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Gonadal lesions in *Anodontites tenebricosus* (Lea, 1834) from Verde River (Paraná, Brazil)

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Abstract: This study aimed at analyzing the frequency of histological alterations in gonads of *Anodontites tenebricosus*. No macroscopic alterations were observed in the valves and visceral mass in the sample constituted of 50 specimens with length class amplitude between 47- and 99-mm. Tissue lesions were observed in males and females in all the length classes. The lesions are characterized by the concentration of pathogenic agent with spherical shape and subcellular size with basophilic reaction. Around these lesions, hemocytes invasion is observed, which evidences a pathological condition. They present progressive characteristic with a decrease in the number of oocytes, loss of tissue structure and gonadal castration, which shows a tendency of population decrease in the study area. Due to the anthropic interference in the study area, with water receiving chemical residues originating from different activities and the presence of the invasive species Corbicula flumninea, different factors may induce or intensify the occurrence of lesions. More studies are necessary to determine the etiological agent and how native bivalve populations are responding to a set of environmental modifications.

Key words: Mycetopodidae. Population. Decline. Gonadal Lesion. Pollutants

Resumo. Lesões gonadais em *Anodontites tenebricosus* (Lea, 1834) do Rio Verde (Paraná, Brasil). No presente estudo foi analisada a frequência de alterações histológicas observadas nas gônadas de *Anodontites tenebricosus*. Na amostra, constituída de 50 exemplares, com amplitude de classe de comprimento entre 47 e 99 mm, não foram registradas alterações macroscópicas nas valvas e massa visceral. As lesões histopatológicas, com 100% de prevalência na população estudada no período de dois anos, foram observadas em machos e fêmeas em todas as classes de comprimento. As lesões se caracterizam pela concentração de agente patogênico, de forma esférica e tamanho subcelular com reação basófila, em torno das quais ocorrem invasão de hemócitos, evidenciando condição patológica. Apresentam caráter progressivo, com diminuição do número de ovócitos, perda da estrutura tecidual e ocorrência de castração gonadal, o que permite inferir que existe uma tendência de declínio populacional na área de estudo. Devido à interferência antrópica registrada na área de estudo, com o curso de água recebendo resíduos químicos originados de diferentes atividades e ocorrência da espécie invasora Corbicula fluminea é possível que diferentes fatores possam gerar ou intensificar a ocorrência das lesões observadas. O quadro atual demonstra a necessidade de mais estudos para determinação do agente etiológico e como as populações de bivalves nativos estão reagindo a um conjunto de modificações ambientais.

Palavras-chave: Mycetopodidae. População. Declínio. Lesão gonadal. Poluentes

Introduction

Unionida are freshwater mussels present in all the continents except Antarctic (Graf & Cummings 2007, Pereira *et al*. 2014). However, the global distribution throughout zoogeographic zones is variable, with high endemism in the Nearctica and Indotropica zones (Graf and Cummings 2007, Bogan 2008; Pereira *et al.* 2014).

According to Pereira *et al.* (2014), the family Mycetopodidae is restricted to Central and South America where the genus *Anodontites* Bruguière, 1792 includes 24 species. Fourteen have occurrence in Brazil.

In Brazil, Machado *et al*. (2008), based on criteria from the International Union for Conservation of Nature (IUCN) and guided by specialists, classified eight species of this genus as vulnerable. However, the new evaluation process adopted criteria only from the IUCN, which considers information about geographic distribution, population data and specific characteristics. There was a reduction of the original list and only *Mycetopoda legumen* (Martens, 1888) and *Diplodon* (*Rhipidodonta*) *koseritzi* (Clessin, 1888) were categorized as endangered species (ICMBio 2016).

This reduction in the list of endangered species is mainly due to the lack of basic information for analysis, since freshwater mussels have been subjected to continuous threats such as habitat modification and spread of invasive species (Downnig *et al*. 2010, Santos *et al*. 2013, Miyahira *et al*. 2017). These threats associated with specific characteristics such as long-life cycle, slow growth and reduced dispersion capacity are responsible for the reduction of populations in all continents (Vaughn & Taylor 1999, Downnig *et al*. 2010).

Environmental factors can act synergistically in the development of diseases in freshwater bivalves, since these organisms usually filter large volumes of water, they can host and concentrate a wide range of pathogens such as viruses, bacteria, larval stages of parasites and crustaceans (Grizzle & Brunner 2009, Morley 2010, Oluseye *et al*. 2013, Carella *et al*. 2016). However, diseases that generate functional incapacity, structural modifications, impairment of energy acquisition capacity and reproduction and growth rates (Grizzle & Brunner 2009, Carella *et al*. 2016) are not generally cited as a direct cause of the reduction in native freshwater bivalve populations.

