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Infection of the muscle tissue of the filter-feeding cichlid, *Chaetobranchopsis orbicularis* Steindachner, 1875, by *Kudoa orbicularis* (Myxozoa: Multivalvulidae) on Marajó Island in the Brazilian Amazon region

[*Infecção por* Kudoa orbicularis *(Myxozoa: Multivalvulidae) na musculatura de* Chaetobranchopsis orbicularis *Steindachner, 1875, oriundo da Ilha de Marajó na região Amazônica do Brasil*]

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ABSTRACT

This study describes aspects of infections caused by the myxosporidian *Kudoa orbicularis* in filter-feeding cichlids, *Chaetobranchopsis orbicularis*, caught in the Arari River in the municipality of Cachoeira do Arari, on Marajó Island, Pará, Brazil. The parasite forms pseudocysts scattered throughout the striated epaxial and hypaxial muscles. Samples embedded in paraffin were analyzed histologically using hematoxylin-eosin, Gömöri, Ziehl-Neelsen, and Giemsa staining. Necropsy of the *C. orbicularis* specimens revealed that 100% (50/50) were infected with K. orbicularis. The specimens presented grossly abnormal muscle texture, resulting in extensive inconsistencies and weakness. Progressive softening of the muscles was observed during necropsy, indicating the rapid enzymatic autolysis of the tissue. The parasite found in the muscle tissue of *C. orbicularis* was identified as *K. orbicularis*, with clinical signs of disease being observed in the fish. The necropsy revealed extensive damage to the host organism, with well-established fibrocystic infections in the muscle fibers, associated with *post mortem* myoliquefaction.

Keywords: muscle, *Kudoa*, Myoliquefaction, filter-feeding cichlid, Myxozoa

RESUMO

O presente estudo descreve os aspectos histopatológicos de infecção causada por mixosporídio da espécie Kudoa orbicularis, *o qual forma pseudocistos dispersos em toda a musculatura estriada esquelética, epi e hipoaxial, de* Chaetobranchopsis orbicularis, *capturados no Rio Arari, município de Cachoeira do Arari, Ilha do Marajó, Pará. Foram realizadas as técnicas histológicas de impregnação em parafina, utilizando-se as colorações de hematoxilina-eosina, Gomori, Ziehl-Neelsen e Giemsa. As análises necroscópicas dos espécimes de* C. orbicularis *revelaram 100% (50/50) de infecção por* K. orbicularis. *Os espécimes apresentavam macroscopicamente musculatura com características anormais de textura, se mostrava inconsistente e frágil. Durante a necropsia, pôde ser observado um progressivo amolecimento da musculatura, o que demonstra um rápido processo enzimático autolítico. Com base nos achados descritos neste trabalho, caracterizou-se uma infecção da musculatura de* C. orbicularis *por* K.orbicularis, *com demonstração de sinais clínicos de doença no peixe; os achados necroscópicos mostraram danos ao organismo hospedeiro, com instalação de infecção fibrosística nas fibras musculares, associada com uma mioliquefação* post mortem.

Palavras-chave: músculo, Kudoa, *mioliquefação, cará, Myxozoa*

INTRODUCTION

The rivers of the Amazon basin contain the world's most diverse freshwater fish fauna in the world, with more than 1500 species described to date (Montag *et al*., 2008). This fauna includes 20 genera and 100 species of cichlids, approximately 6.7% of the total. One of these cichlid genera is *Chaetobranchopsis*, species of which are widely appreciated by aquarium enthusiasts due to the beauty of their coloring and reproductive displays (Kullander, 2003; Lowe-McConnell, 1991).

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Parasites are the main cause of the loss of economic value in fish and their products. These losses are accentuated in the Neotropics, where the local climate favors the rapid and constant propagation of these organisms (Thatcher and Brites-Neto, 1994). The members of the phylum Myxozoa are a prominent group of fish parasites, and include more than 2200 species, representing 65 genera. These parasites are found as microscopic spores in the tissue of many different vertebrate organs. Myxozoans are the causative agents of diseases that affect both freshwater and marine fish, resulting in high mortality rates in many different regions (Lom and Dykova 2006, Azevedo *et al*., 2009).

