RESEARCH ARTICLE

Biomarkers of oxidative stress in the post-embryonic characterization of the neotropical annual killifish

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Abstract Annual killifish are among the most remarkable extremophile species with the shortest vertebrate life span. Few studies have reported on the oxidative balance throughout their life cycle and its association to the natural aging process of these neotropical animals in a natural environment. We standardized and analyzed physiological markers related to the redox balance of the annual killifish (*Cynopoecilus fulgens*) throughout the post-embryonic life cycle (enzyme activity of Superoxide Dismutase, Catalase, Glutathione Peroxidase, and Glutathione *S*-transferase, as well as the determination of the levels of Lipoperoxidation, Carbonylated Proteins, and Total Proteins). We tested the influence of environmental variables on these biomarkers. Individuals were collected, including juveniles, adults, and seniles, in three sampling units around the Parque Nacional da Lagoa do Peixe, located in the Coastal Plain of Rio Grande do Sul. We observed that males and females used different physiological strategies of their redox balance during their life cycle, and their oxidative balance was influenced by their reproductive period and environmental variables (water temperature, abundance of predators, abundance of another sympatric annual killifish species, and abundance of *C. fulgens*). The population of each temporary pond presented different physiological responses to the adaptation of their life cycle, and there was an influence of environmental component as a modulator of this cycle. Our study offers reference values that will be useful for comparison in future research with short-lived organisms.

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Abbreviations
LPNP	Lagoa do Peixe National Park
ICMBIO	Instituto Chico Mendes de Conservação da Biodiversidade

CEUA	Research Ethics Committee for the Use of Animals
PUCRS	Pontifícia Universidade Católica do Rio Grande do Sul
RS	Rio Grande do Sul
P1	Temporary pond 1
P2	Temporary pond 2
P3	Temporary pond 3
ORP	Oxidation reduction potential
NTU	Turbidity
TDS	Total dissolved solids
OD	Oxygen dissolved
ROS	Reactive oxygen species
GST	Glutathione S-transferase
CAT	Catalase
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
LPO	Lipoperoxidation
TBARS	Thiobarbituric acid
CP	Carbonylated proteins
TP	Total proteins

Introduction

Aquatic ecosystems are undergoing constant interference and modifications through anthropic actions, triggering marked changes in habitat quality and biodiversity composition (Saunders et al. 2002). The main global cause of extinction and modification in the abundance of species is the alteration of the natural habitat, leading to a potential inability of interaction between organisms (Tylianakis 2007). Several species present in these aquatic environments may be at risk of extinction and population reduction. The species of freshwater fish are considered one of the most threatened groups in the world (Saunders et al. 2002).

The diversity of freshwater fish faces a substantial crisis worldwide (Collen et al. 2014; Darwall and Freyhof 2015; Dudgeon et al. 2006). The southern region of Brazil presents an intensification of human activity, focused on agriculture and urbanization, which are causing several changes in natural ecosystems (Maltchik et al. 2014). Among these ecosystems, wetlands are considered the most threatened, specifically the ephemeral/temporary environments (Calhoun et al. 2017; Lanés et al. 2018; Maltchik et al.

2010, 2014). These environments are aquatic habitats that dry out periodically and undergo intense physical–chemical variations in water (for example, O_2, light, temperature), as well as changes in biotic components (for example, food availability) throughout their flood cycle-drought (Arezo et al. 2007; Berois et al. 2012).

Remarkably, there is great biological diversity present in these temporary aquatic environments, along with great ecological relevance (Calhoun et al. 2017; Creed et al. 2017). Specialist organisms within these environments, such as annual killifish with restricted and geographically isolated populations, are more exposed to human actions, thus presenting greater vulnerability and risk of extinction (Berois et al. 2014; Fonseca et al. 2013; Volcan et al. 2015). The distribution of annual killifish species in the southern region of Brazil corresponds to areas traditionally used for the cultivation of rice and soybeans. These activities have been causing alteration and destruction of temporary aquatic habitats (Lanés et al. 2018), considering the large amounts of pesticides used (mainly herbicides and insecticides) (Papa et al. 2015; Pignati et al. 2017).

The annual killifish are defined as a group of the order Cyprinodontiformes composed mainly of small and freshwater species (Arenzon et al. 2001, 2002; Fonseca et al. 2013). Their distribution encompasses temporary ponds (ephemeral environments) of the Neotropical region (Family Rivulidae) and Africa (family Nothobranchiidae) (Furness et al. 2015). By inhabiting these regions, they present a short life cycle and exhibit rapid growth, early sexual maturity, continuous reproduction, and production of resistant eggs (Arenzon et al. 2001, 2002; Berois et al. 2012; Errea and Danulat 2001; Fonseca et al. 2013; Lanés et al. 2014). They have been recognized as excellent biological models for aging studies and toxicological tests due to their characteristics and the ease of maintaining and storing their eggs for months under laboratory conditions (Arenzon et al. 2001, 2002; Berois et al. 2012; Hsu et al. 2008; Graf et al. 2010; Polačik and Reichard 2010; Terzibazi et al. 2008).

Aging in fish refers to the deterioration of physiological integrity over time, a process that exhibits the increased risk of mortality related to age due to the decline in vital functions (Blažek et al. 2017; Dong et al. 2016). According to Dong et al. (2016), these declines may be related to mitochondrial dysfunctions

that occur with aging and may induce the exacerbated production of reactive oxygen species (ROS), potentially leading to cellular damage. The annual killifish presents different aging patterns (Di cicco et al. 2011; Liu et al. 2012; Godoy et al. 2019). Liu et al. (2012) studied the changes in age-related markers during the normal aging of the annual killifish *Nothobranchius guentheri*, and demonstrated an increase in lipoperoxidation, protein oxidation, β-galactosidase expression, and the accumulation of lipofuscin with age. In contrast, the activities of catalase, glutathione peroxidase and superoxide dismutase decreased with age, while telomerase activity showed no apparent change with age. These authors then suggested that this annual killifish (*N. guentheri*) could be an adequate model for studies of aging. The development of research on the aging process in vertebrates has been limited by the lack of models with a short life cycle (Genade et al. 2005) therefore, the knowledge of this cycle in annual killifish can collaborate in this area.

When analyzing the aging patterns of species, some aspects should be considered, such as the oxidative damage markers (e.g., antioxidant enzymes, carbonylated proteins, and lipid peroxidation). The beforementioned can assess the roles of different components in this system during aging or under the influence of environmental stressors (Liu et al. 2012). Biological diversity and its ecological implications are often characterized by these physiological tools, illustrating the organismal responses to environmental variations and stressors (Cooke et al. 2013). Godoy et al. (2020) conducted a study with an annual killifish species (*Austrolebias minuano*), in which they demonstrated an efficient antioxidant system throughout the post-hatch life cycle, specifically in males. Mortality in these animals seems to be related to environmental variables that influence the fish aging process (Godoy et al. 2020).

Among the annual killifish, the species *Cynopoecilus fulgens* Costa, 2002 (family Rivulidae) is endemic to the Coastal Plain of southern Brazil, in which agricultural activity is the main economic activity (Lanés 2011). The existence of a single age cohort in their populations has been demonstrated, with the life expectancy of these organisms being approximately eight months in a natural environment (Lanés et al. 2016). The analysis of the population size structure of two annual fish species (*C. fulgens* and *Austrolebias minuano*) suggests the presence of a single-age cohort in different ponds analyzed in southern Brazil; these animals also showed an increase in weight throughout the life cycle (Lanés et al. 2016). For *C. fulgens*, it was observed that the males that make up these populations have a clear sexual dimorphism, being larger and more colorful than females (Lanés et al. 2014). The density of *C. fulgens* populations gradually decreases throughout their life cycle, and differences in the mortality rate between the sexes are observed, with males having a higher mortality rate. These variations may be related to physiological differences between the sexes and the color pattern that increases the risk of predation (Lanés et al. 2014, 2016).

In the ephemeral habitat, these rivulids are considered top predators, generalists, and opportunists, presenting a diet that consists of autochthonous aquatic organisms (e.g., zooplankton, eggs, insect larvae, algae, and diatoms) (Gonçalves et al. 2011; Laufer et al. 2009). The distinction of the annual killifish diet is related to the changes in their body size as they increase in size, larger prey is added to the diet, and smaller prey is suppressed (Keppeler et al. 2013, 2014; Laufer et al. 2009). After periods of intense rainfall, the temporary ponds can connect with permanent bodies of water and be colonized by other species of fish with non-annual life cycles, such as large predators.

Annual killifish are a taxonomic group with high conservation importance (Volcan and Lanés 2018a, b). They represent one of the most threatened vertebrate groups in Brazil and are considered a flag species for the conservation of these temporary ponds (Lanés et al. 2016, 2018; Volcan and Lanés 2018a, b). Thus, understanding the impacts of environmental variations on their life cycle is essential. Investigations done to evaluate the response to physiological stress can benefit from physiological biomarkers, such as the enzyme activity of the intermediate metabolism, oxidative balance, and biotransformation (Braghirolli et al. 2016; Cooke et al. 2013; Dantzer et al. 2014; Pinheiro and Oliveira 2016; Godoy et al. 2020).

