Title **NUTRITIONAL STEATITIS (YELLOW FAT DISEASE) IN CULTURED SILVER CATFISH (*Rhamdia quelen*) ASSOCIATED WITH RANCID FEED.**

Short running title: Nutritional steatitis in *Rhamdia quelen*

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Abstract

Nutritional steatitis (yellow fat disease) caused by vitamin E (vit E) deficiency is typically associated with diets high in unsaturated fatty acids or/and low levels of vit E. This work describes, for the first time, an outbreak of nutritional steatitis in cultured silver catfish (*Rhamdia quelen*) in Argentina. Necropsy, histopathology, bacteriology and molecular studies of the affected fish were performed. In addition, peroxide level and vit E concentration of the fish feed were measured. Affected specimens had firm, yellowish-brown dermo-hypodermal nodules located in the adipose fin and dorsal region of the body. Histologically, lesions consisted of fat necrosis and multifocal granulomatous lobular steatitis, primarily affecting the subcutaneous and intermuscular adipose tissue, and occasionally, the coelomic fat. Feed analyses revealed a high level of peroxide value (41.2 meq/kg), indicative of rancid feed, and low concentration of vit E (2.7 IU/Kg). In this case, fish had been fed rancid food with inadequate value of vit E, leading to the development of nutritional steatitis caused by vit E deficiency.

Keywords

Nutritional steatitis, yellow fat disease, vitamin E deficiency, *Rhamdia quelen*, silver catfish, histopathology.

INTRODUCTION

The silver catfish (Pisces, Siluriformes), is a native freshwater fish distributed throughout Central and South America, from Mexico to northern Argentina. Silver catfish farming is well-established in southern Brazil, whereas in Argentina and Uruguay their production is still an incipient activity. Similar to other Siluriformes, silver catfish has great potential for intensive culture due to its quick adaptation to production conditions, good reproductive and productive parameters, and being an appreciated food source (Baldisserotto & Radunz-Neto 2004).

Nutritional steatitis, commonly known as yellow fat disease or pansteatitis, is caused by vitamin E (vit E) deficiency and is characterized by inflammation of the adipose tissue. Vit E is a lipophilic antioxidant derived from the diet, which protects cell membranes against lipid peroxidation by free radicals (. As an antioxidant, vit E scavenges free radicals, breaking the chain reaction of lipid peroxidation, particularly in polyunsaturated fatty acids (PUFA) of cell membranes, thereby preventing oxidative damage, and consequently the resulting cell injury (Qiang et al 2019). Nutritional steatitis is typically associated to feed diets containing high levels in PUFA and/or deficient in vit E (Huchzermeyer et al. 2013).

This condition may affect subcutaneous adipose tissue (panniculitis), abdominal fat and other fat deposits of the body, such as perirenal or epicardial fat. Pathological findings include fat necrosis, granulomatous or piogranulomatous inflammation, fibrosis, calcification, and deposition of ceroid pigment (Mauldin & Peters-Kennedy 2016). Nutritional steatitis has been described in mammals, the most susceptible species being cat, mink, horse and swine, and it also has been documented occasionally in birds, reptiles and fish species, such as Mozambique tilapia (*Oreochromis mossambicus*), African sharptooth catfish (*Clarias gariepinus*), channel catfish (*Ictalurus punctatus*), and

steelhead (*Oncorhynchus mykiss*) (Goodwin 2006, Huchzermeyer et al 2013, Mauldin & Peters-Kennedy 2016, Twibell et al 2017).

The aim of this study was to describe the gross and microscopic findings in cultured silver catfish (*Rhamdia quelen*) with nutritional steatitis. Analyses of vit E concentration and peroxide value in the food consumed by this population of fish were performed to confirm the nutritional cause.

2. MATERIAL AND METHODS

2.1. Fish and sampling procedure

The outbreak (May-June 2018) affected fish in fattening phase maintained in experimental facilities of the School of Veterinary Sciences of the National University of Rosario (Argentina). Juveniles (15 months old) of silver catfish with no previous reports of any clinical diseases were cultured in circular galvanized metal tanks (9 m^3 of capacity) supplied with ground water and supplementary aeration. Fish were fed twice daily to satiation with a floating commercial feed (29% crude protein, 2,918 Kcal digestible energy/kg). During the disease period the recorded water parameters (mean ± SD) were the following: temperature 15.35 ± 3.16°C; pH: 8.4 ± 0.1; and dissolved oxygen 10.39 ± 0.47 $mg\ L^{-1}$, which were within the tolerable limits for this species. Previous to the outbreak, water parameters did not significantly differ from the above values, except for temperature which was warmer in summer and autumn seasons. Affected fish (67.8 g, mean total length 19.5 cm) displayed multiple yellow, reddish-yellow or red raised subcutaneous nodules, mostly along the dorsal region of the body and in the adipose fins. Morbidity was 87.5%. No animal deaths were recorded during the disease outbreak.