The genus *Kudoa* is part of the Myxozoa group, which includes a number of potentially important species of parasites that have extremely negative impacts on the quality of fish products. In particular, the species of this genus are responsible for *post mortem* myoliquefaction, which renders the meat unacceptable for human consumption. The lysis of the muscle tissue results from the production of proteases by the parasites, which depolymerize the host muscle fibers, and facilitate the access of the parasite to other hosts involved in its life cycle. Although these parasites cause diseases in fish, most studies have found no evidence of any risk to human health (Alvarez-Pellitero & Sitja-Bobadilla, 1993; Mazorra-Manzano *et al*., 2008), Kawai *et al*. (2012) reported poisoning in people who consumed sushi prepared from *Paralichthys olivaceus* that contained spores of *Kudoa septempunctata*.

Kudoa is a myxosporidian genus of the order Multivalvulida, which has four or more valves with radial symmetry and quadrangular spores, when observed apically. The suture lines form an angle of 90°. A few species have short apical microvilli with polar capsules that are either essentially the same size or of different sizes (Eiras, 1994). Here, we describe the infection of the tissue of the skeletal muscles of *C. orbicularis* by *K. orbicularis*, including its pathological features and *post mortem* myoliquefaction.

MATERIALS AND METHODS

For the present study, 50 specimens of *C. orbicularis* were caught in the Arari River in the municipality of Cachoeira do Arari, on Marajó Island in the state of Pará, Brazil (1°00' S, 48°57' W), between 2014 and 2015. The specimens were transported alive in aerated plastic bags filled with water initially to the town of Salvaterra, and subsequently to the Carlos Azevedo Research Laboratory of the Federal Rural University of the Amazon Region (UFRA), in Belém, where they were kept in an aquarium with a water temperature of 28-30°C. The fish were anesthetized using tricaine methanesulfonate (MS222; Sigma) at a concentration of 50 mg/L and were dissected and examined for the presence of parasites and cysts under a stereomicroscope (ethics committee on use of animals nº 013/2014 – UFRA). After the confirmation of the presence of these parasites in muscle tissues, images were captured, and the spores were measured. Small pieces of tissue were removed for observation under an optical microscope. These samples were placed on slides and fixed in place with a coverslip for examination.

When myxozoan parasites were detected, small fragments (approximately 0.5cm thick) of the epaxial or hypaxial muscle of the specimen were extracted and fixed in Davidson's solution (neutral buffered formalin, glacial acetic acid and 95% ethanol in distilled water) for 24 h. The fragments were then processed for embedding in paraffin. Sections of 05µm in thickness were cut from the paraffin block and stained with hematoxylin and eosin, Gömöri, Ziehl-Neelsen and Giemsa (Luna, 1968). These sections were photographed.

For scanning electron microscopy (SEM), spores were fixed in 5% glutaraldehyde buffered with sodium cacodylate (pH 7.2) for 12 hours at 4°C, and then washed overnight in the same buffer solution and post-fixed in 2% OsO_4, buffered with the same solution for 3 hours at 4°C. The samples were then dehydrated in an increasing series of ethanol. The spores were dried to the critical point, metalized with a fine (20 nm) layer of gold, and photographed. The specimens were also photographed in a Hitachi TM 3000 Tabletop electron microscope (Hitachi TM

3000), for which they were prepared by the same procedure, but were not metallized.

RESULTS AND DISCUSSION

The analyses identified pseudocysts interspersed between and within the muscle fibers of the epaxial and hypaxial regions. These cysts were filled with mature spores characterized by pseudo-quadratic radial four-valve symmetry and the presence of a polar capsule valve, which are diagnostic of the genus *Kudoa* (Fig. 1). The necropsy of the 50 *C. orbicularis* specimens revealed that all (100%) individuals were infected with *Kudoa* parasites, in all cases, with pseudocysts interspersed both between and within the muscle fibers.