We expect to see early, and mid-life increases in oxidative damage markers associated with growth, maturation, and copulation, along with an increase in antioxidant enzyme activity to maintain oxidative balance. At the end of the life cycle (senescent phase), a decline in enzyme activity and an increase in oxidative damage is expected, culminating in the

death of the animals. From this perspective, we aimed at establishing and analyzing the physiological markers related to the redox balance (enzyme activity of Superoxide Dismutase, Catalase, Glutathione Peroxidase, and Glutathione S-transferase, as well as the determination of the levels of Lipoperoxidation, Carbonylated Proteins, and Total Proteins) of the annual killifish (*C. fulgens*) at different stages of post-embryonic development in animals collected in a natural environment. Additionally, we tested the influence of environmental variables of different types (physical–chemical parameters of water, habitat, and biotics) on the different biomarkers analyzed.

Materials and methods

All procedures were carried out with proper authorizations (ICMBio: 43251-4; CEUA-UNISINOS: 12.2015; CEUA-PUCRS: 8271), and with registration in the National System for Management of Genetic Patrimony and Associated Traditional Knowledge (SisGen: A732684).

Field procedures

Specimens of *C. fulgens* were collected in three different temporary ponds (temporary ponds: P1, P2 and P3) during the months of June, July, September, October, and November of 2016. June and July represented the juvenile phase, while September and October represented the adult and senile phases (Fig. 1). In November, even though the sample units had a minimum volume of water, there were no more annual killifish. We obtained the geographic coordinates of each sample unit: P1 (W 51° 05′ 15″, S 31° 18′ 13″), P2 (W 51° 05′ 17″, S 31° 18′ 14″) and P3 (W 51° 08′ 03″, S 31° 16′ 46″). Animals were collected in the municipalities of Tavares and Mostardas, around the Lagoa do Peixe National Park (31° 02′–31° 48′ S; 50° 77′–51° 15′ W), located on the External Coastal Plain of Rio Grande do Sul. These regions present high biodiversity and a large number of different wetlands types (Lanés and Maltchik 2010; Lanés et al. 2016).

During each collection campaign, we obtained the physical–chemical parameters of the water (temperature, oxidation potential (ORP), turbidity (NTU), dissolved oxygen (OD), total dissolved solids (TDS), salinity, pH, and conductivity) using a HORIBA U-222 multi-parameter probe. We measured the surface area (m^2) of each temporary wetland by using portable GPS to track their limits, as well as the maximum water depth (cm) with a millimeter ruler. The temperature and luminosity were determined using a data logger (Hobo UA-002-08; Onset Ltd), with a record of 3–3 h, until the complete dissection of each puddle temporary.

The field capture procedure was carried out through active collection with hand nets (60 in length × 40 cm in height and 2 mm mesh size), following methodology already established for this group (Lanés et al. 2016). We collected a total of 443 individuals, including both sexes, at different stages of the life cycle (juveniles, adults, and senile). Afterward, these specimens were sexed and cryoeuthanized at the collection site, according to the CONCEA recommendations (CONCEA Euthanasia Guidelines and Practices 2013). All biological material was labeled, stored in thermal boxes with ice, and transported to the Conservation Physiology Laboratory – PUCRS, where they were stored in a freezer (− 80 °C) for subsequent biochemical analysis.

We also recorded biotic data, including richness and abundance of predators, abundance of *Austrolebias minuano* (a sympatric species of annual killifish often found sintopic in the same temporary wetlands), abundance of *C. fulgens*, and richness and abundance of non-annual killifish. Fish species potentially predatory to rivulids were considered: *Hoplias malabaricus*, *Oligosarcus jenynsii*, *Oligosarcus robustus*, *Cichlassoma portalegrensis*, in addition to insect larvae of the order Odonata, aquatic hemiptera of the family Belostomatidae, and crayfish of the genus *Parastacus*.

Laboratory procedures

The animals were separated by sex, month of collection (stage of the cycle), and temporary pond (P1, P2, or P3) (Fig. 1). All specimens were weighed (body mass) and measured (standard length) with an analytical scale (0.001 g) and digital caliper (0.01 cm), respectively, and were subsequently homogenized and analyzed. We separated the samples to quantify the carbonylated proteins and to generate pools for the quantification of the antioxidant enzymes, total proteins, and levels of lipoperoxidation. We performed between three to eight pools per sex for each

Fig. 1 *Cynopoecilus fulgens* (**A**) male, (**B**) female, and temporary wetland flooded (**C**) and dry (**D**)

temporary pond in each month, which were composed of two to three animals. For individuals from temporary pond 3 (P3) collected in October, the homogenate was made per individual, with a minimum of three individuals being used for each analysis. We adopted this procedure since fewer individuals were collected. As for temporary pond 1 (P1), we did not find any animals in October.

Homogenate for lipoperoxidation and enzyme assays

We homogenized the individuals in phosphate buffer solution (20 mM, pH 7.4, plus PSMF 1 mM and potassium chloride at 140 mM) with a volume of seven times the mass obtained (1 g: 7 mL), and with the aid of Ultra-Turrax® (IKA-WERK) in an ice bath. This protocol was described by Persch et al. (2017) to fish. The homogenate was centrifuged (SORVALL RC-5B Refrigerated Superspeed Centrifuge) in a refrigerated centrifuge (4 °C) at $1000 \times g$ for 10 min. The supernatant was used for the enzymatic assays of

Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx), and Glutathione *S*-Transferase (GST), as well as to quantify total proteins (TP) and lipid peroxidation (LPO). Each pool or sample was determined in triplicate.

Total supernatant proteins

We quantified the total proteins (TP) of the supernatant using the BioTécnica commercial kit. The assay is based on the reaction of proteins present in the sample with copper ions in an alkaline medium. The product of this reaction results in a violet color that is detected at an absorbance of 550 nm. The proteins are expressed in mg of protein. mL^{-1}. Total proteins were used to standardize the results obtained from LPO and enzyme activity.

Lipoperoxidation

Lipoperoxidation (LPO) was determined using the method described by Buege and Aust (1978), a

technique used to measure the damage of lipids by detecting substances that react to thiobarbituric acid (TBARS). To quantify the levels of lipoperoxidation, 300 μL of trichloroacetic acid (TCA), 200 μL of thiobarbituric acid (TBA), 100 μL of Milli-Q water, and 100 μL of sample were added to microtubes. This mixture was heated to 100 °C for 15 min and cooled for 10 min in an ice bath. We then added 600 μL of n-butyl alcohol and centrifuged the samples at 3600 rpm for 10 min (Lima and Abdalla 2001). The supernatant was collected and measured using a spectrophotometer with a wavelength of 535 nm. Results were expressed in nmoles of TBARS. mg of protein^{-1}.

Enzyme tests

To determine the activity of superoxide dismutase (SOD), we used a method based on inhibiting the reaction between the superoxide radical and epinephrine. With the oxidation of epinephrine, the formation of a colored product (adrenochrome) is obtained, which is detected through enzymatic kinetics by spectrophotometry at 480 nm. The reaction medium consists of glycine–NaOH (50 mM, pH 11) and epinephrine (1 mM diluted in HCl) (Boveris and Cadenas 1982). To perform this procedure, the samples were centrifuged at 3000 rpm for 3 min at 4 °C, and we determined the SOD activity by verifying the curves of samples using 20 μL, 30 μL, and 40 μL. The results were expressed in units of SOD. mg of TP^{-1}. min^{-1}, where a unit of SOD (U) corresponding to the amount of enzyme that inhibits epinephrine oxidation by 50%.

Catalase was determined by evaluating the hydrogen peroxide (H_2O_2) decay. We performed this protocol after centrifuging the supernatant obtained from the total homogenate for 5 min at $3500 \times g$ at 4 °C. Subsequently, we used a quartz cuvette with 955 μL of sodium phosphate buffer and 10 μL of sample aliquot was used; afterwards, the spectrophotometer is zeroed and, finally, 35 μL of H_2O_2 (final concentration of 50 mM) is added. The reading on the spectrophotometer is performed with an absorbance of 240 nm for 2 min. For the expression of the results, the proteins in the sample are quantified (Boveris and Chance 1973) and the results expressed in pmoles CAT.mg protein^{-1}.min^{-1}.

We determined glutathione peroxidase by using kinetics through the dismutation of t-BuOOH by oxidation of GSH and formation of GSSG, which are catalyzed by GPx. The oxidation of NADPH is detected at 340 nm. To carry out the protocol, the samples were centrifuged at 3000 rpm for 3 min at 4 °C. Subsequently, a quartz cuvette was used, in which 350 μL of the reaction mixture and 45ul of the supernatant obtained after centrifugation were added. The results were expressed in μmoles of NADPH consumed. min^{-1}. Mg of protein^{-1} (Wendel and Feuerstein 1981).

Glutathione *S*-transferase activity was quantified following the method described by Boyland and Chasseaud (1969), through the conjugation of 1-chlorine 2,4 dinitrobenzene (CDNB) with reduced glutathione (GSH). The samples were centrifuged at 3000 rpm for 3 min before quantifying the supernatant using the spectrophotometer. GST activity is measured in absorbance at 340 nm, and the results were expressed as nmoles of CNDB conjugate. min^{-1}. mg of protein^{-1}.

