"OLD" AND "NEW" BACTERIA ASSOCIATED WITH GRANULOMAS IN AQUARIUM FISH

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Abstract

Cichlids include several fish species having a high economic value in the field of aquaculture. The ornamental fish export trade is mostly based on fish from the african Lake Malawi. Despite their huge economic importance, management of ornamental fisheries is challenged by a paucity of information on the status of the exploited fish stock. The possibility of guaranteeing healthy animals is of paramount importance and has several implications, both for commercial and sanitary reasons. Grossly, cutaneous nodules and black spots are pathological findings frequently encountered in fish, suggesting a meandering disease without a specific etiologic association. Ornamental fish species are plagued by mycobacteriosis, which is quite classically associated with granulomas. This work focuses on debilitated ornamental cichlids presenting cutaneous nodules and black spots and sampled during routinary managing activities held in an aquarium commercial facility; the fish underwent pathological analysis and the presence of pathogens was investigated through a molecular approach. In particular, the presence of lymphocystis disease virus (LCDV), typically associated with cutaneous nodular disease, was excluded.Histologically the granulomas were localized in the spleen, sometimes extending to the other visceral organs. Bacterial Heat-Shock Protein 65 PCR products were detected in tissues associated to granulomas and molecular investigation identified *Mycobacterium* spp. in two samples and *Cutibacterium acnes* in seven samples. Variably sized round "Hamazaki-Wesenberg-like" bodies were immunolabeled with *C. acnes* antibody within macrophages forming the granuloma in the spleen. *C. acnes* has been recently detected by Next Generation Sequencing in the microbiome of internal organs of fish. The role of *C. acnes* within internal fish tissues deserves attention; its role as potential granulomatogenous agent, is taken in consideration.

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Running title: *Cutibacterium acnes* and mycobacteria in cichlids

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Keywords : *Cutibacterium acnes* , granuloma, lymphocystis virus, mycobacteria, ornamental fish trade, *Propionibacterium acnes* , sarcoidosis

Summary

Cichlids include several fish species having a high economic value in the field of aquaculture. The ornamental fish export trade is mostly based on fish from the african Lake Malawi. Despite their huge economic importance, management of ornamental fisheries is challenged by a paucity of information on the status of the exploited fish stock. The possibility of guaranteeing healthy animals is of paramount importance and has several implications, both for commercial and sanitary reasons. Grossly, cutaneous nodules and black spots are pathological findings frequently encountered in fish, suggesting a meandering disease without a specific etiologic association. Ornamental fish species are plagued by mycobacteriosis, which is quite classically associated with granulomas. This work focuses on debilitated ornamental cichlids presenting cutaneous nodules and black spots and sampled during routinary managing activities held in an aquarium commercial facility; the fish underwent pathological analysis and the presence of pathogens was investigated through a molecular approach. In particular, the presence of lymphocystis disease virus (LCDV), typically associated with cutaneous nodular disease, was excluded.Histologically the granulomas were localized in the spleen, sometimes extending to the other visceral organs. Bacterial Heat-Shock Protein 65 PCR products were detected in tissues associated to granulomas and molecular investigation identified *Mycobacterium*spp. in two samples and *Cutibacterium acnes* in seven samples. Variably sized round "Hamazaki-Wesenberg-like" bodies were immunolabeled with *C. acnes* antibody within macrophages forming the granuloma in the spleen. *C. acnes* has been recently detected by Next Generation Sequencing in the microbiome of internal organs of fish. The role of *C. acnes* within internal fish tissues deserves attention; its role as potential granulomatogenous agent, is taken in consideration.

Impacts

- By means of combined laboratory techniques, granulomas observed in fish tissues were associated with m*ycobacteria* , well-known zoonotic agents affecting aquatic species, including ornamental fish.
- The association of granulomas with the bacterium *Cutibacterium acnes* makes possible to consider it a novel and potential granulomatogenous agent, stressing the need to extend the list of bacteria able to induce granulomas in fish tissues.
- The association of bacteria other than mycobacteria with granulomas suggests similarities with human sarcoidosis.

