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Glutathione status and antioxidant enzymes in a crocodilian species from the swamps of the Brazilian Pantanal

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ABSTRACT

In a previous study oxidative damage markers – lipid peroxidation and protein oxidation – were determined in organs of wild *Caiman yacare* captured in winter-2001 and summer-2002 at various developmental stages. An increase in oxidative damage occurred in the hatchling-juvenile transition (but not in the juvenile-adult transition) and winter–summer transition (in juveniles), suggesting that oxidative stress is associated with development and season. Herein the effect of development and season on glutathione (GSH) metabolism and the effect of development on the activity of antioxidant enzymes (catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase) and glucose 6-phosphate dehydrogenase were analyzed. The ratio GSSG:GSH-eq increased in lung, liver, kidney and brain by 1.8- to 4-fold in the embryo/hatchling to juvenile transition. No changes occurred in juvenile–adult transition. GSSG:GSH-eq across seasons was significantly elevated in summer. Total-glutathione content was mostly stable in various organs; in liver it increased in the embryo–juvenile transition. Enzyme activities were only determined in summer-animals (embryos, hatchlings and juveniles). For most antioxidant enzymes, activities increased from embryo/hatchling to juvenile in liver and Kidney. In lung, there was an inverse trend for enzyme activities and total glutathione content. Thus, increased metabolic rates during early caiman growth – in embryo–juvenile transition – appears to be related to redox imbalance as suggested by increased GSSG:GSH-eq and activation of

er–winter nocturnal emistry.

1. Introduction

Limited information exists concerning the free radical metabolism in reptiles, especially in crocodilians. These animals present relatively high tolerance to hypoxia in internal organs prompted by long dives (Axelsson et al., 1991; Soderstrom et al., 1999). Hypoxia and reoxygenation in vertebrates are associated to a redox imbalance that can be accompanied by an increased production of reactive oxygen species (ROS) that can cause oxidative damage to cellular structures (Hermes-Lima and Zenteno-Savin, 2002; Bickler and Buck, 2007; Milton and Prentice, 2007; Ramirez et al., 2007). The connection between dive and oxidative stress in a crocodilian species was indicated in a study by our group (Furtado-Filho et al., 2007). In this study, the relationship between natural crocodilian development and selected antioxidant enzyme activities and glutathione contents was also investigated.

The relationship between oxidative stress and development/aging in animals is currently a topic of considerable research interest (Barja, 2004; Rebrin and Sohal, 2008; Abele et al., 2009; Buttemer et al., 2010), but there is a scarcity of studies with crocodilians. Only three studies have examined parameters related to free radical metabolism in alligators. A study on alligators (Elsey and Lance, 1983) reported the plasma and erythrocyte glutathione peroxidase enzyme activity of animals fed different diets. A second study quantified the hepatic activities of phases I and II biotransformation enzymes (including glutathione S-transferase) in relation to alligator body size (Gunderson et al., 2004). Finally, a third study determined the levels of lipid peroxidation in three alligator tissues, comparing wild animals with captive-reared ones (Lance et al., 2006). None of these studies performed full-length analyses of free radical metabolism in alligators.

In the case of caimans, a previous study conducted by our research group investigated the levels of lipid peroxidation and protein oxidation in animals (*Caiman yacare*) at different stages of development and that were captured in summer and winter in the Brazilian Pantanal swamps (Furtado-Filho et al., 2007). This study showed that oxidative damage increased in the transition from hatchlings to juveniles, but not from juveniles to adult caimans. These observations were correlated with the developmental characteristics of *C. yacare*.

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To complete the picture of changes in oxidative stress indicators associated with age and capture season of caimans, the present study aimed to measure the activities of antioxidant enzymes and glutathione status in selected organs of *C. yacare*, captured as embryos, juveniles or adults in the summer and winter seasons in the swamps of the Brazilian Pantanal region.

2. Methods

2.1. Animal capture and sample collection

Animals were captured during two expeditions to the swamps of the Brazilian Pantanal. One expedition took place in a dry, winter season (July 2001) and another in the rainy, summer season (March 2002). Animals were dissected out in the field laboratory of EMBRAPA, located in the Nhumirin farm, 18°59′S and 56°39′W, 20–90 minutes drive from the sites of capture. The sites of capture included flooded plains, small lakes and dry areas at Nhecolândia, Mato Grosso do Sul state, Brazil. Animals were held blindfolded from 1–3 h from capture to sacrifice. Organs were immediately frozen in liquid nitrogen and transported in dry ice to our laboratory at the University of Brasilia. The following organs were collected: liver, brain, kidney, lung, leg muscle, tail muscle, heart. The samples were kept at −75 °C. Details about animal capture and the procedures for organ dissection are available in a previous publication (Furtado-Filho et al., 2007).

Captures, organ dissections and biochemical determinations in caiman tissues were authorized by IBAMA (licenses 179/2001-DIFAS-DIREC and 019/2002-RAN), the Brazilian Federal Agency responsible for wildlife control.

2.2. Body characteristics of the captured caimans

In the winter-2001 expedition mature adults (n = 6; 17.0 ± 1.6 kg of body mass) and juveniles (n = 8; 5.8 ± 0.5 kg) were captured. In the summer-2002 expedition juveniles (n = 6; 4.0 ± 0.5 kg) and hatchlings (n = 10, hatched within 1–24 h of capture) were captured. Eggs (n = 5) were also collected in 2002 to obtain embryo organs. Eggs and hatchlings were taken by hand from nests and viability was confirmed by detection of movement and heart beat. The difference in body composition between embryos and hatchlings (e.g., body masses of 17.6 ± 0.6 and 49.9 ± 1.6 g, respectively) indicated that embryos were in a different developmental stage than hatchlings, and that they were not about to hatch (for more details see Furtado-Filho et al., 2007). The sex of the embryos was considered indeterminate. For all other developmental stages, only males were captured.

Age in caimans is mostly estimated by means of morphometric parameters that allow only approximate inferences. In addition, temperature, food availability and other environmental factors can influence growth rate (Coutinho, 2000). Therefore growth defined by time is not absolute in the case of crocodilians. Males with a 30–40 cm snout-vent length (SVL) have been described to be 2–3 years old (Coutinho et al., 2005) and were considered juveniles in our study, while those with a SVL greater than 90 cm are at least 10 years old (Coutinho, 2000) and were considered adults.

