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Physiological changes observed in farmed Atlantic salmon (*Salmo salar* L.) with nephrocalcinosis

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ABSTRACT

There is a growing concern for fish health and welfare in the salmon industry in Norway. Nephrocalcinosis, described as mineral deposits within the kidney, is increasingly observed. However, little is known about its frequency and severity in Norway. In this study 810 Atlantic salmon were sampled from 14 different fish groups in nurseries in Mid-Norway and receiving sea farm. Kidneys were examined for nephrocalcinosis by histopathological methods and all fish groups were diagnosed with nephrocalcinosis. The prevalence and severity of the disorder varied extensively between facilities. Most of the fish (68%) had mild forms of nephrocalcinosis, exhibiting at most, negligible tissue damage while fish affected by severe forms of nephrocalcinosis had an almost complete loss of kidney structure. Regardless of the severity of nephrocalcinosis, mineral deposits were mainly found in the form of amorphous cabonate apatite (amCAP), a calcium-dominated mineral. Accordingly, a majority of fish affected by nephrocalcinosis were diagnosed with hypercalcemia. Fish affected by moderate and severe forms of nephrocalcinosis also exhibited high levels of plasma magnesium, glucose, and aspartate aminotransferase (AST). These imbalances in plasma chemistry are likely to be an indication of disturbed osmoregulation and increased stress levels. The results of this study therefore suggest that nephrocalcinosis is a common and serious welfare challenge in Atlantic salmon that needs better monitoring.

1. Introduction

The salmon industry is one of the most important industries in rural Norway (Olaussen, 2018) employing over 8000 people and contributing to a yearly landing value of 6,8 billion EUR (NOK 70 billion) (Directorate of Fisheries, 2019). The industry is known for its innovation and use of new technologies, but welfare of farmed salmon is becoming a growing concern (Sommerset et al., 2020). Nephrocalcinosis is one of the challenges in Atlantic salmon and was among the major diseases listed by fish health professionals in the Norwegian Fish Health Report from 2019 (Sommerset et al., 2020). Although the prevalence of nephrocalcinosis among wild fish is unknown it is likely that the occurrence at production sites is related to the intensive conditions in aquaculture (Applegate et al., 2016; Béland et al., 2020; Bjerknes et al., 1994; Cavrois-Rogacki et al., 2021; Gillespie and Evans, 1979; Klosterhoff et al., 2015; Lewisch et al., 2013; Smart et al., 1979).

To date, there is no systematic registration of nephrocalcinosis in salmon aquaculture, however there is a growing effort to monitor the disease. A scoring form to visually document nephrocalcinosis is currently being validated by Nofima (Noble et al., 2018) and the condition has been more frequently reported by nurseries, fish farms and fish health personnel over the past few years, but without clear indication of severity and etiology (Sommerset et al., 2020). The condition is believed to arise during production on land (nurseries), with a progressive aggravation until transfer to sea (pers. comm. P.A. Sæther). It is generally accepted that nephrocalcinosis is related to increased mortality in the first weeks after sea-transfer and secondary infections with bacteria and fungi are not uncommon.

Nephrocalcinosis is described as deposits of mineral salts within kidney tubules and collecting ducts, which can be visually identified

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(Bruno, 1996). However, macroscopic assessment is not a precise tool, since small deposits are rarely visible to the naked eye (Klykken et al., 2020). To date, histopathology is therefore regarded as the standard in assessing the severity and progress of this condition. Observed changes involve basophilic mineral deposits that may progress into dilation of tubules and collecting ducts, degeneration and necrosis of epithelium of the affected tubular structures, and urine-stagnation causing dilatation and fibrosis in up-stream structures. Upon breach of the tubular structures, fibrosis and inflammation can cause extensive tissue reactions and necrosis in surrounding interstitial tissue (Fivelstad et al., 2018). In addition to the excretory system, the kidney also consist of both hematopoietic, immunological and endocrine tissues, all of which can be damaged upon development of lesions into the interstitial compartment. The sequential progression of histopathological changes occurring during the development of nephrocalcinosis makes it ideal for a scoring system. However, to the authors knowledge, such a scoring system for nephrocalcinosis based on histopathology has not been published. The development of a standardized scoring-system would be crucial to ensure a systematic approach when comparing degrees of tissue lesions both within and between groups, as well as their development over time.

Few studies have been conducted regarding the composition of kidney mineral deposits in farmed Atlantic salmon. To date, there is no peer-reviewed study on the composition of mineral deposits in salmon, but a master thesis from 2019 reported that the kidney deposits consisted of calcium and phosphate minerals (amorphous carbonate apatite, amCAP), carbonate apatite (CAP) and magnesium ammonium phosphate (struvite) (Thomsen, 2019). A survey conducted by Marin Helse AS on post-smolt salmon also reported that kidney deposits consisted of complexes of amCAP, with possible traces of complexes of magnesium, calcium and phosphate (whitlockite) (Sæther, 2019).Among other marine species, a study has been performed on Cobia, (*Rachycentron canadum*), where the kidney stones consisted of pure calcium, oxalate and calcium phosphate (Klosterhoff et al., 2015). In rainbow trout (*Oncorhynchus mykiss*) most studies found that the deposits consisted of calcium, phosphorus, carbonate and magnesium (Bjerknes et al., 1994; Gillespie and Evans, 1979), while FÍkrÍ et al. (2000) found that they contained ammonium urate ($NH_4C_5H_3N_4O_3$) and calcium phosphate.

In humans and domestic (terrestrial) animals, the determination of kidney stone composition play an important role both in treatment and prevention (Koehler et al., 2009; Kourambas et al., 2001; Kravdal et al., 2015; Tepeler and Turna, 2017). It is therefore likely that the mineral composition of nephrocalcinosis in salmon could provide relevant information to prevent the condition.

