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RESEARCH ARTICLE

Age-associated liver alterations in wild populations of *Austrolebias minuano*, a short-lived Neotropical annual killifish

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Abstract Aging processes have become an attractive field for researchers and annual fish have been used as biological models. However, the study on the changes in age-associated markers during the normal aging in wild populations of annual fish remains open. *Austrolebias* is a genus of Neotropical annual killifishes, distributed mainly in ephemeral pools across grassland floodplains of temperate South America and represent an emerging biological model for aging research, but studies investigating rapid aging and senescence in this genus of annual fish are almost non-existent. This study was undertaken to examine the changes in age-associated liver markers at the different developmental stages in wild populations of *Austrolebias minuano*. We demonstrate that *A. minuano* has a number of liver alterations of different severities throughout the life cycle, suggesting that these changes tend to increase with age. Our results revealed that > 70% of the analyzed livers presented alterations. Thus, our study should instigate new approaches on aging using Neotropical annual fish, and could be useful to improve the knowledge already provided by consecrated biological aging models as e.g. *Nothobranchius* killifishes.

Keywords Annual fish · Neoplasia · Steatosis · Temporary ponds · Liver diseases

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Introduction

Aging is a complex processes that cause progressive cellular damage and loss of physiological integrity, leading individuals to death (Kirkwood and Austad 2000; Jones and Vaupel 2017). Aging studies is an attractive research field and diverse organisms have been used as biological models (Reichard 2016; Hu and Brunet 2018), including invertebrates (Guarente and Kenyon 2000; Johnson 2003; Katic and Kahn 2005). However, invertebrates do not provide a solid foundation for studies related to aging and/or cancer formation associated with senescence. (Masoro and Austad 2010; Hu and Brunet 2018).

Vertebrates are more appropriate for understanding aging processes, due to the shorter phylogenetic distance with humans. Zebra fish (Gerhard et al. 2002; Kishi 2004), mice (Rudolph et al. 1999; Hastings et al. 2004), dog (Nasir et al. 2001) and sheep (Davis et al. 2005) have been used as biological models for aging studies. However, these groups mentioned above have relatively long life expectancy, hindering studies in captivity (Lucas-Sánchez et al. 2014). Short-lived vertebrates are rare (Gerhard 2007). Nonetheless, numerous annual killifish species belonging to the family Rivulidae (Neotropical) and Nothobranchiidae (Africa) present the shortest life cycle among vertebrates (Blažek et al. 2013; Berois et al. 2014; Vrtílek et al. 2018), and constitutes more appropriated biological model for aging studies (Genade et al. 2005; Muck et al. 2018). Annual fish share hallmarks of aging with mammals and exhibit histological changes in many age-related tissues (Baumgart et al. 2015; Cellerino et al. 2016; Ackerman and Gerhard 2018; Hu and Brunet 2018).

Histopathological markers have been used to relate aging to histological alterations of annual fishes, indicating a high incidence of age-related diseases, included neoplasm (Valenzano et al. 2006; Hsu et al. 2008; Di Cicco et al. 2011). Studies have reported important relationship between liver histological alterations and age in *Nothobranchius guentheri* (Markofsky and Milstoc 1979). The incidence and timing of onset of neoplasm are related to longevity (Tozzini et al. 2008; Baumgart et al. 2015; Blažek et al. 2017). Cooper et al. (1983) reported incidence of neoplasm only at the final limit of life expectancy in *Nothobranchius guenteri* (medium survival of 12 months). Di Cicco et al. (2011) found neoplasm at age of 11 weeks in *N. furzeri* (Vrtílek et al. 2018).

Most of the studies to identify age-related markers were performed under controlled laboratory conditions. Studies investigating age-related changes are lacking in wild populations. However, environmental conditions in nature vary and the effects of condition-dependence and extrinsic mortality increase (Reichard et al. 2018), generating different degrees of stress when compared to individuals in captivity. Understanding the differences between responses from natural and captive populations is a key process for studies on aging (Reichard 2016).