Both, the biological material used in the study by Furtado-Filho et al. (2007) and the biological material used in the present study, were obtained from the animals captured in the 2001 and 2002 expeditions to the Brazilian Pantanal swamps. The correspondence between the samples descriptions in these two investigations is as follows: "*2001 young-adults*" (*YA-1*), "*2002 young-adults*" (*YA-2*) and "*2001 adults*" (*AD1*) in the paper by Furtado-Filho et al. (2007) are *winter-captured juveniles* (WJU), *summer-captured juveniles* (SJU) and *winter-captured adults* (WAD) in the present study. Hatchlings and embryos were described as such in both studies. There were relevant differences in stomach contents in the WAD, WJU and SJU caimans. For example, fish and invertebrates were found in stomachs of most SJU caimans, while stomach contents of WJU were invertebrates only. More detailed information is provided in Furtado-Filho et al. (2007).

All caimans were evaluated by a crocodilian expert (ecologist Guilherme Mourão PhD, from EMBRAPA—Pantanal) at the capture site. Physical condition was the first parameter used to define health status. Caimans with ectoparasites and with an unhealthy appearance were not used for determinations. None of the sacrificed animals presented pathological alterations at the anatomical level in the organs and tissues.

2.3. Biochemical determinations

Glutathione parameters were determined in all collected organs (brain, lung, liver, kidney, heart, led muscle and tail muscle), from the five caiman groups: WAD, WJU, SJU, hatchlings and embryos. However, enzyme activities were only assayed in 3 organs (liver, lung and kidney) in the summer-animals (SJU, hatchlings and embryos). Brain, heart, leg muscle and tail muscle samples from summer-animals and all organ-samples from winter-animals that were being kept at −75 °C for antioxidant enzyme assays were lost after an electrical power failure in our university in December 2006. All determinations presented herein were performed between 2002 and 2004. Chemicals used in the assays were purchased from Sigma, USA.

2.4. Antioxidant enzymes and glucose 6-phosphate dehydrogenase activities

Protein extracts from each tissue were prepared using a glass hand-held homogenizer. Frozen tissue samples were homogenized 1:20 (w/v) in ice-cold phosphate buffer (50 mM potassium phosphate, pH 7.2, 0.5 mM EDTA) containing 10 μM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 15,000 *g* for 15 min at 4 °C, and the supernatants were removed and used for determination of enzymatic activities. The activities of catalase, selenium-dependent-glutathione peroxidase (Se-GPX), glutathione S-transferase (GST), glutathione reductase (GR) and glucose 6-phosphate dehydrogenase (G6PDH) were determined by spectrophotometric assays as previously described (Ramos-Vasconcelos and Hermes-Lima, 2003; Ramos-Vasconcelos et al., 2005). Briefly, catalase activity was measured by following the rate of H_2O_2 decomposition at 240 nm. Se-GPX activity was assayed (using H_2O_2 as substrate) by following the rate of the GR-catalyzed NADPH oxidation at 340 nm. GST activity was determined by monitoring the conjugation of GSH with 1-chloro-2,4-dinitrobenzene at 340 nm. GR activity was quantified by following the oxidation of NADPH at 340 nm. G6PDH activity was determined by monitoring the reduction of $NADP^+$ at 340 nm.

2.5. Glutathione measurement

Samples of frozen tissues were homogenized 1:10 (w/v) in N_2-bubbled ice-cold 5% sulfosalicylic acid using a glass pestle homogenizer and centrifuged at 15,000 × *g* for 5 min. Supernatants were collected and immediately used to quantify glutathione levels as previously described (Ramos-Vasconcelos and Hermes-Lima, 2003). The total pool of glutathione, including both reduced and oxidized forms was quantified as glutathione equivalents (GSH-eq = GSH + 2 GSSG) by monitoring the rate of reduction of 5,5′-dithiobis-2-nitrobenzoic acid by GSH catalyzed by GR at 412 nm and comparing it to a GSH standard curve. This method relies on the specificity of GR for glutathione determination. GSSG was quantified after samples were treated with 2-vinylpyridine. GSH levels and the ratio GSSG:GSH-eq (%GSSG) were calculated from the experimentally determined GSSG and GSH-eq (Ramos-Vasconcelos and Hermes-Lima, 2003). The enzymatic recycling method has been widely used, being described as accurate, sensitive and reproducible (Rahman et al., 2006; Monostori et al., 2009).

2.6. Statistics

Statistical analyses were made using the software GraphPad Prism 5 (San Diego, CA, USA). Comparisons were made within summer-captured animals (embryos, hatchlings and SJU), within winter-captured animals (WJU and WAD) and between juveniles from summer and winter (SJU and WJU). When samples from three groups of summer-captured animals were available, the analysis was done by means of one-way ANOVA plus Tukey's Multiple Comparison Test (for parametric data) or by means of Kruskal–Wallis one-way ANOVA plus Dunn's Multiple Comparison Test (for nonparametric data). When only two groups were compared, as within winter-captured animals and between juveniles, two-tailed *T* test (for parametric data) or Mann Whitney test (for nonparametric data) was employed. Shapiro–Wilk normality test was applied in all cases. In the case of hepatic GSH levels of summer-captured animals, data were log-transformed prior to application of statistical tests.

Correlations between glutathione status and enzymatic activity were made using data from each summer captured individual by two-tailed Pearson test or by two-tailed Spearman test, respectively when distributions were parametric or nonparametric. Moreover, correlations between oxidative stress markers levels from our previous work (Furtado-Filho et al., 2007) and glutathione status were performed as described above.

Data are reported as means ± SEM and a significance value of $P<0.05$ was employed.

3. Results

3.1. Glutathione status in the liver

Fig. 1A shows that, in summer-captured animals, hepatic GSH-eq concentration increased by 1.9-fold in the transition from embryos to juveniles (SJU). GSH-eq levels in winter-captured juveniles (WJU) were not different from those in winter-captured adults (WAD). Moreover there was no difference in liver GSH-eq concentration when comparing young caimans across seasons (WJU versus SJU). Levels of reduced glutathione (GSH) in caiman liver followed the same trend observed for GSH-eq (Fig. 1B). Hepatic GSH levels in SJU were 2.0-fold higher than in embryos; there was no difference between hatchlings and SJU. GSH concentration in liver of WAD caimans was not different from WJU. Moreover, there was no cross seasonal difference in GSH levels in juvenile caimans.

Hepatic levels of GSSG increased by 1.7-fold in the transition from embryo to SJU (Fig. 1C). Liver GSSG levels in SJU were also significantly higher (by 2.7-fold) than in hatchlings. There was a striking 94% drop in hepatic GSSG concentration in SJU when compared to WJU. These low GSSG levels in WJU were similar to those in WAD caimans (Fig. 1C). The percentage of hepatic GSSG (%GSSG) increased by 1.8-fold in the transition from hatchlings to SJU (Fig. 1D). Curiously GSSG:GSH-eq was 2.0-fold higher in embryos than in hatchlings. Moreover, the percentage of GSSG in SJU (17.3%) decreased (to 1.1% GSSG) in WJU. This result suggests increased hepatic oxidative stress in summer-captured juveniles in comparison with those captured in the winter.

