	24 h	48 h
Gills	0	3	2	3	3
Pyloric caeca/pancreas	0	0	3	3	3
Stomach	0	0	3	3	3
Liver	0	0	3	3	3
Posterior kidney	0	1	2	3	3
Spleen	0	0	3	2	3
Skeletal muscle	0	0	2	3	3
Heart muscle	0	0	1	1	1

4.1 | Post-mortem changes in organs kept at room temperature (21°C)

In fish gills, an organ where the epithelium is covering a large surface area, only a thin epithelial barrier of 0.5–10 μm separates blood from the surrounding water in order to facilitate efficient gas exchange of O_2 and CO_2 (Wilson & Laurent, 2002). The fine structure of the organ, the close proximity to the environment and rapid temperature increase following sacrifice, make the organ very susceptible to post-mortem changes. In this experiment, gills were investigated and post-mortem changes such as epithelial lifting of the lamellar epithelium from the pillar cells, were found as early as 10 min after death. The dead fish was kept at 4°C and at 21°C (RT), leading to a temperature difference of 17°C. This might influence the autolytic changes, especially due to endogenic enzymes of lysosomal origin involved in the process (Wang et al., 2009). Most enzymes in cold-water fish have a functional temperature optimum of 0–6°C (Wang et al., 2009); however, salmonids have shown tolerance up to about 20°C (Elliot & Elliot, 2010; Falconer et al., 2020). A temperature increase of ca. 17°C might increase the enzymatic function of salmonid calpains and matrix metalloproteinases, however the present findings only showed a minor or delayed response in terms of autolysis at 4°C compared to 21°C.

Rapid autolytic changes in the intestine and other organs that have high contents of lytic enzymes, such as pyloric caeca and pancreatic tissues, has been described by Kristinsson and Rasco (2000); Martínez and Gildberg (1988) and Cocariu et al. (2016). Our findings are in accordance with these results. Considering the major function of the intestinal system, which is to degrade food items by hydrolytic and enzymatic degradation, it is not surprising that the intestinal tissues are susceptible to autolytic post-mortem changes. In fish, digestive enzymes can cause extensive autolytic deterioration, leading to rupture of the coelom wall. This rupture of the intestinal or stomach wall can lead to microbial degradation of internal organs located outside of the gastrointestinal tract (Huss, 1995; Mukundan et al., 1986). Normal microbial flora of fish generally includes bacterial species within different genera such as *Pseudomonas*, *Vibrio*, *Alcaligenes*, *Serratia* and *Micrococcus spp.* (Gram & Huss, 2000). It has been described that autolytic changes precedes bacterial degradation especially in studies on fish quality and freshness (Ghaly et al., 2010; Huss, 1976) but epithelial tissues of the intestinal mucosa will be affected by both enzymes and bacteria present within the intestinal lumen. When the fish dies, the bacteria are known to proliferate freely since the immune system collapses and the tissue integrity is weakened. It will take time for the bacteria to spread through the intestinal wall and reach other internal organs that per definition are sterile when the fish is alive. Wolf et al. (2015) mentions rapid fixation as an important step to prevent autolysis of the intestinal epithelium.

In this study, the stomach had more resistance to post-mortem changes as compared to the pyloric caeca. Changes in the stomach were generally not seen until 24 hps. It has earlier been described that organs containing the highest lytic enzyme contents are decomposing more rapidly, while organs rich in collagen is much more resistant (Dettmeyer, 2018). The stomach contains lower pH and is surrounded by more muscle tissue compared to the pyloric caeca. Also, the digestive mucosa has been described to degrade faster than the submucosa and the underlying muscular layer (Cocariu et al., 2016; Dix & Graham, 1999). Organs containing elastic fibres and collagen are generally less prone to autolytic changes (Dettmeyer, 2018). Fibro-connective tissue structures (such as the walls of intraparenchymal blood vessels or tubules), cartilage and bone are resistant to digestion and undergo autolysis at very slow rates, which may not be detected during the inspection (Bradley, 1938; Cocariu et al., 2016). In this study, the skeletal musculature had the greatest delay in development of post-mortem changes. Similar results have also been shown in earlier studies on fish (George et al., 2016) and rats (Tomita et al., 2004).

