RESEARCH ARTICLE

Oxidative stress resistance in a short-lived Neotropical annual killifish

Robson S. Godoy · Luis Esteban K. Lanés · Bruna D. Castro · Vinicius Weber · Natália Wingen · Mateus M. Pires · Guendalina T. Oliveira · Leonardo Maltchik

Abstract Oxidative stress plays an important role in the evolution of aging and life history. High investments in life-history traits and environmental conditions can be associated with increased oxidative stress and aging process. However, to date, most studies that investigated variations in oxidative status were performed with long-lived vertebrates. Studies with short-lived vertebrates in wild are nonexistent. Annual killifishes have the shortest lifespans among vertebrates and inhabit temporary ponds subject to large variations in environmental conditions. In this sense, we investigated whether the high investment in growth and reproduction in a short-lived vertebrate and the large variations in environment has any cost in susceptibility to oxidative stress. We assessed the seasonal variation and the environmental correlates of four different oxidative status markers (lipid peroxidation and activity of the antioxidant enzymes Superoxide Dismutase, Catalase and Glutathione S-Transferase) along the life cycle of wild individuals of the Neotropical annual fish *Austrolebias minuano*. Males showed reduction in all biomarkers (except proteins) along their life cycle, while females showed increased oxidative stress only in the growth period. In addition, we showed that water physicochemical parameters, habitat structure and presence of co-occurring killifish species influenced the seasonal variation of the biomarkers. *A. minuano* showed an efficient antioxidant system for most part of their life cycle (mainly in males), suggesting a well-developed oxidative stress regulation system. We also show that annual fish mortality (mainly in males) apparently is not related to oxidative stress. Thus, environmental factors should drive annual fish aging and mortality.

Keywords Aging · Environmental correlates · Life cycle · Life-history traits · Oxidative stress · Wild population

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Introduction

Oxidative stress is characterized by the unbalance between oxidants and antioxidants, which leads to the disruption of redox signaling and organisms damage (Sies and Jones 2007). The interest in the understanding of the role of oxidative stress in the evolution of aging and life history, as well as the possible influence of environmental drivers has increased in the scientific

literature (Costantini 2019). The "disposable soma theory" stresses that increasing investments in life-history traits (e.g. growth and reproduction) systematically reduces the energy available for self-maintenance, leading to the disruption of redox homeostasis, oxidative stress increase, cell damage, rapid aging and eventual mortality (Kirkwood and Austad 2000; Lemaître et al. 2015).

Different explanations have been proposed to explain the relationship between oxidative stress and life history as well as its role in the aging process. The 'oxidative stress theory of aging' addresses that increased cell damage is related to the aging process (Kirkwood and Kowald 2012). In long-lived species are expected to find less oxidative damage and higher antioxidant capacity, while lower antioxidant defense and higher oxidative damage are expected to find in short-lived organisms (Vágási et al. 2019). The 'oxidative stress hypothesis of life history' points out that oxidative stress mediates trade-offs between life-history traits and self-maintenance. Organisms that allocate high investments in life-history traits show a decline in antioxidant defenses throughout life, thus impairing their survival rates (Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010). Recent studies showed that high investment in growth and reproduction are associated with increased oxidative stress (Costantini et al. 2014; Blount et al. 2016; Smith et al. 2016; Janssens and Stoks 2018) and longer-lived species have higher antioxidant capacity and less oxidative damage (Lemaître et al. 2015; Vágási et al. 2019).

Other studies have highlighted that environmental condition in which organisms face throughout life can also play an important role in the oxidative balance and thus are associated with oxidative stress (Metcalfe and Alonso-Alvarez 2010). Increase in temperature induces oxidative stress in aquatic animals (Lushchak 2011). Under predation risk, odonate larvae have higher lipid peroxidation, lower levels of superoxide dismutase and higher concentrations of superoxide anion (Janssens and Stoks 2013). However, most studies on the ecology of oxidative stress have been conducted with long-lived vertebrates. Costantini (2019) regarded the importance to understand the oxidative status in other organisms, and the information on the variation of the oxidative status from wild populations of short-lived vertebrates can help us to better understand the ecology of oxidative stress.