3.2. Glutathione parameters in kidney and lung

Kidney GSH-eq and GSH levels remained unchanged in the different stages of development of summer-captured or winter-captured caimans (Fig. 2A and B). However, GSSG levels and the %GSSG increased by 2.45 and 3.15-fold, respectively, from hatchlings to SJU (Fig. 2C and D). No differences were observed for GSSG and %GSSG in winter-captured animals (WJU and WAD). Moreover, glutathione parameters were unchanged when comparing WJU with SJU kidney samples.

GSH-eq and GSH contents in lung samples from hatchlings were 30% and 54% lower, respectively, as compared to embryos (Fig. 3A and B). Levels of GSH-eq in SJU were not statistically different from

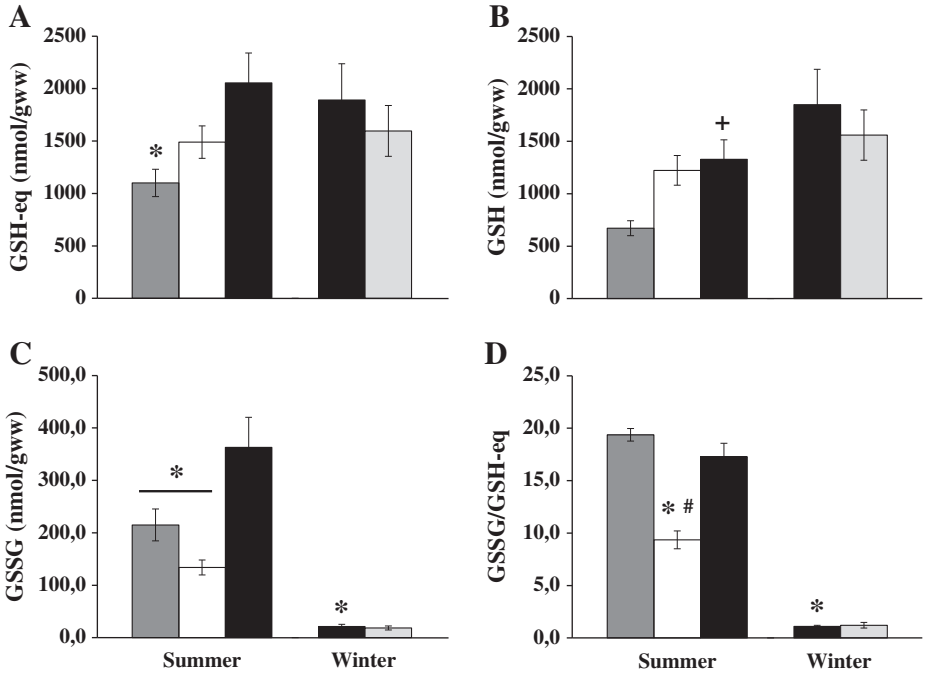

Fig. 1. Glutathione parameters in liver of winter and summer-captured caimans. Embryos: dark gray bars; hatchlings: white bars; juveniles: black bars, adults: light gray bars. * Indicates a statistically significant difference from SJU ($P<0.05$). # Indicates a statistically significant difference from embryos ($P<0.05$). + Embryos versus summer-juvenile were different ($P<0.05$) with log-transformed data (Fig. 1B). n=4–10.

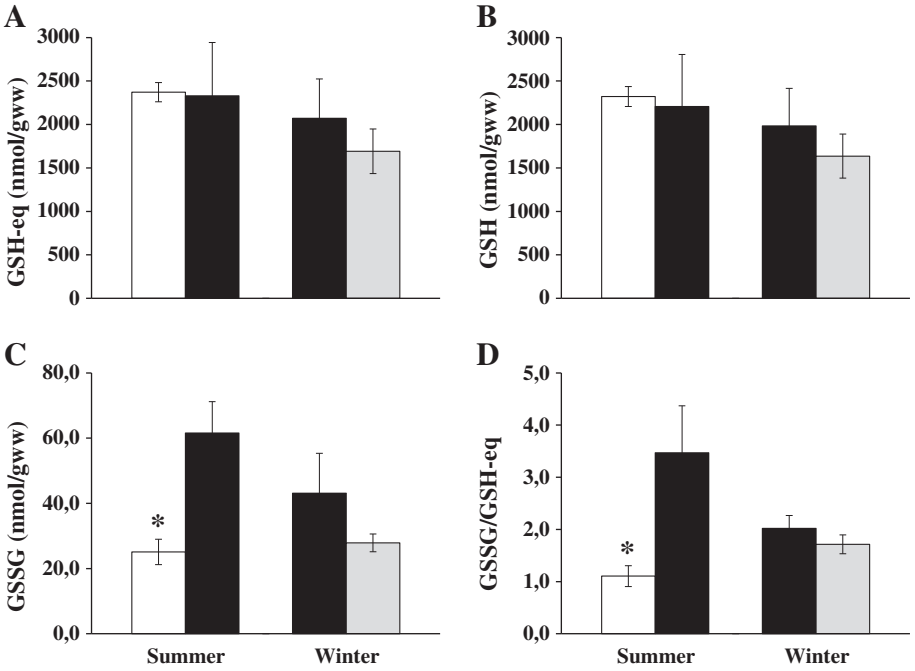

Fig. 2. Glutathione parameters in kidney of winter and summer-captured caimans. Hatchlings: white bars; juveniles: black bars, adults: light gray bars. * Indicates a statistically significant difference from SJU ($P<0.05$). Embryo kidney was not dissected out after collection of eggs. n=5–10.

embryos, while a significant difference was found for GSH (values from SJU were 45% smaller than embryos). Lung GSSG levels increased by 2.5-fold in SJU in comparison with embryos. In addition, %GSSG was 2.6-fold higher in hatchlings when compared to embryos, and 3-fold higher in SJU versus embryos (Fig. 3C and D). Moreover, pulmonary glutathione parameters in winter-captured animals (WAD and WJU) were not significantly different.

When analyzing pulmonary GSH-eq and GSH across seasons, levels in winter-captured juveniles (WJU) were 1.6 and 2.9-fold higher, respectively, than those in SJU. On the other hand, lung GSSG and %GSSG presented contrasting results: the concentrations were 73% and 83% lower, respectively, in WJU when compared to SJU.