Carbonylated proteins

For the quantification of carbonylated proteins, each sample (0.2–0.5 g) was sprayed in liquid nitrogen (N_2) and homogenized in 1 mL of the extraction buffer. The samples were centrifuged at $11,000 \times g$ for 15 min at 4 °C. We used two 500 μL aliquots of the supernatant to quantify the carbonylated proteins, according to the method described by Levine et al. (1994). The quantification was done by spectrophotometry with a wavelength of 370 nm, and the results were expressed in μmoles. mg of protein^{-1}. Each pool or sample was determined in quadruplicate.

Statistical analyses

Before performing the statistical analyses, we evaluated the presence of possible outliers for all data obtained. We tested for normality using the Kolmogorov–Smirnov test, and homogeneity was analyzed using the Levene test. For data with normal distribution ($p > 0.05$), parametric tests were used. We used the one-way analysis of variance test (one-way ANOVA) with Bonferroni correction to analyze the variation of biomarkers, which were compared per month (sampling period). The two-way analysis of

variance test (two-way ANOVA) was used to compare the biomarkers by month and by sex. ANOVA analyses done for the biomarkers were carried out with grouped data from all temporary ponds (P1, P2, and P3) and separately for each temporary pond. The variation in body size and weight of the individuals was tested using one-way ANOVA to compare the results between the months of collection, and two-way ANOVA when considering the period and sex for each temporary pond. The statistical analyses of variance were performed using the SPSS Program, version 20.0 (IBM Corp. 2011). Differences were considered significant when $p < 0.05$, and all results were expressed as the mean $\pm$ standard error.

We applied a multiple linear regression model was used for each of the evaluated response variables (TP, TBARS, CP, SOD, CAT, GPx, and GST) to test the influence of environmental variables on biomarkers. The dependent variables (biomarkers) were transformed into a coefficient of *Hellinger*. A pre-selection was done in each matrix of explanatory variables through the selection *Forward* to eliminate the effects of collinearities between the variables. The final environmental matrix was made up of those explanatory variables not excluded by the variance inflation factor (VIF). VIF is generally used as an indicator of multicollinearity between explanatory variables. Values above 1 (VIF > 1) indicate the variable is not related to any other predictive variable in the model, while values above 10 (VIF > 10) suggest strong collinearity (Zuur et al. 2010). The relative contribution of each category of environmental variable was obtained through Analysis of Partition of Variance (routine VARPARP) (Peres-Neto et al. 2006). The environmental variables were categorized into: (i) "Habitat"; (ii) "Biotics"; (iii) "Physical–Chemicals".

The variables included in the habitat component were constituted by the area/size and depth of the temporary pond. The biotic variables were represented by the presence of predators, the abundance of predators, the abundance of *Austrolebias minuano*, the abundance of *C. fulgens*, the wealth of non-annual killifish, and the abundance of non-annual killifish. The physicochemical variables of the water were temperature, pH, conductivity, NTU (turbidity), and dissolved oxygen (DO). The response matrix was composed of the biomarkers analyzed (TP, TBARS, SOD, CAT, GST), and the explanatory matrices were

constituted of X1—habitat; X2—biotic variables; X3—physical–chemical variables of water. We used the R statistical environment (version 3.5.1) (R Core Team 2018) to analyze the relationship of environmental variables with the investigated metabolism and oxidative stress biomarkers using the following packages: *vegan* (Oksanen et al. 2018) for multiple regression analyzes, and *adespatial* (Dray et al. 2018) for the variance partition. These analyzes were performed with grouped data from all temporary ponds (P1, P2, and P3) and both sexes.

Results

Association between growth, weight, and total protein (TP) levels of individuals

Temporary pond 1 (P1) females had an increase in TP levels in July (Fig. 1a), and this pattern was also observed for P1 males (Fig. 2a). No significant differences were observed between P1 sexes in each month for TP ($F = 0,944$; $p = 0.40$). The growth data indicated that females and males presented an increase in the standard length (SL) from June to July (Fig. 2d). There was a significant difference between sexes over the months in P1 for standard length (SL) ($F = 10,247$; $p < 0.01$). We observed an increase in P1 females body mass in September (Fig. 2g). In P1 males, we observed a decline in body mass, except in September, where there was an increase, presenting a similar initial mass as observed in June (Fig. 2g). A significant difference was detected between sexes over the months in P1 for body mass ($F = 13,682$; $p < 0.01$). In the temporary pond 2 (P2), we observed increased levels of TP in females and males from June to July (Fig. 1b). There was no significant difference between sexes over the months in P2 for TP ($F = 1,667$; $p = 0.19$). The SL results demonstrated that both sexes presented a trend in linear growth over the months; however, females showed a later growth (October) compared to males (September) (Fig. 2e). There was a significant difference between sexes over the months in P2 for SL ($F = 4,623$; $p < 0.01$). We also observed that P2 females seem to maintain body mass during June and July, with an increase in body mass occurring in the final months of the cycle (Fig. 2h). Males, on the other hand, had a decrease in body mass from June to July, and an increase in mass in the last months
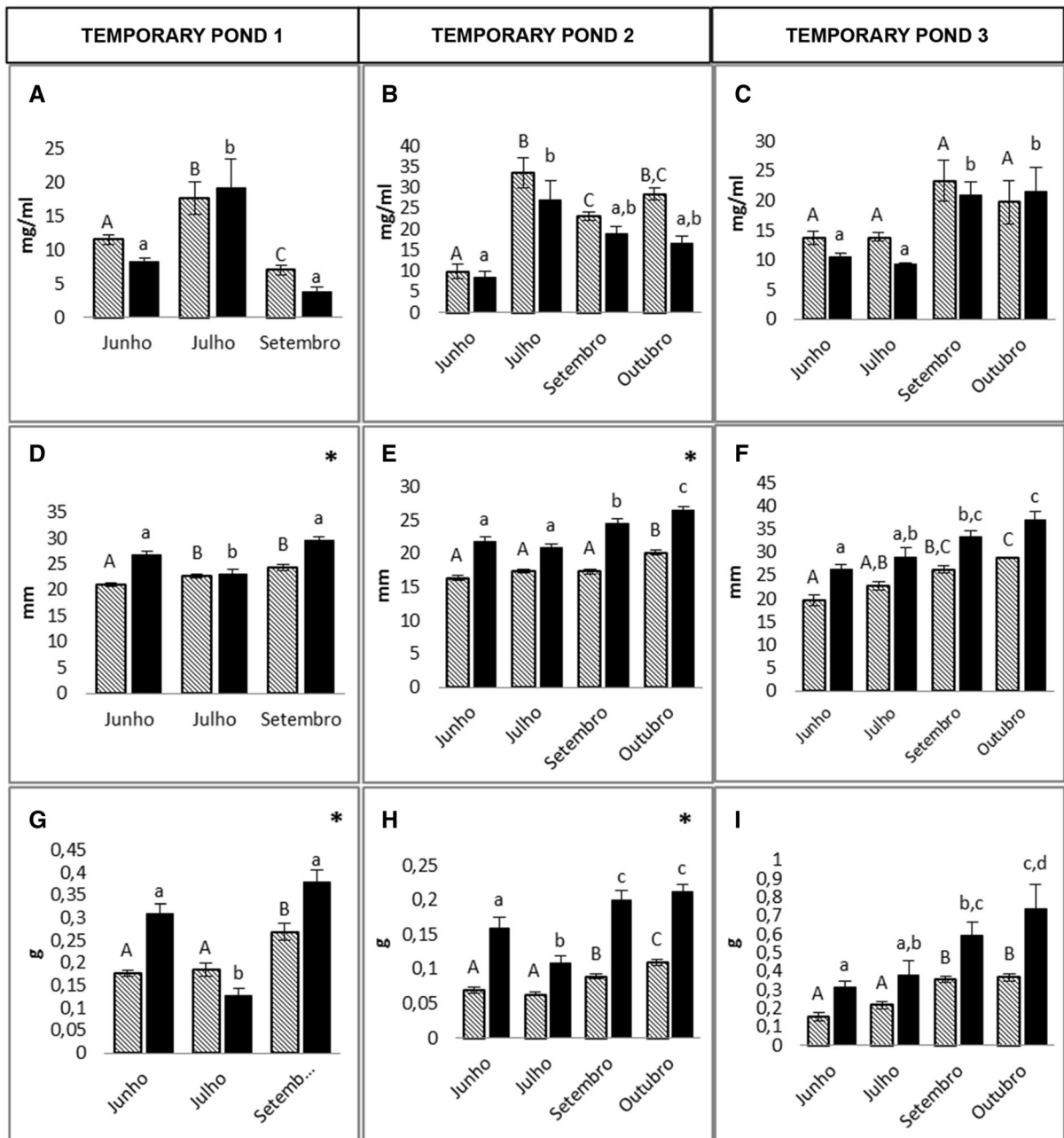
Fig. 2 Total protein levels (TP) (**a**, **b** and **c**), standard length (SL) (**d**, **e** and **f**), and body mass (**g**, **h** and **i**). Bars represent means ± standard error. White striped bars denote females (F), and black bars denote males (M). Different letters represent statistically significant difference (p < 0.05), with uppercase letters used for females and lowercase letters for males. Symbol "*" in the graph represents a statistical difference between males and females over the sampling period

(Fig. 2h). There was a significant difference between males and females along the cycle in P2 for body mass (F = 4,184; p < 0.01).