The family Rivulidae comprises diverse clades of secondary freshwater small-size fishes (max. SL 200 mm), distributed from Mexico to Southern Argentina (Loureiro et al. 2018), and most available information on Neotropical annual fishes derive from research on *Austrolebias* genus (Berois et al. 2014). *Austrolebias* is a large genus of Neotropical annual killifishes, distributed mainly in ephemeral ponds across grassland floodplains of South America. *Austrolebias minuano* is a typical species of the genus in southern Brazil, and solid information on population dynamics (Lanés et al. 2014, 2016), diet (Keppeler et al. 2015), and ecological interactions (Reichard et al. 2018; Silva et al. 2019) was already published. These studies include mark-recapture techniques to estimate the population variation over their life cycle, and data loggers were used to record the duration of the flooded phase and habitat desiccation of temporary ponds. *Austrolebias minuano* presents single age cohort with < 8 months of lifespan (Lanés et al. 2014, 2016). Juveniles hatch relatively synchronously in austral autumn and early winter (May to July) and over the seasonal life cycle, the populations show a continuous decline in abundance and density (Lanés et al. 2016). In contrast to African annual fish, the lifespan of *A. minuano* occur over the low temperatures. The fish mortality increases at the end of seasonal cycle, coinciding with water temperature increasing and habitat desiccation (Lanés et al. 2016). A higher mortality in male is apparently later in the season (October), causing a female-biased sex ratio (Lanés et al. 2016). The high mortality of *A. minuano* males remains unclear. Although males are more colorful and supposedly more vulnerable to predation by birds and predatory fish, Reichard et al. (2018) showed that both sexes suffer significant mortality from avian predation, with no sex-specific adult survival pattern. Lanés et al. (2016) pointed out that more pronounced decline in males was related with environmental deterioration, which could indicate sex-specific aging, with faster functional senescence in males. The life cycle characteristics of *A. minuano* were also observed in other closely related (phylogenetically) of Austrolebias species (Berois et al. 2014; Volcan et al. 2018).

The relationship between histopathological lesions and lifespan of *Austrolebias* was investigated under controlled laboratory conditions in the 1960s (Walford and Liu 1969) and 1970s (Liu et al. 1975). Despite being an emerging biological model for aging research (Herrera and Jagadeeswaran 2004; Berois et al. 2014) recent studies investigating rapid aging and senescence in this genus of Neotropical annual fish are almost non-existent (Lanés et al. 2016). Information on natural populations of Neotropical annual killifish can provide a significant contribution to the advance of evolutionary ecology and aging studies, allowing comparison with available and consecrated aging biological models as African annual killifishes (Cellerino et al. 2016).

In this sense, we analyzed the hepatic histology of *A. minuano* over its life cycle in Neotropical temporary ponds in order to provide a first insight of an age-related marker in natural environment. We predict that frequency and severity of histopathological alterations in annual fishes are age-dependent and sex-specific, with highest incidence in older and in male fishes.

Materials and methods

Study area

Annual fish sampling was carried out in the buffer zone of the Lagoa do Peixe National Park (PNLP) (Fig. 1). The region has a humid subtropical climate with average temperature varies from 13 °C in the winter and 24 °C in the summer. The average annual precipitation varies between 1200 and 1500 mm (Tagliani 1995). The vegetation is classified as representative of the Pampa Biome (IBGE 2004), characterized by subtropical grasslands, but with components of the Atlantic Forest (Lanés et al. 2014, 2016). The presence of water in temporary ponds in South America subtropical grasslands is consequence of balance between precipitation, temperature and evaporation (Lanés et al. 2018) and in the study area the temporary ponds generally contains water for 8 months and remain dry from late austral spring through autumn (Lanés et al. 2014, 2016).

Sampling design

Since the last decade, annual fish dynamic populations are monitored in this region by researchers of Laboratory of Ecology and Conservation of Aquatic Ecosystems—UNISINOS (Lanés et al. 2012;

