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CASE REPORT

Calcium urolithiasis in a breeding population of southern flounder (*Paralichthys lethostigma*) housed in a low salinity environment

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1. Introduction

The southern flounder, *Paralichthys lethostigma*, is a left-eyed flat fish belonging to the family Paralichthyidae that ranges the east coast of North America in the Atlantic Ocean from North Carolina south to eastern Florida and from southwestern Florida throughout the Gulf of Mexico to the Yucatan peninsula (Wenner & Archambault 2005). *P. lethostigma* is the largest member of this group of benthic fishes, is highly sought after as a food fish, and exhibits tolerance to a wide range of salinity in the wild (Watanabe & Carroll 2001). These factors have made the southern flounder attractive as a potential candidate for aquaculture in low salinity and fresh water environments (Daniels et al. 2010). Experimental trials have been conducted rearing and raising southern flounder in low salinity aquaculture with some promising results (Benitti et al. 2001). This manuscript describes urolithiasis in southern flounder brood stock maintained in low salinity aquaculture conditions.

2. Clinical Presentation

Nine southern flounder brood stock, between 2–3 years old, were evaluated for bladder urolithiasis following the incidental finding of bladder stones (Figure 1) in a single animal from the same cohort that was previously sacrificed for unrelated purposes. The cohort was housed in a circular 2.45 meter in diameter 4950 liter tank at a facility 15 km from the site of the diagnostic screening. All 9 fish in the cohort were transported in a 1000 liter transport tank filled with water from the permanent enclosure. The water quality parameters for the brood stock holding facility tank over the months prior to screening were: salinity 1.25 ppt (min 0.9, max 1.46), temperature 19.9 °C (min 19.2, max 20.8), dissolved oxygen 6.67 mgL⁻¹ (min 4.9, max 7.68), pH 7.79

(min 7.54, max 8.12), NH₃ 0.61 mgL⁻¹ (min 0.28, max 1.97), NO₂ 3.91 mgL⁻¹ (min 2.0, max 6.0), total hardness 140.91 grains per gram (gpg) (min 90, max 190), calcium hardness 100.91 gpg (min 70, max 160), magnesium hardness 40 gpg (min 30, max 50).

One fish of the cohort was obviously darker than the other fish and this difference had been noted for some time prior to the work up. This fish will be referred to as fish 2 throughout this paper. Persistent darker color is considered to be an indication of poor health in this species. This fish was one of two in the cohort identified subsequently as having urinary bladder stones. The other eight fish appeared outwardly healthy and robust. Each fish was allowed to settle individually in a shallow plastic tub partially filled with 15 liters of water (water depth 10 cm) from the transport enclosure for up to 5 minutes prior to further manipulation.

3. Diagnostic evaluation

3.1. Ultrasonography

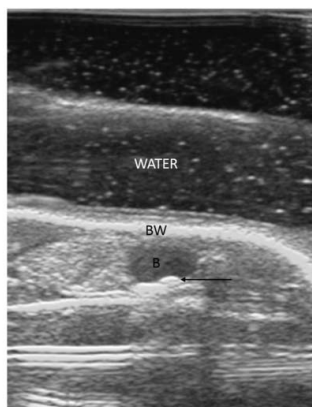
An Ibex® Pro (E.I. Medical Imaging, Loveland, Colorado, USA) portable ultrasound machine with 6.5 MHz linear probe protected by a plastic sleeve lined with coupling gel (Aquasonic 100, Parker Laboratories, Fairfield, New Jersey, USA), positioned below the surface of the water and manually suspended 5cm above the left side of each fish was used to screen for the presence of urinary bladder stones (Figure 2(a)). Two fish that screened positive for bladder stones by ultrasound were kept for further assessment. A third fish was suspected to have urinary sediment on ultrasound evaluation but a stone could not be confirmed. The seven fish without confirmed stones were returned to their primary enclosure.

After confirmatory radiographs were obtained, more detailed diagnostic ultrasound (Logiq E9, GE,

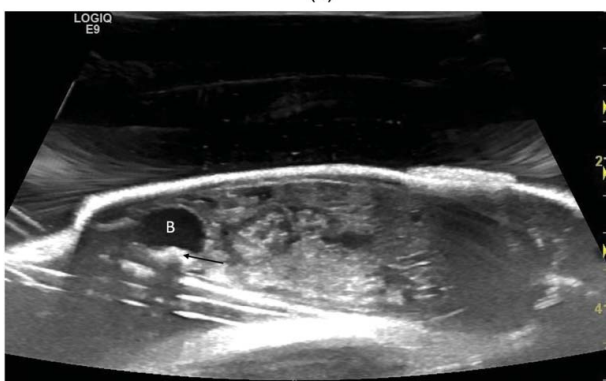


Figure 1. — Urinary bladder calculi from the initial fish compared in size to that of a US penny.