Pauley (1968) attributed lesions that characterize an Spongy disease in *Margaritifera margaritifera* (Linnaeus, 1758) to a viral action. In addition, Zhang *et al*. (1986) reported viral disease for *Hyriopsis cumingii* (Lea, 1852) on Chinese farms which were later characterized by light and transmission microscopy by Zhong *et al*. (2011). According to Grizzle & Brunner (2009), the low number of reports about viral diseases in bivalves is probably due to method limitations for virus detection in these organisms. Carella *et al*. (2016) demonstrated that no significant advances have been made in the registration of viral diseases in unionids in the last decade.

Bacteria are important components of freshwater bivalves' diet (Vaughn *et al*. 2008). However, they are also considered possible disease agents in unionids (Carella *et al*. 2016). Freshwater bivalves which are randomly collected from apparently healthy populations, generally contain a varied set of bacteria including pathogens, making it difficult to establish the direct relationship between the existing bacterial population and the occurrence of diseases (Starliper 2011, Grizzle & Brunner 2009).

Freshwater bivalves can act as primary or intermediate hosts of trematodes (Grizzle & Brunner 2009, Morley 2010, Muller *et al*. 2015). Their larvae affect reproductive physiology since their occurrence is often related to gonadal castration in mussels (Calvo-Ugarteburu & McQuaid 1998, Rantanen *et al*. 1998, Laruelle *et al*. 2002, Gangloff *et al*. 2008, Lajtner *et al*. 2008, Morley 2010, Muller *et al*. 2015).

Disseminated neoplasm (DN) is another group of proliferative disorders with similar morphology and characteristics, diagnosed in a wide variety of mussels. DN was first described by Farley (1969) for marine bivalves *Crassostrea virginica* (Gmelin 1791) and *Mytilus* sp. Since then, it has been identified for many marine and freshwater species (Elston *et al*. 1992, Peters *et al*. 1994, Peters 1998, Alonso *et al*. 2001, Smolarz *et al*. 2005, Gombac *et al*. 2013). It is believed that DNs are the result of hemocytes transformation and, for this reason, they are called hemocytic neoplasia, hemocytic leukemia and leukocytic neoplasia. Like other diseases that affect mussels, the current diagnosis and knowledge about DN has been developed mainly for marine bivalves of commercial interest because they have high prevalence rates in farming, which favors direct transmission among individuals causing significant mortality rates (Elston 1990).

For native freshwater bivalves in the Brazilian territory Meyer *et al*. (2010, 2014), using histological analysis, recorded the occurrence of low frequency gonadal castration in *Diplodon expansus* (Küster, 1856) in Piraquara River (Iguaçu River basin) and *Diplodon ellipticus* (Spix in Wagner, 1827) associated to the presence of parasites in gonadal follicles in an artificial lake in Morretes (Coastal rivers basin of Paraná State).

In South America, especially in Brazil, studies on sublethal diseases and lesions that promote alterations in the reproductive cycle and long-term consequences on native freshwater bivalve populations are not the subject of systematic studies. It clearly follows a trend observed in other continents, where the efforts of research groups are concentrated in mollusks of medical and commercial interests. The lack of information can be attributed to the need for specific methods of study, since the identification of diseases is preliminarily performed through histopathological examination, which is an important tool in the evaluation of individuals' health and populations once it allows the identification of cellular and tissue alterations, detection of pathogens and proliferative disorders that can determine morbidity and mortality rates (Levine *et al.* 2012).

The aim of the present study was to analyze the frequency of tissue lesions in *A. tenebricosus* in the environmental protection area of Verde River, state of Paraná, Brazil.

Materials and Methods

Study area: The environmental protection area of Verde River established by the state government (decree number 2.375/2000), is located in the metropolitan area of Curitiba, state of Paraná, Brazil. It includes four municipalities: Araucária, Campo Largo, Campo Magro and Balsa Nova (Caneparo *et al.* 2012) (Fig. 1). The Verde River sub-basin is part of the Iguaçu River that runs to Paraná River.

Four sample collections were performed from June 2012 to August 2014, in the same stretch of the river, under the license (SISBIO 37400). In June /12, October /12 and March /13, 10 specimens located in the river bed were captured; and in August 2014, 20 specimens of *A. tenebricosus* were collected.

