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Note

Investigations on the incidence of cystic calculus in female cobia *Rachycentron canadum* (Linnaeus, 1766) broodfish leading to spawning failure and mortality

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ABSTRACT

The morphological, chemical and high resolution electron microscopic analysis of a cystic calculus (urinary bladder stone) from a ready-to-spawn female broodfish of cobia *Rachycentron canadum* (Linnaeus, 1766) is reported. The stone was elliptical in shape with 31 mm dia having chalky, amorphous and fragile consistency with several concentric major layers covering the core. Chemically the stone was uniform in composition with 52.63% calcium oxalate monohydrate (COM), 31.58 % uric acid and 15.70% hydroxyapatite (HAP). However, the peripheral layer differed significantly from the middle and the core in elemental composition. Ultrastucturally, each of the individual layers were made of multiple fine sub-layers. Additionally, all the layers/portions of the stone displayed a hard, coral-like structure entrapping spherical sacs and contained isolated and fused spherules inside and outside the sacs. However, hexagonal forms of COM crystals were unique to the peripheral layers. While the obstruction of the oviduct due to the large sized stone was probably the cause of spawning failure. Continuous but futile contraction of the musculature during the unsuccessful spawning effort might have caused energy exhaustion and ultimate death of the animal.

Keywords: Cobia, Cystic calculi, Physico-chemical nature, Spawning failure

Research and documentation of urinary stones from aquatic animals is rare in comparison to terrestrial vertebrates and mostly limited to incidental reporting in certain marine mammals (Dennison *et al.,* 2007; Venn-Watson *et al.,* 2010) and turtles (McKnow, 1998). Reports on natural occurrence of stone in the urinary tract of fish are extremely rare (Lewisch *et al.,* 2013) or absent, especially in finfish species of aquaculture importance.

We report here the first ever incidence and physico-chemical nature of a cystic calculus (urinary bladder stone) in a female broodfish of cobia *Rachycentron canadum* (Linnaeus, 1766) and the consequent spawning failure and death of the animal. Cobia is a popular species for mariculture globally and in India, seed production and farming of the species was initiated recently and is emerging as the finfish species of choice for marine cage aquaculture (Gopakumar *et al.,* 2011).

The female cobia broodes was originally collected from the commercial hook and line catches off Mandapam Coast and reared in a circular HDPE sea cage of 6 m dia and 3.5 m depth installed in the Gulf of Mannar ($9^0$16'06.13" N; 79^0 07'55.58"E) for raising broodfish from sub-adult stage (5-6 kg) along with 38 such animals. The fishes were fed on chopped sardines, squid and portunid crabs @ 2 -5% of their body weight, in two split doses. The animal was evaluated for gonadal maturity a day before hormonal induction, through intra-ovarian cannulation and was kept in a 100 t cement spawning tank. The female cobia brooder, after evaluation of its gonadal maturity stage for readiness for spawning induction *i.e.*, the presence of intra-ovarian eggs of around 700 µ size, was induced with human chorionic gonadotropin (HCG) @ 500 IU per kg body weight, but was found dead on the expected date of spawning.

At necropsy, the total length of the animal and its ovary, recorded were 120 and 30 cm while the total weight of the fish and ovary were 16 and 2.17 kg respectively with a gonado-somatic index (GSI) of 13.24%. The profuse vascular innervations, turgid hydration and the good ova diameter frequency (1.00 mm) of the ovary indicated that the fish was indeed ready for spawning. The presence of matured egg mass inside the ovary and absence of any egg in the spawning tank testifies that it was indeed a case of spawning failure. However, no gross lesions were found on the external body surface, visceral organs (including kidney and urinary bladder) and internal

tissues of the fish except for the presence of a spherical stone of 31 mm dia and 20.76 g weight in the urinary bladder. The stone was chalky and fragile in consistency with a mulberry-like rough surface. It was located at the neck of the urinary bladder which was closely adhered to the serosal membrane of the groove formed by the dorso-caudal parts of the ovarian lobes (Fig. 1), close to the beginning of the oviduct. Normally, presence of large foreign bodies in the peritoneal cavity imparts additional stress and results in improper spawning in fish (Berejikian *et al.,* 2007). In the present case, the large sized stone was closely situated near the beginning of oviduct which might have been completely obstructing the release of eggs leading to the failure of spawning. Furthermore, in the already stressful condition of spawning (Berejikian *et al.,* 2007), continuous but futile muscular contractions might have resulted in irrecoverable fatigue and consequent death of the animal.

