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Biochemical-functional parameters of red swamp crayfish *Procambarus clarkii* (Girard, 1852) Crustacea, Cambaridae female throughout a seasonal cycle in southeast Brazil

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ABSTRACT*Procambarus clarkii* is a freshwater crayfish native to the southern central United States and northern Mexico. In Brazil, it is only present in natural environment in the state of São Paulo. Nutritional and reproductive markers were quantified and characterized, as well as intermediate metabolism and oxidative balance in females of P. clarkii collected in a natural environment over a seasonal cycle. Samples of hemolymph and different tissue were obtained. The degree of gastric repletion presented the highest rates in the spring. An investment in reproduction was detected in the summer, when the energy reserves of the hepatopancreas were mobilized for gonadal maturation, and a higher percentage of mature gonads were observed. In the same period, we observed an increase in lipoperoxidation, despite the increased activity of superoxide dismutase and glutathione S-transferase in muscle and gonads. An increase in the levels of lipoperoxidation and glutathione S-transferase was observed in winter.

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Crayfish; female; reproduction; degree of stomach repletion; intermediate metabolism; oxidative balance

1. Introduction

Crustaceans exhibit seasonal variations in behaviour, growth and reproductive activity (Thongda et al. 2015). In general, reproduction or reproductive peak occurs in mild average temperature months, during the autumn or spring in the southern hemisphere, showing depletion of energy reserves in order to maintain reproductive activity. At lower temperatures, there is a decrease in the metabolic rate and an allocation of nutrients from the digestive tract to the tissues, specifically for hepatopancreas and muscles (Oliveira et al. 2007; Silva-Castiglioni et al. 2007, 2008, 2012; Thongda et al. 2015; Pinheiro and Oliveira 2016).

Reproduction is a high energy cost process, especially in females as there is a need for significant nutrient transfer, sometimes from diet and/or different tissues for

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gonadal maturation (gametogenesis, yolk synthesis and other oocyte components) and parental care (Oliveira et al. 2007; Jimenez and Kinsey 2015; Pinheiro and Oliveira 2016). The energy spent on reproduction is usually associated with an increase in metabolic demand and oxygen consumption, leading to an increase in food intake, decreased growth and increased susceptibility to predators (Guadagnoli et al. 2005: Berglund and Rosenqvist 2016). Several studies have shown allocation of lipid and protein reserves from the hepatopancreas and/or digestive tract to the gonads during gonadal maturation (Girish et al. 2014; Jimenez and Kinsey 2015; Silva-Castiglioni et al. 2015). However, the high energy demand in the gonads and the increased oxygen uptake to support reproductive behaviour may lead to the higher formation of reactive oxygen species (ROS) (Lesser et al., 2006). The production of ROS is an important biochemical-functional response for aquatic animals exposed to different environmental stressors, acting in normal cellular functions as intra/extracellular flags or as second messengers modulating the expression of specific genes (Lesser et al., 2006). Since ROS has a strong oxidative property, when in high concentrations, they can lead to apoptosis, as well as metabolic, tissue and systemic dysfunctions, and are usually related to damage to proteins, lipids and nucleic acids (Martínez-Cayuela 1998; Halliwell 1999; Gil-del Valle et al. 1999).

Procambarus clarkii is a freshwater crayfish native to the southern central United States and northern Mexico, which has high physiological plasticity (Powell and Watts 2010; Bissattini et al. 2015; Peruzza et al. 2015; Bush et al. 2016; Goretti et al. 2016), high reproductive investment and rapid development (Suko 1956; Momot 1995). The species is considered invasive in many countries and is related to the decline of amphibian and arthropod communities (Cruz et al. 2008). According to Loureiro et al. (2015a) this crayfish is one of the most widely introduced freshwater species in the world, mainly due to its high economic importance, being responsible for major changes in invaded environments, causing irreparable ecological and economic damage. In Brazil, it has already been introduced in a natural environment, and populations are observed in the state of São Paulo (Loureiro et al., 2015b). The ability of this crayfish to successfully colonize a wide variety of environments seems to be related to its behaviour and biological characteristics, which gives it a remarkable ecological plasticity. Thus, studies aiming to obtain and standardize functional markers for this species, in the different colonization sites, are necessary and can constitute a tool for the management of these populations.

Freshwater crayfish are animals that have high energy investment linked to reproduction (Oliveira et al. 2007; Silva-Castiglioni et al. 2007, 2008, 2012; Pinheiro and Oliveira 2016; Yazicioglu et al. 2016). This higher energy demand possibly leads to higher ROS formation as already demonstrated by Fanjul-Moles and Gonsebatt (2011), and Pinheiro and Oliveira (2016). ROS levels are usually controlled by an antioxidant system consisting of enzymatic components (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic, which are low molecular weight components. Among the non-enzymatic antioxidants, we have those endogenous (such as glutathione, melatonin and uric acid) and exogenous (such as lipo and hydrosoluble vitamins, trace elements and bioflavonoids, among others) (Lesser 2006). Thus, oxidative status markers can provide not only a currency to quantify adequacy costs but can also provide information on individual or population characteristics.