In the laboratory, the specimens were anesthetized with menthol crystals, fixed in 10% formaldehyde for 48 hours and stored in 70% ethanol. The specimens were numerically identified and examined by magnifying glass to determine the presence of full marsupium and superficial macroscopic alterations in the valves, visceral mass and foot. Additionally, shell morphometric data (length, height and width) were obtained according

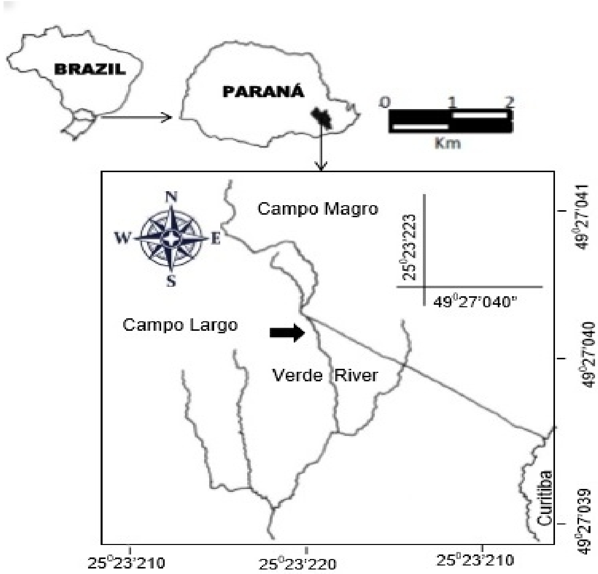

Figure 1. Study site map showing the Verde River basin and the sampling site location (black arrow).

to Mansur *et al*. (1987). For the correlation determination between pairs of variables, Pearson's correlation was applied. Sturges' rule (Toledo & Ovale, 1995) was applied for the amplitude of length class analysis.

Cross sections of the central region of the visceral mass of the specimens were submitted to routine histological processing with dehydration, diaphanization and inclusion in paraffin and historesin. Sequential histological sections of 2 and 5µ were stained by the Hematoxylin and Eosin technique. The quantification of female gametogenesis was performed by counting oocytes, according to the methodology described by Jones *et al*. (1986) and Meyer *et al*. (2010). In each histological section oocytes were counted in the lumen of 30 gonadal follicles. Data were analyzed using the non-parametric Kruskal-Wallis test with significance at $p \leq 0.05$.

Regarding the lesions, four levels of alterations were determined to quantify the extent of the gonadal lesions: degree zero, for absence of alterations in the gonadal tissue; degree I, presence of punctual alterations, with preserved gonadal tissue; degree II, alterations with partial loss of gonadal tissue morphology and degree III with extensive lesions that unable sex determination, called gonadal castration.

Voucher specimens were identified by Luis R. Simone (MZUSP) and were deposited in the Zoology Museum of the São Paulo University as *A. tenebricosus* (MZUSP 106190).

Data Analysis: The Protocol for Rapid Evaluation of Habitat Diversity (Callisto *et al.* 2002) was used to investigate the anthropic impact in the collections of 2012 and 2014. According to this protocol, the total score obtained in each area represents the ecological condition, in which scores from 0 to 40 indicate impacted areas, from 41 to 60 indicate altered areas, and above 61 indicate natural areas. In addition, the following environmental parameters were adopted: depth, superficial water flow speed (following Bicudo & Bicudo 2004), environmental temperature (using a mercury thermometer) and pH (litmus paper).

Results

The studied area presents in its central portion gravel rocks with different granulometry. On the other hand, its bank shows a sludgy clay soil due to a mixture of silt and organic matter from the riparian forest. Abiotic parameters are summarized in Table I.

Table I. Abiotic parameters of Verde River, Paraná State, Brazil, obtained during sample collection.

	Jun/12	Oct/12	Mar/13	Aug/14
Atmospheric temperature	14.5°C	24°C	26°C	17°C
Water temperature	16°C	17°C	18°C	16°C
pH	7.0	7.0	7.0	7.0
Current speed	3.2 m/s	3.9 m/s	3.6 m/s	3.7 m/s
Average depth	0.25m	0.30 m	0.27 m	0.28 m

Fifty specimens of *A. tenebricosus* (Fig. 2) were captured in the sample collections with length class ranging from 47 to 99 mm and a modal class from 79 to 87 mm. The frequency distribution by length class is shown in Figure 3.

The length of the specimens ranged from 47 to 99 mm (average: 78 ± 11.08 mm); width from 11.04 to 33.19 mm (average: 22.12 ± 2.79 mm) and height from 22 to 43 mm (average: 35 ± 5.4 mm). Positive correlation was recorded between the variables: length and width (r^2=0.777); length and height (r^2=0.889) and height and width (r^2=0.724). Considering the mean values, the height corresponds to 44% of the valve length.

