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Original Article

Biochemical-functional differences between reproductive and non-reproductive males of *Procambarus clarkii* (Girard, 1852)

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ABSTRACT

Intermediate metabolism and oxidative balance markers help to better understand environmental dynamics and how they influence the physiological patterns of organisms. *Procambarus clarkii* (Girard, 1852), a crayfish native to the United States of America and Mexico, represents an appealing case study for understanding invasive species' metabolic dynamics. This species has sexual dimorphism and two male morphotypes: reproductive (M1) and non-reproductive (M2). We evaluated the seasonal variations of biomarkers in M1 and M2 males, collected in each season of 2016 at Parque Alfredo Volpi (São Paulo, Brazil). Hemolymph, hepatopancreas and abdominal muscle samples were extracted to determine markers of intermediate metabolism, oxidative balance, the hepatosomatic index and the stomach repletion degree. The results showed differences between the two male morphotypes. M1 showed a predominance of medium to full stomachs throughout the year, with an allocation of energetic substrates mainly used in reproduction (gametogenesis and reproductive behaviors). They also presented increased lipoperoxidation, SOD and GST activities. M2, on the other hand, had a lower capacity to allocate energy reserves in the period

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leading up to and during reproduction, which may be associated with a lower degree of gastric repletion. However, M2 maintained alternating antioxidant strategies that helped preserve low levels of lipoperoxidation in the hepatopancreas throughout the year. This pattern observed for the degree of gastric repletion indicates an intraspecific competition between the categories of males, which, together with the profile of energy reserve usage, seems decisive for M2 to reproduce.

KEYWORDS

Gastric repletion, Intermediate metabolism, Morphotypes, Oxidative balance, Red swamp crayfish, Reproduction

INTRODUCTION

Environmental variations impact the physiology and fitness of different animals (Mathieu-Resuge et al., 2020). In crustaceans, annual cycle variations in abiotic factors, i.e., temperature and salinity, regulate reproduction, growth, and periods of lesser or greater activity (Silva-Castiglioni et al., 2012; Jimenez and Kinsey, 2015; Valgas et al., 2020). Subsequently, these changes can alter their intermediate metabolism, which somehow tries to cope with any stressor source. Glucose is the primary circulating monosaccharide in the hemolymph of crustaceans and is used for the synthesis of many compounds, such as mucopolysaccharides, chitin, nicotinamide adenine dinucleotide phosphate (NADPH), glycogen, and pyruvate formation (Verri et al., 2001; Zhang et al., 2021). Glucose usage in the hemolymph is closely associated with biotic and abiotic variables, including molting, feeding, seasonality, salinity, and dissolved oxygen Jimenez and Kinsey, 2015).

A wide range of metabolites play critical roles in how organisms react to environmental changes. Lipids are essential in the metabolism and cell membranes of crustaceans and their primary energy source. Fatty acids are stored and preserved in tissues as triglycerides, with hepatopancreas being a key storage site (Chang et al., 1983; Riquelme-Bugueño et al., 2020). Moreover, amino acids are found in free forms or the form of proteins, with muscle being the leading protein storage site (Jimenez and Kinsey, 2015). The capacity of metabolic processes and routes in crustaceans is distributed in different tissues, where gluconeogenesis can occur in the hepatopancreas, gills, muscles (Oliveira and Da Silva, 1997; Jimenez and Kinsey, 2015). The hepatopancreas, however, is the main organ for synthesizing glycogen, lipids, and lipoproteins (Jimenez and Kinsey, 2015). Several crayfish species display seasonal variation in the anabolism/catabolism cycles for energy reserves (triglycerides, glycogen, and proteins) in different organs/tissues, which are associated with biotic and abiotic variables (Silva-Castiglioni et al., 2007, 2012, 2016; Buckup et al., 2008; Valgas et al., 2020).

Environmental changes can also affect the oxidative balance of crustaceans, which sometimes compensates for stressors (Pinheiro and Oliveira, 2016; Valgas et al., 2020; Fernández-Cisnal et al., 2018). During oxidation, reduction and hydrolysis processes of energy reserves, oxygen by-products with strong oxidative properties, known as reactive oxygen species (ROS), can be formed (Costantini, 2014; Dzal et al., 2015). ROS can cause damage to proteins, lipids, and nucleic acids (Gil-del Valle et al., 1999; Martínez-Cayuela, 1998), leading to metabolic dysfunction, apoptosis, and tissue damage (Halliwell et al., 1999; Wang et al., 2020). It can also act as an intracellular beacon modulating biochemical pathways (Schieber and Chaudel, 2014) and be used by the immune system in its interaction with pathogens, as well as in autoimmune reactions and the oxidation of chemical agents exogenous to the body (Apel et al., 2004; Guo et al., 2020a). Mitochondria and peroxisomes are important production sites of ROS (Blier et al., 2001). Animals have developed an antioxidant system to prevent damage caused by ROS, which is constituted by enzymatic and non-enzymatic systems (Sies, 1991; Stegeman et al., 1992; Nappi and Ottaviani, 2000; Schlenk and Di Giulio, 2002; Lesser, 2006; Acarlı et al., 2023). Superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) are among

the most well-studied antioxidant enzymes. These enzymes act by conjugating an endo or xenobiotic molecule to another endogenous molecule, causing less toxicity and facilitating its excretion (Richardson et al., 2010; Dantzer et al., 2014). Using metabolic and oxidative balance biomarkers helps to better understand the environmental dynamics and how they govern the physiology of animals throughout the year. Intrinsically, they are essential for conservation biology as they illustrate how periods of greater or lesser susceptibility affect organisms in a natural environment and help outline management strategies for invasive species (Beaulieu et al., 2013; Madliger et al., 2016).