Biochemical analysis of plasma is a widely used diagnostic tool in human and veterinarian medicine. There is an increasing interest in transferring this methodology to the fish farming industry (Fazio, 2019), but the lack of standardized reference values from healthy fish makes it challenging to detect abnormalities (Clauss et al., 2008; Wade et al., 2019). One study proposed normal ranges of plasma chemistry for adult Atlantic salmon (Sandnes et al., 1988), but reference intervals have yet not been established for parr and smolt. Studies based on controlled experiments have demonstrated changes of plasma biochemistry in response to a variety of stressors (Calabrese et al., 2017; Iversen and Eliassen, 2009; Iversen et al., 2009, 1998; Stiller et al., 2020; Veiseth et al., 2006), toxins (Berntssen et al., 2018, 2021; Nieves-Puigdoller et al., 2007) and subtoxic concentrations of water quality compounds (Knoph and Thorud, 1996). Atlantic salmon with coldwater vibrosis (Waagbo et al., 1988) and cardiomyopathy syndrome (CMS) (Yousaf and Powell, 2012) also exhibited changes in blood biochemistry compared to healthy fish. Establishing biochemistry as a non-lethal diagnostic tool for early detection of nephrocalcinosis would represent a valuable tool for revealing risk factors and ultimately preventing the condition.

The objective of this study was to determine the prevalence and severity of nephrocalcinosis in farmed Atlantic salmon in Mid-Norway. To do so, we (1) sampled an extensive number of fish from commercial production groups, (2) examined the kidney tissue with histopathologic methods, (3) determined the composition of mineral deposits found in the kidney and (4) compared the plasma chemistry of individuals affected by nephrocalcinosis with healthy salmon.

2. Materials and methods

2.1. Data sampling

A total of 420 farmed Atlantic salmon were sampled from 14 fish groups in twelve different nurseries, both flow through (FT) and recirculation aquaculture systems (RAS) in Mid-Norway from October 2019 to April 2021. In addition, 390 fish originating from these nurseries were sampled from the receiving sea farm. Group 14 was not sampled after transfer to sea due to capacity issues at the facility. No fish were exposed to experimental manipulation, and the sample material therefore represents fish under conventional farming conditions (see Table A1 and A2 in supplementary material). A total of 30 fish from each facility were randomly sampled among visually healthy (normal swimming behaviour, absence of external injuries/lesions, no sign of emaciation) individuals within 2 weeks before transfer to sea and 1 month after transfer to sea. All sampling were performed in the morning (before 11 am). The fish were not starved before sampling, and they were euthanized with an overdose of Benzoak VET (200–400 mg/l) followed by a sharp blow to the head according to Norwegian legislation (Akvakulturdriftsforskriften, 2008). A general health assessment and physical observations including an evaluation of morphological changes related to parr-smolt transformation was performed at each sampling (parr-smolt transformation; body silvering, darkening of fin margins, loss of parr marks).

2.2. Blood collection and determination of plasma chemistry

Blood samples were collected from 420 fish that were sampled in nurseries. Vacutainer tubes (Becton-Dickinson, Rutherford, NJ, USA) with lithium heparin as anticoagulant were used to collect blood from the caudal vein, immediately after euthanasia. After thorough mixing, the samples were centrifuged at 13500 rpm for 5 min (VWR Mikrostar 12, 12 × 1.5/2.0 ml) and the plasma was transferred to Eppendorf tubes and kept frozen (minimum −20 °C) until analysis.

The following parameters were measured at Aqua Kompetanse's laboratory using an automated dry chemistry analyser (CatalystOne, IDEXX Laboratories, Westbrook, ME, (Boes et al., 2018)): alkaline phosphatase (ALKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA), calcium (Ca), sodium (Na), potassium (K), chloride (Cl), magnesium (Mg), inorganic phosphate (PHOS), glucose (GLU), lactate (LAC) and urea. Other parameters that were derived included the following: sodium-to-potassium ratio (Na/K) and Osmolality (mmol/kg) calculated as:

$$1.86(Na + K) + 1.5GLU + UREA + 14$$

according to Martín-Calderón et al. (2015). Each of the assays used a standard kit developed for the automated analyser (CatalystOne, IDEXX Laboratories, Westbrook, ME). The plasma occasionally showed activities for inorganic phosphate exceeding the instrument ranges, and sporadically for other parameters: in these cases a physiological saline solution was used to dilute the samples (1:4).

2.3. Mineral deposits

When visually identified, mineral deposits were carefully collected from fish ureters and stored in Eppendorf tubes and frozen until analysis (−20°C). The samples were washed in ethanol (EtOH) followed by centrifugation and acetone to remove lipids and water from the samples and air dried for 5 min prior to analysis. The mineral composition was

examined by attenuated total reflection (ATR) Fourier transform infrared spectroscopy (FTIR) in a Nicolet iS10, operated by the Omnic9 software. The spectra obtained were interpreted by OmnicSpecta using an in-house library constructed for human kidney stones, which has been modified for salmon nephrocalcinosis analysis.

2.4. Histopathology

Tissues from the mid-kidney were sampled from all individuals for histopathological analysis of nephrocalcinosis. The kidney tissues were fixed in 4% formaldehyde solution, embedded in paraffin wax and routinely processed (Suvarna et al., 2019). All sections were stained by haematoxylin and eosin and a selection of sections were stained with von Kossa stain (Rungby et al., 1993). The histopathological diagnosis of nephrocalcinosis was defined as the presence of amorphous (structureless), basophilic deposits in tubules, collecting ducts and excretory ducts. Histopathological sections were analysed, and presence of deposit and degree of tissue damage was evaluated and given a score based on the type and distribution of changes. The nephrocalcinosis score defines and categorises the presence of deposits, degree of tissue damage in structures with deposits, glomerular alterations and pathology in the interstitial tissue. Pathological changes were divided into 4 subcategories (Table 1). Each category was weighted by the effect the different pathological changes are believed to have on the development of the condition and the time it will take to heal.