The macroscopic analysis has not revealed alterations in the valves, visceral mass and foot surface. Six specimens collected in August/2014, ranging from 83 to 89 mm in size, presented full marsupium occupying the whole extension of the inner demibranch (Fig. 4).

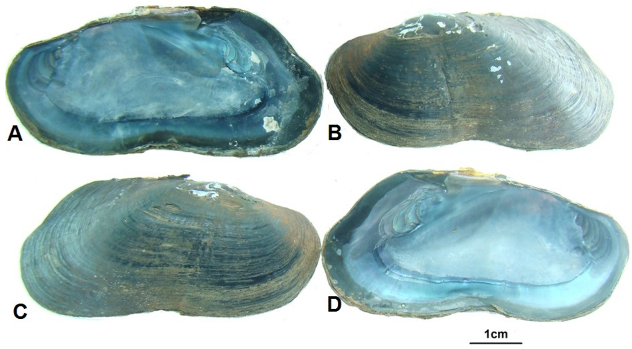

Figure 2. Shells of *Anodontites tenebricosus* (Lea, 1834) collected in Verde River, Paraná State, Brazil, between June 2012 and August 2014. Internal view, left shell (A). External view, right shell (B). External view, left shell (C). Internal view, left shell (D).

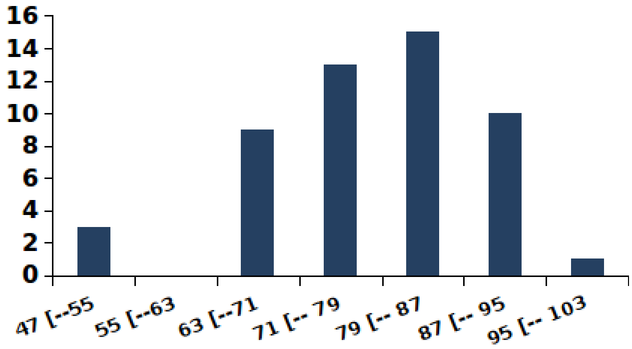

Figure 3. Frequencies by length classes (mm) of *Anodontites tenebricosus* (Lea, 1834) in Verde River, State of Paraná, Brazil.

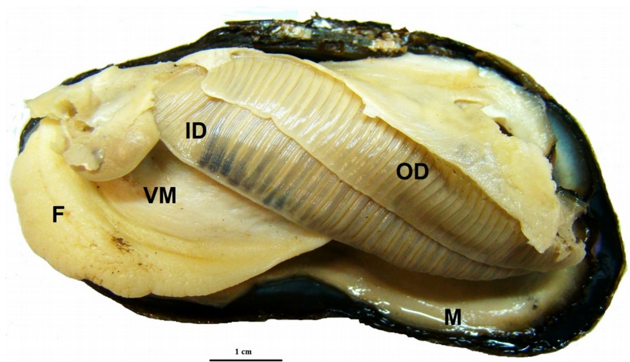

Figure 4. *Anodontites tenebricosus* (Lea 1834) collected in Verde River, Paraná, Brazil, between June 2012 and August 2014. Inner demibranch (ID); outer demibranch (OD); M: mantle; VM: visceral mass; F: foot. (scale = 1 cm).

Through histological analyses, 22 males and 20 females were identified. Eight specimens with gonadal mass alterations could not be identified as male or female.

Histopathological alterations characterized by spherical and subcellular pathogenic dimensions were identified in all the samples and showed a strong reaction to the basic dye (Figure 5a).

Degree I histopathological alterations present infiltrative appearance and are initially observed close to vascular spaces, around adipocytes and gonadal ducts (Figure 5a-5b). Considering the total sample (n=50), these alterations were observed in 12 males ranging from 47 to 87 mm and 13 females from 63 to 87 mm, corresponding to 50% of the analyzed samples, in which the gonadal follicles morphology and the gametogenic activity are visually preserved.

In specimens with degree II alterations, the pathogenic agent is accumulated around gonadal follicles, which are atrophied and reduce the thickness of the germinal epithelium. In these sites, a progressive concentration of hemocytes is observed, indicating a strong phagocytic activity (Figure 5c-5d). Degree II alterations were registered for 11 males and 7 females ranging from 63 to 87 mm, which corresponds to 36% of the analyzed population (n=50).