Annual killifish species present the shortest life cycle among vertebrates (Cellerino et al. 2016), and inhabit temporary freshwater ecosystems such as ponds (Loureiro and De Sá 2015). The short hydroperiod of ponds compels these fishes to allocate high investments in rapid growth to reach sexual maturity prior to drought (Vrtílek et al. 2018a). The reproductive period of annual fishes is intense and occurs daily from sexual maturity to death (Vaz-Ferreira et al. 1964; Gonçalves et al. 2011). Males maintain intense agonistic behaviors and females produce eggs energetically costly (Passos et al. 2014; Wootton and Smith 2015). At the end of the wet phase, earlier mortality is detected in males, leading to a female-biased sex ratio in wild populations (Lanés et al. 2016). The higher mortality rates in males have been related to earlier functional senescence due to intense agonistic behavior and higher predation pressure (Reichard et al. 2018; Godoy et al. 2019). Neotropical annual fish disappear from temporary ponds before the ponds dry up completely (Lanés et al. 2016), and the cause of early annual fish mortality may be related to oxidative stress. However, the relationship between annual fish mortality and oxidative stress remains unclear. In addition, temporary ponds are subject to large variation in the environmental conditions over their wet phase such as increased temperature and reduced dissolved oxygen (Lanés et al. 2016).

The information on how the seasonal changes in the environmental conditions of ponds influence the oxidative status of annual fish remains scarce, especially in wild populations. In this sense, taking into account that the influence of organism life history and environmental characteristics on the oxidative stress is not well understood (Costantini 2019), annual fish can be excellent model organisms for testing the effects of the high investments in life history and environmental drivers on the ecology of oxidative stress. Studies on the ecology of oxidative stress with annual fishes can provide useful information on aging and mortality related processes in natural environments (Reichard 2016; Costantini 2019), enabling a better understanding of physiological aspects in extreme environments.

Here, we investigated whether the high investment in life-history traits such as rapid growth and intense reproduction is associated with oxidative stress in short-lived vertebrate. For this purpose, we assessed the seasonal variation and the possible role of environmental predictors (e.g., water physicochemical

variables, habitat structure and presence of competitors) in the oxidative status markers in wild individuals of a Neotropical annual fish. We hypothesized that the trade-off between investment in rapid growth, reproduction and self-maintenance would lead to variations in the levels of oxidative stress biomarkers and antioxidant defenses. We expected to find (i) increased oxidative stress and decreased antioxidant defense along the killifish life cycle. Based on previous studies that found different functional declines between sexes in killifishes (Godoy et al. 2019), we also expected (ii) the oxidative stress to be higher in senile males rather than females as a consequence of different effects of aging-related processes.

Materials and methods

Model organism

Austrolebias minuano (Costa and Cheffe 2001) is a native annual killifish species from southern Brazil. Previous knowledge of the species' biology include data on population dynamics, ecological interactions and aging-related effects (Lanés et al. 2014; Keppeler et al. 2015; Reichard et al. 2018; Godoy et al. 2019). Wild populations of *A. minuano* are generally composed by single age cohorts with eight-month average lifespans (Lanés et al. 2016). Juveniles have relative synchronized hatching from mid-autumn to early winter (May to July; austral seasons) and, over the seasonal life cycle, the populations show a continuous decline in abundance and density (Lanés et al. 2016). The fish mortality increases at the end of the wet phase, coinciding with increasing water temperatures and habitat desiccation (Lanés et al. 2016).

Study populations and sampling

Collections were carried out in two temporary ponds (distant from each other by ∼ 5 km) in the buffer zone of Lagoa do Peixe National Park in southern Brazil (50° 77′–51° 15′ W, ′S 31° 02′–31° 48). Fish

samplings were carried out at intervals ranging from 37 to 44 days over the post-hatching life cycle of *A. minuano* (between June and November of 2016). Sampling events covered different development stages of the species: the first and second events (June and July) covered the growing phase of juveniles, while the third and fourth events (September and October) covered the adult (in the peak reproductive) and senile phases, respectively (Lanés et al. 2016; Volcan et al. 2019). Pond 1 dried out in October. In November, although pond 2 was still wet, no specimen of *A. minuano* was found. Generally, natural populations of *A. minuano* disappear even before the ponds dry completely (October/November) (Lanés et al. 2014, 2016; Godoy et al. 2019). In this sense, the senile individuals were collected shortly, before the total lack of surface water.

Fishes were collected with hand nets (D-shape; 60 cm in length; 40 cm in height, 2 mm mesh size). Afterwards, they were anesthetized with clove oil, submitted to euthanasia in ice bath and transported in thermal boxes to the laboratory. A total of 20 individuals of each sex were collected in each pond at different sampling period (June, July, September and October).

Environmental variables

The following environmental variables were taken at each pond over the sampling period: water temperature (°C), dissolved oxygen (mg/L), water turbidity (NTU), pH and electrical conductivity (Ms/cm), obtained with a HORIBA U-222 Multi-Parameter probe (Table 1). Additional measures of water temperature and luminosity were also monitored by data logger (Hobo UA-002-08; Onset Ltd) every 3 h throughout the life cycle of the species until total dissection of temporary ponds. The surface flooded area (m^2) was measured in situ using a portable GPS (precision three meters) after walking the pond perimeter. The maximum depth of the ponds (cm) was measured with a graduated ruler.