Five specimens were euthanized by anaesthetic overexposure using benzocaine solution (250 ppm), and subsequent severing of the spinal cord. After that, complete necropsy was performed, and samples for bacteriology and histopathology were collected.

2.2. Bacteriology

Samples from kidney and spleen, and scraping of the surface of skin lesions were extracted under aseptic conditions. Tissue samples were placed in a thioglycolate broth and were incubated for 48 h at 32°C. Then, 50µl volumes were spread onto a blood agar plate and a PolyViteX chocolate agar plate (bioMérieux, France). Scraping material was directly inoculated onto blood agar plates and a PolyViteX chocolate agar (bioMérieux).

The plates were incubated under a CO_2-enriched atmosphere for 48 h at 32°C. At the end of the incubation period, the plates were observed for bacterial growth and representative colonies. Bacterial identification was performed by a commercial miniaturized API 20 NE rapid test kit (bioMérieux) according to the manufacturer's instructions and checked in APIweb. In parallel, the commercial identification system VITEK II (bioMérieux) was used with a Gr(-) id card.

2.3. Histopathology

Samples of skin and subcutaneous nodules, skeletal muscle, gills, brain, heart, stomach, pyloric caeca, intestine, kidney, liver, spleen, pancreas and coelomic fat were fixed in 10% phosphate-buffered formalin, processed routinely and embedded in paraffin wax. Sections (3-4 µm thick) of paraffin embedded tissues were stained with haematoxylin and eosin (HE). Moreover, with the aim of revealing microorganisms, sections of subcutaneous/muscle and coelomic lesions were also stained with periodic acid Schiff´s, Ziehl–Neelsen, Gram's and Grocott's methenamine silver techniques.

2.4. PCR

A total of five formalin-fixed paraffin-embedded (FFPE) tissue samples obtained from the paraffin blocks of subcutaneous lesions were analyzed and total DNA was extracted from each sample using the QIAamp DNA FFPE Tissue Kit (QIAGEN). The protocol was performed according to the manufacturer's instructions. On the other hand, a suspension of 200 µl of 24 h bacterial isolate was treated at 100ºC for 10 min in a hot water bath and cooled in an ice water bath. The suspension resulting from the thermal shock was centrifuged at 10000 g for 5 min. DNA concentration and quality were determined by a Nanodrop spectrophotometer (Thermo Scientific Inc., USA). The primers She211: 5′-CGCGATTGGATGAACCTAG-3′ (forward), and She1259: 5′-GGCTTTGCAACCCTCTGTA-3′ (reverse) were used to amplify the 16S rRNA gene of

Shewanella, as was previously published by Todorova and Costello (2006). Amplification of the 16S rRNA gene was carried out in a reaction mixture of 25 µl, with 10 × buffer (Thermo Fisher, Waltham, MA, USA), 1,25 mM each deoxynucleotide triphosphate, 20 pmoles of each primer, 5 U/µl of Taq DNA polymerase (Thermo Fisher) and 5 µl of template DNA (10 ng/µl). PCR assays were performed in a DNA thermal cycler (IVEMA, Buenos Aires, Argentina). The amplification program consisted of an initial denaturation at 94 ºC for 2 min, followed by 24 cycles of denaturation at 94 ºC for 1 min, annealing at 53 ºC for 1 min, and an elongation phase at 72 ºC for 1 min, and ending with a final extension at 72 ºC for 5 min. Proper negative controls for all components of the PCR mix, except for the DNA template, were included in each PCR run. Amplification products were visualized on a 1.0% agarose gel stained with SYBR Safe® (Invitrogen, Carlsbad, CA, USA). Amplicon sizes were estimated by comparing them with the bands of a molecular weight marker (Thermo Scientific). The PCR reactions with the She211 and She1259 primers generated a 1048-bp amplicon of the 16S rRNA gene. The reference strain *Shewanella putrefasciens* ATCC 8071 was used as a positive control. Negative control using sterile distilled water instead of template DNA was included.

2.5. Analysis of feed

Sample of feed was sent to a specialized external laboratory in Rosario (Santa Fe, Argentina) to determinate the concentration of vit E (analyzed by high-performance liquid chromatographic), and the peroxide value (PV) to assess lipid peroxidation (analyzed by acetic acid-isooctane method).