In specimens with degree III alterations, there is a total loss of the gonadal structure morphology, which makes it impossible to identify gonadal follicles and sexual determination, characterizing gonadal castration (Figure 5e-5f). In these specimens, the pathogen predominates throughout the visceral mass destructing muscular septa and connective tissue of the support organ such as intestine and digestive glands. Visceral mass presents similar lesions to hyaline degenerations, which are the result of physico-chemical alterations of protein components and are characterized by the presence of amorphous material with a vitreous appearance and strong reaction to eosin (Figure 5f). Gonadal castration was observed for 7 specimens ranging from 81 to 94 mm, which corresponds to 14% of the analyzed population (n = 50).

Degree I alterations were more frequent in males ranging from 71 to 79 mm (Figure 6a) and degree II in females from 79 to 87 mm (Figure 6b). Degree III alterations with gonadal castration were observed in specimens with length classes greater than 79 mm (Figure 6c).

Degree II alterations were more frequent for males and females (Figure 7). There was increase of specimens with degree I and decrease of specimens with degree II, in 2014.

During the sampling period, the female and male gonads of specimens with degree I and degree II alterations presented gametogenic activity. Males presented follicles with seminiferous epithelium containing cells of spermatogenic lineage and sperm morulae in the closest region to the follicular lumen. The female gonads presented follicles containing oocytes at different oogenesis stages, with developing oocytes attached to the epithelium through cytoplasmic peduncles.

For the total sample of females (n=20), quantitative analyzes of oocyte numbers showed significant alterations. Females with degree I alterations presented an average number of oocytes of 6.9 ($\pm$ 2.2) per gonadal follicle and females with degree II alterations presented 5.1 ($\pm$ 1.1) oocytes (p= 0.0393).

Data obtained from the Protocol for Rapid Evaluation of Habitat Diversity showed a score of 56 points, which categorize the evaluated portions as altered. Sedimentation by the removal of ciliary forest, presence of residences with domestic animals and fish tank with no commercial purpose with water disposal in the river are the main alterations observed in the sample area.

Discussion

In *A. tenebricosus* collected in Verde River, the histopathological alterations presented 100% prevalence in the study population during the study period, affecting males and females in all the length classes. Degree II alterations are more frequent in females with greater valve length, indicating that the degree of alteration is dependent on age and gender, also reported for parasitic infestations by *Anodonta anatina* (Linné, 1758) (Muller *et al*. 2015). According to Taskinen *et al*. (1997), this relationship is a common phenomenon for organisms that feed by filtration due to the volume of water processed, which allow a greater concentration of aggressive agents. Females are more affected since they have a higher energy expenditure due to the fertilization and embryonic development (Heino & Kaitala 1996).

According to Jokela *et al*. (1993), gonads represent a source of energy resources due to vitellogenesis. They are organs frequently affected by pathogens because they are not vital for the maintenance of basic physiological conditions, which justifies the observation of lining epithelia of the digestive tract with preserved tissue structure in specimens with degree III alterations and gonadal castration.

The oocyte count statistically demonstrates significant reduction in oocyte number for females