Table 1 Average values of water physicochemical and habitat structure variables in the temporary ponds studied over the sampling period

Sampling period	June		July		September		October	
Pond	P1	P2	P1	P2	P1	P2	P1	P2
Environmental variable								
Water temperature (°C)	12.8	14.2	15.6	8.2	17.3	14.6	Pond dry	23.3
pH	6.5	5.5	5.1	5.9	5.1	5.9	Pond dry	4.3
Electrical conductivity (Ms/cm)	0.16	0.11	0.05	0.12	0.04	0.12	Pond dry	0.25
Dissolved oxygen (mg/L)	14.9	10.8	8.9	4.5	6.1	5.2	Pond dry	2.9
Water turbidity (NTU)	86.3	210.3	17.7	103.3	22.5	37.7	Pond dry	774
Area (m^2)	871.2	2308	932	2472	1384	3306	Pond dry	4.7
Maximum depth (cm)	25	22	29	25	37	37	Pond dry	20

OBS area is expressed in absolute values

Population density estimates

In both studied ponds, we estimated the abundance of *A. minuano* and a co-occurring species of annual fish (*Cynopoecilus fulgens* Costa 2002) through mark-and-recapture techniques. These estimates were taken for posterior use as explanatory variables, since population density influences resource availability as well as fish growth and mortality (Hixon and Jones 2005; Lobón-Cerviá 2012). The sampling effort at each pond was standardized per unit time. Two equally-trained people performed sweeps parallel to the bottom of the ponds with hand nets (D-shape; 60 cm in length; 40 cm in height, 2 mm mesh size) during six 15 min periods, thus totaling a sampling effort of 1 h 30 min at each pond. All available habitats at each pond were sampled. In each sampling event, the collected specimens were kept separated in 20 L buckets. The specimens were not released between sampling events. Annual fishes were sexed according to external character patterns (e.g., size, shape and fin color). Fish standard length (mm) was measured with a plastic ruler. Fish were marked by removing a small piece of caudal fin before the release. In the next day, the same sampling procedures were repeated in both ponds. The numbers of tagged and unmarked annual fish caught were recorded and all fishes were released.

We also estimated the abundance and richness of non-annual fishes and potential predators (e.g., turtles, piscivorous fish and aquatic insects such as Belostomatidae (Heteroptera) and larval Odonata) after the sampling procedures. Potential predators were determined to the lowest taxonomic level possible. The

population density estimates was performed based on Lanés et al. (2016). Non-annual fishes were identified to specific level according to Malabarba et al. (2013) and annual fishes, according to Costa (2002, 2006). Fish systematic classification followed Betancur-R et al. (2013).

Biomarker analyses

Overall, 137 individuals of *A. minuano* (69 males and 68 females) were collected over the sampling period (June: N = 61, July: N = 38, September: N = 24, October: N = 14) and used in the biomarker analyses. The collected fishes were frozen at − 80 °C prior to the analyses. Fishes were weighed on an analytical balance and had their standard length (from the tip of the muzzle to the end of the caudal peduncle; mm) measured with a digital caliper.

Two whole individuals were pooled in two-one single sample for homogenization in phosphate buffer solution (20 mM, pH 7.0) with fluoride phenyl-methanesulfonyl fluoride (PMSF 1 mM) and potassium chloride (140 mM) in a ratio of 1 g wet weight to 6 mL buffer, with the aid of a Turrax® disperser, according to the method described by Persch et al. (2017). Total homogenate was centrifuged at 1000 rpm for 3 min in refrigerated centrifuge (4 °C). The supernatant from this centrifugation was used to quantify total protein (TP) and lipid peroxidation (LPO). The activity of antioxidant enzymes Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione S-Transferase (GST) were determined from each group of individuals by sampling lake, gender

and sampling period. All samples were determined in quadruplicate. Total proteins in the supernatant were quantified using a commercially available kit (Biodiagnostica). Samples were read in a spectrophotometer at 525 nm in triplicate for each pool. The result is expressed in mg protein/g of wet weight.

Lipid peroxidation (LPO) was measured by quantification of thiobarbituric acid-reactive substances (TBARS) as modified by Persch et al. (2017). A medium with 200 μL thiobarbituric acid (0.67% TBA), 300 μL trichloroacetic acid (10% TCA), and 200 μL distilled water were added to 100 μL of sample. This mixture was heated at 100 °C for 15 min, and 600 μL *n*-butanol was added to extract the nonpolar product from this aqueous solution. This was followed by centrifugation at 3600 rpm for 10 min. The resulting supernatant was placed in a black quartz cuvette and read in a spectrophotometer at 535 nm. Results were expressed as nmoles TBARS mg TP^{-1}.