3.3. Glutathione parameters in brain, muscle and heart

Brain contents of GSSG were 3.2-fold higher in SJU in comparison with embryos; %GSSG was 4.0-fold higher in SJU than in hatchlings

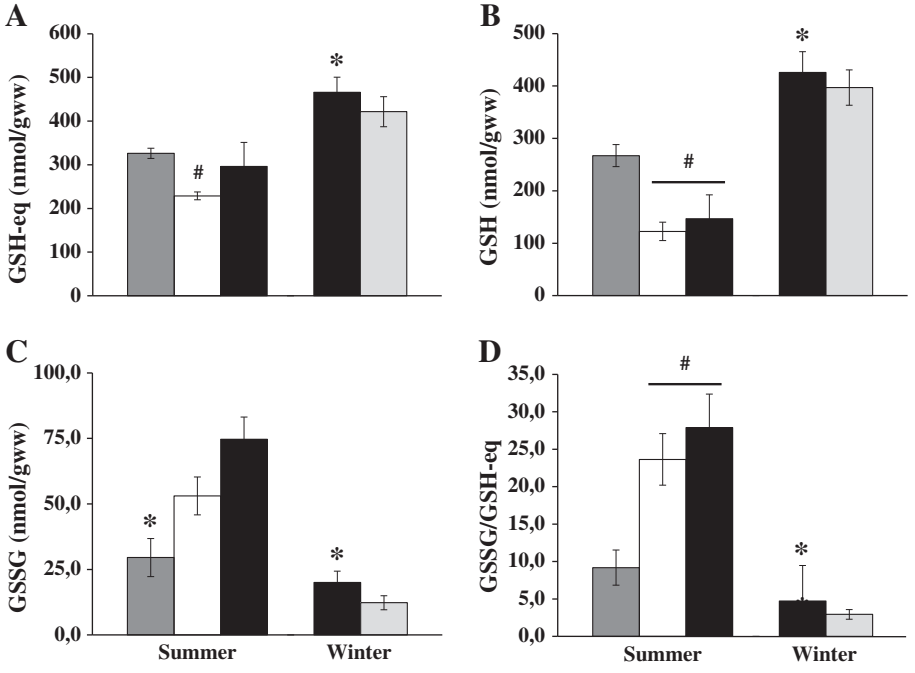

Fig. 3. Glutathione parameters in lung of winter and summer-captured caimans. Embryos: dark gray bars; hatchlings: white bars; juveniles: black bars, adults: light gray bars. * Indicates a statistically significant difference from SJU ($P<0.05$). # Indicates a statistically significant difference from embryos ($P<0.05$). n=5–10.

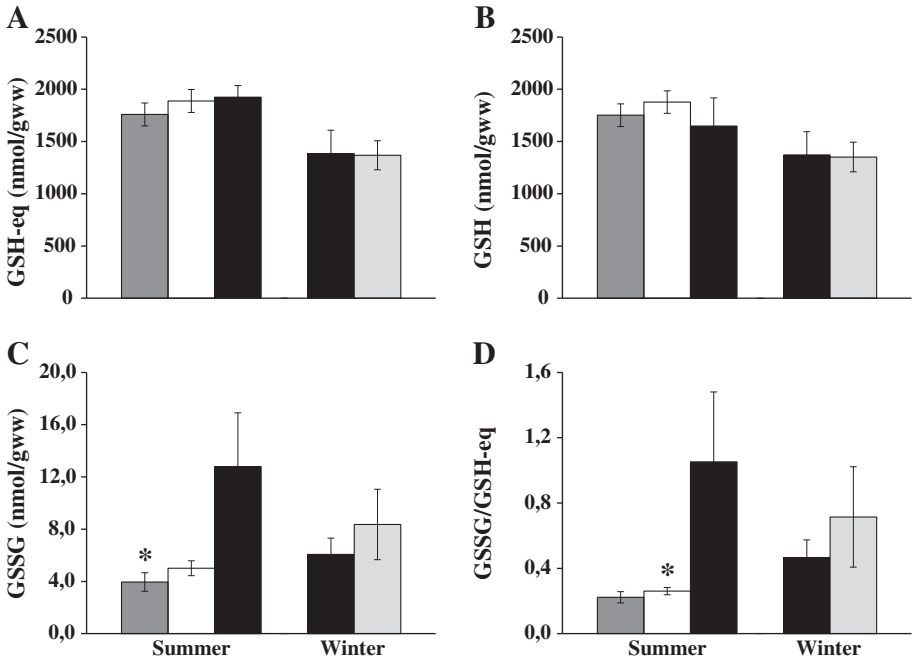

Fig. 4. Glutathione parameters in brain of winter and summer-captured caimans. Embryos: dark gray bars; hatchlings: white bars; juveniles: black bars, adults: light gray bars. * Indicates a statistically significant difference from SJU ($P<0.05$). n=5–10.

(Fig. 4C). There were no significant differences in the other parameters across seasons or caiman development.

Glutathione parameters were not significantly different in leg muscle and tail muscle in caimans from different developmental stages or different seasons (Table 1). In the case of heart (Table 1), no statistically significant changes were observed in the comparison of SJU with hatchlings (GSSG and GSH were not determined in the heart of winter-captured caimans).

3.4. Glutathione parameters across seasons (SJU versus WJU)

Fig. 5 summarizes the changes in glutathione status in caiman organs. GSSG levels and %GSSG were elevated in five organs (by 1.7 to 17.2-fold, in liver, kidney, lung, brain and leg muscle) of SJU in comparison with WJU. Significant differences were observed in hepatic GSSG (17.2-fold) and %GSSG (15.7-fold) as well as in pulmonary GSSG (3.7-fold) and %GSSG (5.9-fold). In the case of GSH-eq and GSH, no statistically significant changes were observed in caiman organs when comparing animals from different seasons, except for lung (see Section 3.2).

Table 1
Concentrations of GSH-eq, GSH, GSSG and % GSSG in caimans tissues at different developmental stages and captured in the summer or winter season.

Tissue/ experimental group	n	GSH-eq	GSH	GSSG	%GSSG
Leg muscle					
Hatchlings	(4)	205.5±37.5	195.4±37.0	5.06±1.03	2.63±0.66
SJU	(6)	240.8±14.4	226.8±14.8	6.99±2.88	2.89±1.15
WJU	(8)	291.3±24.7	283.8±24.7	3.75±0.58	1.35±0.22
WAD	(4)	375.5±47.0	370.7±47.1	2.37±0.28	0.68±0.16
Tail muscle					
Hatchlings	(10)	209.0±14.4	202.9±14.0	3.06±0.65	1.47±0.27
SJU	(3)	242.5±28.5	239.8±29.5	1.34±0.54	0.62±0.29
WJU	(8)	209.1±19.1	205.8±18.9	1.62±0.30	0.79±0.16
WAD	(6)	216.4±25.1	213.8±24.9	1.29±0.19	0.62±0.10
Heart					
Hatchlings	(3)	845.0±106.4	835.7±105.4	4.65±0.80	0.55±0.07
SJU	(6)	1053.3±67.9	1034.6±67.99	9.34±1.37	0.91±0.14

Data are means±S.E.M. n=3–10 individual animals. Values for GSH-eq, GSH and GSSG are in nmol/g tissue. SJU (summer juveniles), WJU (winter juveniles), WAD (winter adults). Hatchlings were captured in the summer.