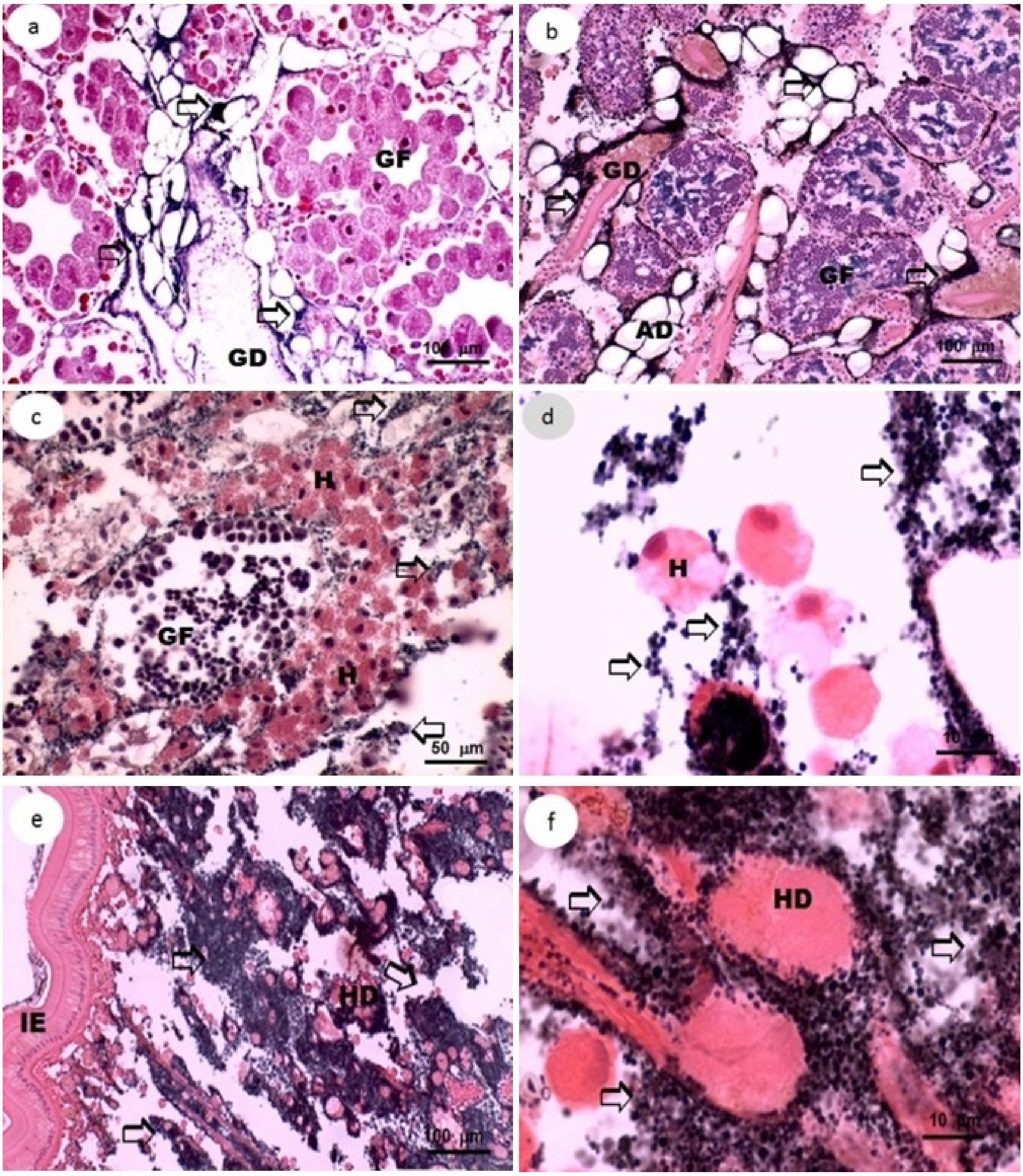

Figure 5. Photo-microscopy of visceral mass sections from *Anodontites tenebricosus* (Lea, 1834) collected in Verde River, Paraná, Brazil, between June 2012 and August 2014. (a) female, degree I lesions. (b) male, degree I lesions. (c-d) male, degree II lesions. (e-f) Specimen with gonadal castration. Gonadal ducts (GD), gonadal follicles (GF), Oocytes (O), adipocytes (AD), hemocyte (H), intestinal epithelium (IE), pathogenic agent (➡), hyaline degenerations (HD).

with degree II alterations, which is apparently related to the presence and extension of the tissue lesions. However, further studies are needed to allow the comparison with healthy populations, once reproductive capacity in freshwater bivalves decreases naturally with age (Haag & Staton, 2003).

Over time, the comparative analysis of lesions frequency shows that there was an increase in the number of specimens with degree I and decrease of specimens with degree II and with castration in 2014. Considering the progressive aspect of the disease, it may be the result of specimen's death due to the apparently irreversible lesions that affect gonads and may be associated with the low density of the species and large number of articulated valves reported by Meyer *et al*. (2017), who compared the