Catalase (CAT) enzyme activity was also measured by kinetic spectrophotometry at 240 nm, as described by Boveris and Chance (1973). The medium contained 955 μL phosphate buffer (50 mM, pH 7.0, 25 °C), 10 μL sample homogenate, and 35 μL hydrogen peroxide (0.3 M, 170 μL H_2O_2 in 5 mL distilled water), kept continuously on ice and protected from light. CAT activity was expressed in nmoles H_2O_2 min^{-1} mg $protein^{-1}$.

Glutathione S-Transferase (GST) activity was estimated according to Boyland and Chasseaud (1969) through the measurement of the conjugated 1-chlore 2,4 dinitrobenzene (CDBN) by the reduced glutathione (GSH). The activity of the enzyme was expressed as mmol conjugate CNDB mg $proteins^{-1}$ min^{-1}.

Superoxide dismutase (SOD) activity was measured by adding to the homogenate a solution of g NAOH-glycine buffer (50 mM, pH 11, 25 °C) and epinephrine (1 mM) (Boveris and Cadenas 1982). Using the kinetic spectrophotometry method, three curves were obtained at 480 nm: the first with 980 μL buffer and 20 μL homogenate, the second with 985 μL buffer and 15 μL homogenate, and the third with 990 μL buffer and 10 μL homogenate, always to a total volume of 1000 μL (plus 17 μL of epinephrine mixture), in a quartz cuvette. One unit (U) of SOD activity is defined as the amount of enzyme which inhibits the rate of reduction of the detector (adrenaline) by 50%. Results were expressed in U SOD/mg TP-1.

All biochemical-functional procedures were authorized by the PUCRS Research Ethics Committee for the Use of Animals (CEUA number 8271) and National System for Management of Genetic Patrimony and Associated Traditional Knowledge (SisGen: A732684).

Data analysis

Differences in the standard length of individuals according to sampling event (months) and sex were tested with a two-way ANOVA. Linear Mixed Models (LMMs) were used to test the effect of sampling period and sex on the seasonal variation of biomarkers. The dependent variables (values of biomarkers) were either log or square-root transformed prior to the analysis. In the LMMs, pond identity was used as the random factor in all models; sampling event, and sex were used as the fixed factors of interest. A set of candidate models was generated accounting for the single and additive effects of the fixed factors as well as the interaction. The full set of candidate models was compared with a null model (composed by intercept and random effect only). The selection of the best model was performed via *single term deletions* and Wald test. Model assumptions were checked for normality and homoscedasticity by visual assessment of diagnostic plots. LMMs and model selection procedures were performed using the functions from the *lme4* package (Bates et al. 2014). Data were visualized graphically using the functions from the *ggplot* library (Wickham 2009).

The influence of environmental variables on the seasonal variation of the biomarkers was assessed using two analytical approaches. Prior to the analyses, the explanatory variables were grouped according to their nature into the following subsets: (1) habitat structure: pond area and depth. (2) Biotic variables: richness and abundance of predators (aquatic insects, turtles and piscivorous fishes), abundance of *A. minuano* and *C. fulgens*, richness and abundance of non-annual fishes. (3) Water physicochemical variables: dissolved oxygen, electrical conductivity, water turbidity, pH and water temperature.

First, we assessed the influence of environmental variables on the full set of biomarkers with a Partial Redundancy Analysis (pRDA) using sampling period

as conditioning matrix to control the effect of time. Prior to the RDA, the response matrix (TP, LPO, CAT, GST, SOD) was Hellinger-transformed. We carried out pre-selection procedures on each subset of explanatory variables. This procedure searches for the subset of variables in the explanatory matrices that best explain variation (R^2 adj.) in the response matrix. In each forward selection procedure, we employed as stopping criteria a significance value of 0.05 and R^2 adj. values of each explanatory variable no higher than the model containing all variables of each explanatory subset. The Monte Carlo test (999 permutations) was used to evaluate the significance of the general model (i.e., the model with the variables retained). We used the variation partitioning approach (Peres-Neto et al. 2006) to assess the influence of individual and shared fractions of by each explanatory subset on the biomarkers.

Second, we assessed the influence of environmental variables separately for each biomarker using multiple linear regressions. We used the variance inflation factor (VIF) to eliminate collinearities among explanatory variables. In brief, low VIF values (VIF = 1) indicate that the variable is not related to any other predictor; on the other hand, VIF > 10 suggests strong collinearity among variables (Quinn and Keough 2002). All analyses were performed in the statistical environment R version 3.5.1 (R Core Team 2018).