3.5. Antioxidant enzymes activities in summer-captured caimans

The activity of hepatic GST increased in SJU caimans in comparison with both hatchlings and embryos by 1.85 and 2.4-fold, respectively (Fig. 6B). Hepatic G6PDH was also increased in SJU (by 2.1-fold) when compared to hatchlings (Fig. 6E). No statistically significant changes were observed in the activities of hepatic catalase, Se-GPX and GR (Fig. 6A, C and D).

In the kidney, GST and catalase activities were higher in the SJU group when compared with hatchlings (by 2.9 and 3.25-fold, respectively) (Fig. 6A and B). No statistically significant changes were observed in the activities of Se-GPX, GR and G6PDH in kidney (Fig. 6C, D and E).

A contrasting trend was observed in the lung, in which three enzymes activities (GST, GR and G6PDH) were found to be higher (by 1.7 to 3.1-fold) in embryos than in hatchlings or SJU (Fig. 6B, D and E). Lung catalase activity, however, was increased in SJU caiman when compared to hatchlings and embryos (by 1.6–1.8-fold) (Fig. 6A). No statistically significant changes were observed in lung Se-GPX (Fig. 6C).

3.6. Correlation analyses

The activities of antioxidant enzymes were correlated with glutathione parameters in three organs of embryos, hatchlings and SJU caimans (Table 2). In the liver, GST activity was positively correlated with the concentrations of GSH-eq, GSH and GSSG. In the kidney, significant and positive correlations were observed in the following cases: (i) GST activity versus GSSG levels and %GSSG, (ii) catalase activity versus GSSG levels and %GSSG, (iii) Se-GPX activity versus %GSSG and (iv) GST activity versus GSSG levels. All other correlations in the kidney and liver were non-significant.

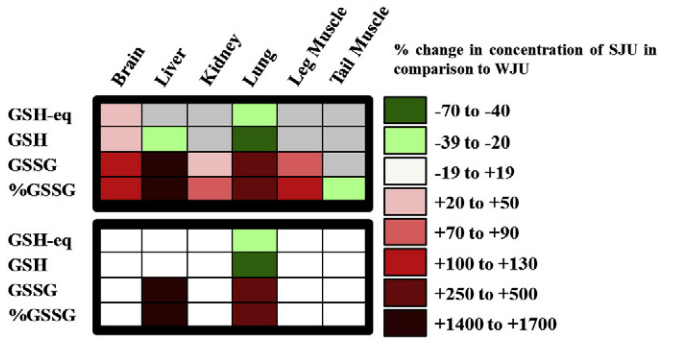

Fig. 5. Comparison of changes in glutathione parameters in tissues of caimans captured in the summer or winter. SJU (summer juveniles) and WJU (winter juveniles). Upper panel indicates all changes and lower panel indicates those that were statistically significant ($P<0.05$).

In the lung, GSSG concentration and %GSSG were inversely correlated with GR activity. GSSG levels were inversely correlated with Se-GPX and GST activities and %GSSG was inversely correlated with G6PDH and GST activities. Moreover, pulmonary GST and GR activities were positively correlated with GSH levels and G6PDH activity was positively correlated with GSH-eq and GSH levels (Table 2). Furthermore, levels of lipid peroxidation (as TBARS; data from Furtado-Filho et al., 2007) were positively correlated with GSSG in liver ($P=0.010$; $r=0.5395$). The same positive correlation was found for %GSSG and TBARS in kidney ($P=0.045$; $r=0.4416$). Other correlations between TBARS and oxidized glutathione (as GSSG or %GSSG) in all organs were non-significant (data not shown).

4. Discussion

4.1. Glutathione levels and oxidative stress in caiman growth and development

In this study we observed that the transition from embryos/hatchlings to juveniles (SJU) in Paraguayan caimans was accompanied by an increase in the levels of GSSG and/or %GSSG in liver, kidney, lung and brain — an exception was the embryo–hatchling transition in liver (see Section 4.2). The increases in these parameters are suggestive of a redox imbalance and oxidative stress in juveniles – who were 2–3 years-old on average (Coutinho, 2000; Coutinho et al., 2005) – in comparison with embryos and hatchlings. On the other hand, the transition juvenile–adult occurred without changes in GSSG concentration and %GSSG.

These results match previous observations that markers of oxidative damage (lipid peroxidation and protein oxidation) increase in several caiman organs in the transition from hatchlings to juveniles — all summer-captured animals (Furtado-Filho et al., 2007). This phenomenon is not observed in the transition from winter juveniles (WJU) to winter adults (WAD). Determinations in various organs of winter-captured caimans show no indication of changes in oxidative damage markers in the comparison between WJU and WAD (Furtado-Filho et al., 2007).