Introduction

The african Lake Malawi/Nyasa represents one of the major underexploited fishery resources, since it contains more endemic fish species than any other lake in the world (Rico and Turner, 2002). African cichlids are highly appreciated "colourful rock dwelling fishes" by aquarists worldwide. This peculiarity induced fishery program which is operating since the early 1970s (Msukwa et al., 2021). The ornamental fish export trade is mostly based on fish from the Lake Malawi, with an average of 28,000 live fish being exported annually and valued at US$218,000 at 2020 prices (Msukwa et al., 2021). Despite their huge economic importance, management of ornamental fisheries is challenged by a paucity of information on the status of the exploited fish stocks (Dee et al., 2014). Fish can dysplay aspecific signs of disease as emaciation, exophthalmia, keratitis and skin lesions, as nodules and black spots. Mycobacterial infection is probably the most common chronic disease affecting aquarium fish species (Noga, 2010). Cichlids are known for a territorial behaviour and sexual aggression, making these animals prone to skin damage, which might become the site of bacterial penetration and transformation of dermal mycobacteriosis into systemic granulomatous infection (Novotny et al., 2010). Regarding viral diseases, those associated with viruses in the family *Iridoviridae* are reported in ornamental fish. Among viruses of this family, *Megalocytivirus* and *Ranavirus* are considered highly pathogenic iridoviruses and are frequently associated with highly mortality outbreaks, whereas *Lymphocystivirus* is mainly associated with a self-limiting cutaneous nodular disease (Whittington et al., 2010; Johan and Zainathan, 2020). Thanks to high species diversity and a broad range of speciation mechanisms, cichlid fish represent a textbook model in parasite evolutionary biology. Despite their importance, among the biological agents of disease, cichlid parasites remain understudied (Vanhove et al., 2016).

The huge relevance of these fish species is not counterbalanced by literature references regarding pathologic conditions with only a limited amount of papers being available (Paperna et al., 2001; Birkbeck et al., 2011;

Lewisch et al., 2016).

This study aims to focus on debilitated ornamental cichlids that were sampled during routine fish management activities. Our goal is to add knowledge of their health status to the scientific community and exotic fish trade industry. To achieve this, a multidisciplinary approach was performed, to investigate pathological patterns and search for the most probable microbiological agents responsible for subtle disease.

Materials and Methods

Animals -clinical history and Sampling

In 2019, during the routine managment activities held by a company specializing in ornamental cichlids trade, some fish showed aspecific signs of disease and were noted. An investigation about possible underlying diseases was conducted.

The fish were kept in 250 L tanks, equipped with independent filters and holed bricks used for shelter by the animals. Two fish samplings conducted in August and October, were carried out. During the first sampling the water temperature was 27°C-28 °C. The second sampling had a consistent temperature of 24 °C. All fish in the first sampling came from breeding activities within the company, whereas the second sampling included a subject that was purchased from another fish farmer and used for breeding.

Mortality outbreaks were not registered in the period before and during the sampling. The selection of the subjects was based on visual and behavioural abnormalities. Thirteen debilitated subjects, which were inclined to be isolated from the group, having nodules and/or dark spots on their skin were chosen. The fish were individually packaged and transported in an insulated container to the laboratory for analyses, then euthanized one at a time with a lethal dose of anaesthetic 2-phenoxyethanol, diluted in the bag used for transport, weighted and measured. Afterwards, sampling was performed with sterile instruments for molecular investigations. Sampling included spleen, liver, kidney, heart, intestine, and any pathological tissue such as cutaneous red or whitish nodules.

Histology and Histochemistry

The remaining parts of each organ sampled for molecular investigations were fixed in 10% (vol/vol) phosphate-buffered formalin according to standard procedures, paraffin embedded, and then cut into 3µm sections. Then, the sections were stained with Hematoxylin-Eosin (H&E). Concurrently sections of the spleen were stained with Gram (Gram Kit, Histoline Laboratories, Milan, Italy) and for the detection of acid-fast bacteria (Zeehl-Neelsen Kit, Histoline Laboratories, Milan, Italy). Archived positive controls for Gram and acid-fast staining were included.

