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Nephrocalcinosis and kidney stones in *Rachycentron canadum*

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Abstract

Nephrocalcinosis cases have been reported in fish, with different causes attributed for calcium deposition in the kidneys. We report the diagnosis of nephrocalcinosis and the presence of kidney stones in cobia, describing the main changes caused by the disease. On gross examination, an increase in kidney size was observed, with the presence of several grouped stones in the renal parenchyma. Microscopically, interstitial fibrosis and tubular dilation with basophil granules due to calcium deposits in the lumen were observed. By infrared spectroscopy, the stones were found to be composed of pure calcium (65%), oxalate (15%), calcium phosphate (16%) and non-specific salts (4%). This report of nephrocalcinosis and kidney stones is the first reported case of the disease in the species *Rachycentron canadum*, but given the range of factors that may contribute to the disease occurrence in aquatic cultivation environments, it was not possible to identify the real cause of the emergence of lesions in the fish, necessitating the study of the predisposing factors.

Introduction

The cobia *Rachycentron canadum* is widely distributed in tropical and subtropical waters of the Atlantic Ocean, the Indian Ocean and the Pacific Ocean (with the exception of the eastern portion) (Brown-Peterson et al., 2001). Interest in this species evolved largely due to its cultivation characteristics, which includes an excellent growth rate (Liao and Leaño, 2007), as these fish attain weights of up to 6 kg in one year (Arnold et al., 2002; Benetti et al., 2008) and between 8 to 10 kg in 16 months (Liao et al., 2004).

Among the various diseases that can affect cobia farms, metabolic disorders represent an important area for research investigation.

In humans, nephrocalcinosis is a metabolic disease of multifactorial etiology, with genetic and environmental contributing factors (Peres et al., 2010). The occurrence of nephrocalcinosis is related to an increase in calcium or urinary oxalate concentrations or a decrease in urinary citrate, with calcium being involved in approximately 85% of the nephrocalcinosis cases. Cases of non-calcium stones involve uric acid and cystine, as well as stones resulting from infection (Gomes et al., 2005).

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Various theories for the development of nephrocalcinosis have been proposed, such as the common occurrence of an increased urinary excretion of the elements that participate in the formation of the stones, as well as a decrease in excretion inhibitors that participate in the process of crystallization of such elements.

Nephrocalcinosis cases have been reported in fish undergoing treatment with sulfamiderazin (Smith et al., 1973) and in Nile tilapia (*Oreochromis niloticus*) reared in a recirculation system (Chen et al., 2001). Cases of nephrocalcinosis were also observed in salmon (*Salmo salar*) (Fivelstad et al., 2003) and spotted catfish (*Anarhichas minor*) (Foss et al., 2003) reared in systems with high levels of CO_2, contributing to the deposition of Ca in the kidney. A high concentration of phosphate, a pH increase in the cultivation system and a mineral imbalance in the diet have also been associated with the occurrence of nephrocalcinosis in marine horses (*Hippocampus reidi*) (Lewisch et al., 2013).

The involvement of the diet in the occurrence of Ca crystals has been discussed by some authors. The presence of calcium oxalate crystals in the renal tubules of clownfish *(Amphiprion ocellaris)* fed vitamin A and E-deficient diets (Blazer and Wolke, 1983) and the occurrence of nephrocalcinosis in rainbow trout fed diets with high levels of selenium have been reported (Hicks et al., 1984).

The mortalities caused by this disease in fish farming are generally low, as it is considered a reversible process (Schlotfeldt, 1981; Fivelstad et al., 1999, 2002). However, such deposits may develop and lead to secondary gastric complications (Schlotfeldt, 1981), i.e., abdominal distension (Harrison, 1979), which modifies the appearance of the fish and hampers its commercialization.

Thus, this study aimed to report the diagnosis of nephrocalcinosis in cobia, describing the main changes caused by the disease, and to investigate the possible causes of its occurrence, as the signs often arise in a subclinical form, therefore making diagnosis difficult.

Materials and methods

Five fish reared in a recirculating system in the Laboratory of Estuarine and Marine Aquaculture at the Marine Aquaculture Station - FURG were studied, which had an average weight of 4.2 ± 2.4 kg and length of 75 ± 3.1 cm. The water from the system was maintained at 24 °C, with a salinity of 26 $0/_{00}$ and dissolved oxygen greater than 6 mg/L.

