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Oxidative and osmotolerant effects in *Salvator merianae* (Squamata: Teiidae) red blood cells during hibernation

Efeitos de oxidação e osmotolerância dos eritrócitos de *Salvator merianae* (Squamata: Teiidae) durante a hibernação

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Abstract

Hibernation is a natural condition of animals that lives in the temperate zone, although some tropical lizards also experience hibernation annually, such as the lizard native from South America, *Salvator merianae*, or "tegu" lizard. Even though physiological and metabolic characteristic associated with hibernation have been extensively studied, possible alterations in the red blood cells (RBC) integrity during this period remains unclear. Dehydration and fasting are natural consequences of hibernating for several months and it could be related to some cellular modifications. In this study, we investigated if the osmotic tolerance of RBCs of tegu lizard under hibernation is different from the cells obtained from animals while normal activity. Additionally, we indirectly investigated if the RBCs membrane of hibernating tegus could be associated with oxidation by quantifying oxidized biomolecules and the activity of antioxidant enzymes. Our findings suggest that RBCs are more fragile during the hibernation period, although we did not find evidence of an oxidative stress scenario associated with the accentuated fragility. Even though we did not exclude the possibility of oxidative damage during hibernation, we suggested that an increased RBCs volume as a consequence of hypoosmotic blood during hibernation could also affect RBCs integrity as noted.

Keywords: tegu, osmotic fragility, erythrocytes, oxidative stress.

Resumo

A hibernação é uma condição natural dos animais que vivem na zona temperada, embora alguns lagartos tropicais também experenciem hibernação anualmente, como é o caso do lagarto nativo da América do Sul, *Salvator merianae* ou "teiú". Embora as características fisiológicas e metabólicas associadas à hibernação tenham sido amplamente estudadas, possíveis alterações na integridade das hemácias durante esse período ainda permanecem obscuras. A desidratação e o jejum são consequências naturais da hibernação por vários meses e podem estar relacionadas a algumas modificações celulares. Neste estudo, investigamos se a tolerância osmótica de hemácias do lagarto teiú sob hibernação são diferentes das células obtidas de animais em atividade normal. Além disso, investigamos indiretamente por meio da quantificação de biomoléculas oxidadas e da atividade de enzimas antioxidantes se a membrana das hemácias dos teiús em hibernação poderia estar associada à oxidação. Nossos resultados sugerem que as hemácias possuem maior fragilidade durante o período de hibernação, embora não tenhamos encontrado evidências de um cenário de estresse oxidativo associado à essa fragilidade acentuada. Embora não tenhamos excluído a possibilidade de dano oxidativo durante a hibernação, sugerimos que um aumento no volume das hemácias como consequência de sangue hipoosmótico durante a hibernação também poderia afetar a integridade de hemácias, tal como foi observado.

Palavras-chave: tegu, fragilidade osmótica, eritrócitos, estresse oxidativo.

1. Introduction

Salvator merianae (Duméril and Bribon, 1839) is a neotropical lizard that annually goes through three to five months burrowed in hibernation (Andrade et al., 2004; Toledo et al., 2008). An endogenously controlled condition, marked by metabolic depression, dehydration, and consequently low oxygen consumption (Andrade et al.,

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2004; Souza et al., 2004). Traditionally, studies that have investigated the unique biochemical and cellular characteristics of hibernators and other hypoxic resistance animals, highlight significant metabolic depression and consequential responses associated with it (Giraud-Billoud et al., 2019). An extensive literature has focused on the redox metabolism of some tissues, such as the liver, intestine, and interscapular adipose brown tissue (IBAT), but little attention has been given to the red blood cells (RBCs), especially on non-mammal hibernators, such as *S. merianae* (Carey et al., 2000; Hermes-Lima and Zenteno-Savín, 2002; Pérez-Campo et al., 1990). Nevertheless, investigation on *S. merianae*'s RBCs dynamics during hibernation can contribute to a broader understanding of how different cell types respond to extreme metabolic states.