The score of the first category was weighted with a factor of 1, while the score of category 2 was weighted with a factor of 2 because dilation of tubules and renal collection ducts causes gradual damage on the epithelium and subsequent chronic changes resulting in fibrosis of the basal membranes of the affected structures. Urine stagnation with dilation of the glomerular space is indicative of down-stream obstruction (Docherty et al., 2006) and develops into periglomerular fibrosis and subsequent thickening of Bowmans capsule. For this reason the score of category 3 was weighted with a factor of 3. The score of category 4 was weighted with a factor of 4 since presence of deposits in the interstitial tissue over time results in necrosis and loss of the affected structures. Deposits in the hematopoietic- and immune tissue of the interstitium leads to granulomatous inflammation surrounding the damaged structures (tubules, collecting ducts and excretory ducts). The inflamed interstitial tissue is gradually replaced by fibrosis.

Total nephrocalcinosis score is calculated as:

$$\sum_{n=1}^{4} C_n \times S_{C_n}$$

Where C is the category, S is the severity and n is category number.

In the nephrocalcinosis score, overall scores 1 to 10 were generally considered mild changes, scores 11 to 20 were considered moderate changes and scores greater than 20 were regarded as severe changes.

Table 1
Histopathological nephrocalcinosis score. The scores are weighted based on the effect the various changes are believed to have on the development of the disease and the time it will take to heal the condition.

Severity	1	2	3
Category 1 Presence of deposits	Sparse amounts in collecting ducts and ureteres - close to absence in tubules and affects less than 10% of the excretory system	Moderate amounts in collecting ducts and ureteres - sparse amounts in tubules and affects between 10% and 50% of the excretory system	Extensive quantities in ureteres, collecting ducts and tubules and affecting more than 50% of the excretory system
Category 2 Epithelial degeneration and/or necrosis	Affects less than 10% of tubules and collecting ducts	Affects between 10% and 50% of tubules and collecting ducts	Affects more than 50% of tubules and collecting ducts
Category 3 Pathological changes in the glomeruli	Dilatation of the glomerular space (urine stagnation) and fibrosis / thickening of the parietal Bowman's capsule, changes in less than 10% of the glomeruli	Dilatation / thickening of the parietal Bowman's capsule, peri-glomerular fibrosis - changes in between 10% and 50% of glomeruli	Dilatation, thickening of the parietal Bowman's capsule, peri-glomerular fibrosis, changes in over 50% of glomeruli
Category 4 Pathological changes in the interstitial tissue	Affects less than 10% of interstitial tissue	Affects between 10% and 50% of interstitial tissue	Affects more than 50% of interstitial tissue.

2.5. Statistical analysis

The reference interval for normal ranges of plasma chemistry parameters were obtained by including all fish without nephrocalcinosis ($n = 234$) and excluding fish with HSS (Haemorrhagic smolt syndrome, $n = 5$), to ensure that only healthy fish were included in the data set ($n = 229$). Extreme values (outliers) were identified using Horn's algorithm with Tukey's interquartile (IQ) fences. The criterion for rejection was values exceeding IQ fences according to Horn et al. (2001). A non-parametric statistical method with a 95% confidence interval of reference intervals was chosen, were the 2.5th and 97.5th fractiles serve as the lower and upper reference limits (Friedrichs et al., 2012).

All statistical analyses were performed using R software 4.0.5 (R Core Team, 2017). Normality was tested with the Shapiro-Wilks test and showed non-normal distribution for the majority of plasma chemistry variables and the non-parametric test Kruskal-Wallis was performed to explore significant differences. Wilcoxon rank sum test was used to compare healthy fish and fish with different severity of nephrocalcinosis. *P*-values were adjusted using Bonferroni correction. P-values ≤ 0.05 were stated as significant.

3. Results

Visual assessment showed that 89% of the fish from the nurseries had developed physical smolt characteristics as described in Langdon (1985). There were no diseases documented among the nurseries, with exception of HSS in 1.7% of the sampled fish (0.5% had both HSS and nephrocalcinosis). The mean weight of healthy fish was 150.4 ± 91.2g, while the mean weight of fish affected by nephrocalcinosis was 270.0 ± 204.4g ($p<0.05$). The gender distribution was 49% males and 51% females in both the healthy group and the group affected by nephrocalcinosis.

3.1. Histopathology

Mild changes in the kidney primarily consisted of amorphous mineral deposits in collecting ducts (Fig. 1b), and tubulus (Fig. 1c) with minor changes in the epithelium of the tubular structures. With increased severity, increased damage to the tubular wall was seen. From degeneration and necrosis of the epithelium (Fig. 1d) to complete loss of epithelium with fibrosis of the basal membrane, and dilatation (Fig. 1e), and further to complete loss of integrity of the wall, often accompanied by extensive tissue reactions in surrounding interstitial tissue (Fig. 1f). Associated glomerular changes involved dilatation of the glomerular space, fibrosis and thickening of the parietal layer of the Bowman's capsule, and varying degree of per-glomerular fibrosis and glomerulitis (Fig. 1g). These changes observed in the glomeruli are thought to be at least in part a result of urine stagnation (Docherty et al., 2006). In advanced cases, acute interstitial inflammation or chronic interstitial fibrosis is seen (Fig. 1h), often in association with misshaped and degenerated tubuli (Fig. 1h) and extensive dilatation of collecting ducts,