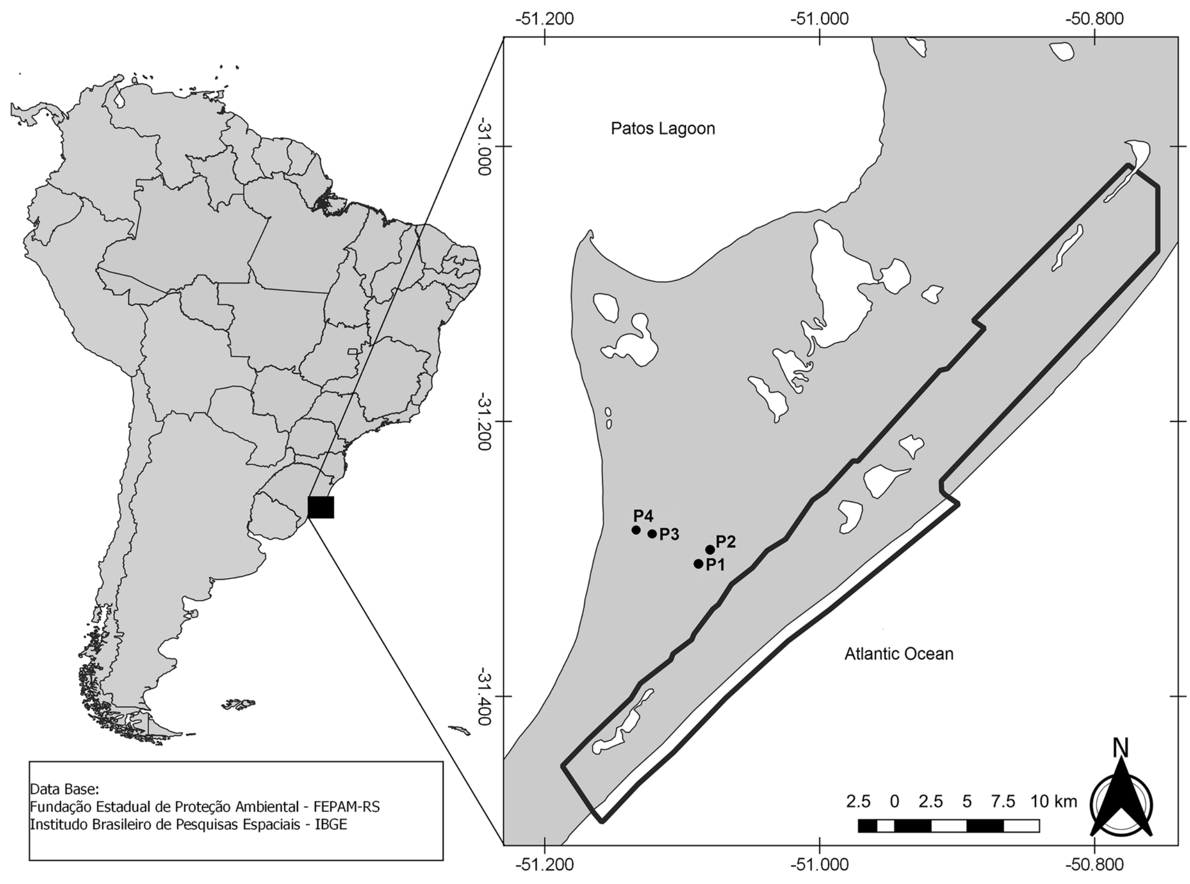


Fig. 1 Location of the study area and sample units

Keppeler et al. 2013; Lanés et al. 2014; Keppeler et al. 2015; Lanés et al. 2015, 2016).

In this study four natural temporary ponds inhabited by *A. minuano* were selected (Fig. 1). All ponds were isolated and at least 500 m apart. 40 individuals (20 males and 20 females) were collected (Pond 1 = 8, Pond 2 = 12, Pond 3 = 12, Pond 4 = 8), distributed in three developmental phases throughout 2014 (16 juveniles—June), 16 adults (August) and eight senile individuals (October). The minimum interval between samplings was 4 weeks at least. We used data from our studies of temporal dynamics of *A. minuano* (Lanés et al. 2014, Keppeler et al. 2015, Lanés et al. 2016), and others closely related *Austrolebias* species (Volcan et al. 2018) to establish the sampling periods. These studies demonstrated that individuals hatch between May and July, and variations may occur between (Lanés et al. 2016; Volcan et al. 2018). We used individuals from populations that based on their size (Lanés et al. 2014, 2016; Volcan et al. 2018) and coloration (Keppeler et al. 2015) had hatched less than a month (they had not acquired the specific color of each sex, a characteristic that also defines them as juveniles). The reproductive peak of the species occurs in August, when the adult individuals were collected. The senile individuals were collected in October, just before the period of greatest mortality and drought of biotopes (Lanés et al. 2016).

The fishes were collected using hand nets (D shape, 60 cm × 40 cm, 2 mm mesh) and submitted to euthanasia with a lethal dose of clove oil and fixed in 10% formalin. The fishes were taken to the laboratory, were weighed through analytical balance (0.001 g) and the total (TL) and standard length (SL) measured using a digital caliper (0.01 cm). The liver of each individual was removed and weighed in analytical balance for histological analysis. The fish collections were authorized from IBAMA/ICMBio (SISBIO process number No. 43.251-1).