We aimed to characterize biochemical-functional aspects related to the intermediate metabolism and oxidative balance of *P. clarkii* females over a seasonal cycle, in order to answer the following questions: (1) What is the reproductive period of this species in Brazil? (2) What is the period of greatest energy demand? (3) What are the energy substrate allocation strategies used at each time of the year to maintain homeostasis? (4) Are there any periods of increased susceptibility to stressors?

2. Materials and methods

2.1. Collection of biological material

Ninety-nine *P. clarkii* females were collected over a seasonal cycle throughout 2016 (Summer = 29: Autumn = 20: Winter = 30 and Spring = 20 individuals) at Alfredo Volpi Municipal Park, São Paulo, SP, Brazil (23°35'16" S 46°42'09" W). The animals were sampled in the central month of each season, which were divided as follows: Autumn – March, April and May; Winter – June, July and August; Spring – September, October and November, and Summer – December, January and February. The traps were placed at the end of the day (around 4 pm) and removed the next day (around 8 am) in order to avoid a prolonged fast. To capture the animals, a fish-flavored cat food was used and placed in a perforated container that allowed the release of chemical clues but prevented animals from ingesting it. At the time of placing and removing the traps, dissolved oxygen levels and water temperature were checked using a digital field oximeter (Lutron DO-5519), the pH was determined using a commercial kit (Labcon Test pH tropical). The meteorological data of air temperature and precipitation were obtained at the National Institute of Meteorology (INMET), through a daily measurement, determined throughout 2016, by a meteorological station, located in the region where the animals were collected.

In all collections, animals were measured for cephalothorax length (digital caliper 0.01 cm precision) and weighed (Pesola® dynamometer 0.25 g precision). Hemolymph samples were extracted using insulin syringes containing potassium oxalate (10%) as an anticoagulant. The animals were placed in an ice bath. Abdominal muscle, hepatopancreas, gonads and stomach were removed in the field under an ice bath, frozen in a − 20°C freezer and transported in thermal boxes to the Conservation Physiology Laboratory at the Pontifical Catholic University of Rio Grande do Sul in Porto Alegre, RS. In the laboratory, the samples were placed in a − 20°C freezer until quantifying the different biochemical parameters. All sample preparations and quantifications were performed no later than 3 months after collection as described by Braghirolli et al. (2016).

2.2. Gonadal maturation

Gonadal maturation was determined according to the coloration of the gonads, as described by McClain et al. (2007): white and cream (immature gonads); brown and black (mature gonads).

2.3. Degree of gastric repletion

The stomachs were categorized into classes according to the degree of stomach repletion (SR), and were visually determined according to the six class scale (Willians 1981): Class 1 = 0% – empty; Class 2 = <5% – partially empty; Class 3 = 5% to 35% – empty to half full; Class 4 = 35% to 65% – half full; Class 5 = 65% to 95% – half full to full; Grade 6 ≤ 95% – full.

2.4. Gonadosomatic and hepatosomatic index

The gonadosomatic index was determined as follows: GI = (Gw/Tw).100, where Gw is gonadal weight and Tm is the total individual weight.

The hepatosomatic index: HI = (Hw/Tw).100, where Hw is hepatopancreas weight and Tw is individual total weight.

2.5. Metabolism markers

The plasma metabolites glucose, total protein, uric acid, triglycerides and total cholesterol were quantified in duplicate for each sample/animal, which was done by using commercial kits from BioTécnica (Advanced Biotechnology LTDA) and spectrophotometry. Total lipids were measured by the sulfophosphovaniline method (Frings and Dunn 1970) and VLDL cholesterol was obtained by mathematical relationship from the values found for triglycerides (TGL/5, where TGL is the value of circulating triglycerides in hemolymph) (Silva-Castiglioni et al. 2015).

All biochemical analyses of tissue metabolites were performed using the same kits as above and determined in duplicate for each organ/animal. Glycogen was extracted according to Van Handel (1965) and quantified as glucose, after acid hydrolysis (HCl) and neutralization with Na_2CO_3, using the commercial glucose oxidase-based kit. Total protein levels were quantified with a commercial assay kit based on the reaction of copper ions with peptide bonds in serum proteins in alkaline medium, producing a purple-colored liquid with a peak absorbance at 545 nm. Lipids were extracted by the method of Folch et al. (1957), and total lipids were quantified by the sulfophosphovaniline method as described by Frings and Dunn (1970). After extraction, triglycerides were hydrolyzed by lipase, producing free glycerol, which is transformed into glycerol-3-phosphate by the action of glycerol kinase. Glycerol-3-phosphate was oxidized to dihydroxyacetone and hydrogen peroxide by glycerol-P-oxidase. Hydrogen peroxide reacts with 4-aminoantipyrine and 4-chlorophenol under the catalytic action of peroxidase, producing a pink compound (quinonimine), which has a maximum absorption at 505 nm. The cholesterol esters in the samples were hydrolyzed by the cholesterol esterase enzyme-producing free cholesterol. The enzyme cholesterol oxidase, in the presence of oxygen, catalyzes the oxidation of free cholesterol, producing hydrogen peroxide. Peroxidase catalyzes the oxidation of the phenolic reagent by the hydrogen peroxide formed in the presence of 4 amino antipyrine, producing a pink compound (4-(p-benzoquinone monoamine)-phenazone) with maximum absorption at 505 nm. The color intensity is proportional to the cholesterol concentration in the sample.