Molecular Investigation for Lymphocystis Disease Virus (LCDV)

Subjects with nodules or skin alterations (n = 9) were analysed for the Lymphocystis disease virus (LCDV) by molecular method. The skin tissue or a portion of the sampled fin was subjected to DNA extraction using approximately 20 mg of tissue. DNA was extracted using the Purelink genomic DNA extraction kit (Invitrogen, USA) according to the manufacturer's directions. The LCDV investigation was then conducted on these samples by a nested PCR assay. A first amplification step was performed using the LF7/LC1R primers (Kitamura et al., 2006; Kvitt et al., 2008), and a second amplification step using the LCDV qPCR F1 and LCDV qPCR R3 primers (Ciulli et al., 2015). For each amplification, a reaction mix containing 1X PCR Buffer, 1 mM $MgCl_2$, 200µM dNTP, 0.4 µM of each primer, 1.25U of Taq polymerase and 5µl of DNA in a final volume of 25 µl was performed. The first amplification step was conducted with 94 ° C for 5 minutes, 45 cycles consisting of denaturation at 94 ° C for 2 minutes, annealing at 55 ° C for 2 minutes, extension at 72 ° C for 2 minutes and a final extension at 72 ° C for 10 minutes. The second amplification step was conducted at 95 ° C for 5 minutes, 45 cycles consisting of denaturation at 95 ° C for 15 seconds, annealing at 50 ° C for 30 seconds, extension at 72 ° C for 30 seconds and a final extension at 72 ° C for 10 minutes. The result of nested PCR was visualized by electrophoresis on 1.5% agarose gel by running the samples together with a reference molecular marker (100 bp ladder, Invitrogen, USA).

Molecular Investigation for Bacteria

Due to the finding of granulomas revealed by the histopathological investigation, the presence of mycobacterial DNA was investigated in frozen (pool made of liver, spleen, heart, and kidney), and in formaldehyde-fixed paraffin-embedded (FFPE) samples. Frozen samples were processed for DNA extraction using the Purelink Genomic DNA kit (Invitrogen, USA) following the manufacturer's instructions. FFPE samples were processed for DNA extraction using the Purelink Genomic DNA kit (Invitrogen, USA) following the manufacturer's instructions with minor modifications. Particularly, unstained sections, serial to sections showing granulomas, were used for DNA extraction: 5–10 mg of sliced FFPE tissue was placed in 1 mL of xylene (J.T. Baker) and a pre-extraction step to remove paraffin from the sample was applied as previously described (Sirri et al., 2018). Samples were deparaffinized in xylene for 5 min; following centrifugation, samples were washed twice in 100% Ethanol. The pellet was dried at 37 degC for 10 minutes, and DNA extraction was subsequently undertaken using the aforementioned kit. Bacterial presence was investigated through a PCR method targeting the HSP65 gene using primers common to all mycobacteria (Telenti et al., 1993). For the amplification, a reaction mixture was assembled containing 1X PCR Buffer, 1 mM $MgCl_2$, 200µM dNTP, 0.4 µM of each primer, 2.5U of Taq polymerase and 5µl of DNA in a final volume of 25 µl. The amplification cycle was conducted at 94 °C for one minute, 45 cycles consisting of denaturation at 94 ° C for one minute, annealing at 60 °C for one minute, extension at 72 ° C for one minute and a final extension step at 72 °C for 10 minutes. The PCR results were visualized by electrophoresis on 1.5% agarose gel by running the samples with a reference marker (100 bp ladder Invitrogen). PCR products of positive samples were purified using the ExoSAP-IT PCR Product Cleanup Reagent (Invitrogen, USA), and sequenced through the Bio-Fab Sequencing Service (Rome, Italy). The sequences were then manually corrected and subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification.