The crayfish species, *Procambarus clarkii* (Girard, 1852), native to the south-central United States of America and northeastern Mexico is an interesting case study to investigate environmental impacts on biochemical-physiological aspects further. *Procambarus clarkii* has high plasticity and fertility with rapid sexual development, growth, and maturation (Suko, 1956; Momot, 1995; Powell et al., 2010; Bissattini et al., 2015; Peruzza et al., 2015; Bush et al., 2016; Goretti et al., 2016; Logarbo and Bonvillain, 2020; Dörr et al., 2020). These characteristics have facilitated its establishment when introduced into several countries, leading to the decrease and extinction of several populations of arthropods and amphibians (Cruz et al., 2008; Oficialdegui et al., 2020). This species has evident sexual dimorphism, and in males, there are differences between reproductive (M1) and non-reproductive (M2) adults. The M1 males have the first two pairs of the well-developed and highly calcified pleopods, adapted as copulatory organs. They also have copulatory hooks on the third and fourth ischiopodites, and the chelipeds are developed. M2 males have the first two pairs of developed pleopods, but they are poorly calcified, and they do not have copulatory hooks on the ischiopodites and the chelipeds are poorly developed, compared to M1 males. Both male stages are present in populations throughout the year and can switch between forms throughout their life cycle (Loureiro et al., 2018). Little is known about the physiological mechanisms and metabolic capacity that govern the change from one morpho stage to another in *P. clarkii*. To better understand these changes, we analyzed parameters related to the intermediate metabolism and oxidative balance in reproductive and non-reproductive males throughout the year.

MATERIALS AND METHODS

Fieldwork

During 2016, we collected 147 adult individuals of *P. clarkii* at the Alfredo Volpi Municipal Park in the City of São Paulo/SP, Brazil (23º35'16" S 46º42'09" W), of which 81 were reproductive males (summer=28; fall=20; winter=13; spring=20) and 66 were non-reproductive males (summer=19; fall=8; winter=20; spring=19). A trap with an attractive fish-flavored cat food was used to capture these individuals. All animals were collected in the central month of each season in Brazil (fall: April; winter: July; spring: October; summer: January) and transported according to the Brazilian legislation under license from Chico Mendes Institute for Biodiversity Conservation (ICMBio) (45775152/36548762). At collection we verified the dissolved oxygen levels and the water temperature using a digital field oximeter (Lutron DO-5519) and determined the pH using a commercial kit (Labcon Test pH tropical). Air temperature and precipitation data were obtained from the National Meteorological Institute (INMET) through access to the daily record of 2016 made by a meteorological station located in the region where the animals were collected.

The animals were weighed in the field using a Pesola (dynamometer with 0.25g precision), and the total length was measured using a digital caliper (Vonder 200mm). We collected the hemolymph using insulin syringes containing the anticoagulant 10% potassium oxalate. The animals were cryo-euthanized, and we excised the abdominal, hepatopancreatic and stomach muscle tissues. The frozen tissues were transported at -20 ºC in thermal boxes to the Conservation Physiology Laboratory at Pontifical Catholic University of Rio Grande do Sul in Porto Alegre/RS. Afterwards, the samples were stored in a -20 ºC freezer.

Morphometric parameters

To investigate their stomach content, we classified them, considering their degree of stomach repletion (SR), which was visually determined by the amount of food in them. We used a six-class scale (Williams, 1981; Silva-Castiglioniet al., 2016) and considered the following: class 1 as empty (0%); class 2 as partially empty (<5%); class 3 as empty to half full (5 to 35%); class 4 as half full (35 to 65%); class 5 as half full to nearly full (65 to 95%); class 6 as completely full (≥ 95%).

The hepatosomatic index (HI) was determined by considering the mass of the hepatopancreas (Mh) and the total mass of the individuals (Mt), and the following equation was used: HI=(Mh/Mt)*100. We compared the size of the individuals using the relationship between body mass and the total length of the animals by applying a power regression ($Y = a.X^b$). Y is the animal weight, a is the intercept, b is the coefficient of allometry, and X is the carapace length. *Procambarus clarkii* data were grouped (M1 + M2) to convert each measure to residues to eliminate the bias of the individual size and the pattern of allometric growth.

Intermediate metabolism

We quantified glucose, total proteins, uric acid, triglycerides, and total cholesterol in the plasma using the BioTécnica: Advanced Biotechnology commercial kits. These plasma metabolites were quantified in duplicates using spectrophotometry. Furthermore, total lipids were measured by the sulfophosphovaniline method (Frings and Dunn, 1970), and VLDL cholesterol was obtained by applying the mathematical relationship of TGL/5, where TGL is the value of circulating triglycerides in the hemolymph.

All biochemical analyses of tissue metabolites were analyzed with spectrophotometry and in duplicate. Glycogen was extracted according to the method of Van Handel (1965) and quantified as glucose after acid hydrolysis (HCl) and neutralization (Na_2CO_3). This was done using the commercial kit based on the BioTechnology glucose oxidase method (Advanced Biotechnology). The levels of total proteins in the tissues were measured using BioTechnics kits (Advanced Biotechnology). Total lipids in the tissues were extracted by employing the method of Folch et al. (1957) and measured by the sulfophosphovaniline method (Frings and Dunn, 1970). Triglycerides and total cholesterol were extracted using the method of Folch et al. (1957) and quantified using the BioTechnical spectrophotometry kits (Advanced Biotechnology).