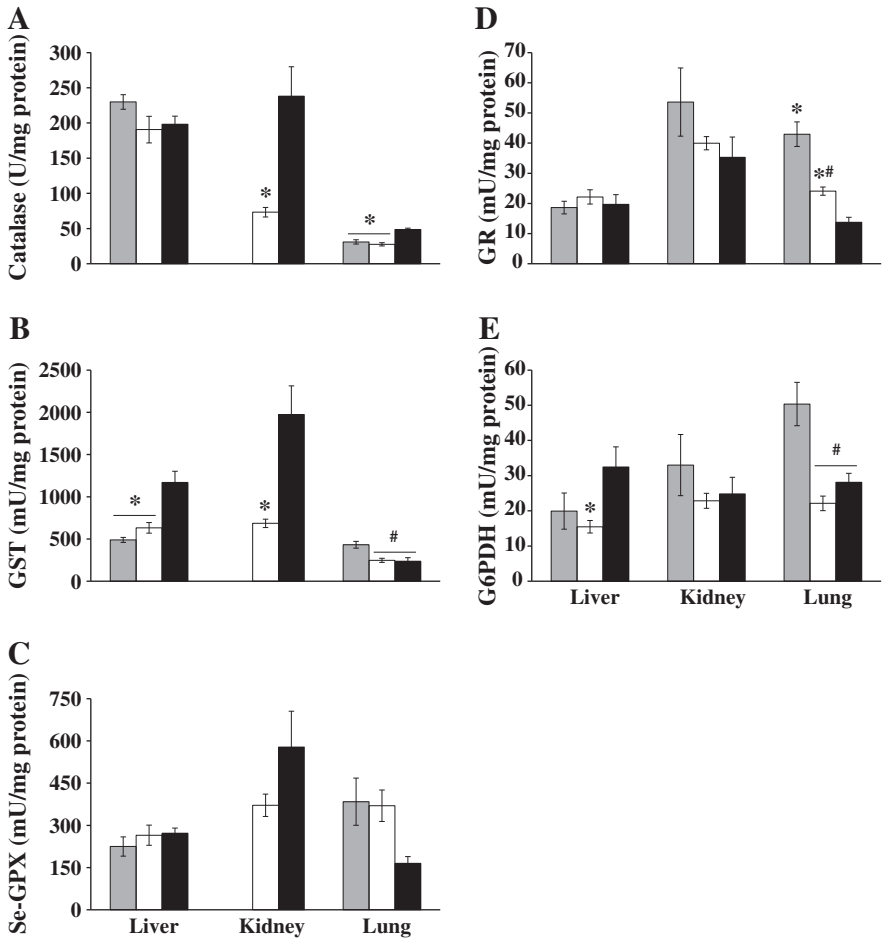

Fig. 6. Catalase, GR, GST, G6PDH and Se-GPX activities in liver, kidney and lung of summer-captured animals. Embryos: gray bars; hatchlings: white bars; SJU: black bars. * Indicates a statistically significant difference from SJU ($P<0.05$). # Indicates a statistically significant difference from embryos ($P<0.05$). ($n=4–10$). Embryo kidney was not dissected out after collection of eggs.

Table 2
Correlation analysis between glutathione parameters and antioxidant enzymes activities of summer captured animals. All development stages were included in the analysis.

	GSH-eq	GSH	GSSG	%GSSG
Liver				
GR	r=−0.4421	r=−0.4376	r=−0.3431	r=−0.1083
	P=0.0510	P=0.0537	P=0.1386	P=0.6496
G6PDH	r=0.1684	r=0.0782	r=0.1971	r=0.1850
	P=0.4778	P=0.7431	P=0.4048	P=0.4350
GST	**r=0.6321**	**r=0.4870**	**r=0.6184**	r=0.2067
	P=0.0028	**P=0.0294**	**P=0.0037**	P=0.3818
CAT	r=−0.0703	r=−0.0946	r=0.0026	r=0.1637
	P=0.7682	P=0.6913	P=0.9911	P=0.4904
GPX	r=0.1038	r=0.0665	r=0.1253	r=0.0452
	P=0.6632	P=0.7805	P=0.5986	P=0.8499
Kidney				
GR	r=−0.4229	r=−0.4015	r=−0.4816	r=0.2103
	P=0.1027	P=0.1232	P=0.0589	P=0.4344
G6PDH	r=−0.3415	r=−0.3337	r=−0.2240	r=0.3691
	P=0.1955	P=0.2065	P=0.4042	P=0.1595
GST	r=0.1151	r=0.0744	**r=0.7009**	**r=0.6273**
	P=0.6830	P=0.7921	**P=0.0036**	**P=0.0123**
CAT	r=0.0836	r=0.0450	**r=0.6593**	**r=0.6468**
	P=0.7668	P=0.8733	**P=0.0075**	**P=0.0092**
GPX	r=−0.1977	r=−0.2266	r=0.4298	**r=0.5896**
	P=0.4800	P=0.4167	P=0.1098	**P=0.0207**
Lung				
GR	r=0.1885	**r=0.4958**	**r=−0.5890**	**r=−0.5618**
	P=0.4262	**P=0.0262**	**P=0.0063**	**P=0.0099**
G6PDH	**r=0.5989**	**r=0.7173**	r=−0.3669	**r=−0.5880**
	P=0.0053	**P=0.0004**	P=0.1116	**P=0.0064**
GST	r=0.0869	**r=0.4612**	**r=−0.6780**	**r=−0.5536**
	P=0.7155	**P=0.0407**	**P=0.0010**	**P=0.0113**
CAT	r=0.2163	r=−0.0629	r=0.4289	r=0.2454
	P=0.3739	P=0.7978	P=0.0669	P=0.3113
GPX	r=−0.0754	r=0.1985	**r=−0.4594**	r=−0.3887
	P=0.7589	P=0.4153	**P=0.0479**	P=0.1000

Values in bold indicate statistical significance (P<0.05).

In the case of caiman liver, there was a significant trend toward an increase in markers of lipid peroxidation (TBARS, lipid hydroperoxides and conjugated dienes) and protein oxidation (carbonyl protein) during animal growth, indicated by animal size, in the transition from hatchlings to juveniles — but not from juveniles to adults (Furtado-Filho et al., 2007). The present observation that GSSG and %GSSG follows the same trend provides support to our earlier proposal that the fast growth observed in the first years of life in *C. yacare* (Coutinho, 2000) is accompanied by increased oxidative damage to lipids and proteins. The fast growth in Paraguayan caimans from hatchlings to juveniles (with an increase of approximately 100-fold in body size) may be accompanied by an increased metabolic rate – which is normally proportional to body size in reptiles (Toledo et al., 2008) – and by a rise in oxidative stress indicators.

The amount of GSSG in the brains of young animals of the rainy season (summer-captured) was about 2.2 and 1.8 times higher than that found in embryos and hatchlings, respectively. These results suggest that the cerebral tissue of young caimans was subjected to greater oxidative stress (and/or redox imbalance) than the same tissue in hatchlings. Brain is very sensitive to ROS attack due to its high amount of phospholipids, main targets of free radicals (Joshi et al., 2010). Thus, the high GSH content found in this organ (second highest value, after kidney) can be interpreted as a strategy for protecting its susceptible structure.

Whether or not the caiman hatchling–juvenile transition is accompanied by an increase in mitochondrial ROS formation, the most important endogenous source of ROS (Tahara et al., 2009) – leading to a physiological oxidative stress – needs to be verified. Several factors modulate the equilibrium in mitochondrial ROS formation and detoxification, including proton leak, levels/activity of antioxidants and metabolic activity (Brookes, 2005; Kowaltowski et al., 2009). Mitochondrial proton leak is known to inhibit ROS formation, and is modulated by mitochondrial membrane composition and uncoupling protein (UCP) activity (Brookes, 2005). Thus, determination of mitochondrial proton conductivity, which has been studied in several ectotherms, including crocodilians (Hulbert et al., 2002), during caiman growth could aid to understand the modulation of ROS formation in the hatchling–juvenile transition.

Our results on glutathione parameters partially agree with other observations (Rebrin et al., 2003) that GSSG:GSH-eq ratio (%GSSG) rises continuously in five mice organs from 5 to 26 months of age. In addition, similar to our observations in caimans, GSH concentration does not change appreciably during mouse aging while GSSG levels increased in most organs (Rebrin et al., 2003). Moreover, erythrocyte GSSG:GSH ratio increases by 2.3-fold and GSH concentration decreases by 27% in 12-month compared to 1-month old rats (Ozturk and Gumuslu, 2004). For the brown trout (*Salmo trutta*), hepatic %GSSG increases with age, from 5 months to 3 years; while no changes occur for liver GSH-eq (Almroth et al., 2010). An increase in GSSG levels and %GSSG is also observed in North Sea *Mya arenaria* clams (with maximum life span of 13 years) from 2 to 9 years of age (Philipp et al., 2005).

