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Transport stress in bullfrog: Hematological and plasma biochemical responses

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ABSTRACT

This work aims to evaluate the effect of transport stress on the hematological and biochemical responses in males and females of bullfrogs (*Lithobates catesbeianus*). Frog conditions were evaluated before and at five times after transport (0, 6, 12, 24, and 48 h) for both genders. Glycemia increased in the frogs evaluated at time 0 h and recovered 24 h after transport. Lactate increased in the animals evaluated at time 0 h, recovered at time 6 h and reduced 48 h after transport. Aspartate-aminotransferase (AST) increased in animals analysed 0, 6, and 12 h and recovered 24 h after transport. Alanine-aminotransferase (ALT) increased in the frogs analysed at time 0 h, recovered at time 6 h and decrease 48 h after transport. Total proteins, albumin and globulins decreased 48 h after transport. Triglycerides and total cholesterol decrease in the animals evaluated 24 and 48 h after transport. Males had high levels of total cholesterol. Number of erythrocytes increased in the animals evaluated 0 and 6 h after transport and recovered 12 h after transport. Mean corpuscular volume (MCV) of the frogs evaluated 0 and 6 h after transport were lower and recovered 12 h after transport. Hematocrit and hemoglobin decrease 48 h after transport. Mean corpuscular hemoglobin (MCH) decreased in the animals evaluated 0, 6, and 12 h and recovered 24 h after transport. Frogs demonstrated rapid recovery of homeostasis after transport, between 6 and 24 h. Between 24 and 48 h after transport, a decrease in energy reserves and red blood series is observed, caused by prolonged fasting.

1. Introduction

Native to North America, the United States and Canada (Frost, 2008), the bullfrog (*Lithobates catesbeianus*) has a meat of excellent nutritional quality, with adequate balance of amino acids and low levels of triglycerides and cholesterol (Casali et al., 2005; Pires et al., 2006). Frog meat is appreciated in several countries (Hsu et al., 2011) where it is sold whole or only the legs, the most noble part of the animal.

Most of the literature available on the raising of frogs in captivity is about the bullfrog, as it is the most cultivated species (Álvarez and Real, 2006). However, there is still a lack of studies on the cultivation of frogs, especially those that prioritize the development of new technologies, such as management techniques that prioritize the welfare of animals during the routines performed in the bullfrog farming. Among the

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management techniques for raising frogs, the transport of live animals is a quite common practice. Frogs must be transported in closed containers that allow only small movements (Cribb et al., 2013) to avoid injuries during transport. These containers must have small holes that allow air to enter, thus preventing the animals from suffocation and death. The frogs normally are transported inside puncture polyvinyl chloride (pvc) tubes of 100 mm diameter, nylon bags and punctured plastic boxes with the frogs free or inside nylon bags.

Transport can be considered stressful procedure, since it exposes the frogs to a series of management such as the capturing of the frogs in the pens, the handling animals, densification, and transport itself. In response to the stressing agent, catecholamines are secreted by the chromaffin cells of the interrenal gland of amphibians after activation of the sympathetic nervous system, which promotes rapid increase of these hormones (Herman, 1977). Amphibians also activate the hypothalamus-pituitary-interrenal axis in a stressful situation and this axis make mediation of the animal's response to its environment (Moore and Jessop, 2003; Rollins-Smith, 2017). After the perception of stressor agent, the hypothalamus produces the corticotrophin-releasing hormone (CRH), which stimulates the pituitary to produce the adrenocorticotrophic hormone (ACTH). In turn, ACTH acts on cortex cells of interrenal gland, where it stimulates steroidogenic cells in the production of corticosteroid hormones (Rollins-Smith, 2017).

In response to the stressor, a series of rapid structural, metabolic and hematological physiological adaptations is necessary for the restoration of the organism's homeostasis and its survival. In these circumstances, there is an increase in energy demand, with catecholamines and glucocorticoids acting in the mobilization of energy reserves. This mobilization occurs mainly in hepatic glycogen (Mbangkollo and deRoos, 1983; Broughton and deRoss, 1984; Rosenthal and deRoos, 1985) and muscular (Mbangkollo and deRoos, 1983), as well as in the tissues that store lipids (Farkas, 1969; Harri and Puuska, 1973; Migliorini et al., 1992; Sheridan and Kao, 1998) to supply the energy demand due to the attempt to restore the organism's homeostasis. With the increase in energy demand, there is also an increase in oxygen consumption by the tissues. Therefore, hematological changes, such as changes in hemoglobin concentration (Palenske and Saunders, 2003), the number of erythrocytes, as well as the volume of these cells (Boutilier and Shelton, 1986; Peng et al., 2016) are necessary to increase efficiency transport of oxygen to the tissues.