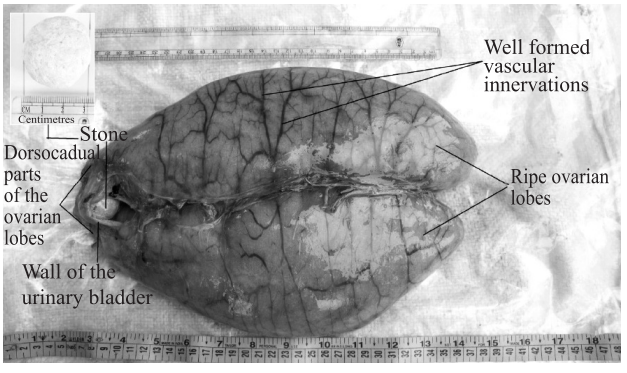

Fig. 1. Vascular innervations and turgidity of the ovary and position of the stone inside the urinary bladder, Inset: external morphology and the size of the stone

For analysing the physico-chemical nature of the recovered stone, it was cut carefully through its centre into equal hemispheres with the help of a jeweler's saw. The powder resulting from cutting was collected and subjected to chemical analyses as per Hodgkinson (1971) for gaining preliminary idea about its chemical nature. Stereomicroscopy of the cut surface of the stone showed grossly 7-8 concentric layers surrounding an elliptical core. Upon further magnification, multiple fine sub-layers were observed within each of the layers (Fig. 2). Shreds of the stone from the most peripheral layer (mentioned "periphery" hereafter), powder from the core (mentioned "core" hereafter) and shreds of the stone from a middle layer between the core and the periphery of the stone (mentioned "middle" hereafter) were removed carefully and each were subjected to high resolution scanning electron microscopy (HR-SEM), energy-dispersive X-ray spectroscopy (EDX) and Fourier transformation Raman spectroscopy (FT-RS) analyses. Furthermore, a portion of the stone representing all the major layers together (mentioned "whole" henceforth) was taken out by cutting out a sector from the cut surface of one of the hemisphere and was subjected to HR-SEM.

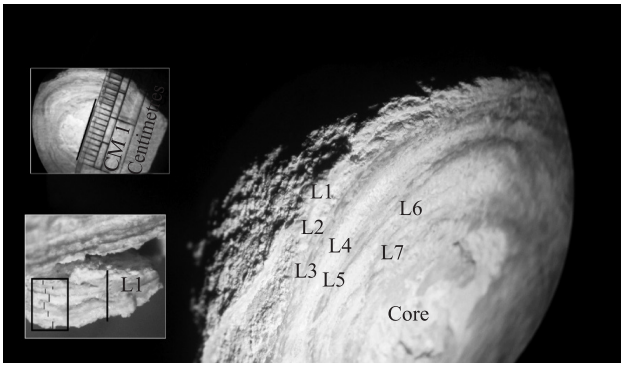

Fig. 2. Photomicrograph of the cystic calculus several concentric layers surrounding the core of the stone L1, L2… L7 indicate presence of seven layers starting from the periphery to the core of the stone. Upper inset: diameter of the core. Lower inset: multiple sub layers (vertical black bars inside the square within the first (L1) layer (long vertical black bar)

Chemical tests of the powder indicated oxalate and urate as the two major chemical components of the stone and this was confirmed by FT-RS analyses. FT-RS analyses of the periphery, middle and the core of the stone and *a priori* comparison of respective FT-R spectra revealed little chemical difference among them and on an average the major part of the stone was composed of calcium oxalate monohydrate, COM (52.63%) and uric acid (31.58%) while hydroxyapatite (HAP) formed a minor (15.70%) part of the stone. The FT-R spectra and corresponding peak areas of the periphery of the stone are presented in Fig. 3.

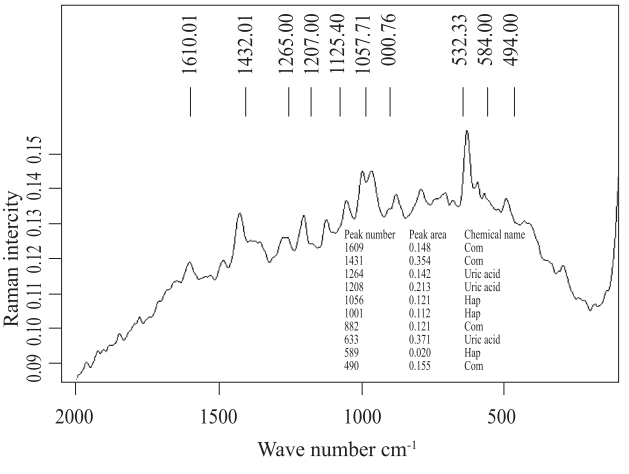

Fig. 3. FT-Raman Spectra of the periphery of the stone and the number, area and identity of its peaks.