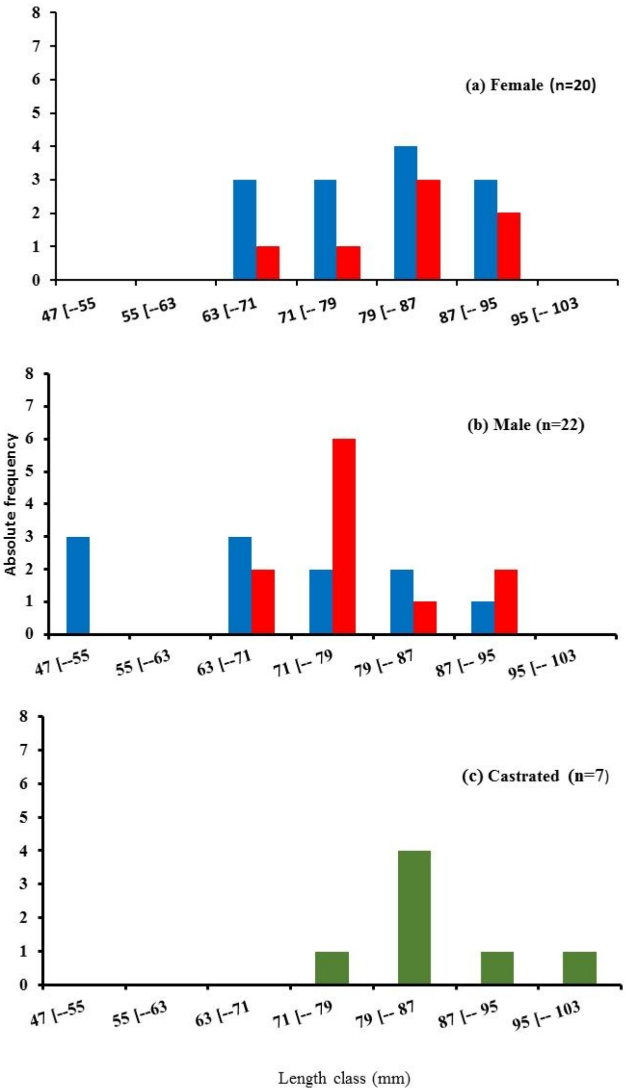

Figure 6. Frequency of degree I (blue), degree II (red) and degree III (green) alterations by length classes (mm) in *Anodontites tenebricosus* (Lea 1834) collected between June 2012 and August 2014. (a) females. (b), males (c), castrated.

occurrence and density of other mussels in the same study area.

Mussels have only the innate defense system mediated by hemocyte activation, which have a wide variety of physiological functions and are related to tissue repair and immune response, including recognition mechanisms, phagocytosis, intracellular digestion encapsulation, and the production of cytotoxic substances (Cheng 1981, Matozzo 2016). According to Carella *et al*. (2016), defense system responses present three histotypes: focal infiltration, nodulation, and encapsulation. In *A. tenebricosus*, degree I lesions are observed as a diffuse infiltration in vascular spaces with progressive increase of hemocyte invasion and concentration in lesions with degree II and III, whose structural characteristics are in agreement with what was described by Hine (1999) for granular hemocytes with phagocytic function, indicating severe pathological condition. However, there is no record of nodulations or encapsulations in the visceral mass. The observed characteristics do not allow the association with a specific pathological agent, since the concentration of hemocytes is common in different pathological conditions. For example, Peters *et al*. (1994) described neoplasms in marine bivalve in a comparative study; Lajtner *et al*. (2008) found trematode infestations in *Dreissena polymorpha* (Pallas 1771), Müller *et al.* (2015) in *Anodonta anatina* (Linnaeus, 1758), and Carella *et al*. (2013) found bacterial infections.

Considering that the application of the Rapid Assessment Protocol of Habitat Diversity (Callisto *et al*. 2002) classified the area as altered, there are many factors that can act in isolation or synergistically in the observed scenario. Freshwater bivalves are bioaccumulators of heavy metals, hydrocarbons and pesticides derived from domestic, industrial and agricultural wastes, which may increase susceptibility to pathogens (Machado *et al*. 2014, Vaughn 2017). In laboratory conditions, freshwater bivalves exposed to heavy metals present histopathological alterations in the gills, compromising filtration and the mechanisms associated with energy acquisition (Sonawne 2015, Moëzzi *et al*. 2013); digestive gland and foot (Chandrudu & Radhakrishnaiah 2008, Yasmeen *et al*. 2012). Kumar *et al*. (2012) have shown that pesticides from the organophosphate class determine severe tissue alterations in the digestive gland of *Lamellidens marginalis* (Lamarck, 1819).

Morley (2010) reviewed the interactive effects of pollution on viral, bacterial, and parasitic infestations in mussels demonstrating the complexity of these studies. Under laboratory conditions, Wang *et al*. (2007) demonstrated that freshwater mussels present different degrees of sensitivity to toxic contaminants depending on the stage of life, with glochids presenting a greater sensitivity than juveniles and adults.