Results

Seasonal variation of biomarkers

Fish body length significantly differed between sampling periods (F = 16.547, p < 0.001), but did not differ between sexes (F = 0.382, p = 0.583). Total proteins (TP) levels in males and females varied differently over the sampling periods (interaction between sex and sampling period). Males showed a continuous increase in TP along their life cycle, peaking in the senile phase (October). Females showed lower TP levels in the growth phase (June and July), followed by an increase in the reproductive phase (July and September), and eventual reduction in the senile phase (October) (Fig. 1a).

Lipid peroxidation (LPO) differed according to sex and sampling period (p = 0.01). LPO continuously decreased in males along their life cycle, while it peaked in females in July and October. LPO was higher in females in the growth period (June and July). In the reproductive phase (July and September), LPO reduced in both sexes and was higher in males than females. In the senile phase, LPO decreased in males and increased in females with the opposite pattern (October; LPO was higher in females than males; Fig. 1b).

Catalase (CAT) activity was influenced by sex and sampling period (p = 0.002) but not by the interaction between the two factors. CAT activity was higher in the growth phase (June and July) in males, followed by a decrease in the reproductive and senile phases (September and October). In females, CAT activity also decreased from the growth to the reproductive phase (June to September). Within this temporal range, the decrease in females was larger compared to males; however, CAT activity increased in the senile phase of females (October) (Fig. 1c).

Glutathione S-Transferase (GST) activity was solely influenced by the interaction between sex and sampling period (p = 0.0002). GST activity in males continuously decreased along their life cycle (from the growth to the senile phase). GST was higher in males in the growth period (June and July). In females, GST activity also decreased from the growth to the reproductive phase (June to September). Within this temporal range, the decrease in GST activity in females was larger compared to males; however, GST activity increased in the senile phase of females (October) (Fig. 1d).

Superoxide dismutase (SOD) activity was influenced by the interaction between sex and period (p < 0.001). SOD activity continuously decreased in males along their life cycle (from the growth to the senile phase). SOD activity in females increased in the growth phase (from June to July) and decreased in the reproductive phase (September) followed by an increase I the senile phase (October) (Fig. 1e).

Influence of environmental variables on biomarkers

The output of the pRDA indicated that water physicochemical, habitat structure and biotic variables significantly explained ∼ 43% of the seasonal variation of the full set biomarkers (F = 13.877, p = 0.001). Water physicochemical variables (pH
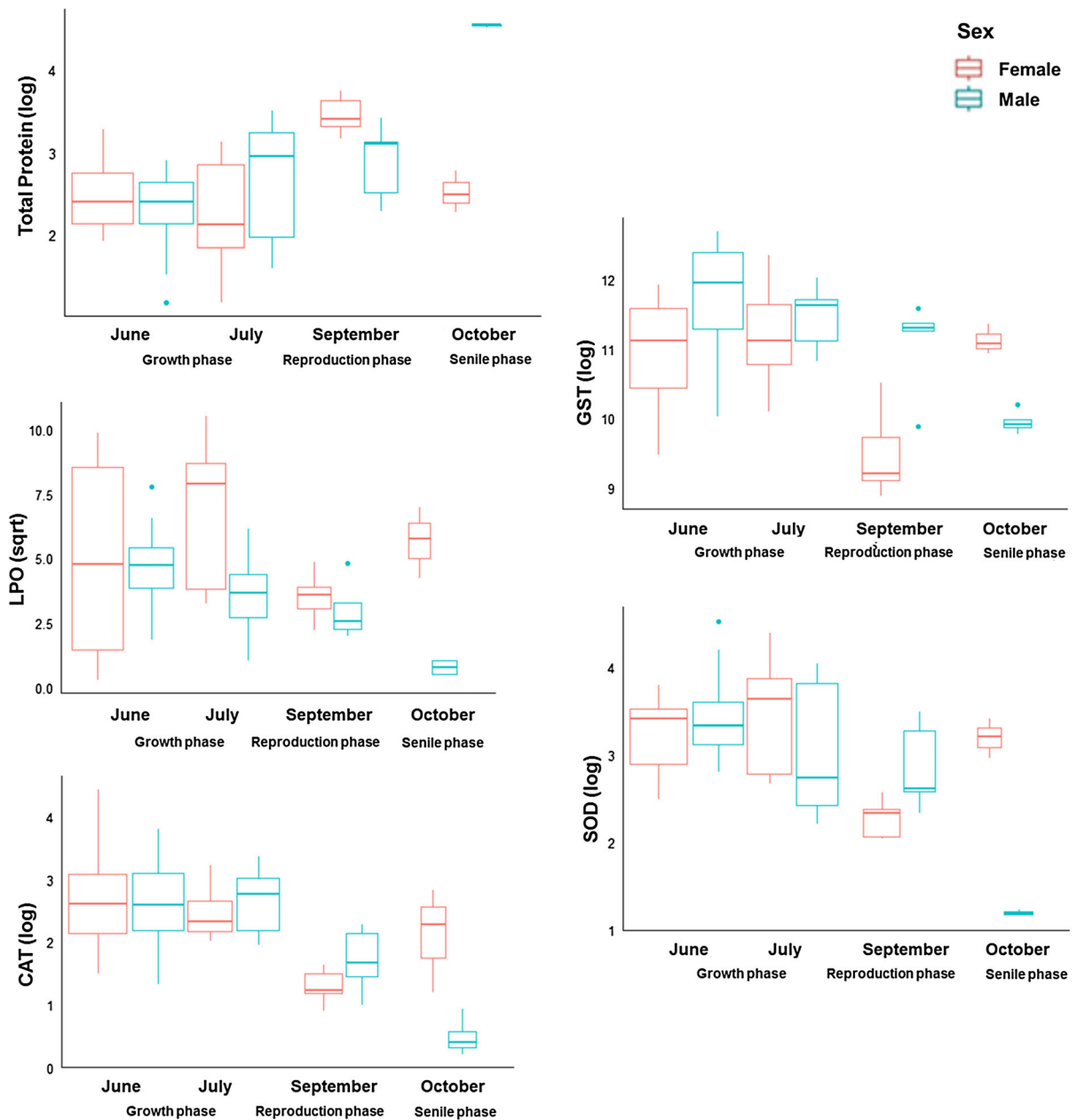
Fig. 1 Seasonal variation of the oxidative status markers and total proteins in wild populations of *Austrolebias minuano*