In contrast to mammalians, ectotherms vertebrate's RBCs are nucleated and highly metabolic active, with functional mitochondria and other cellular organelles (Giacomo et al., 2015). This characteristic is particularly relevant, considering that for other categories of metabolic functional cells, alterations in the whole organismal metabolism associated with oxygen availability variation can be a trigger of reactive oxygen species (ROS) overproduction (Ramos-Vasconcelos and Hermes-Lima, 2003). The consequences of exceeding ROS if there is no balance with antioxidant molecules and enzymes can be an oxidative stress state and, consequently, induction of cellular functional loss. Especially because ROS can interact with a wide range of biomolecules within the cell, including fatty acids from the cellular membrane, which leads to aldehydes products (Barrera et al., 2018). In the case of RBCs, if cellular structure, such as the membrane is damaged by the interaction with ROS, it could lead to RBCs loss of integrity and facilitate hemolysis (Clemens and Waller, 1987; Dikmenoglu et al., 2008). The efficient redox metabolism consisted of antioxidant enzymes and non-enzyme molecules that play an important role in scavenging the oxygen radicals and prevent tissue damage in all cell types (Grundy and Storey, 1998; Halliwell and Gutteridge, 2015). An important defense system in erythrocytes, as in other cells, is partially consisted of enzymatic means such as glutathione reductase (GR) and glutathione peroxidase (GPx) and low molecular weight non-enzymatic antioxidants, such as tripeptide glutathione (GSH). This redox metabolism plays an important role in scavenging the oxygen radicals that defends against oxidative stress. The antioxidant function of GSH is accomplished by GSH peroxidase (GPx)-catalyzed reactions, which reduce hydrogen peroxide (H_2O_2) and lipid peroxide as GSH is oxidized to GSSG. GSSG, in turn, is reduced back to GSH by GR at the expense of NADPH, forming a redox cycle (Halliwell and Gutteridge, 2015). Still, there is no consistency regarding the oxidative status and the antioxidant response in the hypometabolic state in ectotherms vertebrates, varying between species and tissues. It has been evidenced that even some animals are capable of sufficiently control oxidative damage when hibernate, others still experience considerable oxidation in some tissues. As it occurs in toads during estivation, which has their tissues in oxidative stress status due to an overall decrease of the antioxidant system (Hermes-Lima and Zenteno-Savín, 2002)

Despite the energetic metabolism, studies showed that water depletion can promote overly ROS accumulation, potentially damaging, even more, the biomolecules, as it reduces the hydration shell of biomolecules and the biomembranes become more susceptible to be attacked by ROS (Schliess and Häussinger, 2002). These changes could induce dysfunction of specific enzymes or trigger chemical reactions that in normal hydration would not occur (Schliess and Häussinger, 2002). Under normal metabolic conditions, the antioxidant system would scavenge the ROS, although, when dehydrated these mechanisms might be compromised (França et al., 2007). Furthermore, RBCs of animals that live in arid environments and experience long dry periods, demonstrated that despite oxidative damage, can also be affected by biophysical stress because of dehydration (Yagil et al., 1974). As dehydration prolongs for a long time, blood osmolality changes, affecting osmotic pressure gradient and K+/ Na+ transport, affecting the RBCs resistance (Brugnara, 1993). In such a context, we aimed to investigate if *S. merianae* ´s RBCs have their integrity affected during the hibernation period, characterized by hypometabolism and dehydration conditions. Therefore, we measured membrane integrity of RBCs through the osmotic fragility test, allowing us to infer osmotic hemolysis degree of the studied specimens. We also quantified oxidized biomolecules generated from the RBCs in order to infer oxidative lesion in these cells and measured the activity levels of antioxidant enzymes, both during the hibernation period and after hibernation when the animals have returned to activity.

2. Materials and Methods

2.1. Ethics statement

All procedures in this work were in accordance with the precepts of Law nº 11,794, of October 08, 2008, of Decree Nº 6,899, of July 15, 2009, and with regulations supervised by the National Council for the Control of Animal experimentation (CONCEA). Moreover, this study was approved by the Committee on Animal Use Ethics (CEUA), of Instituto de Biociências, Letras e Ciências Exatas, UNESP. Registration N ° 149/2016 – CEUA.