2.6. Oxidative balance

While still frozen the abdominal muscle, hepatopancreas and gonads were homogenized in ultra-turrax, using phosphate buffer solution (20 mM) plus potassium chloride (140 mM) and phenyl methyl sulfonyl fluoride, a protease inhibitor (PMSF: 1 mM) in a ratio of 1 g per 5 ml of this solution. After homogenization, the samples were centrifuged at 1000xg and 4ºC for 10 min, the supernatant was collected, aliquoted and frozen at −20°C for further quantification of oxidative balance parameters.

The determination of superoxide dismutase (SOD) was based on the inhibition of superoxide radical reaction in the presence of adrenaline. In this reaction, SOD competes for the superoxide radical with the detection system forming a stained compound (adrenochrome) that was spectrophotometrically quantified at 480 nm and expressed as U. mg protein^{-1} (Boveris and Cadenas 1982). As the enzyme activity in terms of substrate consumed per unit of time cannot be determined, quantitation in relative units was used. Catalase (CAT) activity was quantified by the hydrogen peroxide decay of the sample, detected at 240 nm and expressed in pmol. mg protein^{-1}. min^{-1} (Boveris and Chance 1973). Glutathione S-Transferase (GST) activity was measured by conjugating 1-chloro 2,4 dinitrobenzene (CDNB) with glutathione reduction (GSH), detected at 340 nm and expressed in mmol. mg protein^{-1}. min (Habig and Jakoby 1981). Lipoperoxidation (LPO) levels were quantified by detection of thiobarbituric acid reactive substances (TBARS), which are detected at 520 nm and expressed in μmol. mg protein^{-1} (Lima and Abdalla 2001).

2.7. Statistical analysis

The results obtained were analyzed in the programs SPSS 20.0 and Bioestat 5.0. Data normality was verified by the Kolmogorov–Smirnov test, and for those that did not show a normal distribution, we used the Kruskal–Wallis comparison test with Dunn's complementary test and considered significant for $p < 0.05$ (Zar 2010).

3. Results

Throughout the collection year, we verified a significant variation for the air and water temperature of the stream, as well as for the precipitation levels. In summer, the highest values were observed and in winter, the lowest for these variables. The dissolved oxygen and pH levels in the stream water did not vary significantly over the year (Table 1).

The animals did not show significant differences as to the values observed for the length of the cephalothorax (summer = 84.08 ± 1.23, autumn = 87.01 ± 0.98, winter = 85.61 ± 1.32, spring) = 87.65 ± 1.73 mm) nor for body weight (summer = 21.03 ± 0.78, autumn = 18.5 ± 0.78, winter = 19.9 ± 0.81, spring = 19.56 ± 1.27 g).

Regarding gonadal maturation, in the summer we observed 52% of mature gonads, followed by 27%, 10% and 20% in the autumn, winter and spring, respectively (Figure 1(a)). Regarding the degree of gastric repletion, we observed a predominance of stomachs in categories 1 (empty) to 3 (empty to half full) in the summer (73%), 4 (half full) to 6 (full) in the winter (60%) and spring (90%). In the autumn, the stomachs were in categories 1 to 3

Table 1. Abiotic variables obtained during the year of animal collection.

	Summer	Autumn	Winter	Spring
Air temperature (°C)	$23.1^{a} \pm 0.21$ (79)	$19.9^{b} \pm 0.46$ (93)	$17.8^{c} \pm 0.31$ (96)	$20.4^{b} \pm 0.3$ (90)
Water temperature (°C)	$22.75^{a} \pm 0.75$ (2)	$22.3^{a} \pm 0.57$ (2)	$16.8^{b} \pm 0.1$ (2)	$20.8^{a} \pm$ (2)
Water pH	$6.2^{a} \pm 0.1$ (2)	$6.4^{a} \pm 0.2$ (2)	$6.2^{a} \pm 0.1$ (2)	$6.4^{a} \pm 0.2$ (2)
Oxygen dissolved ($mg.L^{-1}$)	$7.6^{a} \pm 2.98$ (2)	$5.9^{a} \pm 0.39$ (2)	$7.7^{a} \pm 0.32$ (2)	$7.6^{a} \pm 0.1$ (2)
Preciptation (mm)	$6.18^{a} \pm 0.04$ (81)	$3.84^{ab} \pm 0.03$ (93)	$3.14^{b} \pm 0.03$ (96)	$3.21^{b} \pm 0,03$ (90)
mean/day	568.8	358	292.4	295.2
accumulated				

The values represent the mean ± standard error, the number in brackets represents the number of checks and the different letters represent significant differences for a $p < 0.05$.

(50%) and 4 to 6 (50%) (Figure 1(b)). Gonadosomatic (Figure 1(c)) and hepatosomatic index (Figure 1(d)) showed a significant decrease from summer to spring.