The above mentioned studies suggest that GSSG levels and %GSSG are good indicators of age/development effects in organisms. The unchanged GSSG and %GSSG in the juvenile–adult transition, together with unchanged markers of oxidative damage (Furtado-Filho et al., 2007), suggest that such transition happens without oxidative stress (as indicated by lipid peroxidation and protein oxidation) in Paraguayan caimans. It is also relevant to the positive correlation between oxidized glutathione (as %GSSG or GSSG) and TBARS in liver and kidney (see Section 3.6).

4.2. Hepatic glutathione parameters in embryos

The increased %GSSG in embryo liver (versus hatchlings) agrees with observations of increased levels of lipid hydroperoxides and TBARS in this developmental stage (Furtado-Filho et al., 2007). These observations suggest that embryo liver is in a chronic state of oxidative stress, which could be caused by a mild hypoxic environment inside the eggs (see below). Exposure to low oxygen tensions increase mitochondrial ROS formation in some biological systems, leading to oxidative stress (Clanton, 2007; Kolamunne et al., 2011). Thus, a putative increase in ROS formation plus the low levels of hepatic antioxidant defenses (see Fig. 6A) would favor GSH oxidation and a rise in lipid peroxidation in embryo liver.

There are no determinations of oxygen tension in crocodilian embryos in nature, however there are several ecological circumstances, typical of the Pantanal swamps, that may cause hypoxia in *C. yacare* eggs, including (i) oxygen-consuming organic matter decomposition in the nests, which has been investigated for nests of *Crocodylus porosus* made of mounds of decaying vegetation (Booth, 2000) and (ii) poor oxygenation of caiman eggs in the interior of nests that could set a condition of hypoxia for these embryos, in comparison with eggs in peripheral regions, as indicated by studies with leatherback turtle eggs (Reina et al., 2005). Lack of ventilation for eggs in the nest interior could set an increase in pCO_2 (evidence of hypoxia) as reported for chicken eggs in incubators (De Smit et al., 2006). Moreover, a decline in pO_2 has been observed in nests of leatherback turtles with increasing time of embryo development (Wallace et al., 2004). Chicken eggs also suffer a decrease in pO_2 of venous blood at late development, rising again only after pipping (Freeman and Mission, 1970). Furthermore, a decrease in $\dot{V}O_2$ was also observed in the last weeks of embryonic development of various crocodilian species (Thompson, 1989; Whitehead and Seymour, 1990; Aulie and Kanui, 1995).

4.3. Caiman development and antioxidant defenses in liver and kidney

The activities of three antioxidant enzymes increased in liver and kidney in the transition from embryos/hatchlings to juveniles. This was the case of kidney catalase and GST, and liver GST and G6PDH (Fig. 6). Moreover, a positive correlation between animal size and kidney GST activity is observed in summer-captured caimans ($P = 0.009$, $r = 0.613$; (Machado, 2005)), strengthening the trend towards the increase in enzyme activity in SJU versus hatchlings. In the lung, only catalase followed the same embryo/hatchling–juvenile tendency to increase. Other enzymes (GST, GR and G6PDH) presented an inverse trend (see Section 4.4 for this). Superoxide dismutase activity was not determined in our caiman tissues.

The increase in the activities of antioxidant enzymes in liver and kidney of juveniles (as well as catalase in lung) in comparison to embryos/hatchlings can be interpreted as a response to the more oxidative intracellular environment of these tissues. A proposed higher rate of ROS formation in juvenile organs could inflict a chronic increased expression of antioxidant enzymes to control the redox potential of liver and kidney cells, maintaining oxidative damage at "manageable" levels.

Moreover, summer-captured juveniles presented higher hepatic GSH-eq concentration than embryos (as well as higher GSH levels than hatchlings), which could be the result of increased GSH biosynthesis through increased carbon flux (for GSH formation) and/or increase in the expression of enzymes involved in GSH biosynthesis (glutamate-cysteine ligase and glutathione synthase). Higher levels of GSH can not only function as an "extra" non-enzymatic antioxidant defense (Hermes-Lima, 2004), but can also increase the in situ activities of GST, Se-GPX and some peroxiredoxins (because GSH is a substrate of these enzymes). Increased levels of GSH could also result from its release from glutathionylated proteins (deglutathionylation), which plays a relevant role as a reservoir of cellular GSH (Dalle-Donne et al., 2008). Changes in glutathionylated protein levels occur in various tissues during mouse development/aging, from 5 to 26 months (Rebrin et al., 2003).

The proposal that increases in oxidative stress could up-regulate antioxidant enzymes in liver and kidney is supported by the positive correlations of hepatic GST activity versus GSSG levels and of renal catalase, Se-GPX and GST activities versus %GSSG and/or GSSG levels (Table 2). In these cases, the increased GSSG concentration may signify that intracellular ROS steady state level was elevated, bringing about the activation of gene expression of antioxidant enzymes.

One possibility to explain how development-induced oxidative stress could modulate antioxidant enzyme expression is by up-regulating Nrf2 (NF-E2-related factor 2, a transcription factor) gene expression and/or triggering its release from its repressor protein Keap1 (Lewis et al., 2010; Pamplona and Costantini, 2011). This could hypothetically up-regulate the expression of GST in kidney and liver of Paraguayan caimans. Increased expression of Nrf2 raises the expression of GST (Pamplona and Costantini, 2011), as observed in African clawed frogs upon dehydration stress (Malik and Storey, 2009). On the other hand, in the case of hooded seals, muscle Nrf2 expression decreased from pups to adults (Vazquez-Medina et al., 2011).

4.4. Caiman development and antioxidant defenses in the lung

In the lung, the decrease in activity of three antioxidant enzymes – as well as GSH levels – in the embryo/hatchling–juvenile transition came as surprise because %GSSG and GSSG levels are increased in juveniles (SJU). On the other hand, pulmonary markers of oxidative damage (lipid peroxidation and carbonyl protein) are not elevated in SJU caimans when compared to embryos or hatchlings (Furtado-Filho et al., 2007). Moreover, the activities of GST, Se-GPX, GR and G6PDH in lung were inversely correlated with %GSSG and/or GSSG levels. These correlation data indicate that the antioxidant enzymes are acting to maintain the lowest possible state of GSH oxidation in pulmonary cells, via GSSG recycling in the case of GR and G6PDH, and through the control of H_2O_2 levels by Se-GPX. However, because the activities of these enzymes are reduced in the embryo/hatchling–juvenile transition, this could cause the increase in GSSG concentration in SJU lung (less antioxidant enzymes could set up a state of redox imbalance, as indicated for increased %GSSG).