Temporary pond 3 (P3) data demonstrated increased levels of TP in females and males in the final months (September and October) when compared to the initial months studied (Fig. 2c). Moreover, we observed that both sexes presented a linear growth over the months in P3 (Fig. 2f). This same pattern was observed for body mass in P3 females and males,

which followed a linearity over the months. However, we observed an increase in the body mass of males in July, while females only in September (Fig. 2i). There was no significant difference between sexes over the months for TP (F = 0,684; p = 0.06), SL (F = 0,143; p = 0.93), and body mass (F = 1,587; p = 0.20) in P3.

When we compared the results of all temporary ponds, we observed that the levels of TP increased in July for P1 and P2 for both sexes (Fig. 2a, b), while this increase was only observed in the last months for P3 (Fig. 2c). The standard length presented a trend towards linear growth in females and males of P2 and P3 (Figs. 2e and 1f), whereas P1 presented increased growth in July (Fig. 2d). We observed an increase in female body mass in all temporary ponds in September (Figs. 2e and 1e–i). However, in P1 and P2 males, a decline in body mass was observed in July, followed by an increase in the following months (Fig. 2g, h). In P3, males had a linear increase throughout their life cycle (Fig. 2i).

Levels of oxidative damage

The level of lipoperoxidation (TBARS) in P1 females does not differ between the different moments of collection, although we observed an increasing trend throughout the post-hatch life cycle (Fig. 3). P1 males showed a significant increase in September when compared to the initial months (Fig. 3). There was no significant difference between sexes over the months in P1 for TBARS (F = 1,122; p = 0.34). The levels of carbonylated proteins (CP) in females and males of P1 were high in June, followed by a decline until the last month studied (September) (Fig. 3). There was a significant difference between males and females of P1 over the months for CP (F = 10,536; p < 0.01).

Females and males from temporary pond P2 presented a decrease in TBARS levels throughout the cycle (Fig. 4). The tests showed no significant difference between P2 sexes over the months for TBARS (F = 0,513; p = 0.68). Regarding CP, we observed a peak at the beginning of the cycle in both sexes, followed by a decline and a subsequent increase in October (Fig. 4). There was a significant difference between males and females over the months for CP (F = 35,225; p < 0.01).

We observed a peak in the TBARS results of P3 females during June, followed by a decline until September, which then reached similar values as

observed in the initial months (Fig. 5). In males, these levels were constant throughout the cycle (Fig. 5). The observed results of CP in P3 females and males fluctuated over the months, in which both sexes showed a peak in June and a decrease in these levels in the last month (October) (Fig. 5). There was no significant difference between P3 sexes over the months for TBARS (F = 2,441; p = 0.09) e CP (F = 2,891; p = 0.06).

When comparing the TBARS levels in all temporary ponds, we observed different patterns for P1, P2, and P3. The results of P2 and P3 indicated an increase in TBARS during June and a subsequent decline in these levels (Figs. 4 and 5). We observed a tendency to increased TBARS levels over the months in P1 (Fig. 3). The CP levels of females and males in all temporary ponds presented a peak in June and a reduction in July (Figs. 3, 4 and 5). In P2, we also observed an increase in CP levels during the last month (October) in males and females (Fig. 4). Whereas in P3, this increase occurred in September for females and males, with a subsequent decrease in the last month (Fig. 5).

Activity of antioxidant and biotransformation enzymes

We observed a peak in SOD activity in the last month (September) in P1 females and males (Fig. 3). There was a significant difference between the P1 sexes over the months (F = 3,656; p < 0.04). In addition, CAT activity levels in both P1 sexes remained constant throughout the cycle (Fig. 3). There was no significant difference in P1 for CAT (F = 0,726; p = 0.49). GPx activity in P1 females was constant throughout the cycle (Fig. 3), while P1 males presented a decrease in GPx activity in July and an increase in the activity at the end of the cycle (September) (Fig. 3). There was a significant difference between the P1 sexes over the months for GPx (F = 6,466; p < 0.02). The GST activity in P1 sexes followed the same pattern as SOD, with a peak in September (Fig. 3). There was a significant difference in P1 for GST (F = 4,652; p < 0.02).

The activity of SOD demonstrated that P2 males and females have higher enzyme activity at the beginning of the cycle (June), followed by a decline in the following months (Fig. 4). CAT in P2 females showed constant enzyme activity throughout the
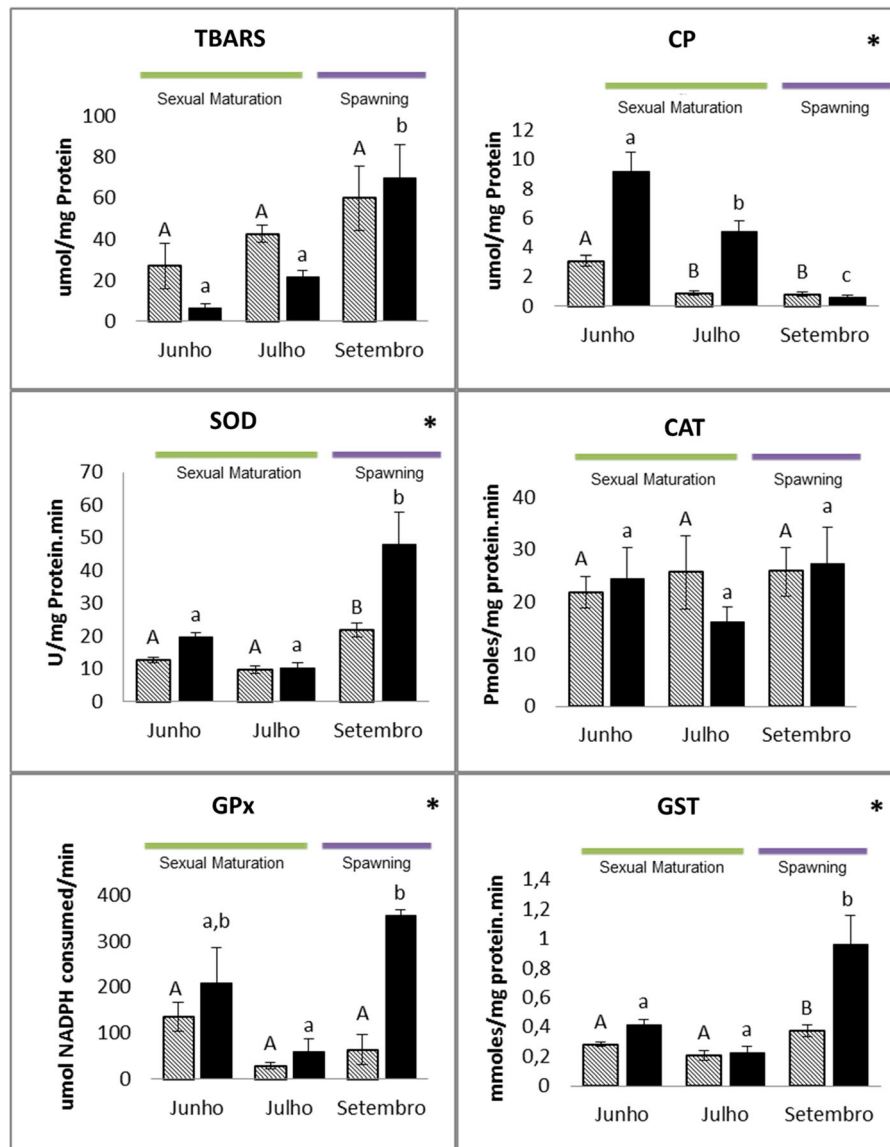
Fig. 3 Levels of lipoperoxidation (TBARS) and carbonylated proteins (CP), as well as the activity of antioxidant enzymes (SOD, CAT and GPx) and biotransformation (GST) in females and males of the species *Cynopoecilus fulgens* in temporary pond 1 (P1). Bars represent means ± standard error. White striped bars denote females (F), and black bars denote males (M). Different letters represent statistically significant difference ($p < 0.05$), with uppercase letters used for females and lowercase letters for males. Symbol "*" in the graph represents a statistical difference between males and females over the sampling period

cycle; however, in males, CAT activity peaked in June and decreased over the months (Fig. 4). The GPx activity for both P2 sexes remained constant throughout the cycle (Fig. 4). We observed that the GST enzyme presented the same pattern as SOD for both P2 sexes, with high activity at the beginning of the cycle, followed by a decrease (Fig. 4). There was no

significant difference between the sexes over the months for SOD ($F = 0{,}099$; $p = 0.96$), CAT ($F = 1{,}512$; $p = 0.22$), GPx ($F = 0{,}503$; $p = 0.69$), and GST ($F = 0{,}196$; $p = 0.90$) in P2.