For hemolymphatic metabolites (Table 2), circulating glucose levels were maintained throughout the year. Total protein decreased during winter and spring compared to the other seasons. As for the levels of uric acid, we observed an increase during spring. Total lipids remained constant throughout the year ($p > 0.05$), while triglycerides decreased significantly in the winter and spring, and total cholesterol and VLDL cholesterol levels decreased in the winter and spring.

For tissue metabolites (Table 2), a significant increase in abdominal muscle glycogen reserves was observed in the winter and spring. There was an increase of this reserve during the winter for hepatopancreas and the spring for gonads. Total proteins remained constant throughout the seasons in muscle tissue ($p > 0.05$), but in hepatopancreas, there was a significant reduction in the winter, and increase in the summer, autumn and spring levels. Proteins decreased in the gonads during the winter and spring and were constant in the summer and autumn. In the muscle, total lipid levels showed a significant increase during spring, while in hepatopancreas cells they remained stable throughout the year ($p > 0.05$). Total lipids showed a reduction in the winter for gonads with a subsequent increase in the spring. A significant decrease in triglyceride reserves in muscle and hepatopancreas was observed in the autumn and spring. The gonads showed triglyceride peak in the summer. Total cholesterol increased in muscle and hepatopancreas in the autumn, while in the gonads there was a gradual decrease until spring (Table 2).

Muscle SOD activity decreased during autumn and spring, while remaining constant for hepatopancreas throughout the year, and decreasing for gonads in the autumn (Figure 2(a)). CAT activity in muscle and hepatopancreas decreased in the autumn and winter, but there was no significant difference throughout the year for gonads (Figure 2 (b)). GST activity in muscle decreased in the autumn and spring, hepatopancreas increased in the winter and decreased in the spring, and gonads decreased in the autumn and spring (Figure 2(c)). Muscle LPO levels were high in the summer, hepatopancreas was high in the winter and summer, and gonads were high in the winter (Figure 2(d)).

4. Discussion

This paper is the first to show the seasonal variation of some biochemical and physiological markers in females of *P. clarkii* collected in a natural environment in Brazil. It was found that the species reproduces throughout the year since there are females with

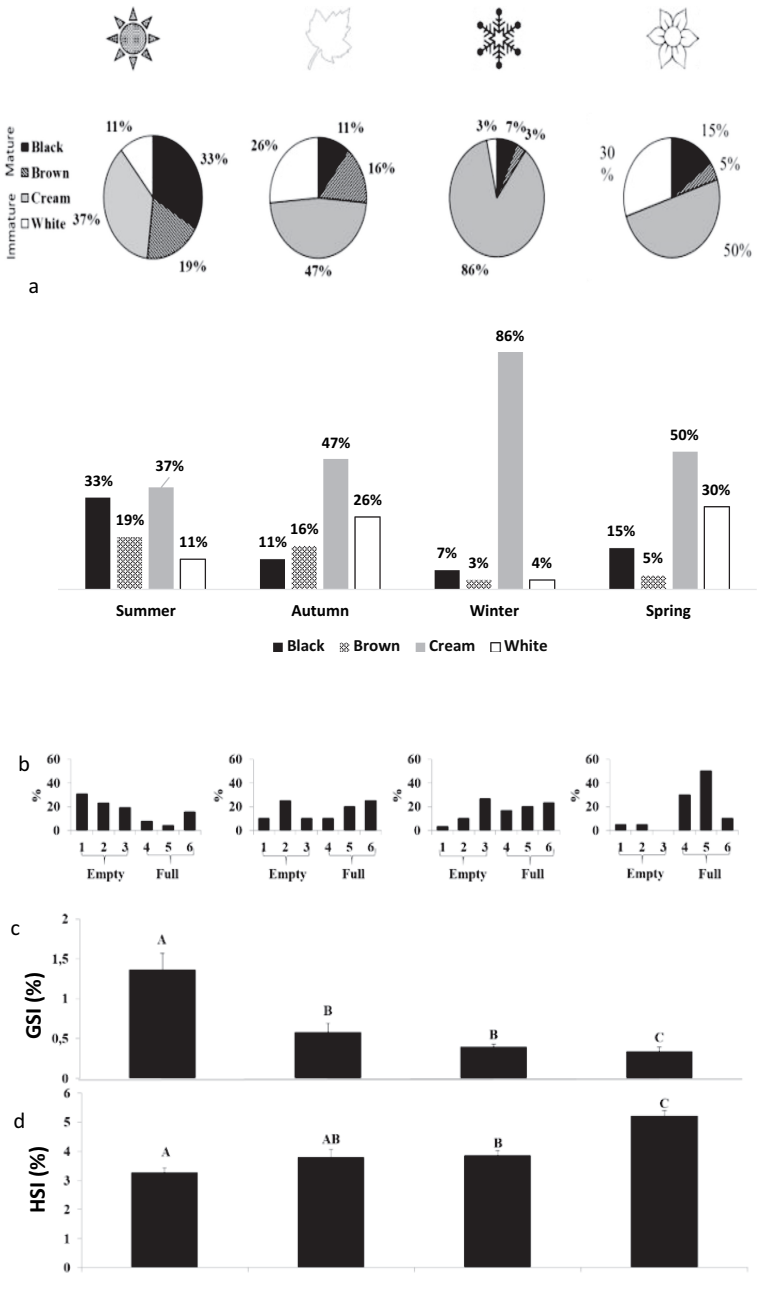

Figure 1. Gonadal maturation, degree of gastric repletion, gonadosomatic index, hepatosomatic index of *Procambarus clarkii* females throughout the seasons in 2016 in Brazil: Sun-Summer; Leaf – Autumn; Snowflake – Winter; Flower – Spring; (a) Gonadal maturation; (b) Degree of gastric repletion; (c) gonadosomatic index (GI); (d) hepatosomatic index (HI). Different letters statistical differences (Kruskal–Wallis followed by Dunn's test, $p < 0.05$).

