



Atrophy and Muscular Fibrosis of Unknown Etiology during the Raising of *Xiphophorus maculatus* on an Ornamental Fish Farm

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Authors' contributions

This work was carried out in collaboration among all authors. Author LAR made the diagnosis and managed the writing and research of literature. Authors VFP and AFFM managed the sample processing for histopathological analysis. All authors read and approved the final manuscript.

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ABSTRACT

During the last two decades, the implementation of histochemical, immunohistochemical, electron microscopy, and recently developed molecular techniques has greatly contributed to our knowledge of skeletal muscle, both normal and sick. This article reports the presence of muscular atrophy and fibrosis in *Xiphophorus maculatus* from an ornamental fish farm. We do not know the origin of this muscular pathology and the purpose of this work is to summarize some of the findings with optical microscopy and electron microscopy shared by all. Although we cannot establish the etiology of this atrophy, we will try to correlate the ultrastructural alterations with the muscular histopathology. Muscular atrophy is a pathology characterized by loss of normal muscle mass. It is caused by a decrease in the total number of muscle cells or by a substantial reduction in the substance of individual muscle cells. It is likely that the cases reported here represent a pathology involving causes concurrent with nutritional problems and disorders of muscle innervation. Therefore, future studies should investigate further about the potential of neurodegenerative disorders. Several experimental models can use muscular atrophy and are suitable for investigations of the underlying mechanisms of this pathology.

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1. INTRODUCTION

During the last two decades, the application of histochemical, immunohistochemical, electron microscopy, and recently developed molecular techniques has greatly contributed to our knowledge of skeletal muscle, both normal and diseased [1,2]. In mammals and humans, during this time, much has been learned about the functional importance of muscle structure and how subcellular organization enables energy transformations that convert chemical energy into mechanical work [3].

Part of this new information has clarified the significance of the structures under the optical microscope and therefore helped the pathologist in the diagnosis of diseases affecting the skeletal muscle [4]. This article reports the presence of muscular atrophy and fibrosis in *Xiphophorus maculatus* from an ornamental fish farm. We do not know the origin of this muscular pathology and the purpose of this work is to summarize some of the findings with optical microscopy and electronic microscopy that are shared in the literature. Although we cannot establish the etiology of this atrophy, we will try to correlate the ultrastructural alterations with the muscular histopathology. The major diagnostic problem for the pathologist when observing muscle lesions is to establish the differential diagnosis between a strictly muscular pathology and a neurological or neuromuscular pathology [5,6].

In mammals, a relatively small percentage of striated muscle tissue samples present dramatic pathological changes under optical or electron microscopies [7]. In fish, common diagnostic problems include muscle atrophy, muscular dystrophy, or myositis. Since histopathological changes are often non-specific, it is necessary to take into account the entire clinical history, growth, and extent of the pathology in one or several fishes, the food ingested, and the water quality, among others. In many cases, the pathologist is often disappointed by the absence of significant microscopic abnormalities in the muscle. In such cases, the study of the muscle ultrastructure can also provide information that contributes to the diagnosis.

2. MATERIALS AND METHODS

Thirty-three *Xiphophorus maculatus* (16 females and 17 males) were sent to our laboratory by an

ornamental fish farmer, which presented a change in the linear posture of the body. Their body was arched with dropped cephalic and caudal regions and they lost their rectilinear posture during swimming. The average length of these animals was 3.7 +/- 1.1 cm. All these animals were raised in the same pond at a temperature ranging between 26 and 28°C. On the other hand, 10 other *Xiphophorus maculatus* from the same breeder, raised in another pond and presenting a normal appearance, were studied (Fig. 1). All fish were fed with Tetramin Flakes Tetra© food. The animals were euthanized in a 100 ppm M-222 bath (Western Chemical, USA). The fish were processed whole, with a previous longitudinal cut from the cephalic to the caudal regions, fixed in Bouin liquid for 12 hours, and then transferred to 70% ethyl alcohol, included in paraplast. Then, they were sectioned at 3 µm, and stained with hematoxylin and eosin, Masson's trichromic, and Panotic of Del Rio Hortega, modified for sections embedded in paraplast [8].