and water temperature) accounted for 18% of the explained variance, followed by biotic (abundance of *A. minuano* and non-annual fishes; 6%) and habitat structure variable (pond area; 3%). The shared fraction between water physicochemical and habitat structure variables explained 12% of biomarker variation, followed by the shared fractions between water physicochemical and biotic variables (2%) and habitat structure and biotic variables (2%). The explained fraction shared by all subsets of explanatory matrices corresponded to 1% of biomarker variation (Fig. 2).

Multiple regressions showed that different environmental variables influenced the variation of each biomarker (Table 2). Abundance of *A. minuano*
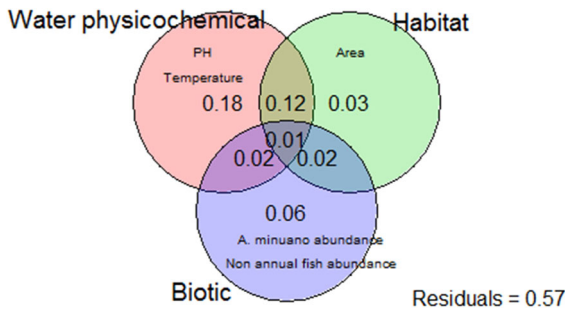
Fig. 2 Environmental variables that significantly explain the variation of oxidative status markers and total protein levels

Table 2 Results of redundancy analysis (RDA) and multiple regression with the selected environmental variables that explained the markers variation analyzed

Biomarker	Environmental variables	Adj. R^2	p
All markers	Water temperature	0.507	0.01
	pH	0.475	0.024
	Area	0.454	0.004
	A. minuano abundance	0.321	0.001
	Non-annual fish abundance	0.418	0.001
TP	*A. minuano* abundance	0.539	< 0.001
LPO	Predator abundance	0.483	< 0.001
CAT	Dissolved oxygen	0.292	0.001
GST	Electrical conductivity	0.097	0.005
SOD	pH	0.539	< 0.001

positively influenced TP, which explained almost 54% of the variation. Predator abundance positively influenced LPO, which represented 48% of the variation. The pH positively influenced SOD, which explained almost 49% of the variation. Dissolved oxygen positively influenced CAT, which explained 29% of the variation. Electrical conductivity negatively influenced GST, which explained about 10% of the variation.

Discussion

Austrolebias minuano is an annual fish species with short lifespan that shows high investments in rapid growth and reproduction to maximize survival in temporary ponds (Lanés et al. 2016, Reichard et al. 2018). However, studies on the effects of high investments in life history and the possible role of environment in the oxidative status in wild short-lived

vertebrates are scarce. This study provided the first field-based assessment of the oxidative status in wild populations of a short-lived vertebrate.