mature gonads in all seasons (summer: 52%; autumn: 27%; winter: 10% and spring: 20%). However, there is a reproductive peak during the summer when the percentage of mature gonads is higher than in other seasons. Unlike what was observed for this animal in its

Table 2. Levels of metabolites quantified in hemolymph (glucose, proteins, uric acid, total lipids, triglycerides, total cholesterol, VLDL cholesterol), abdominal muscle, hepatopancreas and gonads (glycogen, total proteins, total lipids, triglycerides and cholesterol total) of *Procambarus clarkii* females collected throughout the year.

	Summer	Autumn	Winter	Spring
Hemolymph (mg.dL^{-1})				
Glucose	18.29 ± 1.86^{a}	21.83 ± 2.93^{a}	20 ± 4.37^{a}	28.72 ± 3.74^{a}
Protein (mg.mL^{-1})	45.81 ± 3.41^{a}	54.15 ± 8.01^{a}	36.87 ± 7.60^{b}	25.80 ± 2.95^{b}
Uric Acid	0.98 ± 0.14^{a}	1.22 ± 0.19^{a}	1.28 ± 0.13^{ab}	1.75 ± 0.18^{b}
Lipids	107.75 ± 15.13^{a}	115.49 ± 14.14^{a}	106.45 ± 6.52^{a}	126.66 ± 13.38^{a}
Triglycerides	35.79 ± 3.9^{a}	40.2 ± 8.14^{a}	19.29 ± 2.00^{b}	18.78 ± 1.55^{b}
Cholesterol	25.74 ± 7.65^{a}	28.64 ± 6.08^{a}	15.39 ± 2.56^{ab}	9.08 ± 1.24^{b}
Cholesterol VLDL	7.15 ± 0.78^{a}	8.04 ± 1.62^{a}	3.85 ± 0.40^{b}	3.75 ± 0.31^{b}
Muscle (mg.g^{-1})				
Glycogen	0.23 ± 0.05^{a}	0.07 ± 0.02^{a}	1.13 ± 0.28^{b}	0.93 ± 0.25^{b}
Protein	50.99 ± 4.90^{a}	55.25 ± 13.27^{a}	34.52 ± 9.20^{a}	57.44 ± 7.70^{a}
Lipids	1.93 ± 0.25^{a}	0.86 ± 0.26^{b}	1.65 ± 0.28^{ab}	0.39 ± 0.16^{b}
Triglycerides	0.62 ± 0.13^{a}	0.0099 ± 0.0019^{b}	0.40 ± 0.02^{a}	0.15 ± 0.01^{b}
Cholesterol	0.27 ± 0.08^{a}	1.94 ± 0.10^{b}	0.40 ± 0.05^{c}	0.24 ± 0.02^{ac}
Hepatopancreas (mg.g^{-1})				
Glycogen	0.33 ± 0.06^{a}	0.43 ± 0.07^{a}	1.55 ± 0.23^{b}	0.52 ± 0.08^{a}
Protein	20.29 ± 5.66^{a}	21.49 ± 3.30^{a}	5.27 ± 0.60^{b}	34.76 ± 8.35^{a}
Lipids	17.37 ± 2.35^{a}	14.78 ± 0.28^{a}	16.87 ± 2.63^{a}	15.55 ± 3.94^{a}
Triglycerides	0.43 ± 0.03^{a}	0.07 ± 0.01^{b}	0.38 ± 0.04^{a}	0.15 ± 0.01^{b}
Cholesterol	0.18 ± 0.03^{a}	1.07 ± 0.12^{b}	0.37 ± 0.05 ^{c}	0.38 ± 0.04^{c}
Gonads (mg.g^{-1})				
Glycogen	0.92 ± 0.18^{a}	0.74 ± 0.20^{a}	1.07 ± 0.21^{a}	3.64 ± 1.14^{b}
Protein	0.84 ± 0.24^{a}	0.76 ± 0.24^{a}	0.26 ± 0.08^{b}	0.30 ± 0.10^{b}
Lipids	11.86 ± 2.42^{ab}	13.39 ± 2.45^{ab}	5.65 ± 0.70^{b}	34.16 ± 8.376^{a}
Triglycerides	6.44 ± 0.92^{a}	0.01 ± 0.0051^{b}	0.23 ± 0.05^{c}	0.10 ± 0.03^{b}
Cholesterol	0.23 ± 0.60^{a}	1.82 ± 0.26^{b}	0.54 ± 0.24^{c}	0.40 ± 0.05^{c}

Different letters represent significant differences (Kruskal–Wallis followed by Dunn's test, $p < 0.05$).

place of origin, where reproduction begins in late spring and extends into summer (Avaut, de la Bretonne and Jaspers, 1970). According to Loureiro et al. (2015a), in Procambarus clarkii mating period, as well as recruitment and sexual maturation, vary according to hydrographic period and environmental conditions, so depending on where these animals are introduced, their reproduction may change.