The moribund fish were collected and euthanized with benzocaine (300 mg/L), then subjected to necropsy according to an existing protocol (Romano et al., 1987).

Histological analysis of organs:

Kidney, liver, spleen and gills samples were fixed in Bouin solution, subjected to histological processing with inclusion in Paraplast, sectioned into 5µm thick sections and stained with hematoxylin-eosin (HE) and Alizarin red S for calcium. Histological sections were observed and examined under a light microscope (Zeiss Primo Star), and the images were captured by a digital camera (Zeiss AxioCam ERc 5s). The slides stained with Alizarin S were analyzed using polarized light filters TIM-107 ref. TA-0159 (SICA-Brazil).

Infrared spectroscopy method

To define the components of the stones, an infrared spectroscopy technique was used, which allows an observation of the crystal structure of the salts. To this end, the samples for infrared measurements were prepared by crushing with an agate mortar, in approximately 1 mg to 30 mg of KBr (spectroscopic grade). The solid obtained was compressed in a hydraulic press to form an infrared transparent tablet. The infrared measurements were performed in a spectrophotometer (Bruker 66, Ettlingen, Germany) in the spectral region of 4000-400 cm-1 with resolution of 4 cm-1.

Analysis of Phosphorus and Calcium

To analyze phosphorus and calcium, the feed was dried at 105 °C for 5 hours in a forced circulation oven and subsequently degreased and macerated with a Soxhlet extractor, using petroleum ether as solvent. The samples were taken to the Laboratory of Hydrochemistry/ IO-FURG and submitted to acid digestion with nitric and perchloric acid, with the resultant solutions diluted in distilled water and analyzed according to the methodology by Silva and Queiroz (2009). The samples were read on an atomic absorption spectrophotometer at a wavelength of 422.7 nm for calcium and in a digital spectrophotometer (Micronal B342II- Brazil) at a wavelength of 725 nm for phosphorus.

Results

Macroscopically, a severe renomegaly was observed, with the presence of several grouped lithic structures in the renal parenchyma occupying a large portion of the kidney (Figure 1). The stones were removed and washed for further analysis, which revealed the stones to be rounded whitish solids with diameters ranging from 0.4 to 1.3 cm (Figure 2).

Microscopically, interstitial fibrosis and tubular dilation with basophil granules due to the calcium deposits in the lumen were observed. In some collecting tubules with abundant calcic material in the lumen, squamous metaplasia was observed (Figure 3 and 4).

By means of the alizarin red S technique, an encapsulated calcium material was observed to be distributed throughout the renal parenchyma, coated by fibrous tissue. The calcium was birefringent when observed with polarized light (Figure 5 and 6).

By the infrared spectroscopy method, it was demonstrated that the nephrocalcinosis stones were composed of pure calcium (65%), oxalate (15%), calcium phosphate (16%) and non-specific salts (4%).

In the analysis of calcium and phosphorus in the feed supplied to the animals, the averages from the triplicates were 1.55% for phosphorus and 5.45% for calcium.

Discussion

The kidney lesions described herein are compatible with a diagnosis of nephrocalcinosis and have been reported in other species of fish, though a variety of causes have been attributed in these cases (Smith et al., 1973; Chen et al., 2001; Fivelstad et al., 2003; Foss et al., 2003; Lewisch et al., 2013).

The interstitial fibrosis observed in the renal tissue of the cobia sampled represents an injury similar to that already reported in some studies in humans with nephrocalcinosis (Wiech et