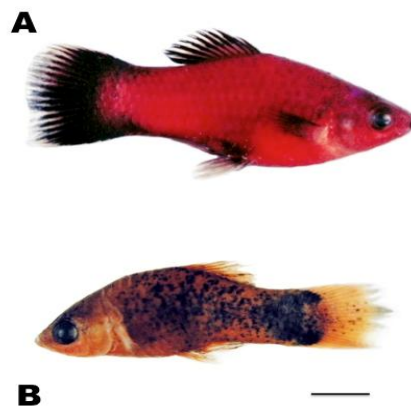


Fig. 1. A: Normal *X. maculatus*, with straight muscular posture. B: *X. maculatus* with muscular posture, where the curved body can be observed. Bar: 1 cm

Fragments of muscle tissue were fixed for transmission electron microscopy in 0.2% glutaraldehyde post-fixed in osmium tetroxide and later prepared according to the protocol described previously [9]. Thick sections stained with toluidine blue were used to locate tumor areas. Next, ultrafine sections were prepared and stained with uranyl acetate and lead citrate, and examined under a Jeol JEM-8T electron microscope (Jeol, Tokyo, Japan).

3. RESULTS

3.1 Optic Microscopy

The different histological sections exhibited a proliferation of fibroblasts among the muscle fibers of all skeletal muscles, both from the abdominal wall and from the dorsal and cranial regions. The muscle fibers were observed "immersed" and surrounded by fibroblasts with abundant intercellular matrix that resulted in atrophied fibers, and even with some fibers almost disappearing and being replaced by fibroconnective tissue (Figs. 2 and 3). The Panotic staining revealed that the fibers began to lose their striations until fragments of fibers without striations were observed (Fig. 4). In animals that did not present macroscopic alterations, the normal skeletal muscle architecture, with their respective striations, was observed. No lesions were observed in the heart, pancreas, or any other organ.

3.2 Electron Microscopy

We observed peripheral loss of myofilaments from myofibrils. In some sectors severely atrophied myofibrils were observed, characterized by fragmentation and the formation of Z-bands. In addition, remains of the myofibers were observed, persisting as a tube of basal

layer that in some cases are phagocytized by macrophages. In other sectors, focal myofibrillar degeneration alternated with myofibrils in regeneration was observed. In other sectors the folding of the myofiber surface membrane was noted, as well as mitochondria grouping and myofibril disorganization. Areas of disorganized myofibrils were observed, marked by residual Z material in the area below the sarcolemma. Severely affected myofibers with a redundant basal lamina, focal fragmentation of the plasma membrane, and changes in the sarcoplasm, with a marked thickening of the capillary base lamina were observed (Figs. 5, 6 and 7).

4. DISCUSSION

The fact that skeletal muscle tissue is susceptible to only few pathological changes is not surprising. The basic histopathological patterns recognizable under optical microscopy are few: normal trophism, hypotrophy, hypertrophy, dystrophy, and myositis [10,11,12]. "Myopathy" is a non-specific term meaning intrinsic muscle disease. Myopathies of various types are produced with varying histopathological patterns. In mammals, during the late stage of various muscle diseases, in which myofiber destruction and replacement by connective, fat, and fibrous tissues are extreme, the underlying pattern of the disease may disappear [13,14].