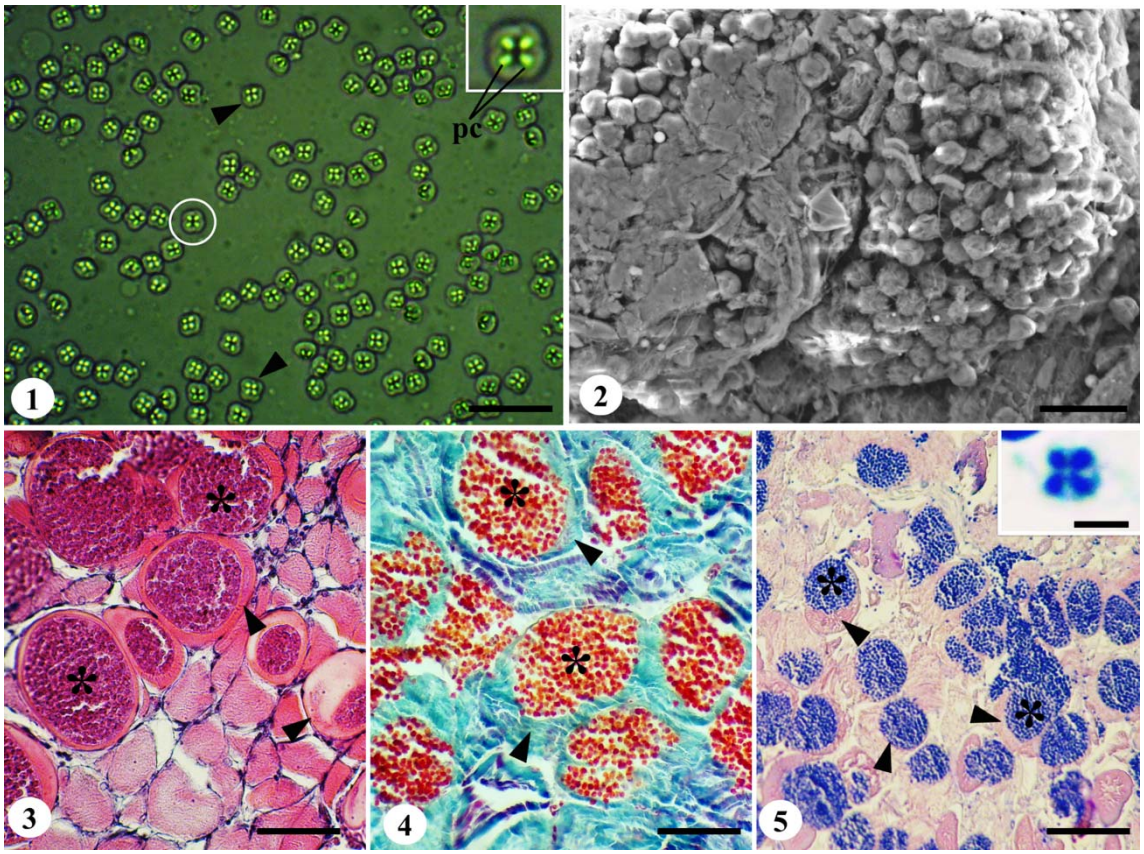

Figure 1. Photomicrograph of a tissue sample from *Chaetobranchopsis orbicularis* showing fresh *Kudoa* sp. spores (arrowhead), highlighting the polar capsules (pc); scale bar: 20µm. Figure 2: Spores of *Kudoa* sp. in pseudocysts revealed by Scanning Electron Microscopy; scale bar: 20µm. Figure 3, 4 and 5**:** Histological sections of skeletal striated muscle with pseudocysts (*) located in the cytoplasm of the fibers (arrowhead), under hematoxylin-eosin, Gömöri and Ziehl-Neelsen staining, respectively; scale bars: 75µm, 60µm and 160µm respectively. Inset: Spore of *Kudoa* sp. stained with Giemsa, especially in the four polar capsules; scale bar: 5µm.

The infected specimens had grossly abnormal muscle texture with fragile tissue and inconsistent features. Progressive softening of the muscles was observed during necropsy, indicating rapid enzymatic autolysis, as noted by Henning *et al*. (2013) in specimens infected by *Kudoa*, who identified the rapid softening of the muscle, which led to post mortem myoliquefaction, as an enzymatic process. However, Andrada *et al*. (2005) and Azevedo *et al*. (2016) did not observe any evidence of myoliquefaction in the specimens they examined.