The EDX data revealed that different portions of the stone consisted mostly of atoms of carbon (39.32-45.1%), oxygen (23.14-33.33%), nitrogen (12.09-22.44%), calcium (2.06-3.97%), phosphorous (2.08-5.43%), magnesium (2.61-3.95%), sodium (0.5-1.78%), potassium (0-0.19%), chlorine (0-0.1%), fluorine (0.46-1.38%) and aluminum (0-0.02%). *Postl hoc* Bonferoni one way ANOVA test revealed that none of the portions of the stone differed significantly ($p>0.05$) among each other with regard to the atom percent composition of carbon, aluminum, calcium, phosphorous and chlorine. However, periphery of the stone differed significantly ($p<0.05$) from both middle and core portions in the atom percent composition of nitrogen, sodium, oxygen and fluorine. Similarly, the periphery differed significantly ($p<0.05$) from the core and the middle of the stone in the atom percent composition of potassium and magnesium respectively.

The HR-SEM of the whole stone also revealed presence of several layers, each of which were formed of multiple fine layers (Fig. 4), conforming the stereomicroscopic observation. The core, middle and periphery of the stone were ultrastructurally similar and comprised various entities such as small discoid crystals embedded in irregular masses, scaffolds resembling a hard coral-like structure containing empty sacs and isolated as well as bunches of fused spherules of variable sizes in and outside the sac-like structure (Fig. 5). Considering the morphological similarities of these spherules to uric acid spherules found in many biological instances (Folk, 1969; Lonsdale and Sutor, 1971; Goh *et al.,* 2013) and uric acid being a major component of the stone in the present case, it is inferred that these spherules were most probably formed of uric acid. However, unlike the other portion of the stone, clear hexagonal crystals characteristically resembling the calcium oxalate monohydrate (COM) crystals (De Yoreo *et al.,* 2006; Goiko *et al.* 2013) were uniquely found in the periphery of the stone (Fig. 5). This uniqueness might be attributed to a unique crystallisation condition in the periphery of the stone as a result of its elemental difference (such as nitrogen, oxygen, fluorine, sodium, magnesium and potassium) from the middle and the core. This conforms to the fact that crystallisation or precipitation conditions such as the presence or absence of electrolytes affect the morphology, phase, dimensions and particle size distribution of calcium oxalate crystals (Wesson *et al*., 1999; Akhtar and Haq, 2013).

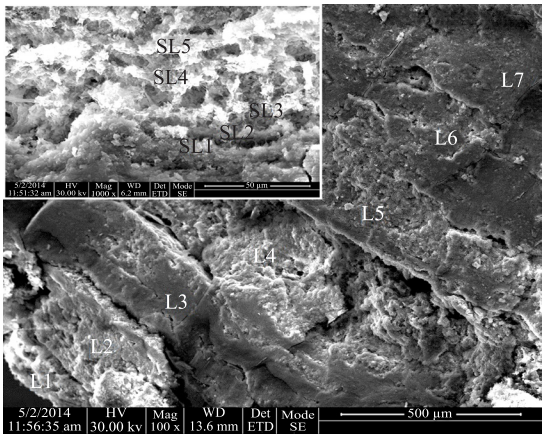

Fig. 4. Several concentric layers (L1 through L7) of the stone surrounding its core observed under HR-SEM. Inset: Multiple fine sub layers (SL 1 through SL5) within each of the concentric layers

The spawning failure caused by stone formation (as in this case) can cause loss of valuable broodfish, reduction in seed production and financial loss to hatcheries. Insights into the cause of stone formation in fish may be helpful to take appropriate preventive measures to avoid such conditions. In this case, we only speculate the causes for formation of such a stone of chemically mixed nature. Nevertheless, the presence of a significant amount of monovalent solutes such as sodium (0.5-1.78%), fluorine (0.46-1.38%), chlorine (0-0.10%) and potassium (0-0.19%) in the stone is indicative of solute imbalance as one of the causative factor for stone formation, since in marine teleosts these monovalent solutes are usually excreted from the gills and not through the urinary tract (Berglund and Foster, 1958; Foster and Goldstein, 1969;