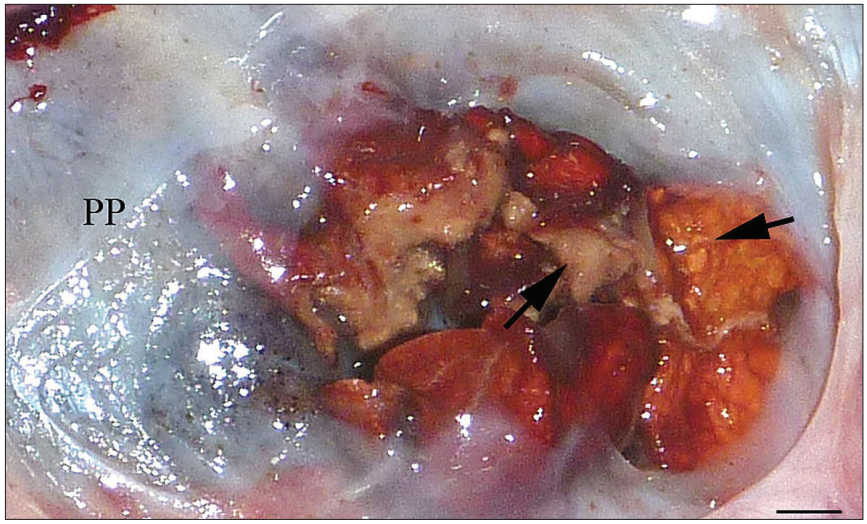

Figure 1. Kidney observed after cutting the parietal peritoneum (PP), where several whitish and yellowish stones could be observed (arrows). Bar = 3 cm.

Figure 2. Six stones taken from the kidney, with diameters between 0.4 and 1.3 cm. Bar = 1 cm.

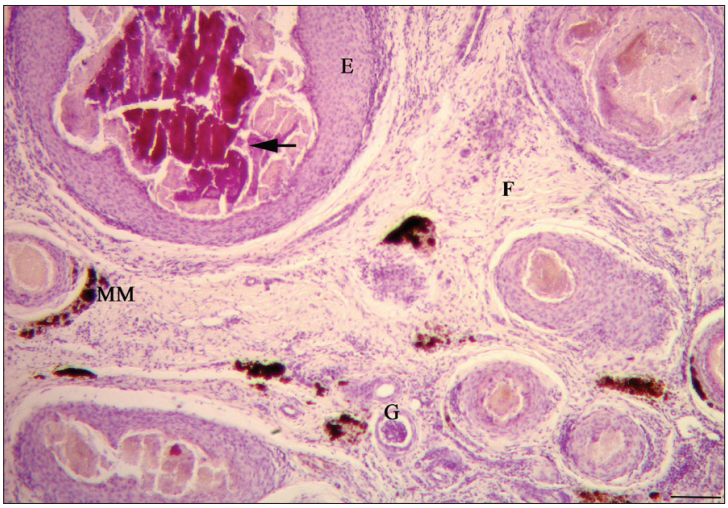

Figure 3. Renal parenchyma with collecting tubules dilated with epithelial squamous metaplasia (E) and abundant deposits of basophilic material with calcic aspect. Interstitial fibrosis (F), melanomacrophages (MM) and glomeruli (G) could be observed. H-E, bar = 100 μm.

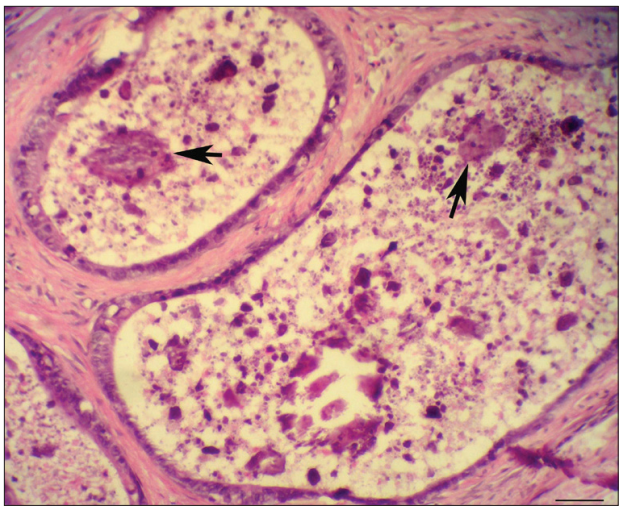

Figure 4. Collecting tubes with calcic precipitate in their lumen (arrows). H-E, bar = 20 µm.