Thus, the knowledge of physiological changes during the handling, such as transport, can bring important results for the establishment of appropriate management practices for these animals, since this type of works is rare in literature. Therefore, the aim of the present study was to evaluate the hematological and biochemical responses of bullfrogs, males and females, after transportation.

2. Material and methods

2.1. Pre-experimental condition and design experimental

The research was carried out according to protocol n° 132/ 2018, approved by the Ethics Committee on the Use of Animals (CEUA) of the Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil.

Seventy bullfrogs (*Lithobates catesbeianus*), 35 males (352.12 ± 34.67 g) and 35 females (375.87 ± 48.10 g), were used. The frogs were from at bullfrog farming at Universidade Federal de Uberlândia (UFU), Uberlândia, Minas Gerais, Brazil. The animals were kept in fattening pens of 10 m^2 (2.5 × 4.0 m), in a flooded system with a stocking density of 50 frogs/ m^2. The fattening pens are inside a covered shed. Natural light illuminates this shed, as it contains areas with transparent roof tiles. The frogs were transferred to these fattening stalls when they still had 50 g and remained there for approximately 4 months until they reached the average weight of the experiment.

The frogs were fed at a feed rate of 1.5 % of body weight, divided into three daily feeds. An extruded feed for carnivorous fish of 8 – 10 mm was used. The feed contained 42 % crude protein, 9 % ethereal extract, and 4 % crude fiber.

The experiment was performed in a completely randomised design in a factorial scheme with an additional treatment [5 × 2 + (1)]. Frog conditions were evaluated before (control treatment) and at five times after transport (0, 6, 12, 24, and 48 h) for both genders. The treatments were consisted of five repetitions, the frog being the experimental unit.

2.2. Transport

After a 42 h fast (Seixas Filho et al., 2017), 10 frogs (five males and five females) were randomly captured for blood collection before transportation (control treatment). The other 60 specimens were placed in five polyvinyl chloride (pvc) tubes of 1.00 m length and 100.00 mm diameter at a density of 12 frogs per tube (six males and six females). To prevent frogs from escaping, the tubes were closed with pvc connections of the same diameter. Throughout the length of the tube, holes were made to allow air to enter and prevent the frogs from dying by asphyxiation.

The frogs were transported by closed car, from the bullfrog farming at UFU to the bullfrog farming sector at the UFMG Veterinary School, Belo Horizonte, Minas Gerais, Brazil. The total route was 535.7 km, lasting approximately nine hours. The animals were transported throughout the day and part of the night. On the day of transport, the minimum temperature recorded was 18 °C and the maximum 28 °C.

2.3. Blood collections, erythrogram and plasma biochemical analyses

Upon arrival, immediately after transportation (time 0 h), blood was collected from 10 animals from one of the tubes. The remaining frogs were removed from the tubes and housed in flooded pens of 1.22 m^2 (1.15 × 1.06 m) in the density of 12 frogs/ pen (six males and six females) until the pre-established evaluation times. For up to 48 h after transport, the animals were not fed.

Blood collection was performed through the posterior limb vessel, by puncture with 3 mL syringes and needles previously moistened in 10 % EDTA. Lidocaine 4 % was used as local external anaesthesia. For each evaluation time, blood was collected from 10 frogs (five males and five females), with 2.0 mL of blood/ frog. With an aliquot of 10 μL of blood, the glycemia of the animals was evaluated using a digital glucometer (ACON, On-Call® Plus, San Diego, USA). The remaining blood was stored in 2 mL microtubes under 4 °C refrigeration for further analysis. The hematocrit (Ht) was determined by the microhematocrit method, by means of centrifugation at 19319 *g* for five minutes (12000 rpm and centrifuge radius – 12 cm) of the microcapillaries containing the blood samples. The rate of total hemoglobin (Hb) was determined by the cyanmethemoglobin method, with a reading on a spectrophotometer with a wavelength of 540 nm. The erythrocyte count (Er) was performed in a Neubauer chamber with the aid of a light microscope, with 400X magnification. Then, the following absolute hematimetric indices were calculated: mean corpuscular volume – MCV (fL) = Ht x 10/ Er; mean corpuscular hemoglobin – MCH (pg) = Hb x 10/ Er and mean corpuscular hemoglobin concentration – MCHC (g dL^{-1}) = Hb x 100/ Ht. All erythrogram variables were evaluated using the methodology described by Ranzani-Paiva et al. (2013)