Oxidative balance

The tissues were homogenized with ultra-turrax in a solution of phosphate buffer (20 mM), potassium chloride (140 mM) and a protease inhibitor (1 mM PMSF) in a proportion of 5 ml of this solution (1 g organ/tissue). After homogenization, the samples were centrifuged at 3000 rpm for 10 minutes at 4°C, and the supernatant from this centrifugation was collected, aliquoted and frozen at -20°C for further analyses of redox balance. All sample preparations and quantification were performed no later than three months after collection, as described by Pinheiro and Oliveira (2016).

We employed a technique based on inhibiting the reaction of the superoxide radical with adrenaline in order to quantify the superoxide dismutase enzyme (SOD). Quantification in relative units was used as it is impossible to determine the concentration of this enzyme nor its activity in terms of substrate consumed per unit of time. We quantified this enzyme using spectrophotometry at 480nm and expressed it as U.mg protein^{-1} (Cadenas, 1982). The activity of the antioxidant enzyme catalase (CAT) was quantified using the decay of the hydrogen peroxide, and it was detected at 240nm and expressed in pmol. mg protein^{-1}. min^{-1} (Boveris and Chance, 1973). Glutathione S-Transferase (GST) was measured by the conjugation of 1-chlorine 2,4 dinitrobenzene (CDNB) with reduced activity of glutathione (GSH), and it was detected at 340nm and expressed in mmol. mg protein^{-1}. min^{-1} (Habig et al., 1981). Lipid peroxidation was quantified using the method described by Buege and Aust (1978), whereby the biotic material for analysis is incubated in an acidic medium and heated to 80–100°C in the presence of thiobarbituric acid (TBA). The condensation of thiobarbituric acid-

reactive substances (TBARS) forms products that can be measured by visible light absorption (532 nm) (Lima and Abdalla, 2001). Concentrations were expressed as µmols TBARS.mg protein^{-1}.

Statistical analysis

The results were analyzed using the statistical programs SPSS 20.0 and Bioestat 5.0. We used the Kolmogorov-Smirinov normality test and considered the data non-parametric when the p-value was <0.05 and parametric when it was >0.05. We employed the Kruskal-Wallis test for the non-parametric data, followed by Dunn's complement test (p-value <0.05) for all biochemical parameters. Whereas for parametric data, the One-Way ANOVA and Tukey's tests were used for all biochemical parameters. To compare the results of adult males M1 (reproductive male) and M2 (non-reproductive male) throughout the year, we used the Mann-Whitney test (p-value <0.05) (Zar, 2010). Power regression was performed using an Excel non-linear regression routine (Microsoft 365), and normality test residues were compared by using Mann-Whitney (p-value < 0.05).

RESULTS

Environmental factors

Significantly lower values of air and water temperature, as well as precipitation, were observed in winter (17.8°C ± 0.31, 16.8°C ± 0.1, 3.14 mm ± 0.03, respectively) compared to summer (23.1°C ± 0.21, 22.75°C ± 0.75, 6.18 ± 0.04, respectively), spring (20.4°C ± 0.3, 20.8°C ± 0.2, 3.21 mm ± 0.03, respectively) and fall (19.9°C ± 0.46, 22.3°C ± 0.57, 3.84 mm ± 0.03, respectively). The oxygen levels (summer: 7.6 mg/L ± 2.98; fall: 5.9 mg/L ± 0.39; winter: 7.7 mg/L ± 0.32; spring: 7.6 mg/L ± 0.1) and pH (summer: 6.2 ± 0.1; fall: 6.4 ± 0.2; winter: 6.2 ± 0.1; spring: 6.4 ± 0.2) of the water remained constant throughout the year.

Morphometric parameters

No significant differences in body weight and total length of the M1 morphotype were found for the different seasons. On the contrary, we observed a significant variation in body weight of the M2 morphotype, where values were higher in the summer and winter when compared to the fall and spring (Table 1). Mean residuals and residual standard deviations derived from the adjusted power regressions (y= 0.0485. Carapace Length^{2,8077} and r^2= 0.7037) are shown in Table 1. The comparison of mean residuals (Mann-Whitney) between M1 and M2 morphotypes was significant (p <0.0001).

We observed a predominance of stomachs as half full to full categories (4 to 6) for M1 (summer: 67%; fall: 65%; winter: 69%; spring: 95%) and for M2 (summer: 52%; fall: 50%; winter: 80%; spring: 47%) (Fig. 1). All individuals showed significant variations in the hepatosomatic index. When comparing the behavior of this variable throughout the year, we observed that reproductive males had a gradual increase in the hepatosomatic index from fall to spring, reaching maximum values in the latter. M2 showed an increase in this variable in the fall, followed by spring (Fig. 1).

Table 1. Average values obtained for body weight and total length of carapace for each morphotype and season of the year. These parameters are expressed as the mean and standard error. Mean residuals and residual standard deviations derived from the adjusted power regressions (y= 0.0485. Carapace Length^{2,8077} and r^2= 0.7037) are presented in B. The different letters represent significant differences for p<0.05. M1: reproductive male. M2: non-reproductive male.

M1	**Summer**	**Fall**	**Winter**	**Spring**
Body weight (g)	23.37 ± 1.03^a	21.54 ± 1.42^a	23.54 ± 1.24^a	21.05 ± 1.34^a
Total length (cm)	8.46 ± 0.16^a	8.68 ± 0.14^a	8.43 ± 0.24^a	8.49 ± 0.22^a
M2	**Summer**	**Fall**	**Winter**	**Spring**
Body weight (g)	17.21 ± 0.98^a	9.95 ± 1.77^b	14.47 ± 0.75^a	11.07 ± 1.07^b
Total length (cm)	7.8 ± 0.2^a	7.73 ± 0.46^a	7.95 ± 0.18^a	7.27 ± 0.26^a
	M1		**M2**	**Significance (mean)**
Residuals	1.987 ± 3.592		-1.646 ± 3.147	p< 0.0001

Figure 1. Degree of gastric repletion and hepatosomatic index of reproductive (M1) and non-reproductive (M2) males. Blue and green bars refer to M1 and M2, respectively. A) Degree of gastric repletion in M1; B) degree of gastric repletion in M2; C) hepatosomatic index in M1; D) hepatosomatic index in M2. Different letters indicate significant differences. * indicates difference between the M1 and M2 curves throughout the year. Sun: Summer; Leaf: Fall; Snowflake: Winter; Flower: Spring.