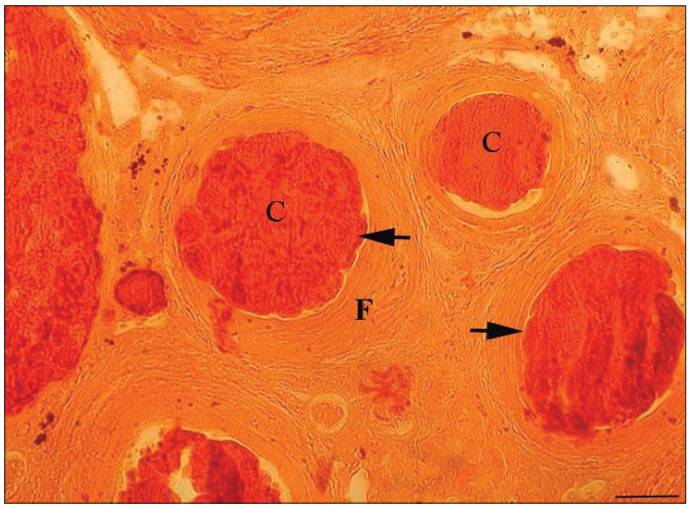

Figure 5. Abundant calcium material (C) stained in orange surrounded by fibrous tissue (F). Alizarin red, bar = 100 µm.

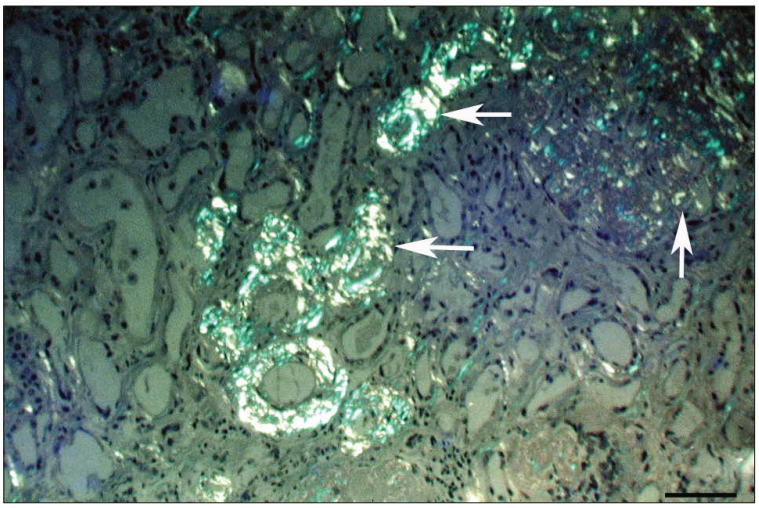

Figure 6. Calcic material displayed birefringence under polarized light (arrows). Bar = 100 µm.

al., 2011). Dilation and renal tubular necrosis with the presence of intratubular mineral deposits have already been reported as injuries in nephrocalcinosis cases in Nile tilapia (*Oreochromis niloticus*) (Chen et al., 2001).

Morphologically, the histological lesions observed in the renal tissue of cobia after HE staining demonstrated a pattern of granular calcification, reported by some authors as prevalent in humans with stones with calcium compounds (Wiech et al., 2011).

Because this disease has a multifactorial origin, there are several causes attributed to the occurrence of nephrocalcinosis. In humans, metabolic and anatomical abnormalities are the two main paths associated with this disease (Miliner and Muphy, 1993; Sakhaee et al., 2012), and although metabolic syndrome is considered a major risk factor for chronic kidney disease, it is not known if it is only associated with nephrocalcinosis or is due to the individual metabolic dysfunctions, specifically obesity, glucose intolerance and hypertension (Mossetti et al., 2008).

Several studies on the dietary factors in the emergence of nephrocalcinosis have been reported in humans. Among them, an excess of purine in the diet causes an overproduction of uric acid, with hyperuricosuria associated with the production of calcium oxalate in the stones (Coe, 1978).

Many of the metabolic disorders associated with nephrocalcinosis require further research to detail the relationship of dietary components with the prevalence of the stones. In the case of our research involving the cultivation of fish, when dealing with a disease arising in a subclinical form, all information regarding the animal management, particularly with regard to the feed supplied, must be researched.

There are many components in the formation of nephrocalcinosis, such as calcium oxalate, uric acid, struvite and cystine, as well as stones resulting from infection (Gomes et al., 2005), and the results of our analysis of the stones were representative of this, particularly when a non-calcic constituent is involved. An evaluation of the metabolic disorder should lead to the adequate prevention of a recurrence of the disease (Wasserstein, 1998).

The application of the Alizarin Red S technique has been discussed in some nephrocalcinosis studies in humans, where it was possible to differentiate the types of deposits observed in kidney damage (Wiech et al., 2011). Through this technique, we observed calcium birefringent material distributed throughout the renal parenchyma, covered by fibrous tissue.