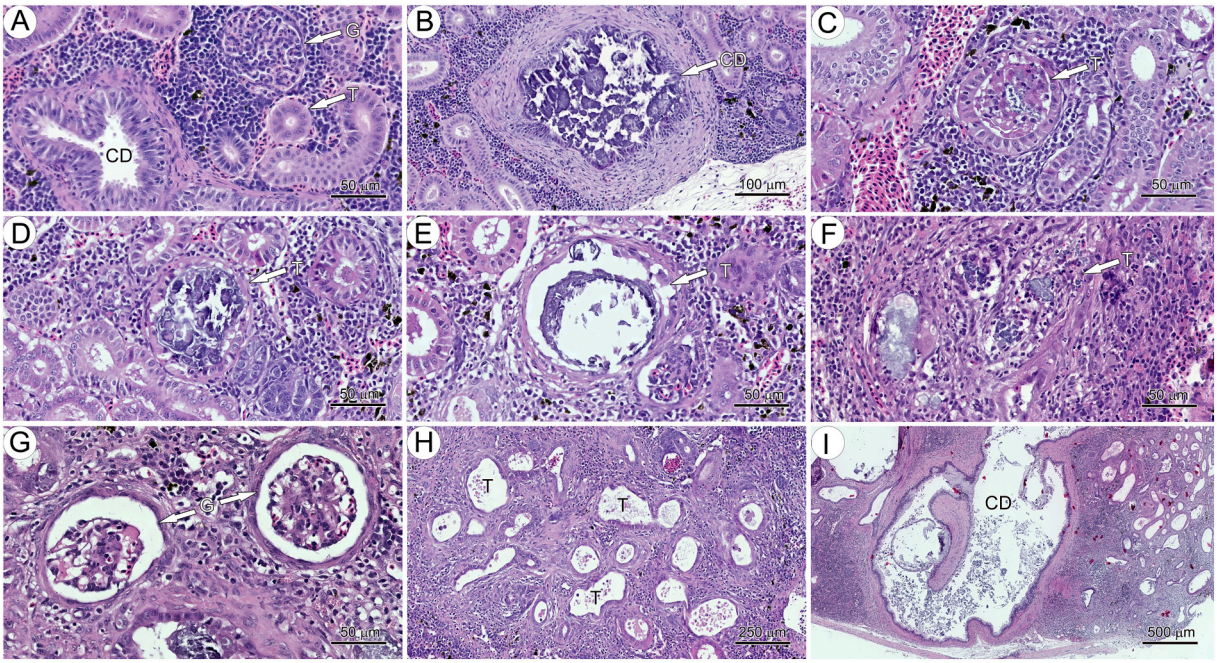

Fig. 1. Histopathological changes associated with nephrocalcinosis. A) Normal tissue showing a healthy glomerulus *(G)*, tubulus *(T)* and collecting duct *(CD)*. B) Early lesion, with basophilic, amorphous deposits in a collecting duct. Note that the epithelium of the duct is intact, and that surrounding tissue appears without remarks. C) Early lesion in a tubule, with slight occurrence of degeneration and necrosis of the tubular epithelium. Deposits together with necrotic epithelial cells are seen in the tubular lumen. D) Moderate lesion in a tubule, with marked degeneration and necrosis of the epithelium and rich amounts of deposits in the lumen. E) Extensive, chronic lesions in a tubule, with complete loss of epithelium, fibrosis of the basal membrane, and dilatation of the lumen containing deposits. F) Advanced, chronic lesion with complete destruction of the tubular structure including the basal membrane, allowing the deposits getting in contact with the interstitial tissue inducing massive inflammation. G) Chronic changes seen in glomeruli, with dilatation of the Bowman's space, fibrosis and thickening of the parietal layer of the Bowman capsule, peri-glomerular fibrosis and moderate glomerulitis. H) Chronic changes, with degeneration of epithelium and dilatation of tubules, surrounded by extensive fibrosis replacing normal interstitial tissue. I) Chronic changes, with extensive dilatation of a collecting duct, degeneration and necrosis of epithelium and presence of deposits together with necrotic cells in the lumen.

with degeneration and necrosis of associated epithelium (Fig. 1i). The changes observed in the interstitium appear when the mineral deposits formed within the excretory system breaks through the tubular wall and interacts with the immune tissue of the interstitium.

Black staining of deposits by von Kossa stain indicated high contents of calcium salts in the deposits associated with nephrocalcinosis (Fig. 2).

The most severe cases of nephrocalcinosis were easily visually identified, as extensive amounts of deposits cause extensive loss of kidney structure (Fig. 3).

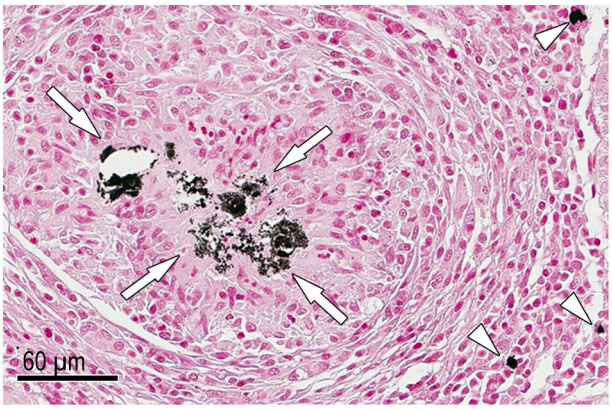

Fig. 2. von Kossa stain of kidney tissue with deposits and tissue reaction associated with nephrocalcinosis. Black staining (arrows) indicative of presence of calcium salt, surrounded by a granulomatous inflammatory process. Arrowheads points to melanin-containing cells; as for calcium salts, melanin reduces silver from the von Kossa stain into black deposits.

Fig. 3. Severe nephrocalcinosis in Atlantic salmon - extensive amounts of deposits in the kidney accompanied by swollen tissue and loss of normal structure.