Intermediate metabolism in the hemolymph

The comparisons made for M1 and M2 throughout the year showed an intermorphotypic difference in any studied metabolites. Regarding M1 males, we observed the maintenance of glycemic levels (p-value >0.05) throughout the year and a peak of total proteins in the summer, followed by a reduction in the spring. There was an increase in uric acid and total lipids in the spring and a reduction of total lipids in the winter (Fig. 2). Triglycerides and VLDL cholesterol, however, did not present significant differences. In contrast, total cholesterol decreased in the spring (Fig. 2). M2 males showed a decrease in circulating glucose in the winter. Their total proteins showed the highest concentrations in the fall, with a subsequent decrease in the winter and spring. Moreover, uric acid levels decreased in the fall, increased in the winter, and peaked in the spring. Total lipids showed their highest concentrations in the summer and spring. Triglycerides and VLDL cholesterol had the lowest values in the winter and total cholesterol in the spring (Fig. 2).

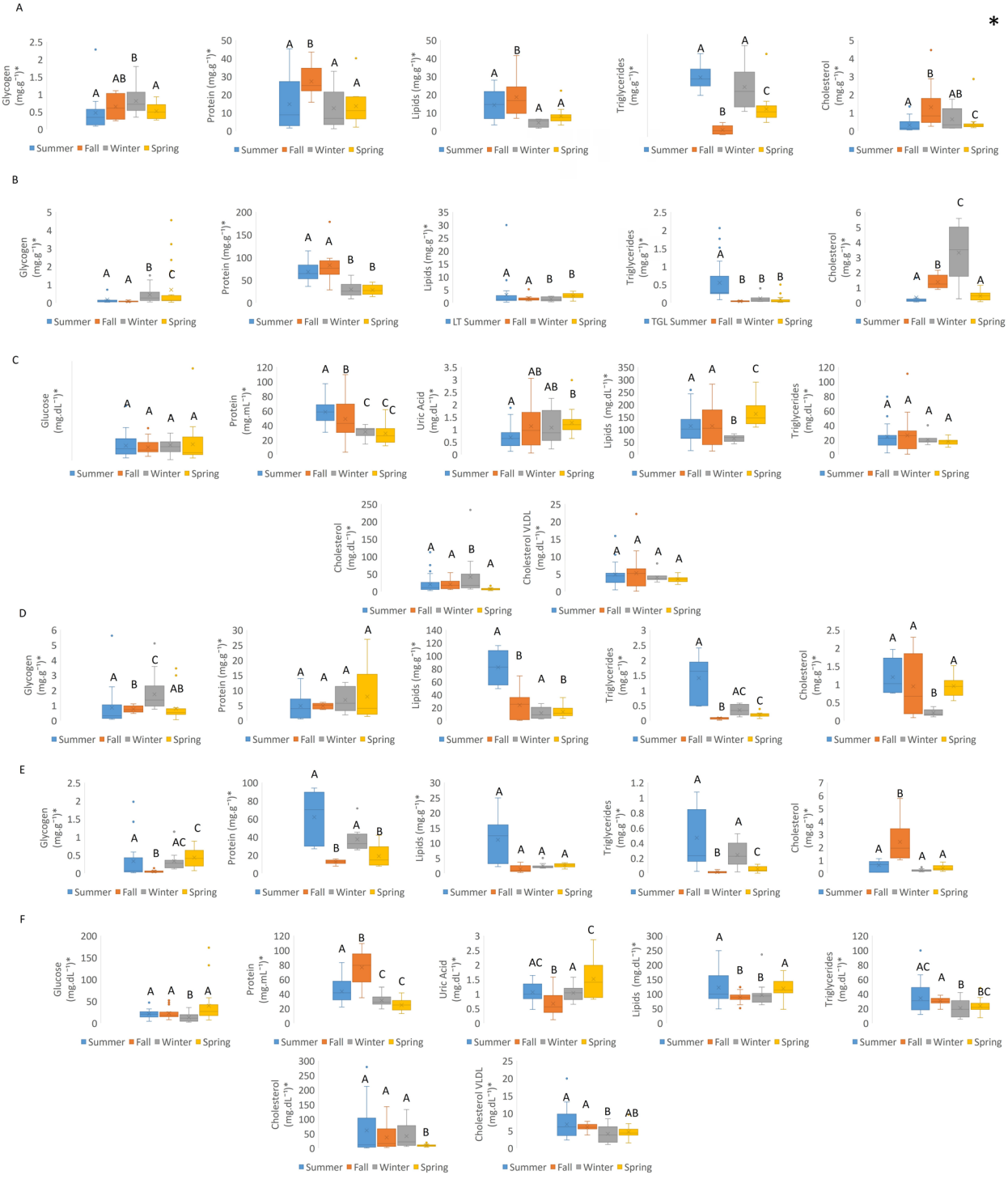

Figure 2. Intermediate metabolism comparisons of reproductive (M1) and non-reproductive (M2) males of *Procambarus clarkii* throughout the seasonal cycle. A – Hepatopancreas metabolites of M1 along the seasonal cycle; B – M1 abdominal muscle metabolites throughout the seasonal cycle; C – Hemolymphatic M1 metabolites throughout the seasonal cycle; D – Hepatopancreas metabolites of M2 along the seasonal cycle; E – M2 abdominal muscle metabolites over the seasonal cycle; F – Hemolymphatic M2 metabolites throughout the seasonal cycle; Different letters indicate significant differences. * indicates difference between M1 and M2 curves throughout the year.