The high investment observed in *A. minuano* life history was not associated with oxidative stress increase along its life cycle. Our results suggest that Neotropical annual fishes have a well-developed oxidative stress control system, adapted to the temporary condition of seasonal ponds. The expected trade-offs between investments in life-history traits (represented here by rapid growth and high reproductive effort) and self-maintenance was solely detected in females during the growth phase. The influence of environmental variables in the seasonal variation of oxidative status markers over the life cycle of *A. minuano* showed the importance of environmental factors in the aging process and mortality. The higher male mortality in the end of annual fish life seems not be related to oxidative stress increase, but with the environmental factors.

Seasonal variation of oxidative stress biomarkers

Oxidative status markers fluctuated over the life cycle of annual fishes and differed according to sex. In females, the activity of antioxidant enzymes was higher in the growth phase compared to other phases. Nevertheless, LPO levels were higher in this phase, which suggest the occurrence of oxidative stress. In turn, the growth phase in males was unrelated to increased oxidative stress, suggesting that the energy used for growth does not affect their self-maintenance. This difference between the sexes may be associated with greater reproductive effort of females due to preparation for reproduction. In annual fishes, studies detected reduced growth rates due to the allocation of energy reserves for reproduction (Fonseca et al. 2013; Vrtílek and Reichard 2015). The evidence of oxidative stress in growing females can be explained by the early investments in reproduction, which is intertwined with the short lifespans of annual fishes (Vrtílek et al. 2018b). The trade-off between growth and reproduction can be related to oocyte production that requires large amounts of energy and have been related to oxidative stress increase (Behrman et al. 2001).

However, there was no evidence of oxidative stress in the period of most intense reproductive activity (September). Reductions in the levels of LPO and in the activity of antioxidant enzymes were observed in

both sexes. These results suggest efficient oxidative balance and oxidative stress control in the effective reproductive phase, especially in females. Previous studies showed none or weak association between oxidative stress and reproductive efforts in long-lived organisms (Nussey et al. 2009; Olsson et al. 2012) and that reproduction can reduce oxidative stress (Garratt et al. 2010; Costantini et al. 2014). In this study, we found a reduction in oxidative stress markers in the reproductive phase, accompanied by increased levels of total proteins (TP). This result may be related to the increase in stress proteins, known as heat shock proteins (HSPs). These proteins are expressed as a cellular response to oxidative damage (Minois 2000), and they may act as alternative to antioxidant enzymes. In addition, the high activity of early life antioxidant enzymes may act as a signaling mechanism, reflecting the reduction of LPO evidenced in the reproductive phase. An adaptive mechanism to sublethal doses of reactive oxygen increases the resistance to oxidative stress (Costantini 2014; Gladyshev 2014).

Males had decreased activity of oxidative stress markers in the senile phase, while females had increased activity. In African annual fish species, under laboratory conditions, the senile phase was associated with increased LPO and decreased antioxidant enzyme activity (Hsu et al. 2008; Liu et al. 2012; Milinkovitch et al. 2018). The increased LPO in females in this period may be related to reproductive efforts, as energetic allocation to reproduction does not decrease with aging process in annual fishes (Blažek et al. 2017). These species reproduce on a daily basis, and females produce eggs until to death (Vaz-Ferreira et al. 1964; Gonçalves et al. 2011). In addition, is possible that annual fishes show terminal investment events, characterized by increased end-of-life reproductive efforts, generally upon experience of immediate survival threats (Bonneaud et al. 2004). The increased end-of-life LPO levels in females were accompanied by increased activity of antioxidant enzymes, which reflects an efficient oxidative balance of the species.

The decrease in LPO levels over the life cycle of males showed that annual fishes have an efficient control of redox homeostasis in natural environment. We found contrastant results for LPO variation compared to previous studies of African annual fish species under controlled laboratory conditions. In nature, environmental conditions 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 (Godoy et al. 2019). On the other hand, laboratory conditions impose other sources of stress (e.g. movement restrictions and non-varied diet). In this sense, the responses obtained from different experimental conditions can be influenced the unexpected result. Both, the lack of studies based on controlled conditions for *A. minuano,* and lack of data from wild populations of other annual fish species compromise a more detailed comparison. Reichard (2016) regarded that the lack of understanding senescence process for matched populations exposed to natural and laboratory conditions constitute a major challenge for generate advance on aging process. According to Speakman et al. (2015), laboratory-based studies offer opportunities for experimental manipulation, but may not expose the organism and thus their physiology to the effects of life history, due to the contorted laboratory environment.