In this study, a variation in the gonadosomatic index from 0.3% to 1.4%, with the highest values seen in the summer when we have a predominance of mature females (52%), was observed ; the highest GSI observed in a mature female was 4.2%. Alcorco et al. (2008) studied reproduction in *P. clarkii* collected in the Lower Guadalquivir Basin (SW Spain) and found a gonadosomatic index ranging from 0.06% to 0.3% for immature gonads and from 2.3% to 8% for mature gonads. In the summer months, when mature gonads are predominant, the authors determined an average GSI of 1.20%. This discrepancy in terms of values of the gonadosomatic index can perhaps be explained by the difference in conditions faced by the animals, that is, the limnological differences (temperature, water flow, and precipitation) and food availability and quality. Another important factor that can contribute to this difference is population density. In the region where the animals were collected (Spain), the climate in the area is Mediterranean with a slight Atlantic influence, and the average annual rainfall is 500–600 mm, 87% of which falls between October and April (Alcorco et al., 2008). In São Paulo, Brazil, the climate is humid subtropical. In the year of development of the study, the accumulated

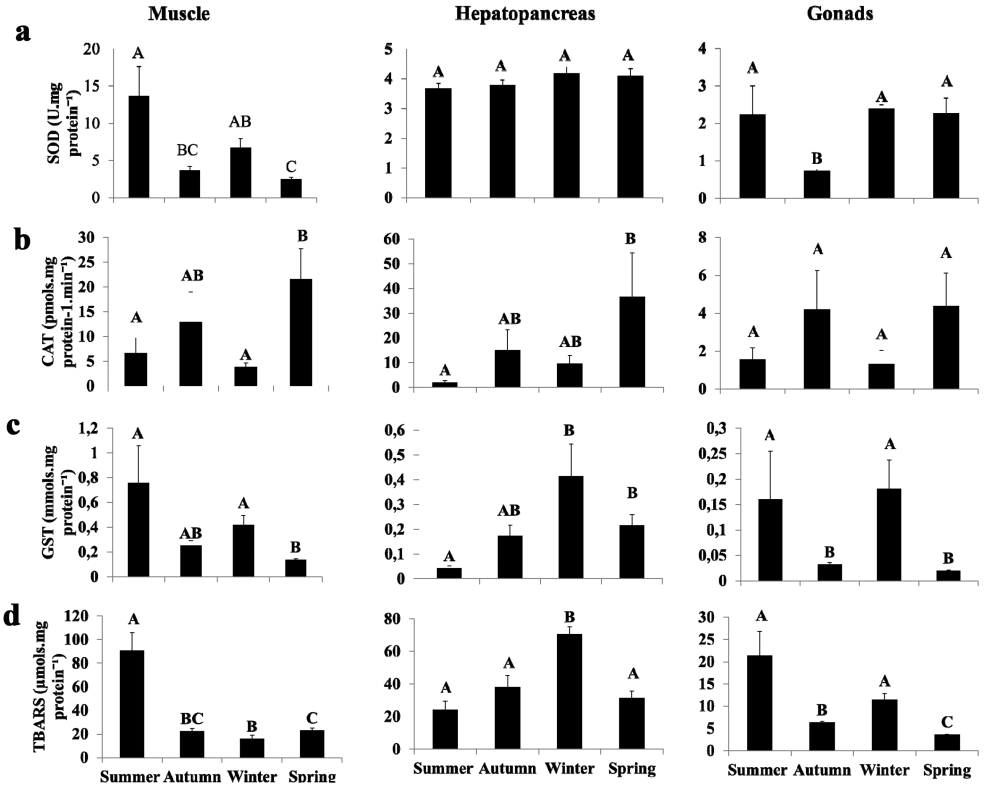

Figure 2. Enzyme activity and lipoperoxidation of *Procambarus clarkii* females throughout the seasons in 2016 in Brazil: (a) Superoxide Dismutase; (b) Catalase; (c) Glutathione S-transferase; (d) Lipoperoxidation. Different letters mean statistical differences (Kruskal–Wallis followed by Dunn's test, $p < 0.05$).

precipitation was 1514.4 mm and the highest volumes of rain were verified in the summer (568.8 mm).

During the summer, there was also a predominance of empty stomachs, lower hepatosomatic index, higher gonadosomatic index and high percentage of mature gonads. It is known that female decapods reduce their feeding activity during the reproductive period, prioritizing reproductive behaviors, prenuptial moulting and parental care (Swetha et al. 2011). Decreased hepatosomatic index and increased gonadosomatic index appear to be closely associated with the allocation of energy reserves from the hepatopancreas to the gonads during the reproductive period, as seen for the *Oziothelphusa senex* crab (Girish et al. 2014) and *Parastacus promatensis* crayfish (Pinheiro and Oliveira 2016). These results corroborate the gonadal maturation data that sets the summer as the reproductive peak of species in Brazil.