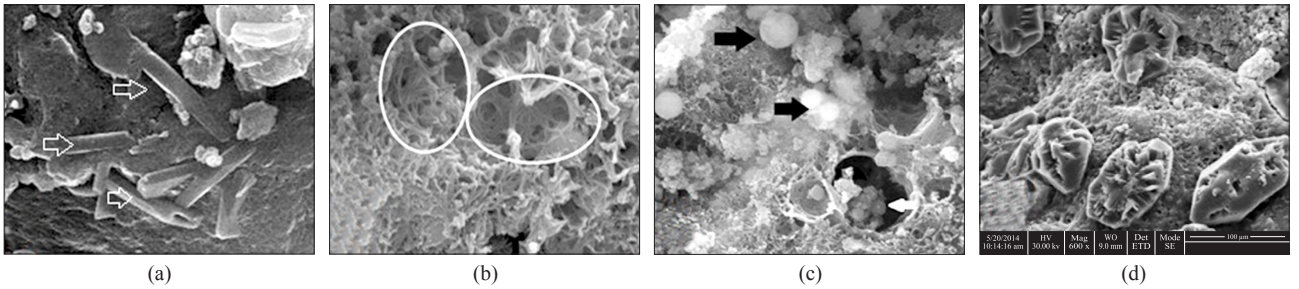

Fig. 5. Ultrastructure of the stone. (a) small discoid crystals (blank arrow); (b) Hard coral-like scaffolds that encompass empty spherical sacs (white circles); (c) spherules of variable size present in (white arrow) and outside (black arrow) of the sac like structures; (d) hexagonal crystals present only in the periphery of the stone

Wedemeyer, 1996). It is notable that fluorine is a promoter of bladder and kidney stone (Anasuya, 1982; Ludlow *et al*., 2007) and therefore, its presence in the stone also implicates it to be one of the lithogenic factors in this case.

The presence of uric acid in the cystic calculus from cobia, a teleost fish, is quite interesting. Normally, only a small fraction of the metabolic wastes are eliminated through urine in fish, since most of the metabolic nitrogenous wastes such as urea and ammonia, being highly diffusible in water, are excreted directly into the water through the gills without going into the urine. Moreover, the minor amount of nitrogenous waste in urine do not contain much uric acid since uric acid, a product of purine metabolism, is converted metabolically first into allantoin which is subsequently converted to allantoic acid (Denis, 1913-1914; Forster and Goldstein, 1969). Therefore, it is possible that the fish might have been experiencing an anomalous purine metabolism wherein it failed to convert the uric acid into the usual allantoic acid.

Despite insufficient information regarding the source and metabolism of oxalate in fish, it is believed to be similar to that in the terrestrial vertebrates (Blazer and Wolke, 1983). It has been reported that an abnormal oxalate metabolism due to deficiency of pyridoxine, a co-enzyme for alanine-glyoxylate aminotransferase that rescues glyoxylate from entering the path of oxalate production in the liver (Nath *et al.,* 1990; Baker *et al.,* 1996; Marengo and Romani, 2008) results in the deposition of oxalates in trout (Smith *et al.,* 1974). In this case also, an abnormal oxalate metabolism in the fish might have contributed to the excretion of excessive amount of oxalate into the urine and its subsequent incorporation into the stone. A look into the normal oxalate metabolism in vertebrate liver (Coulter-Mackie, 2006) may help to explain what went wrong in the fish to produce excess amount of oxalate. Usually, in the liver mitochondria, glyoxylate is transformed to glycolate and glycine by the catalysing action of the enzyme alanine-glyoxylate amino transferase (AGT) which needs the coenzyme pyridoxine for its action (Nath *et al.,* 1990; Baker *et al.,* 1996; Marengo and Romani, 2008). Deficiency of the enzyme AGT or its cofactor pyridoxine or both will lead to the build-up of glyoxylate in the liver mitochondria. The accumulated glyoxylate is thus bound to be channelised into the alternative pathway of transformation into oxalate in the liver peroxisome with the help of the enzyme glycolate oxidase (GO) or in the cytoplasm by the help of the enzyme lactate dehydrogenase (LDH). Thus, either the deficiency in the enzymes of the normal glyoxylate metabolism (*i.e.,* AGT, or its coenzyme pyridoxine or both) or the over-expression of enzymes of the alternate pathways (*i.e.,* GO and LDH) or both might have caused the excess oxalate accumulation in the fish in the present case.

Systematic future research, as we intend to carry out, into the purine and oxalate metabolism pathways in the normal and stone-forming cobias may clarify the etiology of uric acid and oxalate stone formation in the species. Furthermore, the possible role of external factors such as the diet (Smith *et al.,* 1974; Holmes *et al*., 2001) and microbial infections (Kajander and Ciftcioglu, 1998) in the formation of urinary calculi in cobia also deserves due research attention.

In hatcheries, if the broodstock are few in number and if a bladder stone formation is suspected, the fish can be subjected to confirmatory diagnosis by methods like X-ray radiography. If the presence of stone is confirmed, appropriate methods, as used in terrestrial animals, including surgical removal and non invasive extra corporeal shock wave lithotripsy (Bartoletti and Cai, 2008; Stratico *et al.,* 2012) can be employed to correct the condition prior to hormonal induction of spawning. The recovered brooder may subsequently be employed for successful spawning.

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