3.2. Prevalence and severity of nephrocalcinosis in mid-Norwegian nurseries and sea farms

Nephrocalcinosis was observed in all the nurseries in this survey, even though the prevalence varied greatly between the nurseries (Fig. 4a), ranging from less than 5% to 100% of sampled fish. More than half of the individuals with nephrocalcinosis had mild changes in the kidney tissues (68%, Fig. 4a). The total proportion of fish with nephrocalcinosis was 45% in the nurseries. Among fish that were sampled one month after transfer to sea, the prevalence of nephrocalcinosis decreased to 18%. In fish groups with primarily mild degrees of nephrocalcinosis in the nursery, the prevalence had decreased (30% vs. 11%),

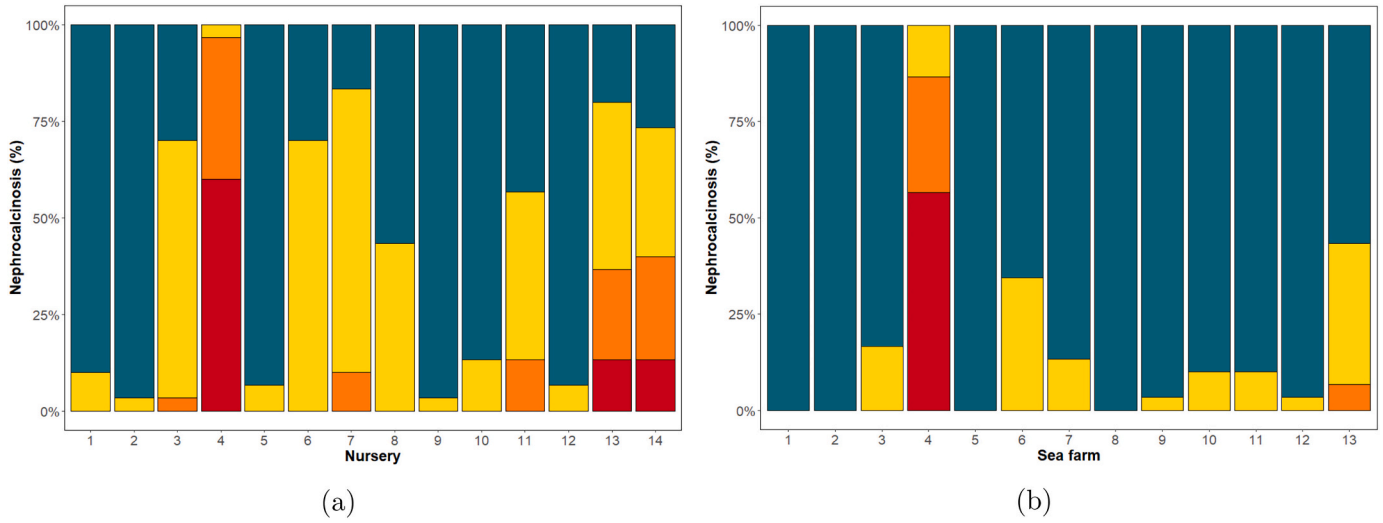

Fig. 4. Nephrocalcinosis in a) nurseries in Mid Norway($n = 420$) and b) receiving sea farm ($n = 390$). Yellow colour indicates mild changes, orange shows moderate changes and red indicates severe changes in the kidney tissue as found from histopathological examination. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

while in the groups with severe nephrocalcinosis the changes in the kidney persisted (6% vs. 4%, Fig. 4b).

3.3. Characterization of mineral deposits

A total of 69 samples of deposits were analysed by FTIR. The results revealed the presence of five different minerals: amorphous carbonate apatite (amCAP), struvite, brushite, whitlockite and newberyite. Amorphous carbonate apatite (amCAP) was the most prevalent mineral and was found in all but one of the mineral samples (Table 2). Struvite, brushite, whitlockite and newberyite were found in different combinations together with amCAP. From half of the nurseries only amCAP was found, while the remaining had different combinations of mixed minerals.

3.4. Plasma chemistry

A considerable proportion of fish affected by nephrocalcinosis showed plasma chemistry that differed from unaffected fish in several of the analysed parameters, with elevated calcium and magnesium being the most predominant changes (Table 3). A notable proportion of fish affected by nephrocalcinosis also displayed elevated levels of AST, inorganic phosphate, glucose and lactate, and lower levels of sodium and chloride, with reduced osmolality.

Smolts with severe changes in the kidney displayed significantly higher concentrations of AST, calcium, creatinine, glucose, magnesium, Na/K-ratio, inorganic phosphate, and urea. While plasma concentrations of chloride, potassium and sodium were significantly lower compared to the healthy group (Fig. 5). The plasma chemistry of fish with a moderate amount of nephrocalcinosis showed significantly higher concentrations of AST, calcium, magnesium, NA/K-ratio and inorganic phosphate, while chloride and potassium were significantly lower in this group compared to the healthy group (Fig. 5). The group with mild changes in the kidney had significantly higher concentrations of AST, calcium, glucose, lactate, magnesium, Na/K-ratio, inorganic phosphate and urea compared to the healthy group (Fig. 5).

Table 2
Mineral complexes found in Atlantic salmon reared in commercial nurseries in Mid-Norway ($n = 69$).

Mineral complex	Chemical formula	Number of samples
Amorphous carbonate apatite	$Ca_{10}(PO_4)_6CO_3$	68
Struvite	$MgNH_4PO_4$ x 6 H_2O	6
Brushite	$CaHPO_4$ x H_2O	7
Whitlockite	$MgCa_8(PO_4)_6$	10
Newberyite	$MgHPO_4$ x 3 H_2O	8

Table 3
Normal intervals of selected plasma chemistry parameters obtained from 229 healthy Atlantic salmon, with a comparison to data collected from salmon affected by nephrocalcinosis.