Histopathology

A total of 40 livers were dehydrated in crescent alcohol solutions (80%, 90%, 96%, 100%). After dehydration, the organs passed through three different baths in isopropyl alcohol, isopropyl alcohol solutions with paraplast and only in paraplast, respectively. After this procedure, the livers were encapsulated in paraplast blocks, cutted in 7 µm slices and mounted on slides. The slides were immersed in Neo-clear baths to remove the paraplast. The sections were hydrated in decreasing alcoholic series (Isopropylic 96%, 90%, 80% and 70%) and stained with Hematoxylin/Eosin. The slides were analyzed under an optical microscope.

A total of 192 slides of 30 livers were analyzed, as ten livers were damaged throughout the procedure. The presence of steatosis was classified according to degree of severity: light, moderate and severe. Light steatosis was characterized by the accumulation of lipids in small droplets located in the hepatic tissue. Moderate steatosis was characterized by the presence of medium-sized fat droplets spread through the hepatic tissue. Severe steatosis was classified by the presence of large diffuse droplets and fat balloons in the tissue, often displacing the nucleus of the central region of the cells (Di-Cicco et al. 2011).

Liver alterations were classified according to the degree of liver function impairment: light, moderate, and severe. Light was characterized by reversible changes and no impairment of tissue function. Moderate was characterized by detrimental changes to the organ. Severe was characterized by changes that generate irreversible organ damage (Poleksic and Mitrovic-Tutundzic 1994). Histological procedures were authorized by the PUCRS Ethic Committee (CEUA PUCRS 8271).

Data analyses

We tested the association of hepatic alterations and steatosis frequencies in relation to degree, sex, and periods of development, through Chi Square tests. All tests were performed using the statistical software Systat 13 (Systat 2007).

Results

The standard length (SL) of the individuals analyzed ranged from 20.9 to 40.8 mm. The SL variation among juveniles (May and July) was 20.9 to 29.9 mm. The size of adult fish ranged from 24 to 40.4 mm (August). Senile individuals measured between 31.4 and 40.8 mm (October) (Table 1).

A total of 22 individual presented alterations in the livers (73.3%), distributed in 12 males (54.5%) and 10 females (45.5%) (Table 2). From 22 individuals with alterations, 63.6% were adults and senile (Table 2).

Table 1 Total of analyzed individuals and body size amplitude (Standard Length—SL in mm) at different development stages of *A. minuano*

Stages	Total	Male (mm)		Female (mm)	
		Minimum	Maximum	Minimum	Maximum
Juvenile	8	21.5	29.9	20.98	28.24
Adult	15	29.19	40.4	24.06	37.02
Senile	7	31.49	37.62	35.68	40.8

Table 2 Absolut and relative frequency of alteration found in *A. minuano* throughout their development stages

Stages	Total	Alteration N (%)		Male N (%)		Female N (%)	
	N	Presence	Absence	Presence	Absence	Presence	Absence
Juvenile	8	8 (100)	0 (0)	4 (50)	0 (0)	4 (50)	0 (0)
Adult	15	10 (66.6)	5 (33.3)	6 (40)	2 (13.3)	4 (26.6)	3 (20)
Senile	7	4 (57.2)	3 (42.8)	2 (28.5)	1 (14.2)	2 (28.5)	2 (28.5)

Individuals with liver alterations had a higher frequency of occurrence than individuals without alterations ($\chi 2 = 6.533$, $p = 0.011$). All juvenile individuals (n = 8) presented alterations in the liver. In relation to the other stages of development, 66.6% (n = 10) and 57.1% (n = 4) of the total adult and senile individuals presented alterations in the liver, respectively (Table 2). The presence of hepatic alterations was not associated with a specific developmental phase ($\chi 2 = 4.188$, $p = 0.140$) or sex ($\chi 2 = 4.466$, $p = 0.690$).