In the temporary pond P3, the SOD activity data showed that females and males had higher enzyme activity in the initial months (June and July) and a
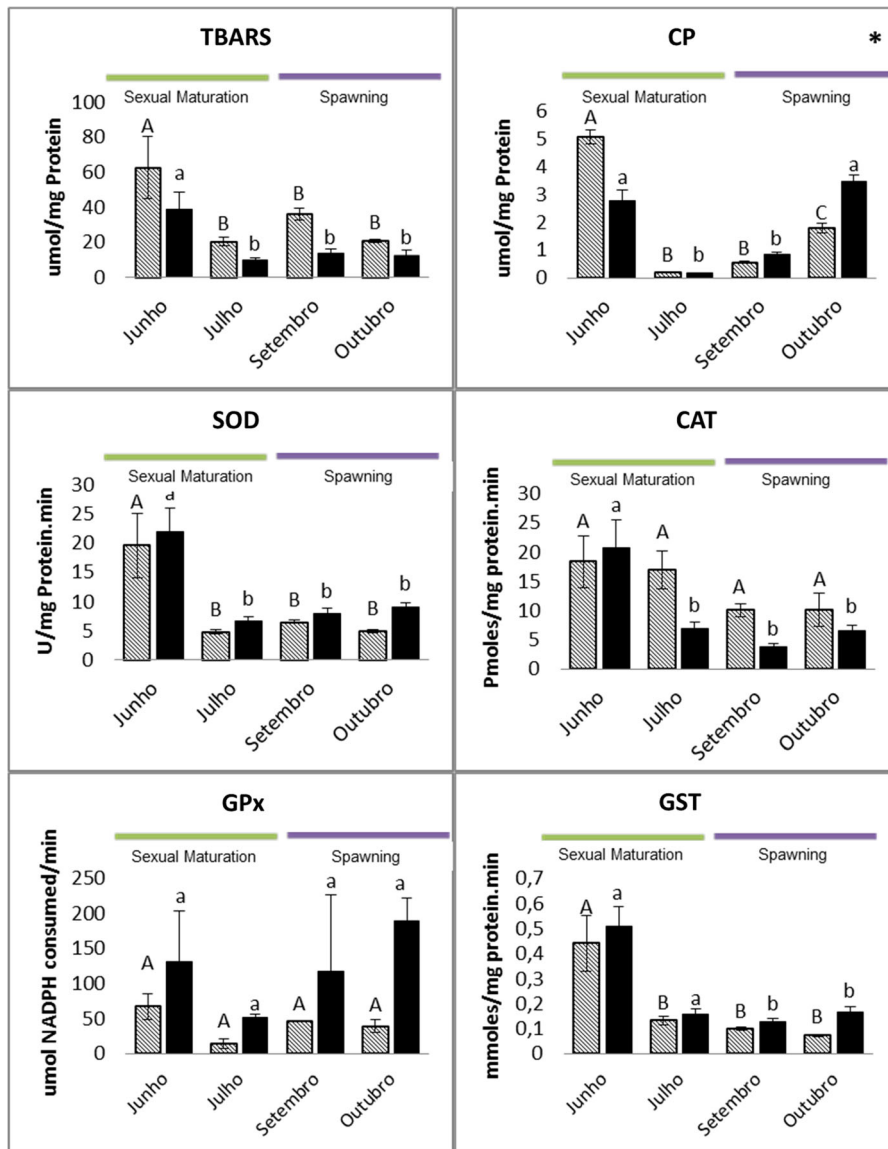
Fig. 4 Levels of lipoperoxidation (TBARS) and carbonylated proteins (CP), as well as the activity of antioxidant enzymes (SOD, CAT and GPx) and biotransformation (GST) in females and males of the species *Cynopoecilus fulgens* in temporary pond 2 (P2). Bars represent means ± standard error. White striped bars denote females (F), and black bars denote males (M). Different letters represent statistically significant difference (p < 0.05), with uppercase letters used for females and lowercase letters for males. Symbol "*" in the graph represents a statistical difference between males and females over the sampling period

decrease in the last months studied (September and October) (Fig. 5). We observed a significant difference between the P3 sexes over the months (F = 4,273; p < 0.02). CAT results remained constant for both P3 sexes (Fig. 5). There was no significant difference for CAT at P3 (F = 1,128; p = 0.36). Likewise, GPx activity in P3 females remained

constant throughout the cycle. In males, this activity peaked in July and declined in the following months (Fig. 5). There was a significant difference between the P3 sexes over the months for GPx (F = 6,475; p < 0.01). We observed that GST presented the same pattern of activity as SOD for both sexes, with a peak in the first two months and a decline in the final months
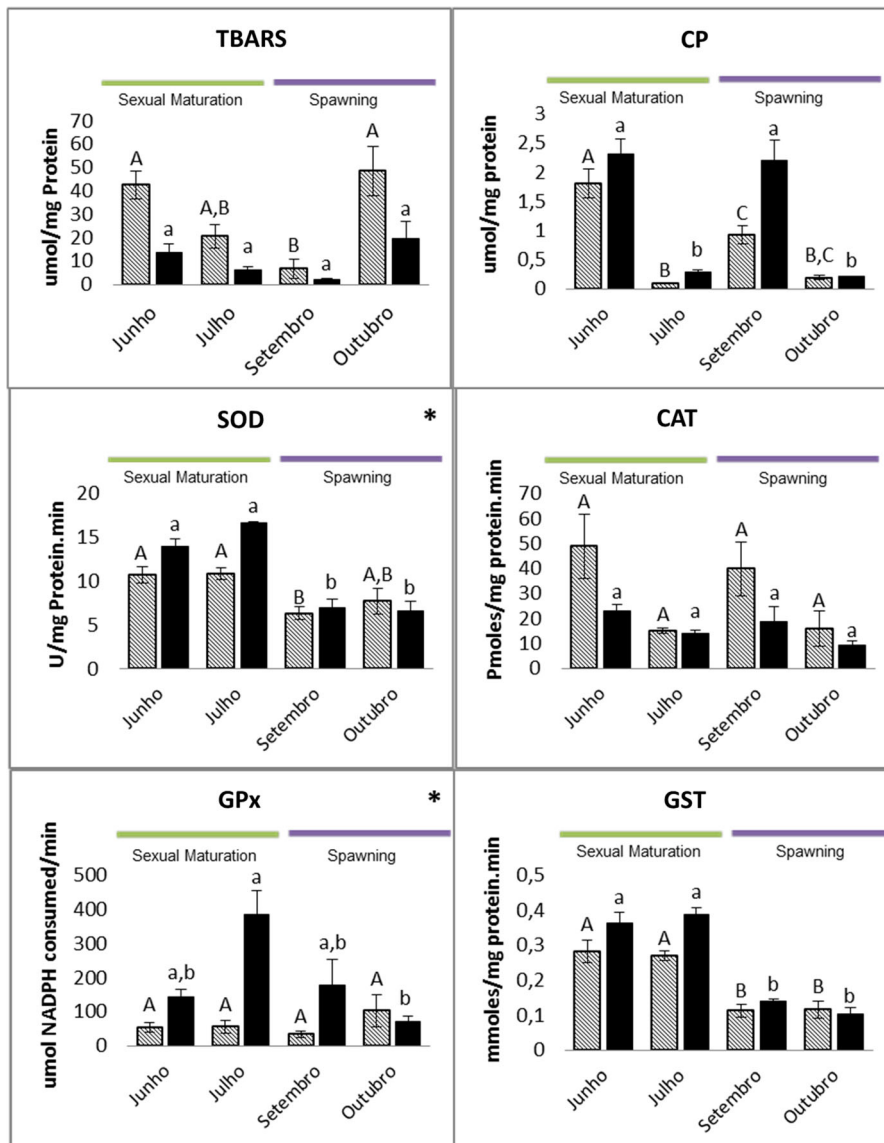
Fig. 5 Levels of Lipoperoxidation (TBARS) and Carbonylated Proteins (CP), as well as the activity of antioxidant enzymes (SOD, CAT and GPx) and biotransformation (GST) in females and males of the species *Cynopoecilus fulgens* in temporary pond 3 (P3). Bars represent means ± standard error. White striped bars denote females (F), and black bars denote males (M). Different letters represent statistically significant difference ($p < 0.05$), with uppercase letters used for females and lowercase letters for males. Symbol "*" in the graph represents a statistical difference between males and females over the sampling period

(Fig. 5). There was no significant difference in P3 for GST (F = 2,081; p = 0.13).

By comparing the temporary ponds, we observed that P2 and P3 presented similar enzyme activity in males and females (Figs. 4 and 5). In P1, we demonstrated a different behavior in some of these biomarkers, mainly in SOD and GST, for presenting a peak of activity in the last month (September) in both sexes (Fig. 3). In P2 and P3, these enzymes (SOD and GST) showed a peak of activity in the initial months in males and females (Figs. 4 and 5).

Finally, when all the temporary lagoons were grouped, we observed a change in the variation of the parameters of each temporary lagoon, showing that

each collection unit was differentiated (Fig. 6). The tests did not show any significant difference between the sexes over the months for each biomarker when the data from the three sample units were grouped (Fig. 6); however, differences were observed when we analyzed the temporary ponds separately (Figs. 3, 4 and 5).