Parameter	Normal interval	Nephrocalcinosis		
		N	Higher	Lower
ALKP(U/L)	66–667	182	16 (9%)	7 (4%)
ALT (U/L)	10–38	101	14 (14%)	0 (–)
AST (U/L)	22–729	185	41 (22%)	1 (1%)
Creatinine (U/L)	9–80	119	15 (13%)	9 (8%)
Calcium (mmol/L)	2.1–3.4	186	69 (37%)	15 (8%)
Magnesium (mmol/L)	0.7–1.5	185	71 (38%)	7 (4%)
Phosphate (mmol/L)	2.4–8.1	184	29 (16%)	5 (3%)
Sodium (mmol/L)	149–169	186	9 (5%)	34 (18%)
Potassium (mmol/L)	1.5–4.9	182	5 (3%)	2 (1%)
Na/K	30–103	182	7 (4%)	4 (2%)
Chloride (mmol/L)	113–137	186	5 (3%)	34 (18%)
Osmolality (mmol/kg)	305–343	178	7 (4%)	30 (17%)
Glucose (mmol/L)	3.7–8.7	184	50 (27%)	2 (1%)
Lactate (mmol/L)	2.1–10.1	177	27 (15%)	2 (1%)
Urea (mmol/L)	0.6–1.7	182	23 (13%)	0 (–)

The comparison is shown as number and percentage of fish having a value higher or lower than the normal interval, out of the total number of valid samples (N).

4. Discussion

Nephrocalcinosis has in later years emerged as a major disease complex in Atlantic salmon reared in fresh water in Norway. To identify early markers, non-lethal diagnostic methods are important to reveal risk factors and support disease prevention. Further, determination of the composition of the kidney stones can provide clues about causation and potential ways to prevent stone formation. In the present study, histopathology, plasma chemistry and mineral deposit analysis were employed to get more insight into this disease in farmed Atlantic salmon.

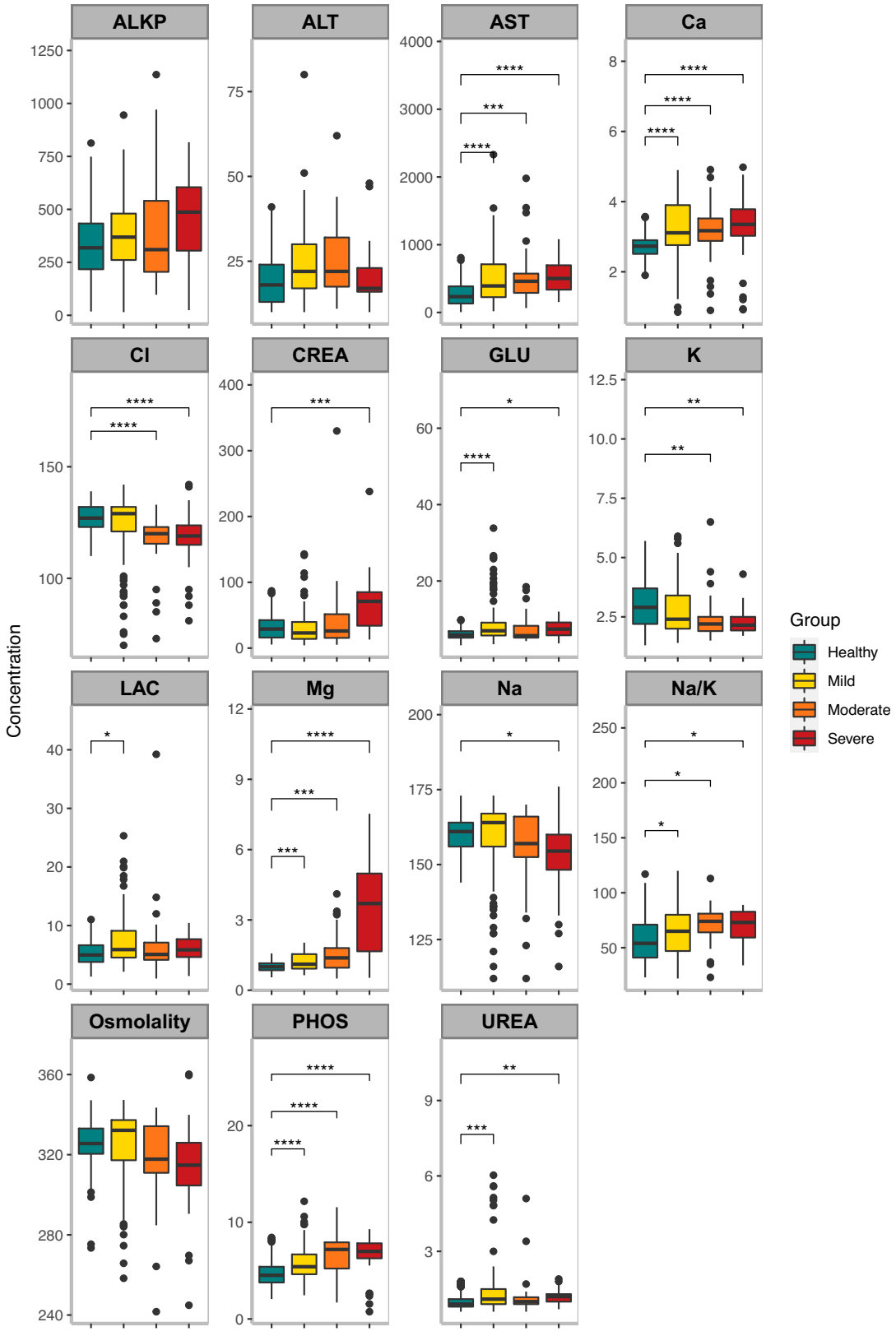

Fig. 5. Plasma chemistry parameters in Atlantic salmon with different severity of nephrocalcinosis (mild, moderate and severe, as assessed from histological evaluation), compared to healthy individuals as defined in Table 3. Significant differences denoted with */**/***/**** ($p<0.05/0.01/0.001/0.0001$). Measured parameters: Alkaline phosphatase (ALKP, U/L), Alanine aminotransferase (ALT, U/L), Aspartate aminotransferase (AST, U/L), Calcium (Ca, mmol/L), Chloride (Cl, mmol/L), Creatinine (CREA, μmol/L), Glucose (GLU, mmol/L), Potassium (K, mmol/L), Lactate (LAC, mmol/L), Magnesium (Mg, mmol/L), Sodium (Na, mmol/L), Osmolality (mmol/kg), Phosphate (PHOS, mmol/L) and Urea (UREA, mmol/L).