2.2. Animal care and blood collection

Animals used in this work were bred in captivity and maintained in UNESP, Campus São José do Rio Preto - SP, Brazil, under the supervision of the Laboratory of Comparative Zoophysiology of Vertebrates. We studied blood samples collected from six males specimens of *S. merianae*. Only male specimens were included because of the availability of specimens in UNESP. Even though erythrocytes of human and mice have been demonstrated to present sex differences in resistance to lysis (Kanias et al., 2016), other animals, like pigeons (*Columba livia*; Oyewale, 1994), peafowls (*Pavo cristatus*; Oyewale, 1994), Sahel goats (*Capra aegagrus hircus*; Igbokwe et al., 2016), and including

Salvator merianae, erythrocytes susceptibility to lysis do not indicate to be sex-related (Troiano et al., 2008). Blood samples were collected at two time points, the first was in July, corresponding to the period of hibernation, in which the animals were inactive for two months. Subsequently, the second collection occurred in August, corresponding to the active period, ten days after the animals returned to normal activity pattern with constant feeding and hydration during all these days. Individual weights varied from an average of 0.9 kg during hibernation to 1.25 kg while the active period and temperature varied from approximately 19ºC to 21ºC in hibernation and active periods, respectively. The animals were monitored daily, aiming to identify the moment that they have entered into hibernation and subsequently awoke. Behavioral changes indicatives (entering and awakening from hibernation) were based on previous studies (Sanders et al., 2015) and these were mainly related to voluntary feeding and hydration. Thus, during the periods of activity, the animals moved frequently, in addition, to be fed and hydrated. On the other hand, the onset of the hibernation period was characterized by apparent lethargy and disinterest in foraging. During the period of hibernation (May to August), the animals were caged in plastic boxes (60cmx40cm) lined with dry foliage and without the availability of water and food. In August the animals were gradually awakening, as they were active and looking for food. With the return to activity, the animals were kept in external enclosures, with water and food available. During the activity, animals were feed based on chicken meat and egg.

2.3. Biochemical analyses

2.3.1. Preparation of concentrated RBCs

Peripheral blood samples were obtained from the ventral coccygeal vein in heparin tubes and were immediately stored on ice. Blood volume varied from 2 to 5 mL depending on the individual weight at the time of collection, always corresponding to 8 to 10% of the total weight. Each blood sample was centrifuged at 850g for 20 minutes in order to obtain concentrated RBCs. Plasma was gently separated while erythrocytes were carefully washed three times with cold phosphate-buffered saline (PBS - 136mM NaCl, 3mM KCl, 10mM Na2HPO4/KH2PO4, pH 7.4) for buffy coat removal. Precipitate cell samples were then divided and directed to two distinct pathways. One part was submitted to hemolysate solution preparation and another part was directed to the osmotic fragility test right after buffy coat removal.

2.3.2. Preparation of hemolysates and membrane extraction

It was prepared hemolysates by adding a fraction of precipitated RBCs into 20 volumes of cold ultrapure water. On the same day of blood collection, subsequent enzymatic activity analyses were performed with the first portion of the hemolysis solution. In order to quantify the oxidative markers from biomolecules in the membrane, from the second portion of hemolysate, an aliquot of 100 µL hemolyzed rich membrane was subjected to high-speed centrifugation (25000g for 30 minutes at 4°C), and the precipitated membrane was washed three times (25000g for 10 minutes at 4°C) with Dodge buffer (Na_2HPO_4/KH_2PO_4 to 20 Mm; pH 7.4) (Dodge et al., 1963), supplemented with 0.1 Mm of fluoride of phenylmethylsulfonyl (PMSF) (Rocha et al., 2015). The precipitate of the membrane obtained was resuspended with 200 µL of Dodge buffer plus 5% of Triton X-100 (V/V) and separated into two aliquots frozen at -80°C for further analysis.

2.3.3. Glutathione Peroxidase (GPx) and Glutathione Reductase (GR) activities

Enzymatic activity of GPx was determined based on Sies et al. (1979) protocol, and GR based on Carlberg and Mannervik (1985) protocol; both measured by spectrophotometry technique.

It is worth noting that all the biochemical assays had temperature controlled according to the average body temperatures (tegu) of each experimental period (hibernation and activity) during the collection time.