One of the mechanisms involved in the formation of nephrocalcinosis is the precipitation of crystals, resulting in urinary supersaturation influenced by the elimination of solutes excreted through the kidneys, pH and urinary volume. Nucleation and crystals are formed when supersaturation occurs. This nucleating event serves as a surface to deposit new crystals, leading to the development of nephrocalcinosis (Branco et al., 2009).

Diets with high oxalic acid content have also been associated with the occurrence of nephrocalcinosis in amphibians from the Ranidae family, which were observed to have renal injury in the form of renal tubular necrosis,

fibrosis and squamous metaplasia and the appearance of intraluminal crystals that were birefringent under polarized light, characterized morphologically and histochemically as calcium oxalate compounds (Forzán et al., 2014).

Nephrocalcinosis is a common disease in aquatic environments rich in carbon dioxide and phosphates (Stoskopf, 1993). The prevalence of nephrocalcinosis in fish is not necessarily caused by the carbon dioxide content in water, but rather is attributed to nutritional factors, i.e., selenium toxicity (Hicks et al., 1984), magnesium deficiency (Knox et al., 1981) and an unbalanced mineral content in the feed in general (Hilton and Hodson, 1983). It is therefore possible that the feed currently used in cobia cultivation do not have the ideal mineral composition for this species.

Nephrocalcinosis in salmonids is a chronic inflammatory condition of unknown etiology, in which calcium and other minerals precipitate as hydroxyapatite within the distal renal and collecting tubules. This condition is commonly seen in water high in carbon dioxide and phosphates. Advanced cases can be recognized grossly by whitish streaks and spots in the kidney. Microscopically, dilation of real tubules and degeneration of tubular epithelium can be observed. Mineral-laden tubular casts, which are histochemically positive for calcium salts, are usually present (Bendele and Klontz, 1975). The main difference with the calcinosis of salmonids is that real stones in *R. canadum* occur in rather thick deposits generally observed in nephrocalcinosis.

Recirculation systems are being used more often to produce fish, and to withstand the high densities, these systems must be able to effectively add oxygen to supersaturated levels and to remove solids, ammonia, nitrite and carbon dioxide. Suboptimal water quality can directly impact fish health. Environmental problems can cause direct mortality or act as stressors that can precipitate disease outbreaks (Noble and Summerfelt, 1996).

The effect of fish exposure to high levels of CO2 in the cultivation environment contributes to the appearance of nephrocalcinosis, with a consequent reduction in the growth and feed conversion rate, as has already been described for the rainbow trout (Eddy et al., 1979) and Atlantic salmon smolt raised in an environment with stable pH in freshwater rich in calcium bicarbonate (Fivelstad et al., 1999). The effect of CO2 on nephrocalcinosis, ionic regulation, hematology and growth parameters in Atlantic salmon smolt was also reported by Fivelstad et al. (2002). Given that the level of dissolved carbon dioxide depends on the pH, alkalinity and temperature of the cultivation water (Butler, 1991), the maintenance of the water parameters within optimal levels for the culture of the species is an important point in the prevention of diseases.

The addition of an extra source of alkalinity to the aquatic environment in recirculating systems may help to maintain the pH and minimize the toxic effects of ammonia and carbon dioxide (Summerfelt, 1996). In the cultivation environment of the cobia in our research, daily doses of sodium bicarbonate were used to maintain the alkalinity at greater than 150 ppm. The use of calcium carbonate replacing the sodium bicarbonate to increase the pH in the cultivation water contributes to the occurrence

of nephrocalcinosis in Nile tilapia (Chen et al., 2001), though it was not used in the cultivation environment in the present study.

In the latest case of nephrocalcinosis reported in seahorses, this occurrence was associated with changes in water parameters, including a high phosphate concentration, along with an increase in the pH and mineral imbalances in the diet offered to animals (Lewisch et al., 2013), demonstrating the relevance of the role of phosphate in the emergence of the disease. This report of nephrocalcinosis is the first case of the disease in the species *R. canadum* (cobia), which is very relevant particularly for some research initiative projects being carried out in Brazil that aim to improve the techniques for the cultivation of the species.

Regarding the range of factors that may contribute to the occurrence of the disease in aquatic environments of cultivation, it was not possible to identify the actual cause of the appearance of lesions in the fish, therefore necessitating the study of the predisposing factors.

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