4.1. Methodology

Histopathology was used to assess the severity of tissue lesions and a weighed scoring system was developed for a more systematic approach for semi-quantification of the disease progression. The criteria and scoring method were designed to describe the changes in the kidney according to the known principles of pathology. The weighting factor was arbitrarily increased by one for each consecutive category to make sure that the more severe tissue-changes with increasing category number, resulted in higher contribution on the overall score. One limitation of the method is that the amount of tissue damage and the amount of deposits found in the histopathological sections do not always correlate. There are two main reasons for this: (1) the histopathological section represents a two-dimensional picture of a relatively large organ and there might be deposits in a plane that is not included in the section and (2) nephrocalcinosis in fish can heal (pers. obs.) and thus the chronic changes could remain whilst deposits could be reduced in quantity. Furthermore, primary and secondary causes of pathological changes in the kidney are challenging to differentiate histologically, as secondary changes caused by increasing inflammatory responses could be a result of chronic irritation due to nephrocalcinosis or other pathological conditions like Haemorrhagic Smolt Syndrome (HSS, pers. obs.). The accuracy of grading and the weighted scoring method is so far untested, but observations made in this study support the relevance for a weighed scoring, fish affected by nephrocalcinosis exhibited a wide range of histopathological changes. Fish affected by severe forms of nephrocalcinosis exhibited an almost complete loss of kidney structure while fish affected by the mildest forms of nephrocalcinosis exhibited at most negligible tissue damage.

Even though our results show that histopathology is a good diagnostic tool for nephrocalcinosis, non-lethal methods or early markers should be developed for a better monitoring of the disease. Blood chemistry analysis is often used as a diagnostic tool in several terrestrial species, but to our knowledge this methodology is infrequently used in farmed Atlantic salmon smolt. This is probably due to the lack of reference intervals for plasma chemistry in healthy individuals in different life stages, making it difficult to detect abnormalities. However, it would be advantageous if this method could be applied to farmed salmon as it would allow for rapid diagnostics without requiring euthanasia.

In this study, we used blood samples collected from 229 fish from 12 different nurseries to establish reference intervals for plasma concentrations for Atlantic salmon smolt reared in freshwater. Reference intervals were calculated following the guidelines from The American Society for Veterinary Clinical Pathology (ASVCP) (Friedrichs et al., 2012). Although of a similar magnitude, the normal intervals established in this study were wider than reference values previously reported by (Sandnes et al., 1988) for 10–20 adult Atlantic salmon from a single sea farm in Norway (Sandnes et al., 1988). One explanation may be that our data included a larger number of specimens as well as several facilities. Matsche et al. (2014) found statistical differences in plasma chemistry parameters in Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*) from different production conditions. The variations in water chemistry and feeds between salmon facilities in our study may therefore have influenced the plasma chemistry. The wider interval of the plasma parameters may alternatively be linked to the time of sampling that coincided with the end of parr-smolt transformation in the nurseries (3905 $\pm$ 537 day degrees from roe to sampling point). Parr-smolt transformation is a complex process that results in a changed physiology in salmon (Folmar and Dickhoff, 1980), and is unlikely to be 100% synchronised in a population, causing differing physiology among the sampled fish. Still, there was no correlation between the different plasma parameters and weight (see Table A3 in supplementary material), and only minor correlation between plasma concentration of AST and gender (see Table A4 in supplementary material). We therefore believe that the reference intervals we propose are relevant for Atlantic salmon undergoing smoltification (60–210 g) in nurseries with rearing conditions in accordance with Norwegian regulations.

4.2. Nephrocalcinosis

The majority of salmon affected by nephrocalcinosis displayed mild changes in the kidney, characterized by deposits in renal collecting ducts and excretory ducts. Histopathological analyses showed substantial accumulation of mineral deposits in kidney tissues of fish affected by nephrocalcinosis. The deposits were mainly identified as amCAP, a calcium-dominated mineral. This is in line with Béland et al. (2020) which stated that calcium phosphate and struvite calculi appears to be overrepresented in fish, such as rainbow trout *Oncorhynchus mykiss* (Bjerknes et al., 1994; Gillespie and Evans, 1979; Smart et al., 1979), wolffish *Anarhichas lupus* (Béland et al., 2020) and southern flounder *Paralichthys lethostigma* (Applegate et al., 2016). Amorphous carbonate apatite indicates rapid precipitation. The formation of carbonate apatite (CAP) begins at pH $\geq$ 6.8, with an increasing ability to aggregate with increasing pH (Olszynski et al., 2015). The pH of the urine was not measured in this study, but Roy and Lall (2004) determined that normal values for urine pH in Atlantic salmon, are around 7.5 (Roy and Lall, 2004). Considering that amCAP and CAP have similar chemical properties, the normal high urine pH of salmon may predispose it for calcium phosphate precipitation in the kidney. In humans CAP is a constituent in about 40% of all stones (Kravdal et al., 2019). They are linked to hypercalcemia, hyperparathyroidism, distal renal tubular acidosis and urinary tract infections (Daudon and Jungers, 2012). In domestic animals calcium phosphate stones are quite rare (Osborne et al., 2009). When observed they are linked to hypercalcemia, hyperparathyroidism, hypervitaminosis D, and dystrophic and ectopic mineralization of vital tissues (Osborne et al., 1995). The underlying mechanisms of nephrocalcinosis in fish do not seem to relate to this, as we did not observe dystrophic or ectopic mineralization of vital tissues in our study. In addition, Tsertou et al. (2020) and Hilton and Ferguson (1982) did not find that excess of vitamin D3 could be related to the incidence of nephrocalcinosis in meagre (*Argyrosomus regius*) or rainbow trout (*Oncorhynshus mykiss*). However, nephrocalcinosis in salmon could be related to hypercalcemia as it was observed in 37% of salmon affected by nephrocalcinosis, and mean plasma concentrations of Ca^{2+} were significantly higher in salmon affected by nephrocalcinosis compared to healthy fish. In addition, the proportion of fish with hypercalcemia increased with increasing severity of nephrocalcinosis. It is not possible to determine whether the observed hypercalcaemia is a cause of the development of nephrocalcinosis or a consequence of nephrocalcinosis based on our data, and this should be investigated further.