The analysis of the images captured of the fresh *Kudoa* spores permitted the establishment of morphometric parameters on the length and

width of the spore and the polar capsule. The parameters recorded in the present study were different from those obtained by Casal *et al*. (2008) for the spores of *Kudoa aequidens* found in *Aequidens plagiozonatus* from a region of the Brazilian Amazon close to the site of the present study, although they were similar to the values obtained by Egusa and Nakajima (1980) for *Kudoa amamiensis* infecting the Japanese fish *Abudefduf sexfasciatus*, *A. vagiensis*, *Chromis isharai*, *C. notatus*, *Chrysiptera assimilis* and *Seriola quinqueradiata,* and those reported by Dykova *et al* (2002) for *Kudoa dianae* found in *Sphoeroides annulatus* in Mexico. These comparisons indicate that spore size is not necessarily related to the geographic distribution of the parasites (Table 1). The general aspects of the morphology of these parasites are similar to those described in the reviews of Moran *et al*. (1999a) and Lom and Dykova (2006).

Table 1. Comparison of shape and measurements of spores species of *Kudoa* sp

	Kudoa aequidens (Casal, 2008)	*Kudoa amamiensis* (Egusa & Nakajima, 1980)	*Kudoa dianae* (Dyková, Avila e Fiala, 2002)	*Kudoa* sp. Present study
Host	*Aequidens plagiozonatus*	*Seriola quinqueradiata*	*Sphoeroides annulatus*	*Chaetobranchopsis orbicularis*
location	In sub-opercular skeletal musculature	Skeletal musculature	In extramuscular sites, in the wall of oesophagus, and less frequently on mesenteries	Epi e Hipoaxial in skeletal musculature
PCs	4 equal size	4 equal size	4 equal size	4 equal size
Length	2.9 – 3.5	4.5-5.0	4.5-5.5	4.4 – 4.8
Width	6.2 – 7.1	5.0-6.0	5.5-6.5	5.0 – 5.6
Spore shape	Quadrate or pseudoquadrate	Quadrangular	Quadrate	pseudoquadrate
Polar Capsule (length x width)	2.0-2.6 x 1.1 -1.5	1.5-2.0 x 1.0 -1.2	2.0 x 1.5	1.4 - 2.0 x 1.2 – 1.6
Country	Brazil	Japan	Mexico	Brazil

The morphology and morphometry of the spores, the host (*C. orbicularis*), the site of infection, and the region in which the specimens were collected, all indicate conclusively that the *Kudoa* species found in the present study was *Kudoa orbicularis* (Azevedo *et al.,* 2016). The presence of the polar capsules was accentuated clearly by the Giemsa staining of the sections obtained from fragments of infected muscle (Figure 6). This technique was also used successfully by Meng *et al*. (2011) in an analysis of the polar capsules of *Kudoa prunusi*.

The SEM (Scanning Electron Microscopy) and histological techniques based on hematoxylin-eosin (HE), Gomori and Ziehl-Neelsen staining (Figures. 2, 3, 4 and 5) showed the relationship between pseudocysts in the muscle fiber and their spores, with the spores being located both inside and between the muscle fibers, as observed by Moran *et al*. (1999b) in the infection of Atlantic salmon by *Kudoa thyrsites*.

The histological-pathological analysis of the infected samples found multifocal necrosis with the presence of fibroblastic tissue adjacent to the pseudocyst regions, together with muscle fibers of irregular appearance, with pyknotic nuclei or a complete absence of the nucleus, and the presence of "tracery" in the sarcoplasm, which is diagnostic of a process of tissue necrosis. Morado and Sparks (1986) described a similar inflammatory process in the muscles of *Merluccius productus*, caused by *Kudoa thyrsites* and *Kudoa paniformis*, which progressed to fibroblastic encapsulation, like that observed in the present study. The hosts analyzed in the present study also appeared apathetic, with lethargic movements, like those observed by

Azevedo *et al.* (2016) in *Chaetobranchopsis orbicularis* infected by *Kudoa orbicularis*.

The histopathological findings of the present study were consistent with the infection of the muscles of *C. orbicularis* by *Kudoa orbicularis*, with clear evidence of clinical disease in the fish specimens. The necropsy revealed extensive damage to the host organism, with fibrocystic infections established in the muscle fibers, resulting in *post mortem* myoliquefaction.

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