The rest of the collected blood was centrifuged at 1006 *g* for 15 min (3000 rpm and centrifuge radius – 10 cm) in a microtube centrifuge (Biovera® model RB1), the plasma was collected and stored at – 80 °C. Subsequently, at the Clinical Pathology Laboratory of the Clinical and Veterinary Surgery Department of the Veterinary School of UFMG, Belo Horizonte, Minas Gerais, Brazil, using the biochemical analyser COBAS MIRA PLUS®, cholesterol, triglycerides, albumin, lactate, total proteins, alanine-aminotransferase (ALT), and aspartate-aminotransferase (AST) were analysed by commercial kits (Biotécnica®). Globulins were determined by the difference between total proteins and albumin. The

albumin/ globulin ratio (A/ G) was also determined. All plasma biochemical variables were evaluated using the methodology described by Burtis and Ashwood (1998).

2.4. Survival

Survival was observed immediately after transport and at others blood collection times, ending 48 h after transport.

2.5. Statistical analysis

At the end, the data were analysed in Software R 3.5.3, being submitted to Shapiro Wilk and Bartlett's tests to assess the normality and homoscedasticity of the variances, respectively. An ANVOA – two way (5×2) was performed to verify the existence of interaction between the factors, collection time and gender, or the isolated effect of each factor. Subsequently, according to the result of the factor analysis, Dunnett's test was used to compare the additional treatment (control) with all the collection times. Significant differences were considered when $P < 0.05$. To meet normality and or homoscedasticity, the variables lactate, triglyceride, AST, and ALT were transformed into natural logarithm (ln), while the variables glucose, MCV, and MCH were transformed into exponential $(x)^{-0,5}$.