The proportion of fish with nephrocalcinosis decreased after transfer to sea in fish with mild forms, which is in accordance with earlier observations (Fivelstad et al., 2003, 1999) This may indicate that mild changes in the kidney are reversible after the transfer to sea. The mechanisms involved in this process are yet unknown and should be considered in future studies. Even though it appears that mild forms of nephrocalcinosis in the fresh-water facility did not adversely affect survival after transfer to sea, it is likely that nephrocalcinosis negatively affects fish welfare. Our data shows that fish with mild changes display, in addition to elevated calcium, increased phosphate, AST, lactate and glucose concentrations in plasma, which are all signs of decreased welfare. AST is an enzyme which is considered a good indicator for tissue damage in fish (Li et al., 2011; Peres et al., 2015; Wagner and Congleton, 2004). Elevated plasma calcium and phosphate points to a disturbed regulation of homeostasis (Vielma and Lall, 1998) and elevated plasma glucose and lactate levels are linked to secondary stress responses (Barton and Iwama, 1991; Iversen and Eliassen, 2009; Iversen et al., 2009, 1998) and metabolic stress in salmon (Li et al., 2011).

Fish that were affected by moderate and severe forms of nephrocalcinosis exhibited much higher concentrations of magnesium in their plasma compared to healthy fish. The affected fish were diagnosed

with severe changes in the kidney, also described as pathological changes in glomeruli and interstitial tissue with extensive epithelial damage in the tubules. The primary site of magnesium excretion in fish is the kidney (Bijvelds et al., 1998) and elevated concentration of plasma magnesium has previously been linked to kidney damage (Nieves-Puigdoller et al., 2007; Singh et al., 2002). Fish with severe forms of nephrocalcinosis also displayed elevated plasma levels of AST indicating once again tissue damage, as well as significantly lower concentrations of potassium, sodium and chloride, which are considered as signals for reduced osmoregulatory capacity (Carey and McCormick, 1998; McDonald and Milligan, 1997).

Fish health personnel at the sea farms included in this study reported nephrocalcinosis to be a contributor to the registered mortality in the groups with severe nephrocalcinosis. To the authors knowledge there is no scientific publication that has investigated the potential direct mortality caused by nephrocalcinosis in salmon, but Jelmert et al. (1995) reported mass mortality in cultured Atlantic halibut larvae linked to the same disease. Unlike a freshwater environment, seawater is hyperosmotic and causes a net water loss by osmosis in fish (Beyenbach, 2004). The transition from freshwater to seawater is a demanding process for anadrome species like salmon and it requires drastic changes in the physiology of several organs such as the kidney (Takvam et al., 2021). Fish affected by severe nephrocalcinosis exhibit a loss of normal kidney structure with vast amounts of calcium deposits and extensive damage in glomeruli and interstitial tissue. These individuals are thus likely to have a highly reduced kidney function, which probably makes adaption to seawater extremely challenging for the organ. This may, in turn, lead to increased mortality after transfer to sea, either directly as a loss of osmoregulatory function in the kidney or indirectly by increased susceptibility to stress and secondary infections by bacteria.

This study revealed that plasma chemistry analyses is not adequate to diagnose nephrocalcinosis. Normal blood chemistry values do not necessarily indicate absence of nephrocalcinosis, since a notable proportion of the fish with nephrocalcinosis displayed plasma chemistry values within the normal intervals. On the contrary plasma chemistry values can be used as preliminary diagnosis for the disease, as changes in calcium, magnesium, glucose and AST were observed in a pronounced part of the fish affected by nephrocalcinosis.The preliminary diagnosis of nephrocalcinosis should thereafter encourage subsequent investigations with histopathological methods to confirm the diagnosis. In this study we investigated 15 different blood parameters and we can't disregard that other components could be relevant for diagnosis of nephrocalcinosis.

5. Conclusion

This study clearly showed that nephrocalcinosis is a common production disorder in farmed Atlantic Salmon in nurseries in Mid-Norway. The fish studied exhibited a broad range of severity, ranging from sparse amounts of deposits in tubules and collecting ducts to pathological changes in glomeruli and the interstitial tissue. Although the majority of the examined fish were only mildly affected, our results revealed that nephrocalcinosis is a welfare challenge. A considerable percentage of the fish affected by nephrocalcinosis had altered plasma chemistry, indicating stress and osmoregulatory disorders. Even though it is not possible to diagnose nephrocalcinosis with a blood sample at this stage, elevated plasma levels of magnesium, calcium, glucose and AST can be used as indicators for disturbed physiology that may be related to nephrocalcinosis. Routine blood samples may therefore be used as a relatively easy detection tool, where detection needs to be followed up by autopsy and histopathology, to confirm a suspicion of nephrocalcinosis.

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Author contributions

CK and LB were responsible for the study conception and design, and funding acquisition. CK carried out the field work and was responsible for data analysis and visualisation. MKM analysed the mineral deposits and AKR and ASD performed the histopathological assessments. CK wrote the manuscript with support from AKR, ASD and LB. LB, KJKA and REO supervised the work and aided in interpreting the results.

Declaration of Competing Interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738104.

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