Oxidative balance and environmental variables

The different environmental variables recorded varied during the months and temporary ponds (Table 1). We observed that the water temperature and oxygen levels greatly changed over time, featuring high water temperature, when the oxygen levels were lower
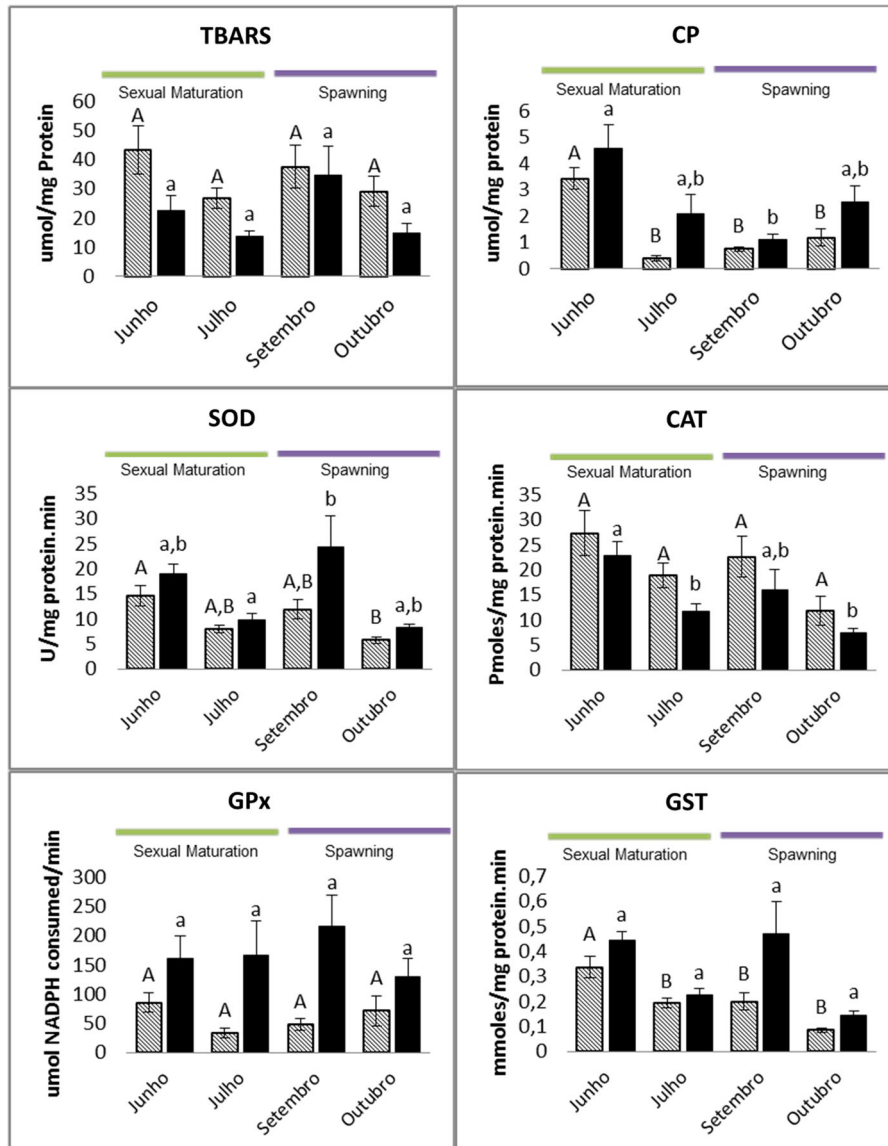

Fig. 6 Levels of lipoperoxidation (TBARS) and carbonylated proteins (CP), as well as the activity of antioxidant enzymes (SOD, CAT and GPx) and biotransformation (GST) in females and males of the species *Cynopoecilus fulgens* with all temporary ponds (P1, P2 and P3) grouped. Bars represent means ± standard error. White striped bars denote females (F), and black bars denote males (M). Different letters represent statistically significant difference (p < 0.05), with uppercase letters used for females and lowercase letters for males. Symbol "*" in the graph represents a statistical difference between males and females over the sampling period

Table 1 Abiotic water parameters in each temporary pond over the sampling period: Temperature (°C), pH, Conductivity (Ms/cm), O_2 (mg/L), ORP (Oxidation reduction potential—mV), Turbidity (NTU), TDS (Total dissolved solids—ppm), and OD (Oxygen dissolved in %)

Sampling Period	Temporary Ponds	Temperature	pH	Conductivity	O_2	ORP	NTU	TDS	OD
June	P1	12.8	6.5	0.16	14.9	240.7	86.3	0.02	149.9
	P2	8.7	5.6	0.04	10.7	268.3	15.9	0.03	93
	P3	14.2	5.5	0.11	10.8	249.3	210.3	0.07	107.7
July	P1	15.6	5.1	0.05	8.9	275	17.7	0.03	90.9
	P2	14.2	5.2	0.04	7.4	255.3	5.1	0.03	73.8
	P3	8.2	5.9	0.12	4.5	225.7	103.3	0.08	38.6
September	P1	17.3	5.1	0.04	6.1	263.3	22.5	0.03	64.4
	P2	19	5.2	0.04	6.5	273	8.2	0.03	71.2
	P3	14.6	5.9	0.12	5.2	279.3	37.7	0.08	51.6
October	P1	–	–	–	–	–	–	–	–
	P2	22.3	6.4	0.05	6.2	250.3	47.5	0.03	72.6
	P3	23.3	4.3	0.25	2.9	133.3	774	0.16	33.5

(Table 1). The recorded temperature values increased over the months, reaching between 8 and 23 °C, and 14 and 2 mg/L of dissolved oxygen, respectively (Table 1). These parameters were related to the size (area and depth) of each temporary wetland (Table 2). Regarding the measurements of each temporary wetland, we observed an increase in surface area and depth until September, followed by a decrease in October. The largest temporary wetland in terms of surface area was P3, while P2 was the greatest in depth (Table 2).

We observed that TP was only influenced by temperature, which explained approximately 11.5% of the variation ($R^2 = 0.1159142$, $F = 15.864$, $p = 0.001$) (Table 3). The TBARS results were influenced by the following factors: physical–chemical (15%), habitat (12%), and biotic (4%). Among the physicochemical, temperature explained 3% ($R^2 = 0.03413963$, $F = 7.096042$, $p = 0.009$), pH 14% ($R^2 = 0.14353308$, $F = 20.278078$, $p = 0.001$), NTU 2% ($R^2 = 0.02605025$, $F = 4.504172$, $p = 0.044$), and conductivity 4% ($R^2 = 0.04845357$, $F = 8.138135$, $p = 0.008$). In the habitat category, we observed an area that explained 9% ($R^2 = 0.09950035$, $F = 15.773540$, $p = 0.001$), and a depth 6% ($R^2 = 0.06272652$, $F = 12.386964$,

Table 2 Measure of each temporary pond over the sampling period: surface area (m²) and maximum depth (cm)

Sampling period	Temporary ponds	Surface area	Maximum depth
June	P1	871.2	25
	P2	544.6	29
	P3	2308.7	22
July	P1	932.6	29
	P2	746.7	25
	P3	2472.4	25
September	P1	1384.5	37
	P2	1378.5	46
	P3	3306.6	37
October	P1	–	-
	P2	244.1	27
	P3	4.7	20

Table 3 Environmental variables that had an influence on biomarkers

Biomarker	Adj. R^2 (set)	Environmental variables	R^2 (environmental variable)	p
TP	0.11	Water temperature	0.11	0.001
TBARS	0.41	pH	0.14	0.001
		Área	0.09	0.001
		Depth	0.06	0.002
		Conductivity	0.04	0.008
		Water temperature	0.03	0.009
		A. minuano abundance	0.03	0.020
		NTU	0.02	0.044
SOD	0.17	Water Temperature	0.10	0.001
		C. fulgens abundance	0.04	0.007
		A. minuano abundance	0.03	0.022
CAT	0.52	*A. minuano* abundance	0.36	0.001
		C. fulgens abundance	0.05	0.001
		Predator abundance	0.05	0.001
		Non-annual killifish abundance	0.04	0.002
		Área	0.02	0.018
GST	0.35	Water temperature	0.26	0.001
		A. minuano abundance	0.04	0.008
		Área	0.03	0.017
		Depth	0.02	0.029

Analyses performed in the R studio statistical program

p = 0.002). Finally, in the biotic factor, only the 3% abundance of *A. minuano* explained the variation ($R^2 = 0.03232224$, F = 5.816746, p = 0.020) (Table 3).

The variation of SOD activity was explained by biotic (7%), and physical–chemical (6%) variables. Among the biotic variables, the abundance of *A. minuano* explained 3% ($R^2 = 0.03523132$, F = 4.944652, p = 0.022), and the abundance of *C. fulgens* 4% ($R^2 = 0.04557978$, F = 6.700949, p = 0.007). In the physical–chemical category, temperature explained 10% ($R^2 = 0.10975228$, F = 14.917225, p = 0.001) (Table 3).