The alterations found in the hepatic tissue of the annual fish were: steatosis (Fig. 2b–d), necrosis (Fig. 2e) and tissue alteration indicative of hepatocellular carcinomas and encapsulated tumors (Fig. 2f). Steatosis were more frequent than necrosis and tumors, present in 72.7% of the individuals (n = 16) ($\chi 2 = 19$, $p < 0.001$), and the presence of this type of alteration was independent of sex ($\chi 2 = 0.229$, $p = 0.892$) and developmental phase ($\chi 2 = 5.111$, $p = 0.530$) (Table 3). Necrosis were present in three adult individuals (30%), two males and one female, and one senile female (Table 3). Tissue alteration indicative of hepatocellular carcinomas and encapsulated tumors were found only in adult individuals (one male and one female) corresponding to 10% of the total adult fish with hepatic alterations (Table 3).

The degrees of steatosis were light (43.8%), moderate (25%) and severe (31.2%). Steatosis were observed in all stages of development and in both sexes (Table 4). All the juveniles presented steatosis, mainly of severe degree. From total adult individuals, 33.3% had light to moderate steatosis, and 42.8% of senile individuals presented only light degree steatosis (Table 4). The frequencies of occurrence of the different degrees of steatosis did not vary between individuals with and without steatosis ($\chi 2 = 1.200$, $p = 0.273$), between sex ($\chi 2 = 0.362$, $p = 0.547$) and developmental stages ($\chi 2 = 3.105$, $p = 0.362$).

The hepatic alterations also presented different degrees of impairment and their frequencies varied between individuals ($\chi 2 = 21$, $p < 0.001$), but not between sex ($\chi 2 = 7.067$, $p = 0.070$) and developmental phases ($\chi 2 = 4.909$, $p = 0.179$). Light alterations were more frequent (72.7%) than severe (27.3%) (Table 5). No moderate hepatic alterations were observed (Table 5).

We found an increase in the relative frequencies of liver alterations along the ontogenetic development and lesions of greater severity in adult fish (Fig. 3), although we did not find significant differences. In the juvenile phase, individuals presented only steatosis, mainly of severe grade. In the adult phase, steatosis, necroses, hepatocyte carcinomas and encapsulated tumors were observed. In senile individuals, a lower