Intermediate metabolism of the abdominal muscle and hepatopancreas

When analyzing tissue metabolites, the abdominal muscle of M1 had a peak of glycogen in the spring, total proteins in the fall, total lipids in the spring, triglycerides in the summer, and total cholesterol in the winter (Fig. 2). In the hepatopancreas, there were higher stocks of glycogen in the winter, total proteins and total lipids in the fall, and triglycerides in the summer. Whereas total cholesterol levels were lower in the summer (Fig. 2). For M2, we observed a decrease in muscle glycogen levels in the fall, with a subsequent increase in the winter and spring, as well as a peak in total proteins in the summer and winter (Fig. 2). We also observed an increase of total lipid levels in the summer, a decrease of triglycerides in the fall and spring, and an increase of total cholesterol levels in the fall. In the hepatopancreas, there were higher levels of glycogen in the winter, maintenance of protein levels throughout the year ($p > 0.05$), and an increase in total lipids, triglycerides, and total cholesterol in the summer (Fig. 2).

Oxidative balance

All parameters of oxidative balance showed an intermorphotypic difference for both M1 and M2 males throughout the year. In M1 males, high SOD activity in the muscle was observed in the summer, with a decrease in its activity in the spring. Whereas CAT activity peaked in the fall and spring, GST had a higher activity level in the summer, followed by a gradual reduction until spring. The LPO levels were higher in the summer, gradually decreasing until winter (Fig. 3). In the hepatopancreas, there was a reduction in SOD activity in the spring, while CAT showed increased activity in the fall and spring. We observed a reduction in GST levels in the summer, and LPO levels increased in the fall (Fig. 3).

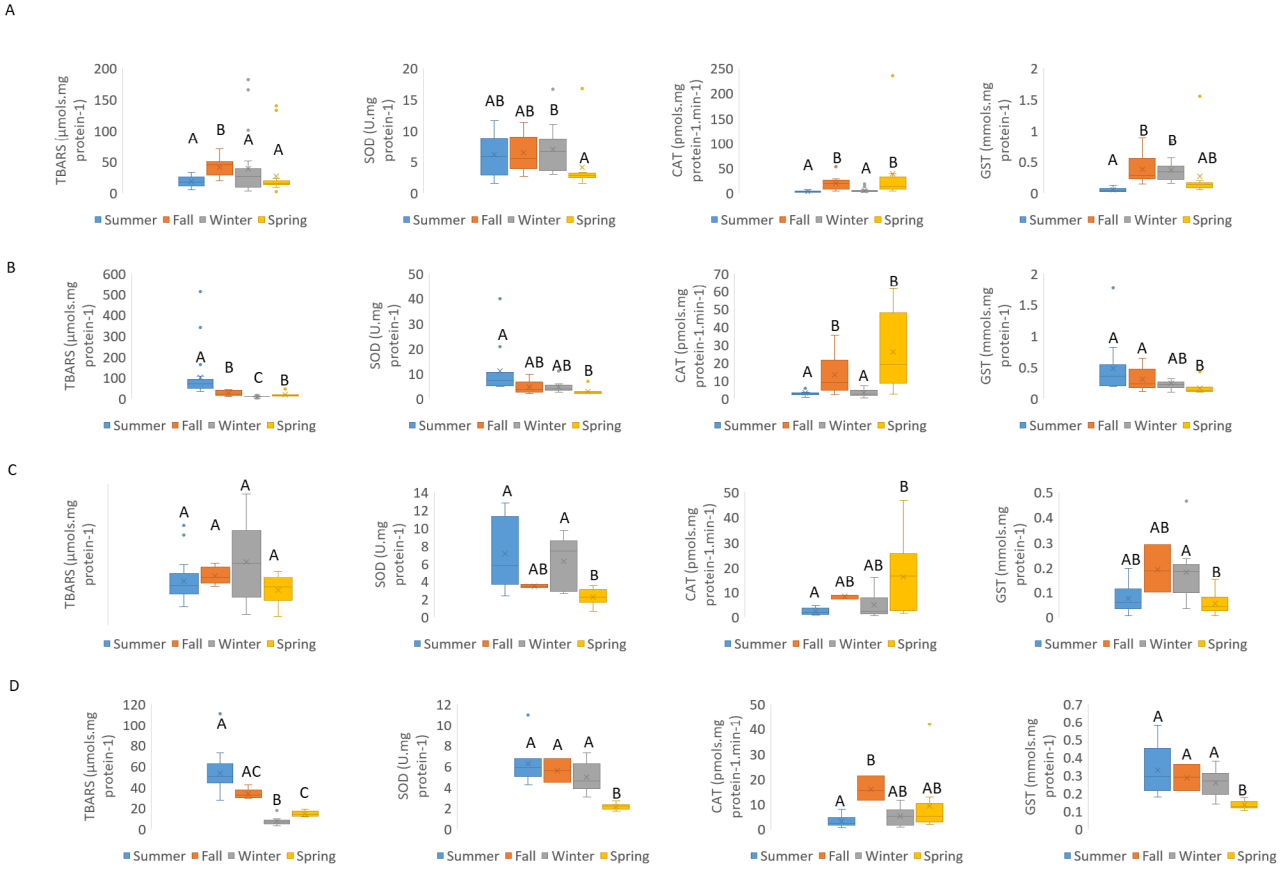

Figure 3. Enzymatic activity and lipoperoxidation level in the hepatopancreas and abdominal muscle of reproductive males (M1) and non-reproductive males (M2). A- Oxidative balance of the M1 hepatopancreas throughout the seasonal cycle; B – Oxidative balance of the M1 abdominal muscle throughout the seasonal cycle; C- Oxidative balance of the M2 hepatopancreas throughout the seasonal cycle; D – Oxidative balance of the M2 abdominal muscle throughout the seasonal cycle; Different letters indicate significant differences. * indicates difference between M1 and M2 curves throughout the year.