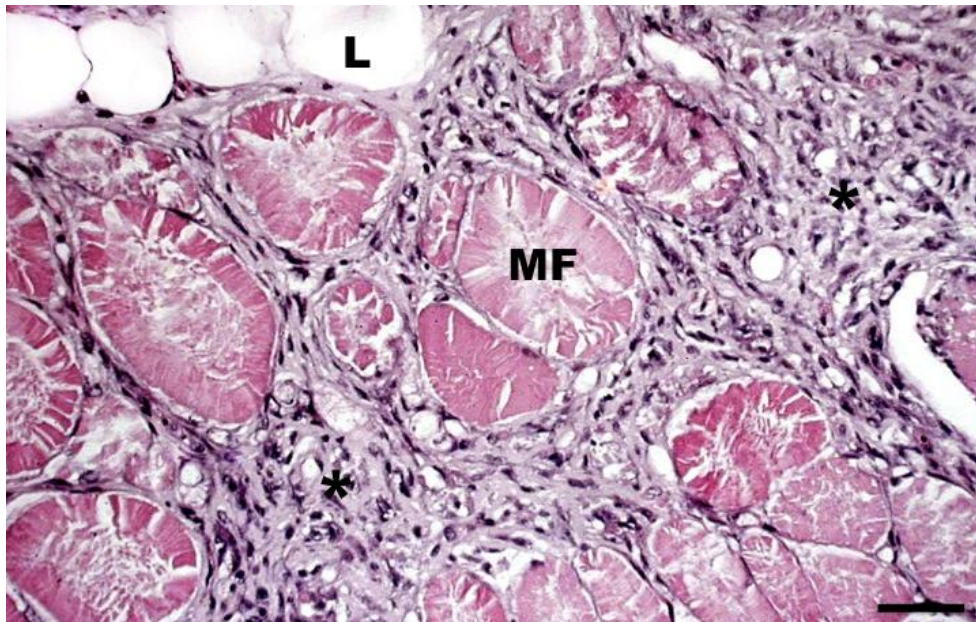


Fig. 2. Muscle of the dorsal region, where proliferation of fibroconnective tissue (*) is observed between the muscle fibers (MF), which are "immersed" between the fibroblasts. Remaining adipose tissue (L) is observed. H-E. Bar: 20 μ

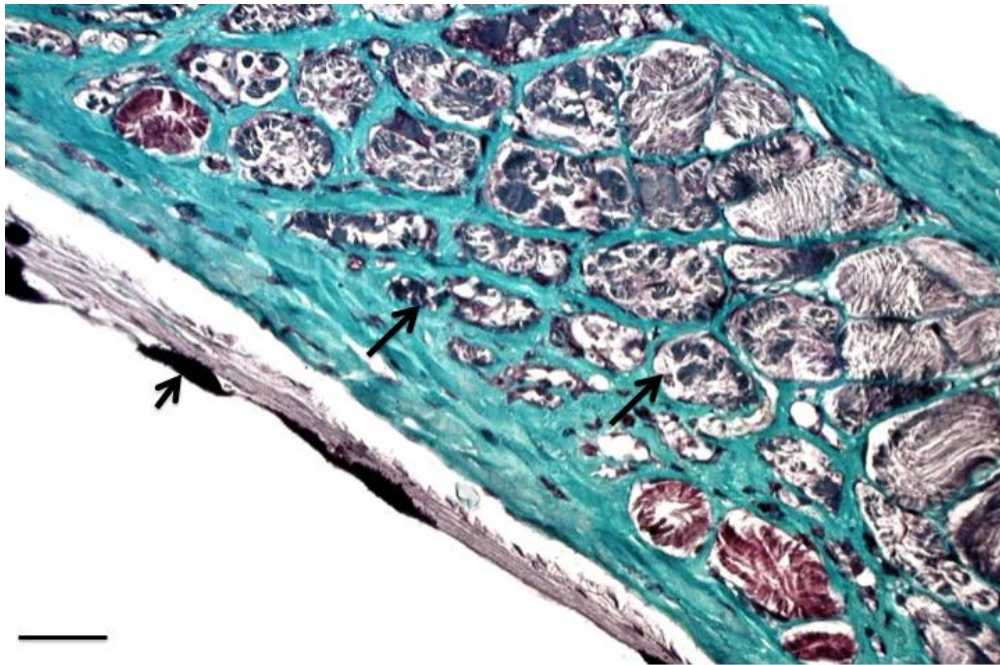


Fig. 3. Abdominal wall with proliferation of fibroconnective tissue that surrounds and compresses muscle fibers (wide arrows). Melanin is seen in the visceral peritoneum (short arrow). Masson's trichrome. Bar: 50 μ