Immunohistochemistry (IHC)

A polyclonal antibody against *Mycobacterium bovis* (Bacillus Calmette-Guerin, BCG, code no. B 0124 Dako, Denmark), that was previously applied in a study about etiological deepening study of granuloma in Doctor fish (*Garra rufa*) (Volpe et al., 2019) was employed. Endogenous peroxidase inhibition was made with 3% H_2O_2 methanol solution. The antigen retrieval was made by using a microwave oven at 750W, 2 cycles x 5min. Preincubation with a blocking solution (10% Normal Goat Serum and Phosphate Buffered Saline solutions) was performed for 30min and then incubated overnight at 4degC with a primary antibody diluted at 1:3,000 in a blocking solution. The secondary anti-rabbit antibody was incubated at 1:200 dilution for 30min, followed by a revelation system with an Avidin-Biotin Complex (ABC) kit developed with diaminobenzidine (DAB) chromogen for 90s and counterstained with Papanicolaou haematoxylin.

To study *Cutibacterium acnes* (formerly*Propionibacterium acnes*), polarized slides were sent to Department of Human Pathology, Tokyo Medical and Dental University, for IHC with a *C. acnes* -specific monoclonal (PAB) antibody that reacts with lipoteichoic acid, the cell membrane constituent of the bacterium, in formalin-fixed and paraffin-embedded (FFPE) tissues. IHC with a commercially available PAB antibody (#D371-3, MBL, Nagoya, Japan) was performed by Leica BOND-III (Leica Microsystems Inc., Tokyo, Japan) using a BOND Polymer Refine Detection kit (#DS9800, Leica Microsystems Inc.) as described in the study by Isshiki et al., 2021. Deparaffinization, peroxidase inhibition, antigen retrieval with BOND Epitope Retrieval Solution 1 (#AR9961, Leica Microsystems Inc.) at 100 *C for 60 min, incubation with PAB antibody (diluted 1:500) at room temperature for 8 min, and counterstaining with Mayer's hematoxylin were performed according to the manufacturer's protocol.

Positive internal and negative control slides were processed in parallel by replacing the primary antibody with a non-reactive isotype-matched antibody.

Results

Animals

The weight of the thirteen investigated fish ranged from 3.1 g to 13.6 g, with the total lenght varying from 8.5

cm to 10 cm. On the basis of the gross findings, in combination with the histological examination referring the gonads, sex was detected in seven out of thirteen fish.

Gross and Microscopical Findings

Overall, the fish were emaciated, thin and had scarce adipose tissue. The information regarding the sex, size and weight, as well as gross and microscopic lesions detected, are summarized in Table 1. Five fish were revealed to have round or irregularly-round black areas on integument and fins ranging from 2 to 5 mm in size. In addition, four fish showed white or pinkish nodules on the skin, fins and/or oral mucosa (Figure 1). In one fish (case 9), white-yellowish multifocal nodules interpreted as granulomas were observed in the spleen and perivisceral adipose tissue. Through histological analysis, the spleen of six fish showed multifocal, coalescent epithelioid granulomas with occasional central necrosis and concurrent hyperplasia of melanomacrophagic centers (MMC). In case 9, the spleen showed acid-fast bacteria within granulomas (Figure 2). In other cases, including 1, 2, 5, 6, and 11, the granulomas were Zeehl-Neelsen negative. All granulomas All granulomas contained Gram positive granular material consistent with bacteria.

The cutaneous nodules histologically were consistent with the presence of mature granulation tissue and concurrent mild, chronic, lymphocytic dermatitis; signs of hypertrophy of fibroblasts, suggestive of an LCDV infection, were absent. The black areas of the skin matched with focal hypermelanosis and were not associated with intralesional parasites.

Molecular Analyses

Concerning molecular detection of LCDV, the tested skin samples of nine subjects were negative.

Regarding bacteria identification, nine samples out of the 13 tested positive to the PCR targeting HSP65 gene of *Mycobacterium* sp. However, sequencing of the PCR products showed that not all of them were ascribable to the *Mycobacterium* genus. BLAST analysis of the obtained sequences identified mycobacteria in two samples (cases 9 and 11) and *C. acnes* in the other seven samples (cases 1, 2, 3, 4, 5, 6, and 8). In particular sequences obtained from samples of cases 1, 2, 3, 4, 5, 6, and 8 showed [?]98.5% nucleotide identity with*Propionibacterium acnes* ATCC 11828 (Genbank accession number CP003084); whereas, sequences from samples of cases 9 and 11 showed the highest similarity with *Mycobacterium chelonae* (100% nucleotide identity with JX154110) and *M. parascrofulaceum* (98,4% nucleotide identity with AY337276) respectively.