4.2 | Post-mortem changes in organs kept in refrigerator (4°C)

Fish removed from their aquatic habitat, such as during organ sampling at fish farms, are often moved from a low temperature ('cold') environment in water, to a higher temperature ('warm') environment on land. It is known that increases in temperature can speed up the process of autolysis (Clark et al., 1997; Cocariu et al., 2016; Sterne et al., 2000). As such, chilling of fish is recommended to maintain a uniformly low temperature and reduce autolysis and bacterial degradation (Ghaly et al., 2010; Rand & Pivarnik, 1992). As shown in this study, storing the samples in a refrigerator at 4°C slowed down the degree of post-mortem development for the liver, spleen, skeletal musculature and heart musculature, but only slightly. This is supported by George et al. (2016), which demonstrated moderate autolytic changes for the liver, pancreas, spleen and kidney kept at room temperature for 24 h, and mild changes in refrigerated fish after 24 h.

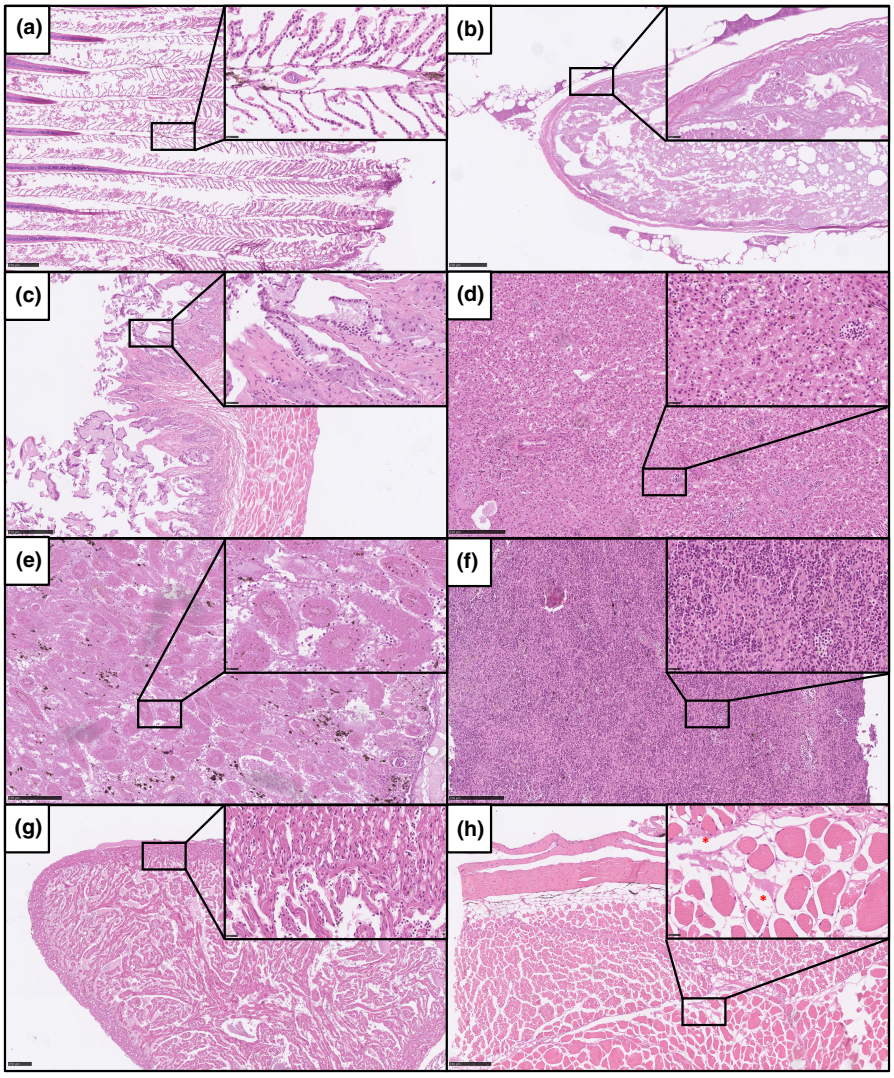

FIGURE 4 Histologic images of frozen samples at 24 hps. (a) Gills with disintegrated filaments and lamellae. Insert shows destruction of epithelial and pillar cells of the lamellae. (b) Pyloric caeca and pancreatic tissue with severe disintegration of pyloric and mucosal cells. Insert shows higher magnification of mucosal and pancreatic destruction. (c) Stomach with severe epithelial destruction in gastric mucosa. Insert shows higher magnification of cell destruction in gastric mucosa. (d) Liver with cellular disintegration and pyknotic nuclear changes. Insert shows higher magnification of cellular destruction. (e) Posterior kidney with severe cellular disintegration and unrecognizable tissue. Insert shows higher magnification with destruction of haematopoietic tissue and remnants of kidney tubular cells. (f) Spleen with moderate to severe cellular changes. Insert shows higher magnification with cellular destruction. (g) Heart musculature with mild cellular damage to cardiac myocytes. Insert shows higher magnification of the ventricular compact layer with mild tissue damage. (h) Skeletal musculature with severe damage to muscular cells. Inserted image show higher magnification of muscle tissue, with red asterisk (*) demonstrating cellular destruction of skeletal myocytes. Scale bar 250 μm for images and 25 μm for the inserted images

The enzyme activity and breakdown of tissues has been described to increase at higher temperature, but they will still be active at lower temperatures (0–6°C) (Wang et al., 2009).

4.3 | Post-mortem changes in organs kept in freezer (−20°C)

When sampling fish organs during freezing winter periods, there are other challenges to take into consideration. Sampled fish may be moved from low temperature conditions in water to sub-zero temperatures on land. This can potentially cause samples to freeze, even when put in fixatives such as formalin. Formalin-fixed samples may also be put in the freezer with good intensions, believing that this will help preserve the samples. As demonstrated in this study, freezing of samples can then lead to development of severe artefacts in the fish tissues. George et al. (2016) also support this finding by demonstrating the development of autolytic changes over time in frozen organs. One possible explanation for this may be drastic temperature changes have shown to hasten autolysis. Glycolysis can be enhanced at temperatures below −1°C, and thus lead to autolysis (Mukundan et al., 1986). The frozen samples must also be thawed