3. RESULTS

3.1. Gross pathology and histopathological findings

Macroscopically, affected fish had multiple yellow, reddish-yellow or red raised dermo-hypodermal nodules, mostly along the dorsal region of the body and in the adipose fins (**Figures a-c).** The nodules ranged from 1 to 4 mm in diameter and some of them were ulcerated. On cut section, the nodules were solid, mildly granular, and yellow to yellowish-brown. Upon opening of the coelomic cavity, one fish displayed diffuse redness and yellow spots scattered (less than 1mm) over the coelomic fat. No grossly visible lesions were noticed in the internal organs.

Histologically, the nodules corresponded to multiple focus of fat necrosis and moderate granulomatous inflammation, involving and expanding the subcutaneous and the deeper intermuscle adipose tissue (**Figures d and e**). The inflammatory infiltrate was predominantly composed by foamy macrophages, sometimes surrounding foci of amorphous necrotic fat; scarce multinucleated giant cells and granulocytes (**Figures f and g**). The specimen with diffuse redness of the coelomic fat, had fat necrosis and granulomatous inflammation like those described for subcutaneous and intermuscle adipose tissue (**Figure h**). No acid-fast positive microorganisms, bacterial clusters, fungal and parasitic structures were identified in any of the lesions or evaluated organs.

3.2. Bacteriology

Shewanella putrefaciens was isolated from scraping of the surface of skin lesions in all specimens. *S. putrefaciens* was identified by using the API 20NE system (identification percentage 99.0%) and Vitek II system (identification percentage 98.4%). The bacteriological culture of the spleen and kidney samples were negative.

3.3. PCR

171 FFPE tissue samples collected from the five affected specimens were tested by the PCR
172 method with She211-She1259 primers. None of the FFPE tissue samples were found to
173 exhibit a visible DNA band (1048 bp) after the amplification. The positive control sample
174 showed positive result for the tested gen, while the negative control showed no positive
175 result. The evaluation of the amplification products by conventional PCR and subsequent
176 electrophoresis did not show the presence of the bands corresponding to the genes
177 sequence of *Shewanella* spp.

178 **3.4. Analyses of feed**

179 The concentration of vitamin E in feed was 2.7 IU kg^{-1}. The peroxide value (PV) was
180 41.20 mEq O_2 kg^{-1} feed.

181

4. DISCUSSION

This work describes for the first time the pathologic findings in an outbreak of nutritional steatitis in cultured silver catfish in Argentina. A comprehensive protocol including necropsy, histopathology, bacteriology and feed analyses, was followed to achieve the disease diagnoses.

Microscopically, the disease was characterized by fat necrosis with multifocal granulomatous lobular steatitis, one of the distinctive pathological manifestations of vit E deficiency in animals (Mauldin and Peters-Kennedy 2016, Twibell et al 2017). In these specimens, steatitis involved the subcutaneous, intermuscular and coelomic adipose tissue, coinciding to the main fat depots, principally hypodermis and coelomic adipose tissue. Etiologically, fat necrosis is classified as nutritional, enzymatic or pancreatic, traumatic, and idiopathic (Gross et al. 2005). In the affected fish, no degenerative or inflammatory changes were observed in pancreatic, gastrointestinal and hepatic tissues. Therefore, enzymatic origin of fat necrosis and secondary vit E deficiency due to liver or gastrointestinal alterations were ruled out. Likewise, no trauma was registered.

On the other hand, certain pathogens cause granulomatous steatitis in fish. For instance, parasites and systemic diseases, such as mycobacterial or nocardial infection can produce coelomitis, involving the fat tissue of the coelomic cavity. Similarly, bacterial (e.g. *Mycobacterium* spp.), fungal (e.g. *Aphanomyces* spp) and parasitic agents (e.g. protozoa *Kudoa* spp.) can produce panniculitis, generally by extension of a contiguous deep dermal process into the hypodermis (Huang et al 2021, Law 2001). In this case, infections agents were ruled out since no bacterial, mycotic or parasitic structures associated to the lesions or other tissues were revealed by histopathology (HE and special stains).

Shewanella putrefaciens was just isolated from the cutaneous surface of nodules in all specimens, but no bacteria were isolated from liver and spleen of affected fish. In

addition, no nuclei material of *S. putrefaciens* was detected into the subcutaneous lesions. These results suggest that bacterial isolate was present only on the surface of skin. *S. putrefaciens* is a ubiquitous microorganism in the water that can act as opportunistic pathogen, and could have incidentally contributed to the ulceration of nodules (Pekala et al 2015).