2.3.4. Marker of oxidized biomolecules

The quantification of oxidized biomolecules was performed on erythrocyte cell membrane extract, using the product formed with thiobarbituric acid (TBA), according to Esterbauer and Zollner with few modifications (Esterbauer and Zollner, 1989). The product was detected by HPLC-FD according to Domijan et al. (Domijan et al., 2015). The calculations were based on an analytical curve previously constructed by injecting authentic standards into the HPLC system.

2.3.5. Osmotic Fragility (OF) test

We used the osmotic fragility test to investigate the integrity of RBCs in both hibernation and active period, allowing us to infer osmotic hemolysis degree of the studied specimens besides the typical results of resistance and/or susceptibility to lysis (following Ansari et al., 2015 and Silva et. al., 2019).

We tested osmotic fragility according to Ansari et al. (2015). The blood portion with fresh concentrated erythrocyte, 50 uL of were washed three times in 1 ml PBS (136mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4/KH_2PO_4, pH 7,4). After each wash, the mixture of concentrate and PBS was centrifuged for 10 minutes at 3500g to separate the cells from the buffer and residues. After the last wash, the cells were resuspended in 450 µL of PBS to maintain the integrity of the cells. Then, 50 µL from this suspension was transferred to 5 ml of lysis buffer (5 mM Na_2HPO_4; pH 7,2) or different saline concentrations, ranging from 20% of NaCl to 70% of NaCl, most hypotonic to an isotonic concentration (referring to the internal osmolarity of the animal). After transferring the cells to different concentrations of salt, it was left to rest for 2 hours at room temperature to ascertain the possible occurrence of lysis. Finally, the samples were centrifuged for 10 minutes at 850g and the supernatant was collected for analysis in a spectrophotometer (Thermo Scientific, Evolution 300) from the reading of hemoglobin in wavelength of 540nm.

2.4. Statistical analyses

Statistical analyses were carried out in Rstudio software, from (R Development Core Team, 2017) using *ez* package (Lawrence, 2016), while the graphics were made in the Software GraphPad Prism Version 5.01 for Windows (GraphPad Software). For the osmotic fragility test, we verified the normality of the data using visual indicative with Normal Probability Plots of Residuals and data homoscedasticity by Levene's test, assuming a significance level of 0.05. For comparisons analysis, we adopted General Linear Model (GLM) in the two-factor repeated measures ANOVA design, because this approach allows different combinations of categorical and continuous variables, thus checking the effects of the groups evaluated, saline concentrations, as well as any interactions between these predictors on dependent variables (the osmotic hemolysis degree). When appropriate, we applied Bonferroni's post hoc test, a robust and conservative method (McDonald, 2014; Quinn and Keough, 2002).

For further biochemical results, we verified the normality of the data using the Shapiro-Wilk test, while homoscedasticity was evaluated by Levene´s test. For non-normally distributed data, subsequent analysis of group comparison was performed using the Wilcox test for non-parametric data, while for the parametric ones, we adopted the paired T-test. The results were expressed as median, range, mean ± SD (standard deviation) in the graphs of their biological values or logarithmic in base 10 values, when appropriate.

3. Results

After the individuals were kept in seclusion for two months without eating and hydrating, they have lost a considerable amount of body mass and body temperature. During hibernation, individuals' average weight fell to approximately 93g and body temperature in 2°C. Animals during hibernation presented significantly higher hemolysis susceptibility. We tested RBCs for hemolysis in a specter of saline solution, following the osmotic fragility protocol.

Animals during hibernation presented on average elevated osmotic fragility compared to the active animals when considered the interaction of periods with different saline concentrations (paired ANOVA: *F= 2.56*; *P= 0.03*) (Figure 1). Such differences were probably mostly influenced by the elevated scores from the saline concentration 0.5 and 0.6 from hibernation in comparison with the almost null scores from the active period, as shown in Figure 1a. Still, symmetrically paired salinity concentrations did not present significant differences between periods after Bonferroni correction. Even though, despite the overall higher susceptibility to lysis in the hibernation period, this was not accompanied by higher production of oxidized biomolecules on erythrocyte membranes collected during hibernation. The levels of oxidized biomolecules measured from the RBCs membrane collected from hibernate individuals did not show to be higher from RBCs collected while animals were awake (Wilcox*: F= 7.23*; *P= 0.31*), actually despite the absence of statistical significance, RBCs from active animals showed a trend to higher levels of oxidized biomolecules (see Figure 2). Furthermore, similar results were found for both antioxidant enzymes investigated where hibernate animals did not show any influence on RBCs GPx and GR activities (paired T-test: *F= 0.06-1*; *P= 0.68* and *F= 0.45*; *P= 0.10*, respectively) (see Figure 3 a, b).