Immunohistochemistry (IHC)

A granular immunoreactivity to anti-Mycobacterium antibody was detected within granulomas in case 9 (Figure 2).

In the spleen of cases 1, 2, 5, 6, and 11, variable sized round "Hamazaki-Wesenberg-like" bodies were immunolabeled with PAB antibody within macrophages forming the granulomas (Figure 3). The Hamazaki-Wesenberg bodies are classically detected in human lymph nodes from patients affected by granulomatous conditions. These spheroidal bodies are interpreted as forms of intracellular bacteria that can be detected by immunohistochemistry (Negi et al., 2012).

Discussion and conclusions

Cichlid fishes (Cichlidae) are one of the most worldwide species-rich and widespread families of vertebrates representing a substantial part of the ornamental fish trade and industry (Msukwa et al., 2021). Despite that, a paucity of information on the status of fish stock health is available.

Ornamental fish can display aspecific signs of disease such as emaciation, exophthalmia, keratitis and skin lesions, as nodules and black spots associated with differente causes.

Regarding viral diseases, in ornamental fish including cichlids, several infections due to iridovirus-like microorganisms are reported (Bucke, 2001). Particularly, lymphocystis outbreaks are characterized by a low mortality rate; however, the obvious cutaneous lesions make the subjects not suitable for sale (Noga, 2010).

The lymphocystis virus generally infects dermal fibroblasts causing their hypertrophy; internal organs or gills are rarely affected (Russell, 1974). LCDV commonly presents with white to pigmented masses grossly visible mainly on the pectoral and dorsal fins (Volpatti and Ciulli, 2022), similar to those observed in some of the fish investigated in this study; however in our cases LCDV was not detected. The cutaneous nodules histologically were consistent with chronic dermatitis that matched with the negative molecular results. Chronic dermatitis was related with repeated post-traumatic events occurred. In fact, these fish species are extremely territorial and frequent fighting amongst each other.

Several bacteria that may cause damage to the fish are found naturally in their microbiota or environment and are usually in balance with their hosts. In cases of worsening environmental conditions, skin injuries and/or host immune system impairment, these bacteria may become pathogenic. Mycobacterial infections are the most common chronic disease affecting ornamental species (Noga, 2010), since so far is reported in more than 150 species (Decostere et al., 2004). This disease may cause also human infections, presenting as cutaneous ulcers that struggle to heal (Noga, 2010). As a matter of fact, fish mycobacteriosis is sustained most of the time by three species: *M. marinum* , *M. fortuitum* , *M. chelonae* (Decostere et al., 2004). Other less frequently isolated species are *M. abscessus* , *M. gordonae* ,*M. conceptionense* , *M. parascrofulaceum* and *M. senegalense* (Shukla et al., 2014). In this study, the results aligned with those reported, with both *M. chelonae* and *M. parascrofulaceum* detected in our fish subjectes. A previous study investigated the presence of mycobacteria in freshwater ornamental fish including six species of Cichlids with signs of chronic disease such as persistent cutaneous lesions, abdominal swelling and overall poor general health. This study pointed out the presence of a granulomatous inflammation associated with acid-fast bacteria in 41% of them. However, characterization of bacteria associated with granulomas was not conducted (Gomez, 2008).

Nevertheless, granulomas can be caused bu a variety of bacteria besides mycobacteria (Colquhoun and Duodu, 2011; Maekawa et al., 2018; He et al., 2020).

In our study, Mycobacterium infections were found in only two out of six subjects with granulomas. In seven cases, of whom four showed granulomas, *C. acnes* was identified from FFPE and/or frozen samples through molecular analysis and immunohistochemistry. This finding was interpreted as an intralesional presence, as it was found within visceral organs from fish placed in sterile conditions for tissue sampling. Recently, this bacterium has been found within fish and aquatic environment (Meron et al., 2020; Lorgen-Ritchie et al., 2021), however its pathological role in fish has not been so far investigated.