We observed an influence in the CAT activity by biotic variables (32%), and habitat (2%). Among the biotic variables, the abundance of *A. minuano* explained 36% ($R^2 = 0.36385923$, F = 69.209472, p = 0.001), non-annual presence 4% ($R^2 = 0.04579076$, F = 9.307852, p = 0.002), abundance of *C. fulgens* 5% ($R^2 = 0.05987385$, F = 13.431308, p = 0.001), and predator abundance 5% ($R^2 = 0.05359829$, F = 13.262510, p = 0.001). We only observed the area in the habitat variables, which explained 2% of the variation

($R^2 = 0.02093794$, F = 5.372941, p = 0.018) (Table 3).

The variation in GST was supported by the physical–chemical (16%), biotic (9%), and habitat (5%) variables. Among the physicochemical, we observed only the temperature (26%; $R^2 = 0.26917321$, F = 44.565907, p = 0.001). In the biotic category, only the abundance of *A. minuano* supported the variation (4%; $R^2 = 0.04294921$, F = 7.492474, p = 0.008). We observed that the area explained 3% ($R^2 = 0.03203049$, F = 5.811764, p = 0.017), and the depth 2% ($R^2 = 0.02529656$, F = 4.733948, p = 0.029) in the habitat category (Table 3). The environmental variables did not influence the carbonylated proteins (CP), and the glutathione peroxidase (GPx).

Discussion

This study contributes to the understanding of the temporal variation of oxidative stress in Neotropical annual fishes, obtained based on longitudinal data from the natural environment and post-hatch life cycle

of *C. fulgens*. Our study constitutes the first approach on the oxidative stress balance for a species belonging to the *Cynopoecilus* genus. These animals have a post-birth life cycle of 5 to 8 months with a proportionately long reproductive period (3 to 4 months), consisting of sexual maturity, spawning, and reproductive behaviors (cohort and agonistic interactions) (Berois et al. 2012; Gonçalves et al. 2011; Lanés et al. 2014). They possibly require a greater energy expenditure to obtain reproductive success, and a higher capacity of the antioxidant system to fight the ROS formed, helping to control homeostasis. These traits have been observed during maturation and reproduction in other animals (Braghirolli et al. 2016; Oliveira et al. 2007; Pinheiro and Oliveira 2016).

Our results show that males and females use different strategies for the redox balance during their life cycle, which is influenced by their reproductive period and by environmental variables. We observed that the individuals present in each temporary wet zone demonstrated different physiological responses to adaptation to their life cycle. Moreover, the environmental component served as a modulator of the life cycle of these animals (Figs. 3, 4 and 5). Bacanskas et al. (2004) also showed that antioxidant parameters were significantly affected by environmental conditions and reproductive status in male and female of *Fundulus heteroclitus*.

We observed a decrease in the number of individuals found in each temporary pond over the months, specially of males. We did not obtain records for October in P1 due to the earlier drying of this pond in relation to the other two (P2 and P3). According to Lanés et al. (2014, 2016), males have a higher mortality rate when compared to females in a natural environment. Considering the end of the post-hatch life cycle in October, we believe that these animals had reached their peak of sexual maturation in June, with reproductive interactions between July and August. We observed an increase in egg-laying in September, followed by a decrease in October, in which these animals reached the end of their life cycle. Lanés et al. (2014, 2016) reinforced such post-hatch life cycle profile.

Generally, females allocate a significant amount of nutrients for the development of megalecithal or polylecithal-eggs, characterized by a large amount of yolk (Wourms 1972; Arezo et al. 2005). Potentially, the yolk synthesis combined with environmental adversities seem to be decisive to prevent the body mass increase observed in P1 females between July and September. Males showed an increase in body mass only at the end of their life cycle (September). Linked to this variation in body mass, the levels of total protein showed maximum values in the post-sexual maturation period (July) in P1, followed by a reduction in September. This response pattern observed for both female and male may reflect an adverse environmental condition in this population.

In P2, we observed a peak of total proteins in July, in which reproduction begun, followed by a reduction in September and a subsequent increase at the end of the life cycle (October) (Fig. 2b). The maintenance of high TP levels in P2 and P3 (Fig. 2b and c) may be associated with the preservation of body mass in the months following maturation, i.e., the increase in TP may be helping and allowing these animals to invest in somatic growth. This hypothesis is reinforced by the result presented for P2 and P3 females since they seem to be investing in somatic growth in June and July. We suggest the usage of their reserves for reproductive events, specifically to sexual maturation and egg production (Fig. 2e, f, h and i). According to Vrtílek and Reichard (2015), due to environmental pressure determined by the imminence of drought, females of annual killifish should invest in breeding even if the food conditions in the environment are low. Females of *Nothobranchius furzeri* have the ability to modulate the allocation of energy for growth and reproduction in response to food conditions (Vrtílek and Reichard 2015).

On the other hand, males maintain a slow and gradual somatic growth, specifically those collected in P3, and an intensification at the end of life cycle (P2 and P3). Increased TP suggests that males invest more in somatic growth than females, and this pattern was indicated by the superior size of males throughout the cycle (body mass and standard length). Vrtílek and Reichard (2015) interpreted the energy allocation for growth as a future investment in reproduction. It can be related to a search for a reproductive partner as females can be very selective by preferring males with larger body sizes (Passos et al. 2013, 2014).

Throughout the post-hatch life cycle of P1 females, we observed a maintenance of lipoperoxidation levels and a decrease of carbonylated proteins from July to September. Increased SOD and GST activity at the end of the cycle coupled with the maintenance of CAT and

GPx activity seems to be decisive for the reduction of oxidative damage. These results suggest that females can maintain an efficient antioxidant system throughout their post-hatch life cycle, regardless of investing part of their energy resources for reproduction. In males, we observed increased lipoperoxidation levels in September while presenting increased antioxidant activity (SOD and GPx) and GST activity. Lanés et al. (2014, 2016) observed that males had a higher mortality rate when compared to females at this stage of development.

Higher levels of lipoperoxidation were observed in the predominant sexual maturation phase (June) of P2 females and P2 and P3 males (Figs. 4 and 5), followed by a decrease until the end of the life cycle. P3 females had a U-shaped variation in lipoperoxidation, where we observed higher levels in June (maturation) and October (senescence). For all temporary ponds, females had higher levels of lipoperoxidation when compared to males, indicating a peak of TBARS in the predominant sexual maturation phase. Moreover, we also observed reproductive behaviors in the period of highest energy demand, where females needed either-or to synthesize and allocate molecules to produce eggs (e.g., vitellogenin, vitamins, antioxidant molecules) (Wourms 1972; Arezo et al. 2005). High levels of carbonylated proteins corresponded to low levels of TBARS in all temporary ponds (Figs. 3, 4 and 5), suggesting an inverse relationship between lipid peroxidation and protein oxidation. Carbonylated proteins can be formed through the action of pro-oxidants, including EROs and lipoperoxidation products, which oxidize proteins by changing their structures and functions, possibly resulting in a situation of oxidative stress (Almroth et al. 2005; Moskovitz et al. 2010). The oxidation of lysine, arginine, proline, and threonine residues generates carbonyl derivatives, with exponential increase in natural aging (Moskovitz et al. 2010). Thus, the increase in carbonylated proteins at the end of the life cycle may be related to the deterioration in their quality of life.

There was an increase in carbonylated proteins in P2 females and males, and lipid peroxidation in P3 females at the end of their life cycle. Such response pattern corresponded to the decline in activity of antioxidant enzymes throughout the cycle. We observed a reduction in the activity of SOD and GST in both sexes and temporary ponds, coupled with a decrease in CAT in P2 and GPx P3 males (Figs. 4 and

5). These results show a close relationship between lipoperoxidation, protein oxidation, and the activity of antioxidant enzymes, which may characterize a possible process of functional deterioration of these organisms in the senescence phase. Once more, the results reinforce the hypothesis presented by Lanés et al. (2014, 2016) that females become prevalent in the environment at the end of the post-hatch life cycle. A similar aging process, but obtained under controlled laboratory conditions, has been recorded for African annual killifish, *Nothobranchius rachovii* and *N. guentheri* (Hsu et al. 2008; Liu et al. 2012). All cases showed an increase in lipid peroxidation and protein oxidation, and a decrease in antioxidant enzymes with advanced age. Godoy et al. (2020) studied the annual killifish, *Austrolebias minuano*, and males presented a reduction in all oxidative balance biomarkers throughout their life cycle, while females showed an increase in oxidative stress (TBARS) only during the growth period. These authors did not quantify the proteins carbonylated.

Annual killifish require high metabolic rates to maintain population survival under extreme conditions in ephemeral environments (García et al. 2008), including the effort spent in the reproductive period over a single season (Passos et al. 2014, 2021). High-energy investment in both sexes is required to allocate endogenous reserves that will be used to support reproduction (sexual maturation, spawning, and reproductive behaviors). Thus, with the mobilization of endogenous energy reserves and the increase in ATP synthesis to support reproductive events, we observe an increase in the production of ROS, which requires an increase in both the activity and possibly the gene expression of antioxidant enzymes to maintain the redox balance, as suggested for a freshwater crayfish (*Parastacus brasiliensis promatensis*) collected in a natural environment (Pinheiro and Oliveira 2016). The concept of hormesis can be applied to ROS that would be formed from a dose of non-lethal stress, but capable of inducing adaptive responses and beneficial effects to the body (Costantini 2014). Thus, when exposed to mild stress, such as sexual maturation, the body promotes an adaptive response based on signal transduction for an increase in the expression of antioxidant proteins and other defense proteins. Repair systems then become more efficient to future stress, allowing these organisms to survive and reproduce (Oliveira et al. 2018).