In *P. clarkii*, the maintenance of circulating hemolymph glucose levels throughout the year may be related to its role as an energy substrate for the nervous system and to support muscle activity as reported by Jimenez and Kinsey (2015). It is noteworthy that glucose is the main circulating monosaccharide in crustaceans (Kucharski and Da Silva 1991). The reduction of circulating protein levels in winter and spring seems to be

associated with the use of amino acids for the synthesis of vitellogenin, ATP and/or gluconeogenesis, as there is an increase in glycogen stored in the hepatopancreas and muscle, associated with the maintenance of glycemia. Oliveira and Da Silva (1997) and Vinagre and Da Silva (2002) demonstrated a gluconeogenic capacity from amino acids in hepatopancreas and muscle, in the estuarine crab *Neohelice granulata*. Concomitantly with the decrease in total proteins, there is an increase in hemolymph uric acid levels, which may indicate protein catabolism with the resulting formation of uric acid. It is not possible to rule out that this uric acid is derived from protein digestion in the gastrointestinal tract, since in the winter (60%) and spring (90%) we observe medium to full stomachs. This maintenance of glycemia may also be related to gluconeogenesis, as has already been reported for another decapod crustacean (*Neohelice granulata*) by Sarapio et al. (2017).

Maintenance of circulating total lipid levels throughout the year may be associated with decreased triglyceride levels in muscle and hepatopancreas in the autumn and spring, as well as the predominance of full stomachs in winter and spring. According to Harvey et al. (2012) and Girish et al. (2014) the decrease of tissue triglycerides determines a release of fatty acids and glycerol to the hemolymph, which would be used, respectively, for the synthesis of ATP and gluconeogenesis. The increase in triglycerides and cholesterol in the summer seems to be associated with the allocation of these tissue metabolites for gonadal maturation, vitellogenin synthesis and sex hormones. This hypothesis is corroborated by the high levels of VLDL, which is a lipoprotein that transports hepatopancreas lipids to tissues through circulation (Buckup et al. 2008; Oliveira et al. 2007; Vinagre et al. 2007; Vinagre and Chung 2016; Silva-Castiglioni et al. 2007, 2012; Musin et al. 2017).

The increase in glycogen stored in the muscle during winter and spring, and in the hepatopancreas during winter indicates a relationship with increased dietary activity during these seasons, since 60% to 80% of stomachs are observed in categories 4 to 6 (half to full), respectively. The high levels of glycogen observed in the hepatopancreas during the winter begin to decrease in the spring, reaching minimum values in the summer. In this period, we can suggest a mobilization of this polysaccharide and allocation of glucose mainly to the gonads in order to sustain reproductive events. This hypothesis is reinforced by studies with other species of decapods (Silva-Castiglioni et al. 2007, 2015; Fatima et al. 2013; Oliveira et al. 2007). The decrease in muscle glycogen seen in the summer and autumn months seems to indicate an association not only with reproduction but also with food activity. In the summer, around 65% of empty stomachs were observed which seems to have led to minimum levels of this polysaccharide in the autumn. As well as, a stimulus for the resumption of feeding activity by the animals since in the autumn we verified about 50% of empty stomachs and 50% full.

Although muscle is the main protein storage site for crustaceans (Jimenez and Kinsey 2015), total proteins remained constant throughout the year. In hepatopancreas, there was a significant reduction in protein levels in the winter, suggesting that they are used for ATP synthesis and/or maintenance of glycemia through gluconeogenesis. Higher protein concentrations in the gonads during summer may be related to reproductive peak and synthesis of vitellogenin in hepatopancreas and/or oocytes as suggested by Guan et al. (2016).

The reduction of total lipids in the muscle during the summer indicates their use by the tissue itself to support parental care and egg cleaning behaviours. This also contributes to energetic homeostasis as summer females have predominantly empty and half-filled stomachs (from 1 to 3: 73%), suggesting the occurrence of fasting and/or decreased eating activity. During the autumn, the increase in hepatopancreas lipid reserves may be related to the return of food activity after the reproductive peak. Lower reproductive activity during winter is seen by a large proportion of immature gonads and low lipid stocks in the gonads, a pattern already reported by other authors (Buckup et al. 2008; Dutra et al. 2008; Figler et al. 2010; Musin et al. 2017; Oliveira et al. 2007; Silva-Castiglioni et al. 2007, 2012).

The lower triglyceride values observed during autumn and spring in muscle and hepatopancreas are possibly related to their catabolism to sustain gonadal maturation, as triglycerides are the main source of fatty acids used for ATP and vitellogenin synthesis (Guan et al. 2016). Peak summer triglyceride stocks in gonads may be related to their use in gametogenesis and vitellogenin synthesis, as seen by Oliveira et al. (2007) and Guan et al. (2016). In the autumn, total cholesterol levels in the abdominal muscle and hepatopancreas appear to be related to increased dietary activity and are important for tissue restructuring after the reproductive period and/or to be mobilized for use in molting that will proceed a new reproductive cycle. Gu et al. (2017) and Han et al. (2018) showed in their studies the importance of cholesterol for the growth of juveniles of *Macrobrachium nipponense* and *Portunus trituberculatus*. It is noteworthy that cholesterol is an important constituent of cell membranes and percussion of ecdysteroid hormones (Jimenez and Kinsey 2015). The high concentration of cholesterol during reproductive peak can be interpreted with its use in gametogenesis, structuring of gonadal tissue during reproduction, and synthesis of sex hormones, as suggested by Jimenez and Kinsey (2015) in their review for other crustaceans.