C. acnes (previously called *Propionibacterium*) is an anaerobic, commensal, lipophilic Gram-positive bacterium. *C. acnes* , classically studied as human bacterial agent of acne vulgaris, is an opportunistic pathogen with a likely underestimated role in the development of disease. In addition to acne, it is associated with other human diseases including prosthetic joint infections, prostate cancer, intervertebral disks surgery and sarcoidosis (Fischer et al., 2020; Mayslich et al., 2021). It is now the second most frequent pathogen, after coagulase-negative staphylococci, isolated from infected internal cerebral ventricular bypasses, and rates of infection with this bacterium have increased from 1.5% to 38%. *C. acnes* has also been fould in blood cultures where it may represent up to 80% of the isolated anaerobes (Mayslich et al., 2021). However, its role in human diseases is still debated, its wide colonization of human organs suggests that the bacterium does not harm the human host, at least not under normal circumstances (Bruggemann et al., 2021).

Analogously, *C. acnes* was recently detected through NGS analysis in internal organs of marine fish without external or internal pathological changes and could suggest its harmless nature; however, three well-known bacterial pathogens, *Photobacterium damselae* ,*Vibrio harveyi* and *Streptococcus iniae,* were also found in these organs (Meron et al., 2020). Despite previous studies stating that healthy fish internal organs should be sterile, lately, studies have reported that bacteria are being foundin healthy kidneys and livers (Sevellec et al., 2014; Meron et al., 2020). However, assuming the organs were macroscopically healthy, a histopathological investigation was not performed in these studies, so the presence of potential tissue reactions as granuloma in association with the bacterial presence was not possible to exclude.

To demonstrate how *C. acnes* causes infection or colonization suggestions have been made to use a combi-

nation of techniques including immunohistochemistry (Capoor et al., 2019). In our study, complementary techniques like PCR, histochemical stainings and IHC were performed to detect bacterial components. According to the experience of these authors (Volpe et al., 2019), the sampling of tissues using combined methods, such as IHC and molecular techniques, is highly recommended to maximize the results. Gram-positive bacterial aggregates, that were concurrently immunoreactive to the anti-PAB antibody and identified by molecular analysis, were detected within granulomas. As a whole, these findings make *C. acnes* a possible advocate that is able to elicit a granulomatous reaction. The immunohistochemical results obtained in our cases were similar to those described in human patients affected by sarcoidosis and represented as "Hamazaki-Wesenberg bodies". Electron microscopy findings suggest that these bodies are intact forms of intracellular bacteria lacking a cell wall structure and occasionally exhibit protrusions (Negi et al., 2012).

Sarcoidosis is an enigmatic multi-systemic human disease of unknown origin: insights into the etiology and pathogenesis have been elusive (Casanova et al., 2020). It is postulated to be a multifactorial disease caused by chronic antigenic stimulation (Mousapasandi et al., 2021). Genetic background may have a predisposing role, and pine pollen, microbial infection, specifically *C. acnes* and*Mycobacteria* spp., as well as air pollutants are increasingly regarded as strong environmental trigger candidates (Wilson et al., 2019). Sarcoidosis is characterized by the development and accumulation of epithelioid, non-caseating granulomas typically found in the lungs; however, sarcoid granulomas can be present almost anywhere in the body (Wilson et al., 2019).

Considering animal models of sarcoidosis, zebrafish (*Danio rerio*) has been employed to study its pathogenesis. Nevertheless, there is no universally accepted animal model for human sarcoidosis, largely because animals other than horses, do not develop spontaneous sarcoidosis. and the link between human gene polymorphisms and disease prevalence has not been established with the aim to be recapitulated in the animal genetic manipulation (Locke et al., 2020).

Regarding the use of the term sarcoidosis in fish, it was used in a recent paper by He et al. (2020) to describe multiple granulomas, dispersed through several organs in a species of commercial interest, the largemouth bass (*Micropterus salmoides)* , and the association with the bacterium *Nocardia seriolae* , a well-known agent able to induce granulomatous reaction in fish.

By using the term sarcoidosis, He et al., (2020) placed emphasis on the relationship among the granuloma and bacteria, other than mycobacteria, trying to identify and recognize this condition.