The animals in P1 showed a response profile of antioxidant enzymes that differs from the ones in P2 and P3. We observed increased activity of SOD and GST in P1 females and males at the end of the life cycle (September). CAT increased only in males. The variation range was wider in the levels of lipoperoxidation, carbonylated proteins, and of all antioxidant enzymes studied in P1 (SOD, CAT, GPx, and GST). These reached higher values than those observed in P2 and P3. Thus, reinforcing that some stress was added to P1, which may be associated with the early drying of the environment. For P2 and P3, SOD activity was higher in the sexual maturation phase (June), decreasing from July to the end of the life cycle (Figs. 4 and 5). This SOD activity profile was observed in both males and females, suggesting a preparation or anticipation of the antioxidant system given the increase in ROS produced possibly by reproductive events. During reproduction, these animals are potentially metabolically more active, shifting a significant amount of energy to reach sexual maturity and perform reproductive behaviors; therefore, maintaining or preventing high levels of TBARS and CP from being generated. These findings agree with the "Oxidative Stress Preparation Theory" proposed by Hermes-Lima et al. (2015) for animals facing extreme situations.

CAT activity in females remained constant in all temporary ponds, while the activity of this enzyme decreased over the life cycle of P2 males and remained constant in P1 and P3 males. CAT is a highly efficient antioxidant enzyme, found mainly in peroxisomes. It is associated with elevated concentrations of H_2O_2 and can decompose millions of hydrogen peroxide molecules in one second; therefore, completing the process initiated by SOD (Ighodaro and Akinloye 2018). The maintenance of catalase activity throughout the post-hatch life cycle seems to represent a high efficiency of the antioxidant system. GPX catalyzes the reduction of H_2O_2 derived from oxidative metabolism, as well as peroxides of lipid oxidation and is considered the most effective enzyme against lipid peroxidation, acting on low substrate concentrations (Trenzado et al. 2006). In our study, we suggested that GPx was working jointly with CAT to maintain TBARS and CP in both sexes (Figs. 3, 4 and 5).

Our study showed high levels of GST activity at the beginning of the post-hatch life cycle (sexual maturation period) in both sexes collected in P2 and P3 (Figs. 4 and 5). We suggest that GST may have an important function during the period of sexual maturation and pre-posture, acting possibly in the process of biotransformation of sexual hormones secreted in this stage. Freitas et al. (2008) and Pinheiro and Oliveira (2016) also suggest GST at this stage of the animals' life cycle; however, with ticks and crayfish, respectively. GSTs are present in different organisms and are important for the intracellular biotransformation of endo and xenobiotics (Papadopoulos et al. 2004). They act in the repair of macromolecules oxidized by ROS, the regeneration of S-thiolated proteins and the biosynthesis of physiologically important metabolites (Armstrong 1997; Sheehan et al. 2001).

In P1, GST levels increased in September in males and females, which may indicate an attempt to repair damage to macromolecules generated by the increase in the formation of ROS throughout the life cycle. Therefore, this helps maintain the levels of LPO in females and carbonylated proteins in both sexes (Fig. 6). Despite this increase in GST, we observed an increase in lipoperoxidation that may be associated with a decrease in glutathione reserves in males. Males presented higher GST activity values than females, which can be justified by territorial and aggressive behavior during reproduction. This pattern can also be explained by an increase in circulating testosterone levels since females of this species incite competition among males to increase the chance of breeding with the dominant one (Passos et al. 2013).

In addition, the oxidative damage generated by the characteristics of their life cycle (early maturation, long and intense reproductive activity, and production of a high number of resistant eggs) can be due to the interaction of these animals with their habitat, considering the earlier death in P1 than in P2 and P3. All oxidative damage markers (TBARS and CP) and antioxidant enzymes studied (SOD, CAT, GST, and GPx) maintain a broader and higher range of activity than those seen in other temporary ponds (P2 and P3). There is a concomitant aging of females and males that will culminate in their death after laying eggs while in the environmental drought period.

In our study, we observed wide variations in the water temperature, dissolved oxygen, and pH of the temporary ponds, in addition to a short duration period of these regions. In annual killifish, the stages of development are influenced by the abiotic conditions,

as these animals live in extremely variable environments, with fluctuations in temperature and availability of oxygen (Fonseca et al. 2013). Depending on the stage of life, these organisms have thermal preferences as seen for fish species (*Austrolebias nigrofasciatus*) that have a higher initial growth rate at higher temperatures (22 °C) and maintain this somatic growth with lower temperatures (Volcan et al. 2012). Likewise, a study carried out with the African killifish species (*Nothobranchius furzeri*) demonstrated that the reduction in temperature delays the onset of locomotor and learning deficits related to age, i.e., increases the life span (Valenzano et al. 2006).

The environmental variables and oxidative balance results demonstrated that the physicochemical and biotic factors influenced the biomarkers. Temperature stood out when analyzing the physical–chemical factors. In an annual killifish (*Austrolebias reicherti*), the temperature is the most critical and variable environmental parameter that adults must deal with (Passos et al. 2021). These authors observed in the final period of the puddles that the fish experienced large daily thermal amplitudes of more than 20 °C, and tolerated maximum water temperatures around 32 °C. These temperature changes at the end of the season were postulated as the crucial factor to induce fish death annually. Among the biotic variables, we observed an abundance of predators: *C. fulgens* (dense-dependent processes) and *A. minuano* (a sympatric species of annual killifish). It is worth mentioning that to obtain these results, the average values obtained for each of the oxidative stress markers in the three ponds (P1, P2, and P3) were grouped (Fig. 6).

The temperature influenced the levels of TP, TBARS, and GST. The increase in temperature stimulates metabolic processes such as the intensification of ROS production by increasing oxygen consumption, and this potentially leads to an increase in TBARS levels (Lushchak 2011). In another study, the increase in temperature was related to an increase in the exploratory activity of the *C. fulgens* and to gain body mass, which was associated with greater food spectrum (Lanés et al. 2016). We observed an association of these factors with the increased GST activity. In ticks, endogenous oxidative damage was generated by the products of the metabolism obtained from food intake, and associated with increased GST activity (Freitas et al. 2008). We also observed that the depth of the temporary ponds was an important factor

influencing the levels of TBARS and GST activity. This response profile was mainly observed in animals collected in P1, which presented drought in October, and where animals with the shortest life cycle were collected. According to Vaz-Ferreira and Sierra (1973), connections from temporary ponds to permanent bodies of water can contribute to the colonization of non-annual killifish species in these ponds. In addition, competitive and predatory interactions by non-annual killifish, macroinvertebrates, and birds can play a relevant role in decreasing abundance and eliminating annual adult fish in seasonal ponds (Lanés et al. 2014; Reichard 2018). In our study, the abundance of predators and *A. minuano* influenced the activity of CAT, as well as the abundance of *C. fulgens* influenced SOD, partially explaining their activities. The progressive decrease in the abundance of annual killifish species throughout their life cycle may be due to mechanisms of competition and predation (Lanés 2011). At the beginning of a rainy season, there is a greater abundance of these animals, followed by a decrease associated with an increase in the abundance of non-annual killifish. (Lanés 2011).

Conclusions

The annual killifish species, *C. fulgens*, inhabits ephemeral environments with highly variable abiotic and biotic conditions. These factors are combined with a post-hatch life cycle characterized by rapid growth, early sexual maturation, as well as intense and accelerated reproduction. These animals present a high energy demand and seem to use strategies to support such challenges. Based on this response profile, we suggest that the antioxidant system of these animals is highly efficient, maintaining low levels of lipoperoxidation and carbonylated proteins. Thus, these factors indicate the animals' ability to sustain homeostasis.

The fishes from P2 and P3 showed similar variation pattern of the biomarkers analyzed throughout the life cycle. While those present in P1 have a shortened post-hatch life cycle possibly due to environmental stressors combined with the peculiarities of this cycle. Consequently, the pattern and the time of the post-hatch life cycle associated with the senescence process can be significantly shaped by environmental aspects,

such as water temperature and the presence of predators.

The variable response of *C. fulgens*, which adjusted their antioxidant system to the different environmental conditions of each temporary pond studied, can be especially useful for investigating and testing modulations of the oxidative balance biomarkers in response to specific environmental conditions, both in studies with wild populations and under controlled laboratory conditions. Therefore, the use of Neotropical annual killifish species as a biological vertebrate model is highly promising and allows for in-depth studies of aging linked to toxicological tests at different stages of their biological development.

Author contributions BDC, LEKL, GTO conceived the study; LEKL, RSG, LM collected the animals, BDC, NMAW performed the biochemical analyzes and produced the results; BDC, LEKL, GTO analyzed and produced the data. BDC, SHS, LEKL, GTO wrote the paper and SHDS translated the paper. All Authors revised the paper.

Data availability Data will be made available on reasonable request.

Declarations

Conflict of interest Authors declare that there are no conflicts of interest.

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