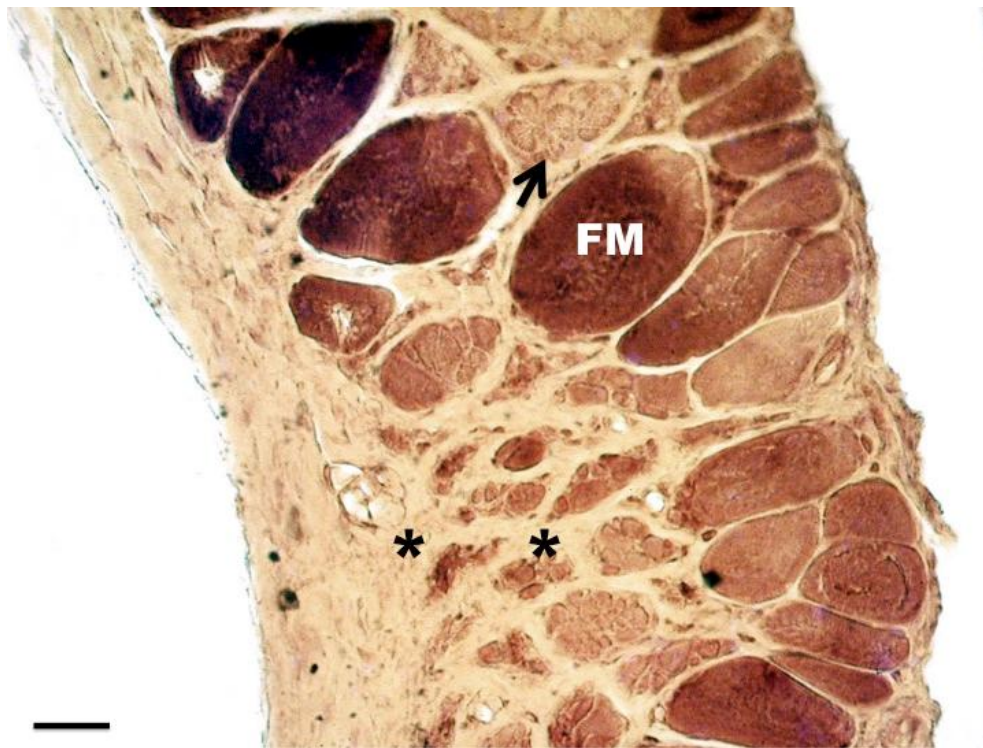


Fig. 4. Abdominal wall with proliferation of fibroconnective tissue (*), atrophic muscle fibers (arrow) and fibers with loss of striations (FM). Panoptic. Bar: 50 μ

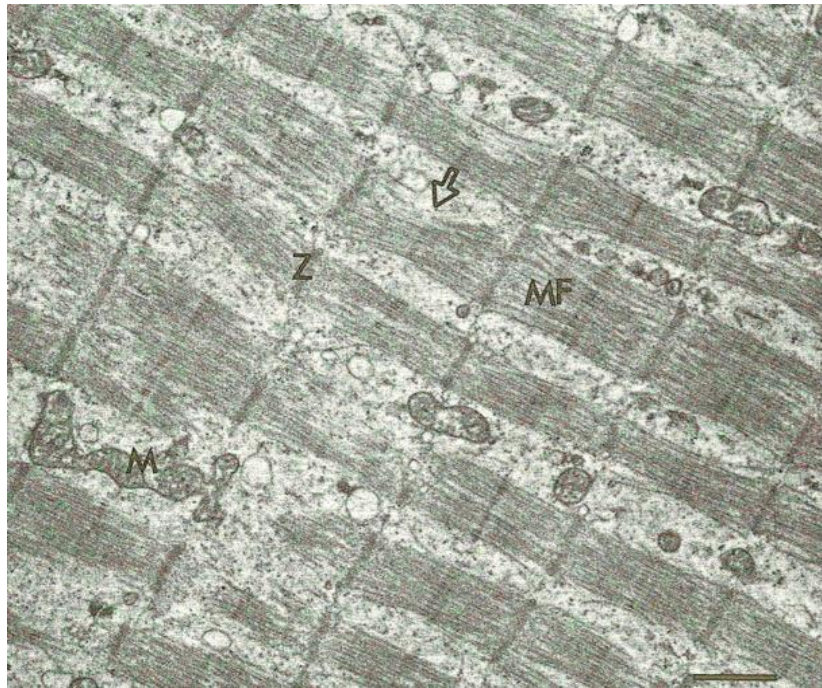


Fig. 5. Electron micrograph illustrating myofibrils with atrophy. Loss of myofibrils (MF) is indicated at the arrow. An elongated mitochondrion (M) is present in the lower portion of the figure. Z bands are observed (Z). Bar: 1 μ