4. Discussion

Information regarding the redox metabolism of *Salvator merianae* related to its annual metabolic cycle has been received recent attention (Moreira et al., 2018). Although

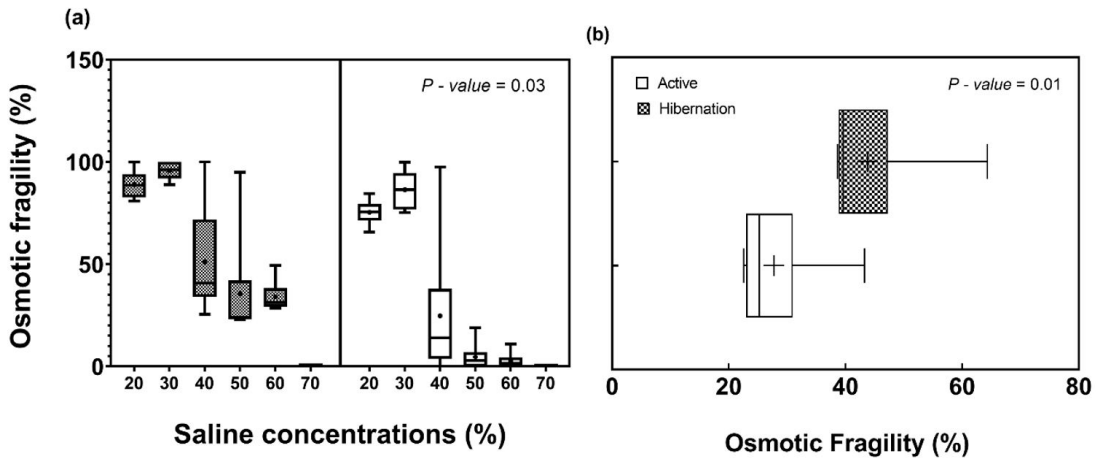

Figure 1. (a) Osmotic fragility in percentage by saline concentration from both periods studied (GLM: $F = 2.56$, $P = 0.03$). No statistical differences were observed between individualized pairs of saline concentrations; (b) Overall osmotic fragility difference between periods considering only periods effect (GLM: $F = 9.72$, $P = 0.01$) ($n = 6$). Sum symbol (+) is the mean and SD are the horizontal lines; median is the vertical line within the boxes and range is distance from the median to the end of the boxes.

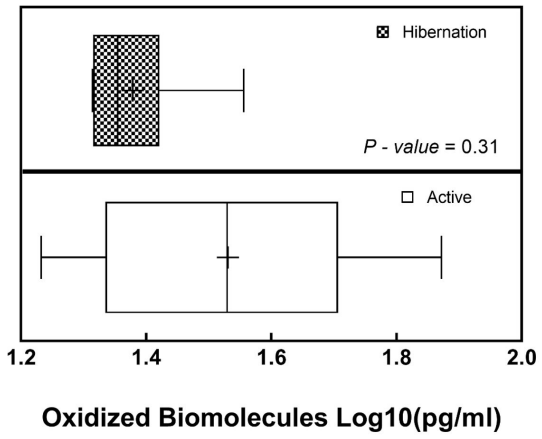

Figure 2. Logarithmic in base 10 values of oxidative biomolecules level for each period collected (n = 6). Wilcox Test: P = 0.31. Sum symbol (+) is the mean and SD are the horizontal lines; median is the vertical line within the boxes and range is distance from the median to the end of the boxes.