Moreover, it is possible that the increased pulmonary GSH-eq levels and activity of antioxidant enzymes in the embryos, when compared to hatchlings, are part of an especial adaptation to hatching. Because embryo caiman cells may be under mild hypoxia (see Section 4.2) they would experience a higher pO_2 as caimans hatch. Such high embryonic antioxidant capacity in lung could minimize reoxygenation stress during hatching — in line with the hypothesis of "*preparation for birth*" in lungs of mammalian species (Frank and Sosenko, 1987). In accordance, an increase in lung catalase activity occurs during late embryo development of chicken and the bearded dragon *Pogona vitticeps* (Starrs et al., 2001). Moreover, the hypoxic environment inside eggs (see Section 4.2) could activate the expression of antioxidant enzymes and GSH biosynthesis, similar to what happens in anoxic/hypoxic tolerant animals during hypoxia/anoxia exposure (Hermes-Lima and Zenteno-Savin, 2002).

4.5. Does the juvenile–adult transition in caimans occur without oxidative stress?

It was unfortunate that we were unable to determine the activity of antioxidant enzymes in organs of winter-captured juveniles and adults that could have been related with the glutathione and oxidative damage data. That would have strengthened our proposal that the juvenile–adult transition happens without oxidative stress (as indicated by lipid peroxidation and protein oxidation) of physiological nature. The slower rates of growth from juveniles (2–3 years old) to adults (which are at least 10 years old) in comparison to the hatchling–juvenile transition may keep the metabolic rate (in relation to body mass) unchanged when comparing the two groups. This would maintain oxidative metabolism and rates of ROS formation at similar levels in adults and juveniles. When looking at oxidative damage markers in liver of hatchlings versus adults, it is clear that they are much higher in adults. Levels of lipid hydroperoxides, conjugated dienes, TBARS and carbonyl protein in adult liver are 2.7 to 4.4 times higher than in hatchlings (Furtado-Filho et al., 2007). This may indicate that hepatic oxidative damage increases in the hatchling–juvenile transition, stabilizing in adults. A relevant bias in the hatchling–adult comparison is that adult caimans were captured in the winter and hatchlings in the summer. Thus, the effect of season was analyzed in animals of similar age group (juveniles) from winter and summer (see Section 4.6).

The analysis of the juvenile–adult transition has an important caveat which is the fact that most captured-adults had empty stomachs and juveniles presented semi-digested fish or invertebrates (Furtado-Filho et al., 2007). It has been shown that food deprivation alters mitochondrial ROS production, thiol redox status and levels of endogenous antioxidant defenses, therefore affecting oxidative stress markers (Di Simplicio et al., 1997; Sorensen et al., 2006; Vazquez-Medina et al., 2010). However, how animals deal with food restriction is dependent on their natural way of life. Those who face long periods of natural food deprivation will behave physiologically different from those who do not. For example, food deprivation happens without major changes in markers of oxidative damage in elephant seal pups (Vazquez-Medina et al., 2010). We observed that markers of oxidative stress and glutathione status were mostly unchanged when comparing adult caimans (under food deprivation in winter) with juveniles (from either summer or winter). This suggests that food deprivation was without effect on these parameters; in any case, these observations must be regarded with care. All the problems we faced in the present field-study represent a common "biological bias" of any study in the areas of ecological physiology and biochemistry.

4.6. Caiman free radical metabolism across seasons

The %GSSG and GSSG levels were increased in summer (versus winter) in five juvenile organs, reaching significance in liver and lung (Fig. 5B). Moreover, TBARS is also elevated (by 1.8 to 2.1-fold) in liver, brain and leg muscle in SJU when compared to WJU caimans (Furtado-Filho et al., 2007). These changes could be hypothetically explained by the 5 °C difference in the average nocturnal temperatures between the summer and winter seasons in Nhecolândia (the site of captures) in the two preceding weeks of captures (~27 versus ~22 °C, at 8 PM; see Furtado-Filho et al., 2007). This may lead not only to higher metabolic rates but to increased oxidative stress in SJU organs. A study with American alligators showed that the Q_{10} for resting VO_2 was 2.8 between 15 and 25 °C and 2.3 between 25 and 35 °C (Emshwiller and Gleeson, 1997). This suggests that a 5 °C elevation in nocturnal temperatures generates an increase of at least 50% in metabolic rate in *C. yacare*. Such an increase in temperature-dependent metabolic rate could set up changes in oxidative pathways and free radical metabolism in an ectothermic vertebrate (Sohal et al., 1985; Hulbert et al., 2007).

4.7. Diving and oxidative stress

Another point to be considered in relation to differences in the activities of antioxidant enzymes is diving. Diving resembles the ischemia/reperfusion process and its consequences because the animals make circulatory adjustments that lead to hypoxia due to shunting of blood flow to vital organs (Axelsson et al., 1991; Kooyman and Ponganis, 1998). As they return to the surface, O_2 consumption and re-establishment of blood flow can lead to an increase in ROS formation. It has been shown that animals in similar situations increase their antioxidant enzymes during the reduction of oxygen (hypoxia/ischemia episode) as a way to prepare for the increase in ROS generation that follows (Hermes-Lima et al., 1998; Hermes-Lima and Zenteno-Savin, 2002; Vazquez-Medina et al., 2006; Gorr et al., 2010; Vazquez-Medina et al., 2012). Moreover, there is some indication that animals that dive have a higher antioxidant capacity when compared with non-diving animals. This has been proposed in comparisons between penguins and non-diving birds (Zenteno-Savin et al., 2010), between pigs and seals (Zenteno-Savin et al., 2002; Vazquez-Medina et al., 2006) and among mammalian divers of different diving capacities (Cantu-Medellin et al., 2011). Furthermore, hooded seals increase their endogenous antioxidant defenses just before they are able to dive in search for food (Vazquez-Medina et al., 2011). That could be considered a kind of preparation for the stress of diving. Thus, taking into consideration our results, the increased antioxidant defenses in juvenile caimans, in comparison with hatchlings, may possibly make them prepared to deal with dive-related physiological oxidative stress.

4.8. Conclusion

In summary, our results suggest that the transition from embryos/hatchlings to juveniles in summer caimans is accompanied by an increase in oxidative stress markers and activities of antioxidant enzymes. Such changes are likely to make juveniles more suited to face conditions that may promote a greater generation of ROS, for example, reoxygenation after dive. Our results also suggest that the juvenile–adult transition occurs with a greater management of oxidative damage when compared with the transition from embryos/hatchlings to juveniles, suggesting that a physiologically functional antioxidant protection is achieved in the juvenile stage.

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