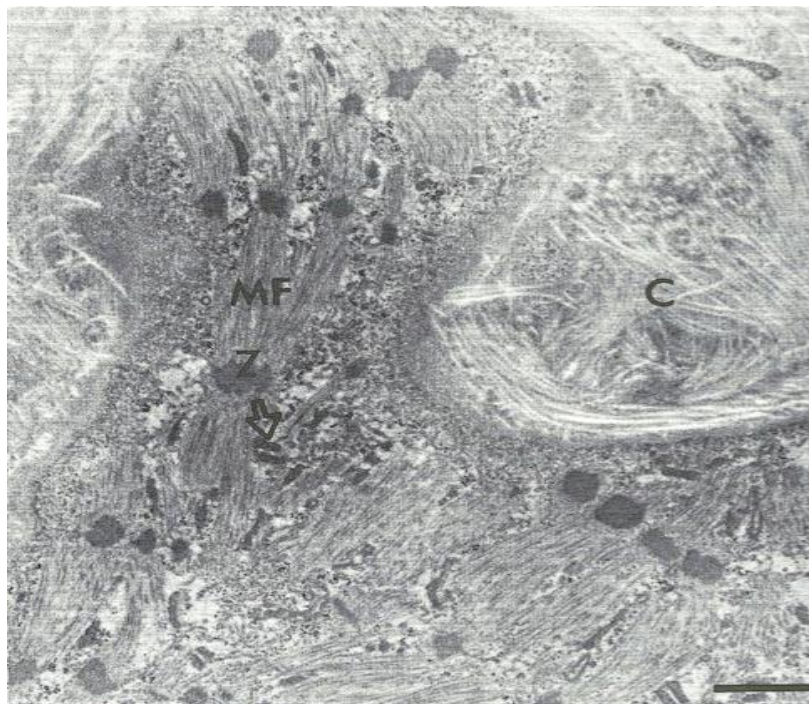


Fig. 6. Electron micrograph of a severely atrophic myofiber illustrating fragmentation of myofibrils (MF) and smudging of Z bands (Z). Note the large amount of collagen (C) surrounding the myofiber. Terminal sacs of the sarcoplasmic reticulum filled with dense granular material are indicated at the arrow. Bar: 1 μ



Fig. 7. Electron micrograph illustrating focal myofibrillar degeneration (arrow). The mitochondria (M) are in the contracted state. Bar: 0.5 μ

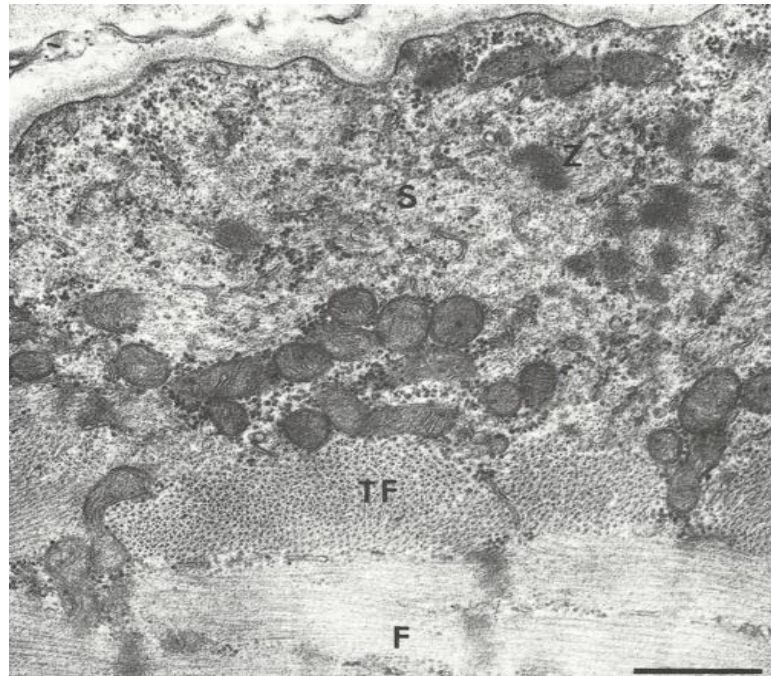


Fig. 8. Electron micrograph illustrating the appearance of a spiral annulet (ringbinden) from muscular atrophy. Note the area of disorganized myofilaments marked by residual Z material in the area beneath the sarcolemma. This area (S) appears in light micrographs as a sarcolemmal pad. The transverse myofilaments of the ringbinden are illustrated at TF. Normally oriented myofilaments are present at F. Bar: 0.5 μ

Due to confusion about the term "muscle atrophy", some word definition is required. As used clinically to describe a muscle, "atrophy" refers to a decrease in the size of a previously normal muscle. When used in reference to individual myofibrils, "atrophy" is generally used to describe abnormally small myofibers [15]. However, small myofibers can result from a variety of pathological processes. For example, motor nerve rupture leads to denervation atrophy. Although the role of the peripheral nerve in maintaining skeletal muscle is clear, the mechanism behind it is unknown [16]. Several studies investigated the causes of muscle atrophy in fish [17,18]. Roberts and his collaborators have extensively studied degenerative and inflammatory changes in skeletal muscles of teleostean fishes [19,20,21,22].

Chronic pancreatitis, which is a characteristic of diseases such as infectious pancreatic necrosis in the late stages of pancreatic injury, presenting marked acinar atrophy, is accompanied by degenerative changes in the cardiac and skeletal muscles [23,24]. In this work we found no pancreatic or cardiac lesions, with the pathology restricted only to the skeletal muscle.