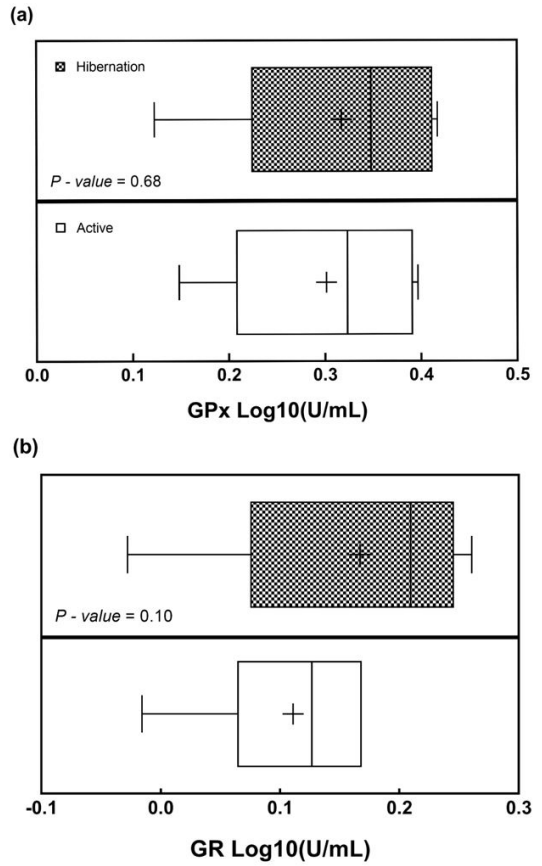

Figure 3. Logarithmic in base 10 values of (a) Enzymatic activity level of glutathione peroxidase – GPx and (b) activity of glutathione reductase - GR of red blood cells in hibernation and active periods (n = 6). T-test, P = 0.68 and P = 0.10, respectively. Sum symbol (+) is the mean and SD are the horizontal lines; median is the vertical line within the boxes and range is distance from the median to the end of the boxes.

similar to other studies with non-mammal hibernators, the focus was inclined to specific tissues, such as the intestine, liver, and lung (Hermes-Lima and Storey, 1998; Welker et al., 2013; Giraud-Billoud et al., 2019), whereas our understanding of the effects that the hibernate state might have on the RBCs remained unknown. As far as we are concerned we were the first that investigated if *S. merianae´s* RBCs integrity is affected on hibernation, and additionally, we searched for possible effects of accentuated oxidation in the RBCs during hibernation. As usual, hibernation was verified by the constant observation of the animals and the hypometabolic condition induced loss of weight and body temperature. Under estivation or dehydration RBCs membrane integrity can be influenced by several factors, such as by the interaction with ROS or because of alteration in osmolality between intra- and extracellular space (Frank et al., 1998; Baloyi et al., 2006; Igbokwe, 2019). For the animals in this study, our findings suggest that RBCs membrane integrity was negatively affected during hibernation, becoming more fragile than in the active period, even though, these results were not accompanied by an alteration in oxidized biomolecules markers or the antioxidant enzymatic activity that we analyzed.

In general, RBCs from ectotherm vertebrates present higher resistance for lysis when compared with those from endotherms, which under osmotic fragility tests often have their hemolysis occurring in much more hypotonic solutions (Aldrich et al., 2006). Nevertheless, when accounting for animals that experience extreme metabolic depression in order to thrive in environmental constraints, RBCs may also be compromised. Long periods of water deprivation or estivation increase RBC's structure susceptibility to oxidative damage (Johnstone et al., 2017). It can be related to an elevation of ROS production, which in turn, is derived from an internal hypoxic status on the animal (Reischl, 1986; Storey, 1996). It has been proposed that ROS production could be accentuated during lower metabolic rates, due to mitochondrial electron leakage (Guzy et al., 2005; Hamanaka and Chandel, 2009). RBCs membranes are particularly susceptible to the effects of ROS as a result of its highly unsaturated fatty acids, which case could compromise the cell integrity (Behn et al., 2007). Despite the fact we could not detect alteration in enzymatic activity, oxidative damage followed by antioxidant activity associated with hypometabolic periods varies among tissues and/or species (Gorr et al., 2010; Hermes-Lima et al., 2015; Napolitano et al., 2019). Whereas some organisms appear to experience higher oxidation accompanied by elevated antioxidant enzymatic activity during hypometabolism (lizards submitted to an 8% of oxygen (Zhang et al., 2015); cold-acclimated frogs (Pérez-Campo et al., 1990); aestivated frogs in the Brazilian Caatinga (Moreira et. al. 2020), there are cases of animals that do not demonstrate increased oxidative damage or antioxidant enzymatic activity during hypoxia in plasma sampling (hatchling turtles, Baker et al., 2007). Still, none of these previous studies investigated RBCs, especially metabolic active RBCs from reptiles.