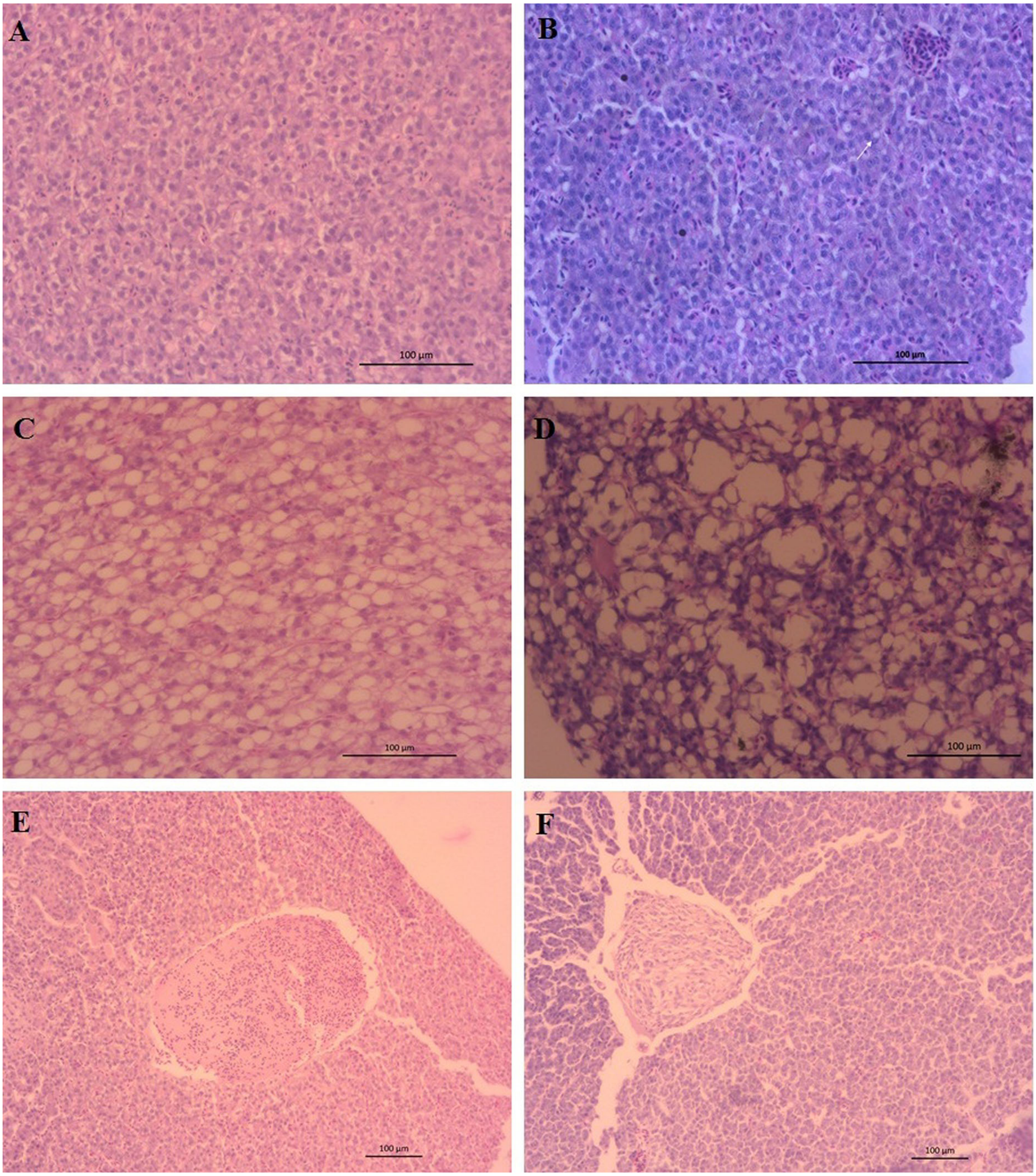


Fig. 2 Alterations found in the liver of *A. minuano*: **a** Normal Liver (20 ×), **b** Liver presenting light degree steatosis, characterized by the accumulation of lipids in small droplets in localized areas of the hepatic tissue (arrow) (20 ×), **c** Liver presenting moderate degree steatosis, characterized by the presence of medium sized fat droplets in areas located or scattered by the tissue (20 ×), **d** Liver presenting severe degree steatosis, classified by the presence of diffuse and large droplets or fat balloons in the organ often displacing the nucleus of the central region of the cells (20 ×), **e** Appearance of a region of the liver presenting necrosis, it is noted that the tissue completely lost its architecture in the necrotic region (10 ×), **f** Region of liver with tissue change indicative of hepatocellular carcinoma and encapsulated tumors (10 ×)

Table 3 Absolute and relative frequency of different alterations in males and females of *A. minuano* throughout the development stages

Stages	Alteration	Steatosis N (%)		Necrosis N (%)		Tumors N (%)	
	N	Male	Female	Male	Female	Male	Female
Juvenile	8	4 (50)	4 (50)	0 (0)	0 (0)	0 (0)	0 (0)
Adult	10	3 (30)	2 (20)	2 (20)	1 (10)	1 (10)	1 (10)
Senile	4	2 (50)	1 (25)	0 (0)	1 (25)	0 (0)	0 (0)

Table 4 Absolut and relative frequency of degrees of steatosis in *A. minuano* throughout the development cycle

Stage	Steatosis	Male N (%)			Female N (%)		
	N (%)	Light	Moderate	Severe	Light	Moderate	Severe
Juvenile	8 (50)	1 (12.5)	1 (12.5)	2 (25)	0 (0)	1 (12.5)	3 (37.5)
Adult	5 (31.2)	2 (40)	1 (20)	0 (0)	1(20)	1 (20)	0 (0)
Senile	3 (18.8)	2 (66.6)	0	0 (0)	1(33.3)	0 (0)	0 (0)

Table 5 Absolute and relative frequency of the different degrees of alterations found in livers of *A. minuano* throughout their development stages

Stages	Alteration	Light N (%)		Moderate N (%)		Severe N (%)	
	N	Male	Female	Male	Female	Male	Female
Juvenile	8	4 (50)	4 (50)	0 (0)	0 (0)	0 (0)	0 (0)
Adult	10	3 (30)	2 (20)	0 (0)	0 (0)	3 (30)	2 (20)
Senile	4	2 (50)	1 (25)	0 (0)	0 (0)	0 (0)	1 (25)

frequency of liver changes was observed in relation to the adult phase (Table 3). In the senile phase, in addition to light steatosis, necrosis was observed in the liver of a female.