In this respect, the association of granulomas with *C. acnes*found in some of our cases can be described as a form of sarcoidosis.

Concluding, the detection of bacteria, which are able to elicit granulomatous reaction, represents a significant but still debatable finding in this study. We advise adding *C. acnes* , as well as the yet widely established *Mycobacterium* spp., to the list of bacteria that are associated with granulomatous reaction. The well-known zoonotic role of mycobacteria found in aquarium fish still raises concern into proper management by fish handlers, aquarists, and dedicated personnel.

Due to zoonotic disease potential and impossibility of elimination of mycobacteria from the aquatic environment, the depopulation and aquaria disinfection are recommended as they are the only current measure for containment disease outbreaks (Decostere et al., 2004). The role of the newly detected *C. acnes* needs to be investigated more in depth, with combined laboratory tecnhiques and on several fish samples.

Several deepenings are necessary to understand pathogenetic mechanisms and the yet unknown, highly probable multifactorial etiologies, that underline the granulomatous condition affecting ornamental fish.

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Conflict of Interest Statement

None

Ethics Statement

No ethical approval was required as the fish included in this study were not exposed to experimental procedures. During the routine management activities held by the personnel that works regularly with these ornamental species, some subjects showing aspecific signs of disease were noted; an investigaton about possible diseases was then set up. Rules to preserve their welfare during transport and anesthesia were rigorously observed (description reported in the M&M section).

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Table 1. Synoptic table of ornamental fish species investigated in this study.

Case number	Species	Weight (g)	Length (cm)	Sex	Gross findings	Gross findings
					Black spots	**Cutaneous nodul**
1	*Aulonocara Jacobfreibergi*	7,9	10	F	present	absent
2	*Aulonocara Jacobfreibergi*	3,1	9,5	F	present	absent
3	*Placidochromis spp.*	6,4	9	F	present	absent
4	*Placidochromis spp.*	7,75	8,5	F	absent	absent
5	*Maylandia estherae*	10,38	9,5	nv	present	present
6	*Maylandia estherae*	11,64	9,5	nv.	absent	absent
7	*Mylochromis guentheri*	11,04	9,5	F	absent	present
8	*Mylochromis guentheri*	5,3	8,5	nv	absent	absent
9	*Maylandia pulpican*	8,61	9	F	absent	absent
10	*Maylandia pulpican*	10,29	8,5	M	absent	absent
11	*Maylandia lanisticola*	9,27	9	nv	present	absent
12	*Petrotilapia xanthos*	13,6	10	nv	absent	present
13	*Symphysodon discus*	na	na	nv	absent	present

na: not available; nv: not valuable; nd: not determined; F: female; M: male

Figure captions

Figure 1. Case 7. Cichilid fish. *Mylochromis guentheri* . Region of the mouth. A pinkish nodule bulging from the oral mucosa.

Figure 2. Case 9. Cichlid fish. *Maylandia pulpican* .a. Viscera. White-yellowish multifocal nodules in the perivisceral adipose tissue and the visceral organs (arrow) interpreted as granulomas. a. Spleen. Multifocal, coalescent granulomas developing from melano-macrophagic centers (MMC) dispersed within the splenic parenchyma. Hematoxylin & Eosin staining, 4x.c. Spleen, Acid-fast bacilli (arrowheads) are present within granulomas. Zeehl-Neelsen stain, 40x.d. Spleen, granular labelling to anti-Mycobacterium antibody within granulomas. Immunohistochemistry, lens 40x.

Figure 3. Case 2. Cichlid fish. *Aulonocara Jacobfreibergi.* a. Skin. Multifocal, irregularly-round black areas of the integument and fins.b. Spleen. Multifocal, coalescent granulomas dispersed throught the splenic parenchyma. Hematoxylin & Eosin staining, 2.5x.c. Viscera. Scarce perivisceral adipose tissue and intestinal viscera showing a serous content.d. Spleen, variably sized round "Hamazaki-Wesenberg-like" bodies immunolabeled with PAB antibody within macrophages forming the granulomas. Immunohistochemistry, DAB staining, 40x.