Ferguson [25] reported degenerative muscle injuries due to vitamin E and selenium deficits, particularly when fish live at temperatures lower than that required by the species. This picture was called "nutritional muscle dystrophy" (NMD), similar to that occurring in other animals [26]. In this case, apparently there is no nutritional deficit. Firstly, because the administered food has a formula that covers the needs of this species and secondly because all the animals were fed with the same food in all the tanks where they were raised.

Some authors reported muscle inflammatory processes, myositis, by bacterial infections [27]. We found no inflammatory infiltrates or necrosis, although we have observed a late stage of the inflammatory process and, therefore, we expected to see at least minimal areas of necrosis and inflammatory infiltrates in some of the 33 *X. maculatus*.

The ultrastructural findings presented in this work are similar to those described in the mammalian literature. These alterations indicate muscular atrophy. Regarding individual myofibers, we observed the following three types of atrophy: "simple" atrophy, characterized by a decrease in

the size of the myofibers, but preserving the normal sarcomeric structure of the individual myofibers; "dedifferentiation" atrophy, in which there is destruction and loss of myofibers, with loss of myofilaments; and "degenerative" atrophy, in which there was a total loss of myofibers and irreversible changes that lead to cell necrosis and subsequent replacement by fibroconnective tissue [28,29]. Therefore, atrophy can result from denervation, disuse, metabolic insufficiency, mechanical compression, and other various causes [30]. The time required for denervation atrophy varies considerably in different animal species. One or two weeks after the cross section of a motor nerve, almost all myofibers show signs of nuclear changes consisting of rounding, increased nucleolar size, and central migration of nuclei [31].

The myofibers become gradually smaller and rounder in cross-section, but striations persist. It is controversial whether number of nuclei increase or not. Since they do not experience mitosis and amitosis is doubtful, it is possible that the redistribution or division of satellite cell nuclei is responsible for the apparent increase in the number of nuclei in the diseased muscle. The occasional myofibers may be fragmented and surrounded by macrophages, suggesting degeneration. It is believed that the presence of "target fibers" is characteristic of denervation atrophy. These myofibers have three distinct zones: a compact central myofibril core that lacks crossed striations; a less dense intermediate zone with few myofibrils; and a less affected outer zone. In prolonged cases of atrophy, hyaline bodies may appear inside the fibers. In late stages, as the myofibers disappear, the interstitial spaces are filled with increasing amounts of connective and fibrous tissue [32]. In some cases, distinct groups of atrophic myofibers are dispersed among larger and more normal looking miofibers. This pattern is a consequence of the innervation of multiple myofibers by branches of a single axon.

This work did not allow the observation of different types of synaptic vesicles in the motor end plates. Nevertheless, Radaelli [33] described different vesicles for *Sparus aurata* and *Anguilla anguilla* under electron microscopy.

The growth of the lateral muscle after hatching has been morphometrically studied in *Sparus aurata* in order to identify and quantify hyperplasia and hypertrophy of the muscle fibers, revealing that hypertrophic growth occurred at all

ages, but was the dominant mechanism of muscle growth only in the juvenile and adult phases [34].

Because of the widespread use of zebrafish in developmental biology studies, a wide range of tools and experimental techniques has been brought together. Recently, it has been evident that these could be used in the analysis of neurodegenerative diseases and muscle pathologies such as atrophy [35,36]. On the other hand, these models also highlight atrophic muscle injuries associated with chemicals such as alcohol or copper oxide nanoparticles [37,38]. However, the damage to muscle tissue in zebrafish and other fish species is different from that found in *X. maculatus*, being characterized by fibrillar and perimisial degeneration, inflammation, vacuolar degeneration, and atrophy [39].

5. CONCLUSION

We did not find an etiological cause for this muscle injury. Muscular atrophy is a pathology characterized by loss of normal muscle mass. It is caused by a decrease in the total number of muscle cells or by a substantial reduction in the substance of individual muscle cells. It is possible that the causes are concurrent to nutritional problems and disorders in muscle innervation.

In this sense, we should investigate further and deepen studies on possible neurodegenerative disorders. Several experimental models can use muscle atrophy and are adequate for investigations of the underlying mechanisms of muscle atrophy.

ETHICAL APPROVAL

All the methods applied in this study were carried out in accordance with the national council for the control of animal experimentation (cebea) and the ethics commission in animal use (ceua) (protocol # 23116.008569 / 2019-78).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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