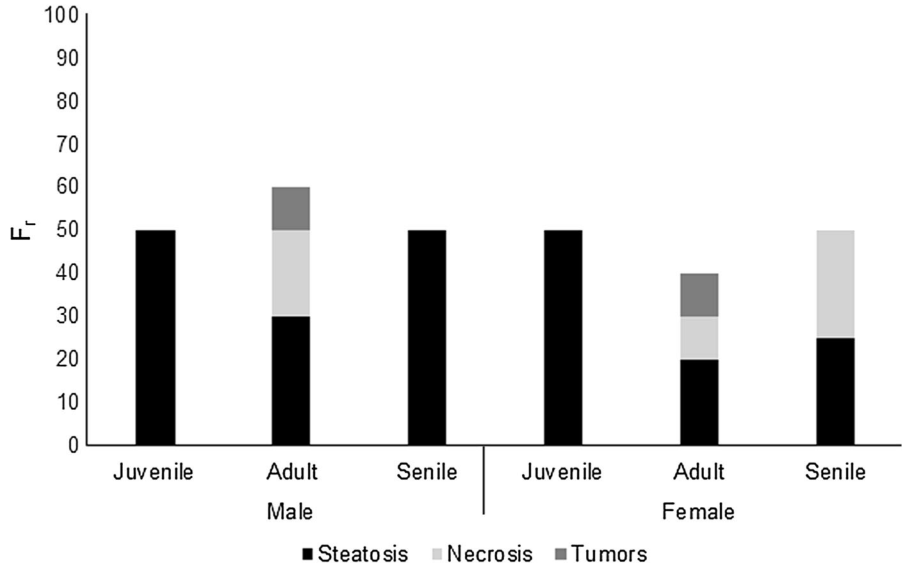
Discussion

Austrolebias minuano is an annual fish species with a short life cycle, with rapid growth and sexual maturation to maximize reproduction (Lanés et al. 2014, 2016). Species with short lifespans are associated with rapid aging and age-related histological changes (Di Cicco et al. 2011; Cellerino et al. 2016). Studies investigating age-associated changes are lacking in *A. minuano* and in natural environments. Here we showed that *A. minuano* has a number of liver alterations throughout the life cycle, suggesting that these changes tend to increase with age.

Our results demonstrated the feasibility of South American annual fishes as a promising biological model for aging studies. However, the absence of data based on well-established laboratory colonies prevents their consolidation as suitable aging model species. In this sense, information on survival curves, life span and other physiological and age-related parameters are required.

Steatosis was the main alteration found in *A. minuano* livers, present in different intensities at all stages of development and in both sexes. Our results showed that all livers of juvenile individuals presented steatosis, and those classified as severe degree were exclusive of this stage of development. The presence of fat in the liver of all juveniles could be related to

Fig. 3 Relative frequency of the different alterations found in the liver of both sexes at different stages of development of *A. minuano*



energy storage for reproduction. Age-related histological changes of two species of guppies (Poecilia reticulata and P. wingei) also showed large quantity of fat in livers of juvenile individuals (Comfort 1961; Woodhead 1998). Fish liver is directly involved in energy production for gamete development and breeding (Stegeman and Lech 1991; Patnaik et al. 1994). Reproductive energy is a key factor for annual fish to reach sexual maturation rapidly, both males and females (Passos et al. 2015). While males allocate large quantity of energy to maintain constant competitive interactions with other males and cohort with females (Passos et al. 2014), females need energy both for egg production and to cope with the strong courtship done by males for breeding (Wootton and Smith 2014).

Annual fish demand great energy for rapid growth in the juvenile stage. Laboratory studies with African annual fish (Nothobranchiiidae family) have shown that allocation of resources for growth and reproduction of *Nothobrachius furzeri* females in the juvenile phase may be associated with food availability (Vrtílek and Reichard 2015). Neotropical annual fish (Rivulidae family) live in temporary wetlands which presents a high abundance of macroinvertebrates throughout the surface water period, including in the re-flooding phase (Stenert et al. 2008). When hatching their eggs, Neotropical annual fish have high availability of food to invest in rapid growth and storage for reproduction. Di-Cicco et al. (2011) demonstrated that a diet based on Chironomidae larvae in captivity provides a large liver fat accumulation (steatosis). Chironomidae is very abundant in the temporary wetlands studied (Stenert et al. 2008) and is one of the main food items of *A. minuano* (Keppeler et al. 2015).