Wauwatosa, WI, USA) was conducted following a protocol identical to that used for the initial ultrasound evaluation. Detailed ultrasound using 10.0–14.0 MHz curvilinear probes was performed to further characterize the abnormal urinary tract findings and better



(a)



(b)

Figure 2 (a). — Ultrasound image of urinary calculi (arrow) within the urinary bladder (B) of southern flounder as seen during screening with 6.5 MHz probe using the enclosure water as contact media. The body wall is indicated by BW. **Figure 2**(b). — Ultrasound image of urinary calculi (arrow) within the urinary bladder (B) of southern flounder creating distal beam attenuation as seen with 14 MHz probe.

evaluate other coelomic structures. Multiple hyperechoic distal beam attenuating calculi were confirmed within the lumen of the urinary bladder (**Figure 2**(b)). Otherwise the urinary tract was ultrasonographically within normal limits for the species. A small amount of free coelomic fluid was noted in fish 2. No other intra-coelomic abnormalities were noted.

The 6.5 MHz probe seemed to be an effective tool to quickly screen multiple fish for urinary bladder calculi, though fish without stones were not sacrificed or further worked up to confirm the sensitivity or specificity of the technique. Calculi down to 1–3 mm in diameter were identified in the work ups of the confirmed positive fish.

3.2. Radiographs

Plain film radiographs (Multix X-ray, Siemens Medical Solutions, Inc., Malvern, PA, USA) confirmed bladder urolithiasis in the two fish identified by ultrasound screening. Each fish was placed in a moist shallow plastic tray with 10 cm high walls that did not contain any appreciable depth of water. Radiographs of each fish were produced within 1 minute of the fish being removed from their water filled transport enclosure. A small focal spot of 0.3 mm was used with subject elevation of 26 cm from the receiver plate to produce magnified left lateral views. A standard source to image distance of 40 cm and technique of 75 kVp and 2.5 mAs were used to obtain images. Orthogonal projections were produced with the use of a movable radiographic head leaving the fish undisturbed in the shallow tray.

Radiographs of both affected fish were remarkably similar. Lateral radiographs revealed multiple radiopaque calculi of mineral density within the urinary bladder (**Figure 3**). Kidney size and shape was considered within normal limits for the species. Orthogonal imaging confirmed the calculi to be within the urinary bladder based on spatial orientation, but were of limited use in discerning soft tissue anatomy based upon the natural extreme lateral compression of the fish. The orthogonal view in this case is actually a ventro-dorsal projection. No radiographic abnormalities other than the presence of urinary bladder stones were apparent in either fish.

3.3. Clinical Pathology

Phlebotomy was performed using a lateral approach to withdraw 0.8 mls of blood from the caudal vein with a 22 g needle on a 3 ml syringe (Monoject™, Covidien, Minneapolis, MN, USA) preloaded with 0.04 mls heparin (Hospira Inc., Lake Forest, IL, USA). The heparinized whole blood was placed in individually labeled dry plastic vials containing no additive and immediately transported to the on-site laboratory for analysis using

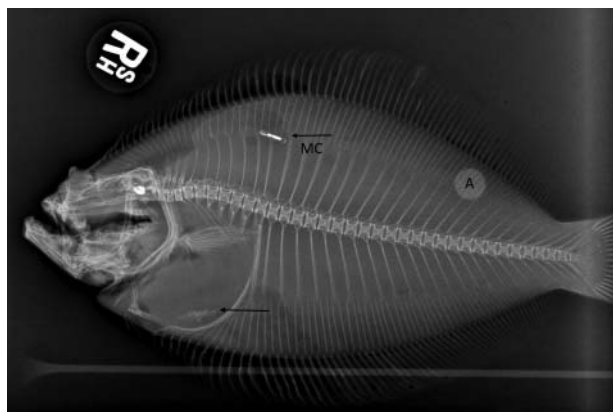


Figure 3. — Right lateral radiograph of southern flounder revealing radiopaque mineral opacity urinary bladder calculi. Note the metal opacity of an implanted microchip (MC) in the dorsal musculature and the round soft tissue opacity artifact in the caudal dorsal musculature (A) due to variation in thickness of the plastic bin in which the fish is positioned.

a Healthcare Diagnostics Advia 120 (Siemens Duluth, Georgia, USA) and a Cobas Integra 400+ (Roche Diagnostics Indianapolis, Indiana, USA) analyzer for the CBC and plasma biochemistries, respectively. Relevant blood count and plasma biochemistries for the two fish are reported in Table 1. All blood analysis was completed within 1 hour of phlebotomy.