In muscle, we observed lower SOD activity during the spring in M2 males and increased CAT activity in the fall and spring. GST and LPO levels were higher in the summer while gradually decreasing until the spring and winter, respectively (Fig. 3). The hepatopancreas presented greater SOD activity in the summer and winter, while CAT showed an increase in the spring, and GST in the fall and winter. The LPO levels remained constant throughout all seasons ($p > 0.05$) (Fig. 3).

DISCUSSION

The present study is the first to describe the seasonal variation patterns of intermediate metabolism and oxidative balance markers in adult males of the crayfish *P. clarkii* from an unnatural distribution of this species. Reproductive males (M1) did not show seasonal variation in body mass or in carapace total length. The non-reproductive males (M2) showed a seasonal variation in body mass, where we observed the lowest values in the fall and spring. When comparing the residuals of the established power function for the body mass-total length relationship, a significant difference was observed, allowing us to conclude that reproductive animals maintain a larger body size when compared to non-reproductive individuals. M1 has a higher degree of exoskeleton chitinization and larger chelipeds (Taketomi et al., 1990), which could account for the observed difference. This pattern has also been previously demonstrated in *P. clarkii* morphotypes (Anastácio and Marques, 1998; Hamasaki et al., 2020). Valgas et al. (2020) found that *P. clarkii*, collected in the same location as the present study, reproduces throughout the year, with a reproduction peak during the summer months. Therefore, we suggest that these observed differences between M1 and M2 may be linked to the reproductive conditions of these males and the biochemical-physiological differences observed between them.

Seasonal effects on morphometric and metabolic biomarkers

M1 population showed greater feeding activity during the spring with 90% of the stomachs classified as 4 to 6 (half full to full),which may be attributed to the abundance of larvae and juvenile stages of dipterans in the Alfredo Volpi Municipal Park (Wilke et al., 2017). As a result, these full stomachs resulted in the storage of energetic substrates, mainly in the hepatopancreas, to be used during the observed reproductive peaks in the summer (Valgas et al., 2020). This pattern was also supported by an increase of HI during the spring and its subsequent decrease in the summer, indicating the allocation of hepatopancreas energy reserves to reproductive events. A similar response has been recorded for other decapods (Silva-Castiglioni et al., 2016; Brodie et al., 2017) and crayfish (Silva-Castiglioni et al., 2012). Non-reproductive males presented the lowest degree of gastric fullness during the fall, in the same period that M1 exhibited the highest values. Zizzari et al. (2016) observed that after the breeding season, reproductive males and females prevented non-reproductive males from food access. Similarly, *P. clarkii* females also showed higher degrees of gastric repletion after reproduction (Valgas et al., 2020). During the winter, the M2 morphotype presented more stomachs in 4 to 6 (80%) categories, while M1 presented 69% and females 60% (Valgas et al., 2020), possibly resulting from reduced intraspecific competition. There was an increase in HI during the fall and spring for M2 when greater glycogen storage in the hepatopancreas was also observed for this morphotype. This storage indicates a preference for using (catabolism) fats and proteins, which has been demonstrated for native crayfish (Silva-Castiglioni et al., 2012; Lu et al., 2020).

The maintenance of circulating glucose levels throughout the year in M1 is possibly due to their constant food intake, representing the predominance of stomachs in categories 4 to 6. In contrast, morphotype 2 presented seasonal variations in glucose levels in the hemolymph, as the lowest values observed in winter seemed to induce the animals to actively seek food. In this period, we observed that the stomachs were half full (80%) to full (4-6), combined with an increase in glycogen storage in the hepatopancreas. These results suggest an increase in glucose uptake by this organ, leading to an increase in glycogen synthesis. A similar pattern of low glucose levels in hemolymph and increased glycogen storage in the hepatopancreas was also observed in the crayfish *Parastacus varicosus* (Silva-Castiglioni et al., 2007).

During the fall, the decrease in proteins in the hemolymph in M1 was related to the potential uptake

of this metabolite by tissues. In this season, protein levels in both muscle and hepatopancreas were high, restoring the stocks of proteins that had been used during the reproductive season (summer). A similar pattern was observed when studying seasonal variation in *Aegla platensis* (Oliveira et al., 2007). However, the decline in protein levels was more pronounced in winter and spring compared to the fall. The catabolism of this substrate and the possible release of gluconeogenic amino acids may have been involved. These amino acids can be used for glucose synthesis, thus helping the maintenance of glycemia and glycogen synthesis since there is an increase in the reserve of this polysaccharide in the tissues, particularly in the muscle. The use of amino acids in gluconeogenic pathways has been demonstrated in other crustaceans (Jimenez and Kinsey, 2015; Lu et al., 2020). The increase in circulating uric acid reinforces the hypothesis of protein catabolism, as uric acid is synthesized from nitrogen through this process (Weihrauch et al., 2017; Guo et al., 2020b). It is worth noting that uric acid also plays an antioxidant role, being an electron donor in the biotransformation process (Squadrito et al., 2000; Glantzounis et al., 2005; Zanette et al., 2015).