Results of feed analyses revealed that fish were consuming a rancid diet, with a high PV: 41.20 mEq O_2 kg^{-1} feed, and containing very low level of vit E (2.7 IU kg^{-1} feed). In general, the desirable PV in fish feeds should be kept below 10 mEq O_2 kg^{-1} feed to ensure good feed quality and avoid adverse effects on fish health; while values above 20 mEq kg^{-1} indicate rancidity (Peng et al 2009, Twibell et al 2017).

On the other hand, even though appropriate dietary vit E levels vary greatly depending on intrinsic factors, such as species, age, physiological condition, etc., and extrinsic factors (mainly diet composition), the published dietary vit E requirements for most fish species are higher than 10 IU kg^{-1} of diet, ranging from 10 to 200 IU kg^{-1} (One IU of vitamin E is defined as the biological activity of 1 mg of dl-a-tocopheryl) (N.R.C. 2011, Twibell et al 2017, Wang et al 2016).

The etiopathogenesis of the disease is complex and depends on several factors mostly related to nutrients interrelationship, such as dietary levels of PUFA, oxidized lipids and other vitamins and minerals essential for antioxidant activity. However, it has been stablished that high levels of dietary PUFA depletes vit E in the feed and increases the vit E requirement in fish tissues (Gao et al 2012, Huang et al 2003). In turn, consumption of the high level of already oxidized lipids results in peroxidation of fatty acids in fish tissues with generation of lipid-free radicals (Gao et al 2012, Yang et al 2015, Zhang et al 2016). In this way, excess of lipid-free radicals in the presence of insufficient/depleted vit E lead to a lipid-damaging chain reaction can cause oxidative damage and necrosis in adipocytes,

to trigger an inflammatory response (Huang et al 2020). In line with this, several studies at enzymatic and genetic levels have consistently shown that consumption of peroxidized lipids and low levels of vit E reduces the antioxidant capacity and increases the oxidative stress and lipid oxidation in fish tissues, such as liver, intestine, kidney, spleen, muscle and skin and adipose tissue, compared with animals fed diets containing unperoxidized lipids and adequate concentration of vit E (Huang 2020, Pan et al 2017, Qiang et al 2019). In conclusion, yellow fat disease in *Rhamdia quelen*, characterized by multifocal granulomatous pansteatitis was attributable to vit E deficiency status, linked to the consumption of rancid feed with a high peroxide value and a low level of vit E. The diagnosis of nutritional diseases, alongside the proper formulation, storage and handling of feed, is essential to prevent deleterious effects on fish health and product quality. In this context, while a definitive diagnosis requires identification of specific nutritional deficiency, histopathological examination of diseased fish serve as a valuable tool for diagnosing and monitoring the presence of nutritional disorders.

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FIGURE LEGENS

Figures a-c. Macroscopic appearance of panniculitis in *Rhamdia quelen* with nutritional steatitis. **a and b**: Multiple small well-circumscribed protruded nodular skin lesions (arrows) on the dorsal region of the body and adipose fin. af, adipose fin, df, dorsal fin. Bars=1cm. **c**: Transversal section of caudal peduncle across the adipose fin of affected fish showing nodular area of yellow-brown discoloration (arrow). The colour is slightly altered (darkened) because the photograph was taken from the formalin fixed tissue and embedded in the paraffin block. Bar=0.5 cm.

Figures d-h. Histological section of lesions observed in *Rhamdia quelen*. **d**: Cross-section of adipose fin showing panniculitis in *R. quelen* with nutritional steatitis, characterized by fat necrosis (*) and focal granulomatous inflammation (arrow) infiltrating and extending the hypodermis (h). Bar=250 µm. ep, epidermis, d, dermis.

Figure e: High-magnification view of panniculitis involving, subcutaneous and muscle tissue, characterized by fat necrosis (*) and focal granulomatous inflammatory infiltrates (arrows) expanding the hypodermis. Bar=100 µm. ep, epidermis, d, dermis, h, hypodermis. **Figure f**: Detail of the inflammatory infiltrate composed mainly by foamy macrophages with pale vacuolated cytoplasm. Bar=25 µm. **Figure g**: Aggregate of macrophages (arrow) around amorphous necrotic fat (*) in the hypodermis. **Figure h**: Coelomic adipose tissue with fat necrosis (*) and replaced by granulomatous inflammatory infiltrate (arrows), mainly composed by macrophages. Bar=25 µm. Insert: detail of foamy macrophages with pale vacuolated cytoplasm. Bar=10µm. HE staining.