3.4. Gross and Histologic Pathology

After phlebotomy, each fish was sacrificed using an overdose (0.4 g/L) of tricaine methanesulfonate (Western Chemical Inc., Ferndale, Washington, USA) buffered 1:1 by weight with sodium bicarbonate. Gross necropsy was performed. Urine from within the bladder of one fish was collected at the time of necropsy and submitted for evaluation, but urinalysis did not add any clinically relevant data. No urine was available for collection from fish 2, however, a moderate amount (1 ml) of straw-colored clear watery coelomic fluid was present in this fish. Urine and free coelomic fluid were analyzed in the North Carolina State University, Veterinary Teaching Hospital diagnostic laboratory (Raleigh, North Carolina, USA) with Chemstrip Criterion II (Roche,

Table 1. Relevant blood count and plasma biochemistries with reference ranges of similar species winter flounder (*Pleuronectes americanus*) (Dye et al. 2001).

	Fish 1	Fish 2	Units	Reference ranges of <i>P. americanus</i>
White blood cell count	12.0	54.3	G/L	13.2 – 145
Packed cell volume	24	20	%	19 – 31
Calcium	3.4	5.5	MMOL/L	5.3 – 7.5
Phosphorus	2.9	4.8	MMOL/L	2.5 – 5.9
Sodium	160	159	MMOL/L	171 – 200
Potassium	4.0	2.8	MMOL/L	0.5 – 2.8
Chloride	138	135	MMOL/L	156 – 194



Figure 4. — Photo at necropsy revealing tan urinary calculi (arrows) within the incised and reflected urinary bladder (B) of southern flounder. The gastrointestinal tract has been removed though the kidneys (K) and heart (H) remain in situ.

Indianapolis, Indiana, USA) with manual sediment evaluation and Shandon Cytospin 4 (Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA) with manual cell count using hemocytometer, respectively. Cytology of the collected coelomic fluid was interpreted as mild to moderate histiocytic and heterophilic inflammation.

Urinary bladder calculi and tissue samples of gill, heart, head kidney, tail kidney, ureter, gonad, urinary bladder, esophagus, stomach, intestine, spleen, skin, eye, brain, and urine or intracoelomic fluid were collected from each fish. All organ samples were fixed in 10% buffered formalin and processed for routine paraffin embedding, sectioned at 7 microns and stained with hematoxylin and eosin, Von Kossa and acid-fast stain for histologic evaluation. Gross and histologic evaluations of all tissues, including the kidneys were unremarkable other than histologic evidence of minimal mural vascular ectasia of the urinary bladder wall in both fish.

The urinary bladder uroliths collected at necropsy were stored in individually labeled dry sample tubes containing no additive and submitted to the Minnesota Urolith Center (University of Minnesota, Saint Paul, Minnesota, USA) for evaluation by means of infrared spectroscopy (Figure 4). The uroliths from fish 1 and fish 2 were both calcium based stones but of different composition. The stone from fish 1 was reported as 100% calcium phosphate carbonate. The stone in fish 2 was reported as 100% calcium hydrogen phosphate dehydrate (brushite).

4. Discussion

The 22% prevalence of stone formation confirmed in this small cohort of 9 fish is notable but not sufficient to warrant advocating routine individual fish screening

for urolithiasis for marine flat fish maintained in low salinity. Identification of higher prevalences in larger cohorts or particularly impacts of the condition on fish well-being and/or production efficiency would affect that interpretation. Should routine screening be of interest, non-contact ultrasound examination with a 6.5 MHz probe appears to be a useful rapid screening method for detecting urinary calculi 1–3 mm or greater in diameter. Because of the clinical nature of the evaluation, the economics of extensive testing and the value of the brood stock, we did not perform radiographic or postmortem evaluations on fish considered negative on the initial ultrasound examination. Therefore, it is possible that smaller stones or grit may have been below detection limits of the technique. Inconsistent positional orientation of the probe could also result in false negative assessments and we cannot rule that out.

Ultrasonographic evaluation (10.0–14.0 MHz) in these two cases was very effective in developing detailed images of the coelomic structures, but the cost of the equipment and the expertise required to effectively develop diagnostic images with these probes would seem to limit their usefulness for routine screening in aquaculture situations. These probes do have great promise for future of diagnostic applications in display and companion fish medicine when economically feasible. The technique of holding the probe suspended free in the water above the fish used for these cases is well reported in the clinical literature (Stoskopf 1993) and was very effective. The water was an effective coupling medium and not touching the fish appeared to avoid outwardly observable stress for the animal.