Regarding the activity of antioxidant enzymes, we observed in the muscle an increase in superoxide dismutase coupled with an increase of lipoperoxidation levels (TBARS) in the summer. This suggests that the increased energy demand and oxygen consumption in this tissue during the reproductive peak was not offset by the increased activity of SOD, thus, leading to an increase in the formation of ROS, which was also observed by Pinheiro and Oliveira (2016) in *Parastacus promatensis*. Decreased SOD activity in the gonads during the autumn seems to be associated with the end of reproductive peak, a profile also verified by Braghirolli et al. (2016) and Fanjul-Moles and Gonsebatt (2011) for other crustacean species. However, this decrease in SOD does not lead to an increase in lipoperoxidation in this tissue, since TBARS levels in the gonads were high in the summer and winter.

Regarding CAT activity, muscle and hepatopancreas showed increased activity during the autumn and spring which may be related to the maintenance of tissue oxidative status, thus avoiding oxidative damage possibly associated with seedling and peak reproductive preparation, a pattern already suggested by Fanjul-Moles and Gonsebatt (2011). The gonads showed no significant difference for CAT activity reinforcing the data found showing an increase in TBARS levels in the summer. Pinheiro and Oliveira (2016) also found an increase in TBARS levels during the reproductive season in all tissues studied (gills, hepatopancreas, abdominal muscle and gonads) and in both sexes of

a neotropical crayfish species (*Parastacus promatensis*). However, this increase was less evident in female gonads and the authors suggest a greater protection of this tissue.

The increase in enzymatic antioxidant capacity before the peak of reproduction that occurs in the summer is accompanied by an increase in circulating levels of uric acid. This result suggests an important role for uric acid as a non-enzymatic antioxidant, possibly being used during the reproductive season. We observed the lowest levels of this molecule which may be minimizing an oxidative stress situation. Uric acid synthesis in ammoniotelic organisms for use as an antioxidant has been reported for insects (Hermes-Lima and Zenteno-Savín 2002) and for tadpoles (de Lima Coltro et al. 2017). This is the first evidence of uric acid usage as an antioxidant molecule in crayfish.

Higher summer and winter GST activity in muscle and gonads may be associated with increased tissue activity and the possible formation of endobiotic by-products from metabolic pathways, as demonstrated by Van Der Aar et al. (1998). This is due to a higher synthesis of sex hormones during the summer related to the reproductive peak, whereas in the winter it is related to an allocation of reserves to these tissues. Increased GST activity in hepatopancreas during winter may be related to higher feeding activity of these animals, since this organ mainly acts in the biotransformation of endogenous and exogenous compounds, such as those derived from diet. Similar pattern was observed in *Parastacus promatensis* (Pinheiro and Oliveira 2016).

High levels of LPO in gonads and muscle during the summer agree with the species reproductive peak and this is possibly due to an increase in energy demand. Therefore, there is a reduction in glycogen tissue storage to support an increase in tissue synthesis activity due to the maturation of oocytes, vitellogenin and sex hormones. This requires an increased demand for ATP, with the subsequent increase in oxygen consumption and the formation of reactive oxygen species. The increase in TBARS levels in the muscle may be related to greater activity in seeking reproductive partners, establishing and sustaining reproductive behaviours such as copulation and parental care of eggs and juveniles, as already suggested by other studies (Fanjul-Moles and Gonsebatt 2011; Braghirolli et al. 2016; Pinheiro and Oliveira 2016). In the hepatopancreas, there was also an increase in TBARS levels during winter, which was also associated with the increased dietary activity, such as digestion, absorption and storage within the organ itself, and availability of nutrients to other organs.

Beaulieu and Costantini (2014) report that ecological studies have shown that oxidative status can have a significant impact on fitness components in animals. This oxidative status not only reflects the environmental conditions that animals experience but it can also predict their chances of future reproduction and survival in this habitat. *P. clarkii* females have a high degree of investment in reproduction, and this event is associated with decreased dietary activity, mobilization of energy substrates and increased TBARS levels in muscle tissue and gonads. This set of results observed in the species reproductive peak leads to oxidative damage and potentially to a situation of oxidative stress. This damage may be a key factor for management strategies where this crayfish is invasive, as females may not be able to maintain reproductive activity or survive when unexpected environmental stressors are associated with this period. Further studies to understand the effects of multiple environmental stressors during the reproductive period should be done to establish management plans that will successfully control the population of this invasive species.

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Disclosure statement

The authors have declared that no potential conflict of interest exists.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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http://www.pucrs.br/saude/programa-de-pos-graduacao-em-ecologia-e-evolucao-da-biodiversidade/

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