In contrast to M1, there was a peak of circulating proteins in M2 during the fall, when there was also a prevalence of empty stomachs and a decrease in muscle proteins, indicating catabolism and the release of amino acids into the hemolymph for possible use in the gluconeogenic pathways and/or ATP production. In a diet experiment, this has also been demonstrated for the estuarine crab *Neohelice granulata* (Dana, 1851) and *P. clarkii* (Oliveira and da Silva, 1997; Tang et al., 2020). Regarding uric acid levels in M2, the lowest circulating levels were observed in the fall, in contrast to the higher hemolymphatic levels of proteins. Uric acid increased progressively in the subsequent seasons (winter and spring), in parallel with the decrease in proteins in the hemolymph in these seasons. This pattern indicates the uptake and metabolism of proteins circulating in the tissues, with subsequent production of uric acid from nitrogen by-products generated in the degradation of proteins (Weihrauch et al., 2017).

The total lipids in the hemolymph of M1 decreased during the winter, which may indicate the usage of this energetic substrate in aerobic pathways for ATP synthesis (Jimenez and Kinsey, 2015). In the following season, a peak of circulating lipids agreed with the greater M1 feeding activity. The allocation of this substrate from the gastrointestinal tract to the hemolymph has been demonstrated in *A. platensis* when subjected to periods of fasting and then refeeding (Silva-Castiglioni et al., 2016). For M2, the lowest levels of total lipids were found in the fall and winter. They may be allocated for growth, as noted in the crab *Portunus trituberculatus* (Han et al., 2017).

Triglycerides and VLDL cholesterol levels in the hemolymph did not change throughout the year in M1. In crustaceans, triglycerides are the main source of substrate for ATP synthesis (Jimenez and Kinsey, 2015). Our findings suggest that VLDL cholesterol may play a role in transporting triglycerides from the gastrointestinal tract to the tissues since the stomachs remained medium to full during all year. In M2, a decrease in hemolymph triglycerides and an increase in tissue reserves during the winter indicated a direct passage of triglycerides from the gastrointestinal tract to different tissues. The advantage of storing triglycerides is that, as they are hydrophobic, the body can store them in an anhydrous way (Nelson et al., 2014). Vinagre and Da Silva (1992) observed that up to 20% of the hepatopancreas mass comprises fats in crabs (*Neohelice granulata*). During the spring, circulating triglyceride levels remained low and insufficient, with a depletion of tissue reserves. The decrease of total circulating cholesterol in M1 during the spring may be related to its usage in the synthesis of sex hormones in preparation for the reproductive peak, as has been suggested for different decapod crustaceans (Lafont and Mathieu, 2007; Silva Castiglioni et al., 2007, 2012; Vinagre et al., 2007; Buckup et al., 2008; Musin et al., 2017; Kumar et al., 2018). In M2, the depletion of cholesterol during the winter can be associated with its usage in ecdysteroid synthesis and the composition of cell membranes (Jimenez and Kinsey, 2015; Fanjul-Moles et al., 2011; Tian et al., 2020).

The reduction of muscle protein stock during the winter and spring in M1 seems to be associated with using this energy substrate for the synthesis of glycogen and/or ATP, with a subsequent production of uric acid, which was high during this period. In the hepatopancreas, the increased protein levels in the

fall could be attributed to greater uptake of amino acids by this tissue, leading to decreased circulating proteins in the hemolymph after the reproductive period. A similar pattern was reported by Dutra et al. (2008) in a study with *Parastacus brasiliensis* (von Martens, 1869). The decrease in protein reserves in the M2 muscle agreed with periods of reduced gastric repletion in the fall. This reduction may be associated with hierarchical agonistic interactions in the dispute for food with the reproductive males (M1) (Issa et al., 1999; Figler et al., 2005; Horner et al., 2008). There was no variation in protein levels in the hepatopancreas, indicating that the muscle tissue serves as the main reserve of proteins for the body, a fact already reported for crustaceans by Musin et al. (2017) and Jimenez and Kinsey (2015).

The allocation of lipids in the muscle has a similar pattern to that observed in glycogen, with the transfer of energetic substrates coming directly from the diet (Gao et al., 2020). Total lipids in M1 increased during the spring likely due to a diet rich in proteins and fats from arthropods (Wilke et al., 2017). Regarding the hepatopancreas, there was an increase in lipid reserves after the end of the reproductive peak (fall). Oliveira et al. (2007) and Jimenez and Kinsey (2015) suggest lipid reserves are reallocated to the hepatopancreas since this is the main site of triglyceride reserves. In M2, there was a peak of muscle and hepatopancreatic lipid reserves in the summer, possibly related to a diet rich in fats (naiads, insect larvae and roots) (Davis and Robinson, 1986; Gutiérrez-Yurrita et al., 1998). A diet rich in fatty acids and proteins accelerates the growth of *P. clarkii* juveniles (Oliveira and Fabião, 1998), which could benefit M2 and prepare them for reproduction in the subsequent season.

The storage of triglycerides in the muscle and hepatopancreas of M1 during the summer revealed a preference for using carbohydrates to support reproductive activities. The increase in this reserve in the hepatopancreas during the winter suggests a preparation for the next reproductive cycle, as Jimenez and Kinsey (2015) pointed out for crustaceans. M1 seemed to use triglycerides at the beginning of reproduction, considering it is a period of high energy expenditure in gamete production. During peak reproduction periods, they store triglycerides and use carbohydrate reserves to sustain reproductive events. The increase in triglycerides in the summer and winter in all M2 tissues may be associated with their feeding behavior and a preference for fatty foods, as evidenced by the predominance of full stomachs and an increased fat content.