Radiographs, though effective in rapidly identifying radiopaque stones, may not be the best approach to screening for urinary bladder stones in fish, particularly where large numbers of fish are involved. Positioning fish for diagnostic quality radiographs generally requires anesthesia. The radiographs in this study were produced with the fish unanesthetized and out of water. Producing radiographs out of the water is a common practice to avoid the need to increase MAS significantly to deal with the effects of the water surrounding a fish. Removal of fish from water without anesthesia is intuitively judged as stressful and even risky for most fish by many clinicians. It is possible that very low quality radiographs taken at lower MAS than would be used for routine radiographs could be sufficient to reliably detect the presence of urinary bladder calculi.

The portability and safety of ultrasound in concert with the ability to leave fish in water and avoid anesthesia are major advantages of ultrasound as a screening modality. Because our workups did not pursue assessment of the entire cohort with alternate forms of stone detection, we cannot speculate on the relative

specificity or sensitivity of the technique. However, the advantages of not needing to contain ionizing radiation, low risk of deleterious effects on roe or milt and non-invasive real time visualization are significant advantages for ultrasound screening if large numbers of animals need to be processed. Studies using harvested flounder and the random application of mock stones in the coelom could be used to establish the specificity and sensitivity of 6.5 MHz probe ultrasound screening for urinary bladder stones in flounder.

The hematology results for fish 1 were unremarkable and well in line with expected values in a wide range of marine fish species. Fish 2 had a distinct leukocytosis and higher than expected serum calcium and phosphorus relative to fish 1. The leukocytosis is consistent with a systemic infection and is much higher than would be expected in a stress leukogram (Stoskopf 1993). The hematologic and serum chemistry perturbations in fish 2 are not necessarily related to the presence or the pathogenesis of the calcium stones found in the urinary bladder of the fish. The elevations in calcium and phosphorus could suggest renal compromise in mammals, but the situation is more complex in fish, particularly marine fish. Divalent cation and anion balance are maintained primarily through biochemical processes in gill with secondary considerable contributions by renal tissues (Stoskopf 1993; Evans 1998; Tipsmark et al., 2008). We were unable to evaluate serum Mg cation and SO_4 anion concentrations, which are particularly useful for differentiating functional physiologic impairment of fish kidneys from that of gills. The changes in serum calcium and phosphorous in fish 2 could be related to the presence of urolithiasis, but the lack of these serum chemistry findings in fish 1 which also had calcium urolithiasis and the wide range of alternate explanations for the finding of hypercalcemia and hyperphosphatemia, including gonadal seasonal hormonal perturbation, potential dysfunction of the corpuscles of Stannius, thyroid and ultimobranchial body dysfunction, magnesium deficiency and gill disease, weaken that argument.

The lack of gross necropsy findings in any major organ system or the tissues of either fish that could be related to the pathogenesis of stone formation, with similarly unremarkable histologic findings, fails to support conjecture that renal or gill pathology are the primary cause of stone formation. The histologic finding of mild mural vascular ectasia of the urinary bladder could be attributed to irritation from the presence of the stones. We were not able to differentiate for certain whether these mild lesions involved lymphatics or small veins. The presence of intraluminal thrombi suggests venous impairment, but we cannot rule out lymphatic dilation related to electrolyte derangement. Mild coelomic effusion was also observed in fish 2. This transudate was unremarkable on direct examination

and could have been a response to renal, gill, heart or liver dysfunction.

The lack of evidence of gross pathology in either fish examined suggests that though urinary bladder stone formation can occur in southern flounder, in the absence of other identified etiologies, the condition could be a response in a subset of fish to low salinity conditions. This is a tempting assumption, however, the pathogenesis of the stone formation found in these cases is not clear. It is possible that low salinity acclimation of fish species evolved to live in high salinity as adults could disrupt overall cation balance. Up-regulation of gill sodium-chloride transport pumps of flounder being housed in low salinity environments is currently being investigated by one of the authors (Gill). Though euryhaline fishes such as southern flounder adapt to fresh and marine environments at different life stages, age related physiologic changes in adult brood stock could limit the ability of the brood stock to compensate for the low salinity environments. We are unaware of evidence for altered feed conversion or decreased reproductive success in flounder found to have urinary calculi and the implications on individual fish health or production efficiency are yet to be demonstrated.

Should higher prevalences or production inefficiency be identified in southern flounder or other marine fish being tested for low salinity aquaculture, there are several options that might be considered to mitigate the problem. About 70% of calcium absorption in *P. lethostigma* is via the intestine (Hickman 1968). Altering dietary calcium by either directly reducing dietary calcium levels or altering calcium and phosphorous ratios could potentially decrease total body calcium content thereby reducing the propensity to form calcium based urinary calculi. Another consideration would be to decrease calcium intake via the gills by decreasing water calcium hardness by substituting other divalent cations to maintain the necessary total water hardness for good feed conversion and productivity.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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