In addition, the presence of *Corbicula fluminea* in the study area (Meyer *et al*. 2017) may acts as a stressor for the native bivalve population, considering that densities above 200 ind./m^2 can determine alterations in ecological conditions due to the accumulation of pseudo-feces, alterations in the percentage of oxygen in the water column, reduction of primary productivity (Mcmahon 2000). Besides,

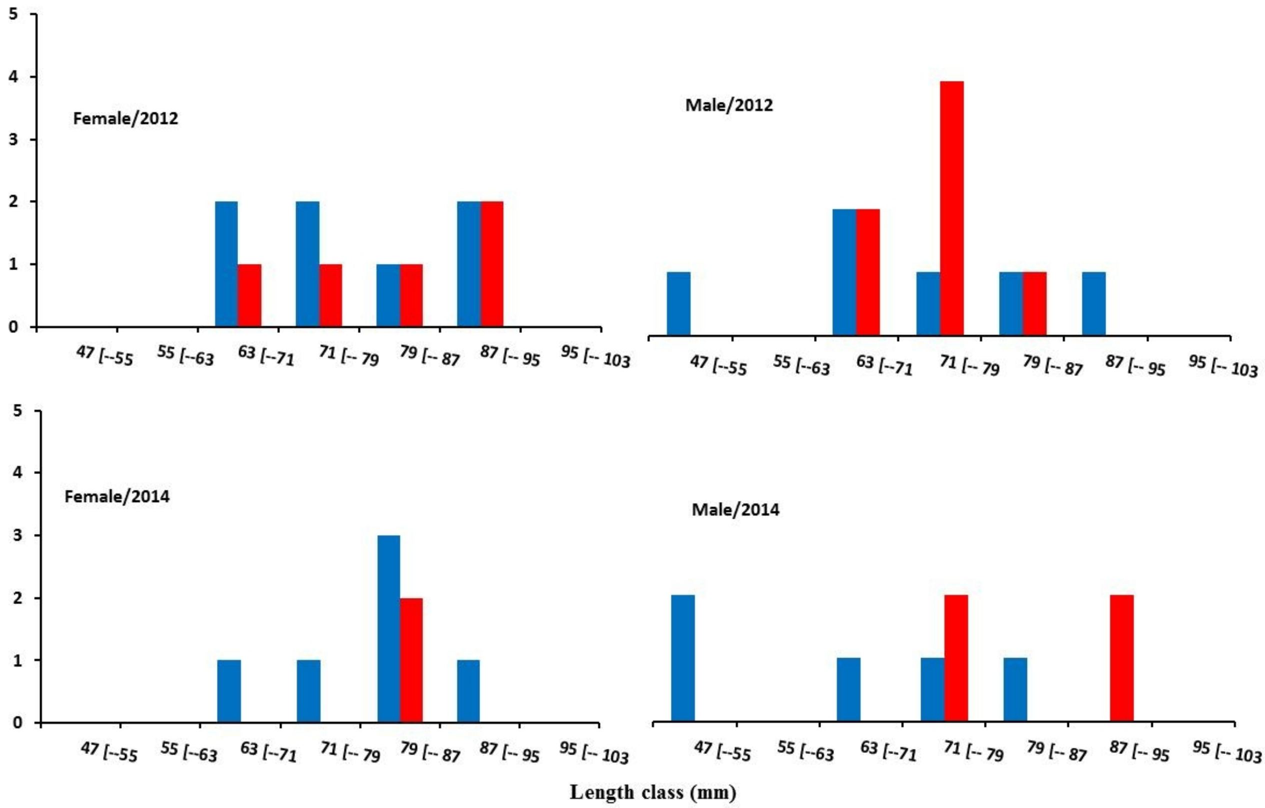

Figure 7. Degree I and degree II frequency of alterations by length classes (mm) of *Anodontites tenebricosus* (Lea 1834) collected between 2012 and 2014. Blue bar = degree I, Red bar = degree II.

they act as vectors in the introduction of new parasites and diseases (Souza *et al*. 2008). Populations of *C. fluminea* present seasonal episodes of mortality related to factors such as decrease of dissolved oxygen, alterations in temperature, pH and water flow (Duarte & Diefenbach 1994), releasing ammonia which can generate toxic effects on native species. Under laboratory conditions, Cherry *et al*. (2005) carried out a study on the effect of ammonia released by the death of *C. fluminea* and demonstrated that juveniles are more sensitive than adults. However, it is difficult to capture juvenile specimens, avoidingthe comparison analyses in the frequency of alterations between adult and juvenile specimens.

The histological analyzes used in the present study did not allow the identification of the pathogenic agent but showed extensive and severe tissue alterations with high prevalence which may compromise the reproductive success of the species and, over time, the population maintenance. However, these lesions are restricted at the cellular and tissue level and are not capable of generating macroscopic alterations in the visceral mass in the valves morphology and in the amplitude of the class length of the population, which is within the parameters for specimens examined by Mansur & Pereira (2006) and Meyer *et al.* (2017) and higher than those observed by Troncon & Avelar (2011) and Rodrigues *et al*. (2012). Therefore, it is important to perform multiple approaches to study native bivalves conservation and to understand how these populations are reacting to a set of environmental modifications.

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