Furthermore, this result helps clarify their higher end-of-life mortality in the natural environment (Reichard et al. 2014). Male mortality is generally attributed to both extrinsic (e.g. predation) and intrinsic (body condition) causes (Reichard et al. 2018; Godoy et al. 2019). Apparently, the higher male mortality observed in the natural environment was not related to oxidative stress, since males at the end of their life cycles had efficient antioxidant defenses. Recent studies have showed the influence of extrinsic factors (i.e. predation and environmental deterioration) in the higher mortality of males of annual fishes (Lanés et al. 2016; Reichard et al. 2018).

The synchronized increases in body size and TP in males can be interrelated, as proteins play important roles in growth (Van der Have and De Jong 1996). Increase in protein is related to rapid growth (Lee et al. 2013; Hooper et al. 2017). The benefits of reaching larger sizes include higher reproductive success (Guimarães et al. 2017) because larger males are usually preferred by females (Passos et al. 2014). TP levels increased in females until September, followed by a decrease in the senile phase. This decrease may be related to gonadal maturation and constant formation of vitellogenin-rich eggs, which lead to smaller sizes compared to males (Patiño and Sullivan 2002). These characteristics require great energy allocation for end-of-life reproduction, as annual fish maintain

reproductive activity until death (Vaz-Ferreira et al. 1964; Gonçalves et al. 2011), as continuous spawning and oocyte production are extremely costly (Wootton and Smith 2015).

Influence of environmental variables on oxidative stress biomarkers

The seasonal variation in the biomarkers of oxidative stress in *A. minuano* was influenced by environmental variables. Environmental conditions greatly vary in temporary ponds and are very associated with water level fluctuations (Polačik and Podrabsky 2015). Decreasing water levels usually lead to changes in physicochemical characteristics (Lanés et al. 2016). In this study, variables such as water temperature, pH, dissolved oxygen and electrical conductivity influenced the markers. As the water level decreases, higher water temperature and lower dissolved oxygen occur in temporary ponds. Such changes are associated with the end of the life cycle of Neotropical annual fish species (Lanés et al. 2016). Water temperature fluctuations are related to increased LPO (Milinkovitch et al. 2018) and favor predation. These factors are related to increased LPO and oxidative damage in other organisms (Janssens and Stoks 2013). In addition, increased temperature has been related to decreased longevity and increased aging markers in annual fishes (Liu and Walford 1975; Valenzano et al. 2006). Positive and negative relationships with oxygen concentration induced increased CAT activity (Lushchak et al. 2001, 2005). The increase in conductivity may be related to the increase in suspended solids. This increase can lead to reductions in dissolved oxygen levels in water, producing a critical oxygen scarcity for organism survival (Ryan 1991) and increased salts that produce toxic effects on fish and their eggs (Weber-Scannell and Duffy 2007).

In addition to the changes in water physicochemical variables, the hydrological cycle (generally related to precipitation regime) is associated with increases in water availability (i.e., pond area). At the peak of the wet phase several wetlands are physically connected with other waterbodies, which allows access to competitors such as non-annual fish and predators to temporary ponds. In fact, pond area, abundance of non-annual fish and predators influenced the markers, while the abundance of *A. minuano* influenced the TP

levels of the fishes analyzed. Competition and predation by non-annual fish has already been acknowledged as important factors in the mortality of annual fishes (Nico and Thomerson 1989; Lanés et al. 2016; Reichard et al. 2018). Regarding the role of competitors, *A. minuano* abundance can exceed more than 1000 individuals at certain life-cycle periods (Lanés et al. 2016) influencing competitive interactions such as resource availability and predation (Ronget et al. 2017).

Conclusions

In this study, we assessed the oxidative status of a short-lived vertebrate in natural environments along their life cycle. We showed that the high investments in life-history traits of *A. minuano* were not associated with increased of oxidative stress along their life cycle. The expected trade-offs between investments in life-history traits and self-maintenance was exclusive to females in the growing period. These results suggest that Neotropical annual fishes, especially males, have a well-developed oxidative stress regulation system adapted to the seasonal conditions of temporary ponds. Besides, we showed that annual fish mortality (mainly in males) apparently is not related to oxidative stress and that the environmental variables may also influence the annual fish aging process and mortality.

To generate a significant advance in the field of aging process and specifically on the basic concepts of two well-known theories of aging (the oxidative stress theory and the disposable soma theory) using the alternative model organism *A. minuano* are necessary further data based on well-established laboratory colonies. New experimental models need to be validated at different levels (genetic, molecular, physiological), before being able to acquire scientific power. In this sense, meticulous studies on the alternative model here used should be performed in the future, both in captivity and at the level of species ecology (Godoy et al. 2019).

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