The total cholesterol levels in the tissues of M1 increased during the seasons preceding and following the periods of greater reproductive activity. High cholesterol levels may be associated with the synthesis of sex hormones and ecdysteroids (Jimenez and Kinsey, 2015; Tian et al., 2020). A spike in cholesterol was observed in M2 during the fall; however, in the hepatopancreas, there was a decrease in the winter, which may be associated with molting. Cholesterol is an important component of the structure of cell membranes and is a substrate for the synthesis of ecdysteroid hormones, making it essential during periods of increased investment in growth as suggested for *Macrobrachium nipponense* and *P.clarkii* (Gu et al., 2017; Tian et al., 2020).

Oxidative balance differences in reproductive and non-reproductive males

The higher levels of lipoperoxidation and activity of the superoxide dismutase and glutathione S-transferase enzymes in M1 muscle during the summer seemed to be related to the stress generated by reproduction. During this time there is an increased metabolic rate as individuals must deal with increased search for reproductive partners and agonistic disputes over territory (Figler et al., 1995; Daws et al., 2002). Despite the increase in SOD and GST activities, they were insufficient to prevent oxidative damage, with low CAT activity and possible damage caused by hydrogen peroxide arising from the dismutation of the superoxide anion by SOD. However, we did not measure glutathione peroxidase activity in these animals. In *Parastacus promatensis* Ferreira Fontoura and Conter, 2008, there is also an increase in SOD and GST activities and lipoperoxidation, and a decrease in CAT (Pinheiro and Oliveira, 2016). The higher CAT activity in the fall could be related to greater post-reproductive feeding activity due to the high metabolism of energetic substrates. In the spring, higher CAT levels are important as they precede the reproductive period and work to prevent oxidative

damage generated by the greater energy demand (Peng et al., 2010). During the fall, the increase in the activity of CAT, GST, and lipoperoxidation levels in M1 hepatopancreas may indicate a greater metabolic activity in this organ, mainly related to the synthesis of energy reserves (glycogen, proteins, and lipids), as demonstrated in *Parastacus defossus* Faxon, 1898 and *Parastacus brasiliensis* (see Silva-Castiglioni et al., 2012), and *Parastacus promatensis* (see Pinheiro and Oliveira, 2016). There was a decrease in SOD and an increase in CAT activities in the spring, suggesting an alternation in the SOD/CAT system to reduce energy expenditure (Fanjul-Moles et al., 2011).The decrease in SOD and GST activities in the M2 muscle occurred along with the lower levels of lipoperoxidation in the spring. On the other hand, CAT presented greater activity during the fall, which could be associated with growth events. During ecdysis, increased tissue activity generates more hydrogen peroxides, and CAT acts to prevent oxidative damage during this period of greater susceptibility (Salaenoi et al., 2014). In the hepatopancreas, there were no variations in the level of lipoperoxidation, which was possibly due to an alternation of antioxidant defense strategies with greater activity of the SOD system in the summer, CAT/GST in the fall, and SOD/GST in the winter. This was linked to the absence of reproduction in this morphotype. In the spring, there was more CAT activity and higher circulating levels of uric acid, a non-enzymatic antioxidant. GST acts on by-products of metabolic pathways by conjugating them to the glutathione molecule (GSH), thus making them less toxic and easier to excrete. This process helps prevent the formation of ROSs (Harayashiki et al. 2016).

Concluding remarks

There was a clear distinction in the seasonal variation profile of intermediate metabolism and oxidative balance between M1 and M2 morphotypes. In addition, M1 has a greater predominance of full stomachs throughout the year and a more efficient allocation of energy substrates to maintain its reproductive activity. On the other hand, M2 has less capacity to store its energy reserves since these are used to sustain possible events of ecdysis and their survival. M1 seems to have a hierarchical domain over M2, as observed by the degree of gastric repletion in M2 and the relationship between body mass and total length. We also observed differences in the balance of anabolism/catabolism of energy reserves. This array of responses can be paramount in restricting the ability of M2 to reach reproductive status during the annual cycle. Further studies are needed to assess the impact of multiple environmental stressors on reproductive capability. Such findings would help establish better management and control plans for the species in environments where it is considered invasive.

ABBREVIATIONS

M1, reproductive males; M2, non-reproductive males; TGL, triglycerides; TL, total lipids; TP, total proteins; GG, glycogen; SOD, superoxide dismutase; CAT, catalase; GST, glutathione-S-transferase; LPO, lipoperoxidation; ROS, reactive oxygen species; *P. clarkii*, *Procambarus clarkii*; ICMBIO, Instituto Chico Mendes de Conservação da Biodiversidade; PUCRS, Pontificia Universidade Católica do Rio Grande do Sul; RS, Rio Grande do Sul; SP, São Paulo.

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ADDITIONAL INFORMATION AND DECLARATIONS

License

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Author Contributions

Conceptualization and Design: AANV, GTO; PBA Performed research: AANV, NMAW, GTO, PBA Acquisition of data: AANV; Analysis and interpretation of data: AANV, NMAW, SHDS, GTO, PBA Preparation of figures/tables/maps: AANV; Writing – original draft: AANV, NMAW, SHDS, GTO, PBA; Writing – critical review and editing: AANV, GTO; PBA.

Consent for publication

All authors declare that they have reviewed the content of the manuscript and gave their consent to submit the document.

Competing interests

The authors have no conflicts of interest to declare.

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All data generated and analyzed during this study are presented in this article.

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