Interestingly, in our study with *S. merianae*, the absence of variation in enzymatic activity in RBCs while hibernation

could possibly lead to two different interpretations. The first is that the production of ROS during hibernation was probably not higher than posteriorly active for 10 days; or in second, that the levels of enzymatic activity observed in both periods are sufficient to control the difference in ROS that could have been occurring. Both possibilities are valid considering that antioxidant enzymes are activated by ROS, integrating a balanced feedback system (Marinho et al., 2014; Sakellariou and McDonagh, 2018). The absence of alterations in ROS levels between periods would not trigger a differentiated production of enzymatic activity, whereas possible fluctuations in ROS could not be enough to request more enzymes. Also, as we only measured the product of biomolecule interaction with ROS and not directly ROS within the RBCs, we are unable to directly interpret fine-scaled ROS alterations. Furthermore, still there was not siggnificant difference, a trend of increased oxidized biomolecules from the active period could be related to the elevated metabolic activity during awakening period from hibernation (August). Still, the capacity of RBCs to sustain the level of enzymatic activity under dehydration and starvation equally to the levels when the animals are normally feeding and hydrating is no less compelling and deserves attention.

Moreover, the elevated fragility from the RBCs collected during hibernate animals could also be related to other factors besides membrane oxidation, for instance, change in cell packed volume (CPV) (Troiano et al., 1998; Jegede et al., 2020). Long periods of organism dehydration can generate osmotic disbalance that affects the form of the erythrocyte, and also, variation in hemoglobin can affect the form as well (Buffenstein et al., 2001; Baloyi et al., 2006). These changes are problematic in animals that naturally pass through dehydration periods, followed by intensive rehydration. It can suffer hemolysis as a result of a rapid shift in the plasma to hypo-osmotic concentration and eventually water permeation in the RBCs. Mechanisms described as being involved in constraining hemolysis derived from osmotic variance have been evidenced for red kangaroo or African camels (Buffenstein et al., 2001; Al-Qarawi and Mousa, 2004). These animals showed similar osmotic fragility baseline during water deprivation and after rehydration state, being interpreted as adaptations to life in arid environments. Particularly the red kangaroos can maintain constant plasma concentration during the water deprivation period, thus maintaining osmolarity (Buffenstein et al., 2001). Compared with our results, the opposite might happen in the tegu lizards from this study, thus resulting in probable increasing RBCs volume due to osmotic variances. Troiano et al. (2008) showed that RBCs of *S. merianae* increase in CPV and hemoglobin concentration (MCH), while hibernation, in contrast with the pattern found in mammal's RBC (see Huisjes et al., 2018) that usually have decreased in CPV when dehydrated. If the RBCs in this study presented the same tendency and become larger during hibernation, the increased membrane tension under extension could explain the hemolysis in less hypotonic solutions and elevated levels of fragility. Still, our hypothesis of CPV association with increased RBCs osmotic fragility during hibernation would benefit from further studies that statistically test the correlation of hemolysis with cell size change between seasonal periods.

5. Conclusions

This study raises new insights about oxidative and osmotolerant effects in RBCs from a hibernator ectotherms, the *S. merianae*, that have been understudied until now. We demonstrated that both BO markers and respective enzymes usually responsible to control the overproduction of ROS are constant in both periods. Still, we highlight that oxidation and osmotic implications could still be related to the accentuated fragility of RBCs collected from animals during hibernation. Therefore, hindering us to completely discard the hypothesis of oxidative influence in the observed fragility during hibernation. More investigations on changes in RBCs membrane are needed to better understand if, during hibernation, membrane molecule structures are altered indicating osmotic consequences or other possible oxidation pathways. Nevertheless, the comprehension of the redox metabolism in RBCs of *S. merianae* can contribute to our knowledge about cellular mechanisms behind hibernation in an ectotherm.

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