The liver plays an important role in allocating energy for the gonadal maturation attempt in gametogenesis in males and females as for the synthesis of vitellogenin in females (Stegeman and Lech 1991). This result seems to indicate a phase of accumulation of lipids in the liver (in juveniles) followed by a period of transfer of these reserves to gonadal maturation and its use as an energy substrate to support reproductive interactions in both sexes (in adults) (Passos et al. 2014; Wootton and Smith 2014). The reproduction of annual killifishes is very intense, extending from sexual maturation to the end of life (Passos et al. 2013, 2014; Vrtílek and Reichard 2015). Some researchers suggest that during the adult phase, all acquired energy is quickly transferred to reproduction (Gonçalves et al. 2011).

Steatosis increased in the senile phase compared to the adults, but this alteration in senile individuals was slight. This result may be due to the fact that in the senile phase, the annual fishes continue to reproduce with a smaller number of males in the population, making reproduction not costly as in adulthood. This dynamic may increase the percentage of fat in the livers over the senile phase.

These results were similar to those of the annual fish *Cynopoecilus melanotaenia*, where low quantity of accumulated fat was always associated with high reproductive rates (Gonçalves et al. 2011). Fat accumulations are very unstable and often associated with nutritional and hormonal status, parasitic infections, and environmental conditions. The accumulation of fat in the liver of annual fish may be an aspect that has evolved over time to maximize its survival in habitats with extreme environmental variations, such as temporary wetlands. However, we cannot affirm that the steatosis found in the liver of *A. minuano* are only related to reproduction and feeding. Steatosis may have other origins, such as related to individual life history and environmental stresses (Da Rocha 2018).

Male annual fish show early mortality, causing a female sexual bias in the end-of-life of these species (Passos et al. 2014; Reichard et al. 2014; Lanés et al. 2016). The highest relative frequency of severe male injuries in August may be related to early male mortality. Probably there is a positive selection for males, which do not present neoplastic lesions, whereas those showing tumors are more prone to die earlier. The high mortality of males was related to predation (extrinsic mortality) (Keppeler et al. 2016; Reichard et al. 2018) due to their more striking colors and larger body size (Belote and Costa 2004; García et al. 2008), as well as environmental deterioration (Lanés et al. 2016), which could indicate aging with a faster functional male senescence (intrinsic mortality). These two factors combined maybe are the explanation for the sex-unbalanced mortality described, due to a putatively higher susceptibility of male to develop liver tumors and higher predation pressure, increasing their mortality rate at the end cycle in relation to females.

High severity lesions were found associated with reproductive period in African annual fish (Cooper et al. 1983; Di Cicco et al. 2011; Baumgart et al. 2015). In our study, in adult (intense reproduction) also found to more severe alterations, such as necrosis and tumors, showing that this development phase can play a crucial role to understanding the aging of the annual fish. However, the number of alterations found in the liver of *A. minuano* was lower when compared to African annual fish. Di Cicco et al. (2011) found 29 altered livers from 31 analyzed, corresponding to 93.7% of individuals presenting lesions. Environmental conditions (mainly related to water temperature and oxygen availability) are factors that can influence the aging of annual fishes (Egami 1980; Patnaik et al. 1994).

The environmental conditions where African annual fish live (tropical zone) are extreme, and the water temperature can reach around 40 °C (Podrabsky et al. 1998; Reichard et al. 2009). The high temperatures generate a greater stress to the African fish, causing a faster and marked physiological deterioration. Neotropical annual fishes inhabit intermittent pools during cold periods of the year (Berois et al. 2014). Some studies have shown that temperature may influence the aging of African and Neotropical annual fish. Low temperatures increased lifetime and delayed the appearance of aging biomarkers in species of the two geographic groups of annual fishes (Liu and Walford 1972, 1975; Valenzano et al. 2006; Hsu and Chiu 2009).

Our study is the first insight on liver alterations, based in longitudinal data obtained from natural populations of a Neotropical annual fish. Although there are well-known models in a controlled environment, a recognized limiting factor for understanding the mechanisms of aging is the scarcity of data on natural populations. We showed that wild populations of *A. minuano* present distinct hepatic alterations of different severities throughout the life cycle. In this sense, our data may be useful for new approaches, which intend to investigate the aging process in annual fishes. Further studies including other age-related histological markers, such as the expression of senescence-associated β-galactosidase and accumulation of lipofuscin, in addition with information on captive colonies are needed to better provide comparison and understanding of aging process in annual killifishes.

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