before it can be further processed for histological evaluation. An elevation of temperatures, such as during freeze–thaw cycles, is known to accelerate autolysis (Carson & Hladik, 2015; Sterne et al., 2000). In addition, freezing may also lead to formation of freezing artefacts. Several studies have demonstrated the effect of freezing in organs, especially in muscular tissue (Strateva & Penchev, 2019). Formation of ice crystals between muscle fibres (intercellular) lead to extensive damage and fibre separation, while ice crystals formed inside muscle fibres (intracellular) only disrupt their integrity to a lesser extent (Gambuteanu et al., 2013; Rahelić et al., 1985). Damage to cellular structures after freezing has also been documented. Freezing cause depolymerization and destruction of the cell membrane, damages to tissue and cellular structures and changes in osmotic pressure (Li et al., 2018). Tissue changes caused by freezing can histologically be recognized as empty vacuoles, causing histological sections to appear punctured such as a Swiss cheese (Love, 1958; Popelka et al., 2014; Strateva & Penchev, 2020), which consequently complicate the histological evaluation.

It must be emphasized that this experiment was conducted using 70 gram farmed Atlantic salmon in fresh water, and the results must be interpreted accordingly. There can be individual differences between fish, as well differences in species, size, water quality and temperature. Such questions should be addressed in future studies.

5 | CONCLUSION

Our results demonstrate that the extent of post-mortem autolytic changes vary between different organs, with gills being most susceptible. Autolytic changes in the gills can be detected as early as 10 min post-mortem. This can lead to complications for histological evaluation of the organ, and sampling should be performed as soon possible post-sacrifice to minimize these changes. Gills and pyloric caeca/pancreatic tissue were the most severely autolysed organs in this study. Epidermal layers of skin was also severely affected. Heart and skeletal musculature were least affected by autolytic changes over time. Keeping samples refrigerated at 4°C will slow the post-mortem changes only marginally, while freezing samples will not prevent autolysis but will lead to development of freezing artefacts within the organs. This study suggests that high effort should be made to minimize the time from sacrifice to sampling. If possible we suggest, gills and pyloric caeca/pancreatic tissue should be sampled first and within 10 minutes of time of fish death. Possible effects from using chemical fixatives other than formalin, and potential differences between fish in fresh- and seawater would be interesting areas to investigate in future studies.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

Data Availability Statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

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