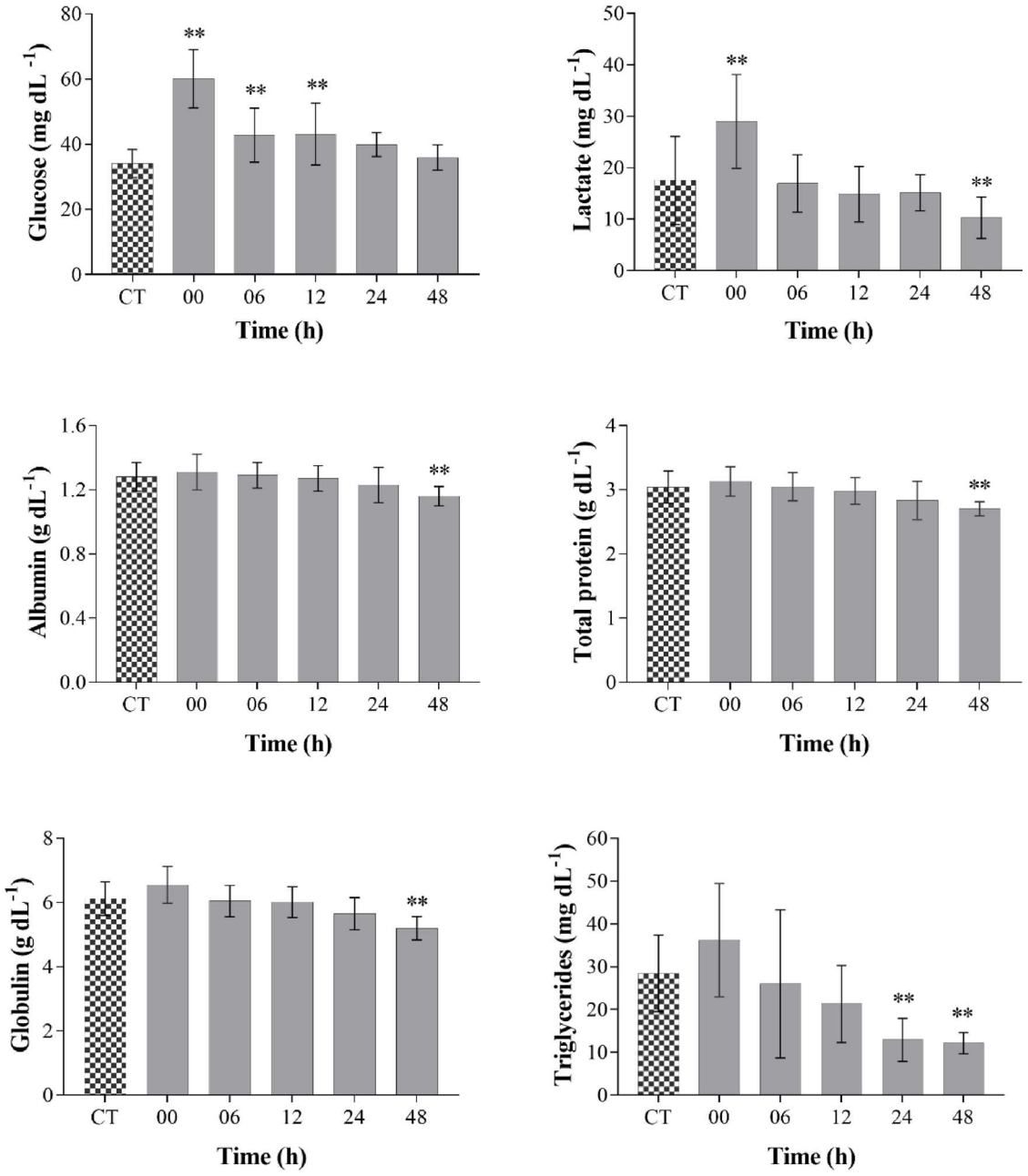

Fig. 1. Bars represent mean ± standard deviation of the variables glucose, lactate, total proteins, albumin, globulins, and triglycerides of bullfrogs before and after transport. CT – control treatment (animals in homeostasis sampled before transport); animals sampled immediately after transport; 06 – animals sampled 6 h after transport; 12 – animals sampled 12 h after transport; 24 – animals sampled 24 h after transport; 48 – animals sampled 48 h after transport. ** Significant difference ($P < 0.01$) in relation to the control treatment by the Dunnett's test.

3. Results

3.1. Survival

There was no mortality during transport and 48 h after transport management.

3.2. Biochemical variables

There was no interaction ($P > 0.05$) between the blood collection times of the frogs after transport and the gender of the animals on the plasma biochemical variables evaluated. The blood collection times did not influence ($P > 0.05$) only the albumin/ globulin ratio (A/ G), demonstrating an effect ($P < 0.05$) on all other measured plasma biochemical. The gender of the animals influenced ($P < 0.05$) only the total cholesterol, demonstrating no effect ($P < 0.05$) on all other variables.

Glycemia increased in the frogs evaluated immediately after transport (time 0 h) and remained high in animals measured at 6 and 12 h after transport. Glucose recovered in the frogs analysed at times 24 and 48 h after transport, not differing from the animals of the control treatment (Fig. 1).

Plasma lactate increased in the animals evaluated immediately after transport in relation to the levels of the frogs of the control treatment. The lactate values of the specimens evaluated at times 6, 12, and 24 h after transport did not differ from levels of the animals of the control treatment. On the other hand, the specimens evaluated at time 48 h after transport showed a reduction in lactate levels compared to the frogs measured before transport (Fig. 1).

The values of total proteins, albumin, and globulins in the frogs evaluated at times 0, 6, 12, and 24 h after transport did not differ from the levels of the animals of control treatment. However, the levels of these variables decreased in the frogs evaluated 48 h after transport in relation to the values of the animals belonging to the control treatment (Fig. 1).

Triglycerides levels and total cholesterol of the frogs evaluated at times 0, 6, and 12 h after transport did not differ from the values of the animals of the control treatment. However, there was a decrease in the levels of these variables in the frogs evaluated at 24 and 48 h after transport in relation to the animals of the control treatment (Figs. 1 and 2A). Bullfrog males had higher total cholesterol values (Fig. 2B).

The plasma aspartate-aminotransferase enzyme (AST) increased in the animals evaluated immediately after transport and remained elevated in the frogs analysed at times 6 and 12 h after transport. AST re-established in the animals evaluated at times 24 and 48 h after transport, not differing from the frogs of the control treatment (Fig. 3).

The plasmatic alanine-aminotransferase enzyme (ALT) increased in the animals evaluated immediately after transport. In the frogs analysed at times 6, 12, and 24 h after transport, there was a restoration of the levels of this variable in relation to the animals of the control treatment. However, in the frogs evaluated at time 48 h after transport, there was a reduction in ALT levels in relation from control treatment specimens (Fig. 3).

3.3. Erythrogram variables

There was no significant interaction ($P > 0.05$) between the blood collection times of the frogs after transport and the gender of the animals on the erythrogram variables. Likewise, the gender of the frogs did not influence ($P > 0.05$) these variables. Blood collection times of the animals did not influence ($P > 0.05$) only the mean corpuscular hemoglobin concentration (MCHC), showing an effect ($P < 0.05$) on all the other variables in the erythrogram.

The number of erythrocytes of the animals evaluated at times 0 and 6 h after transport increased in relation to that of the frogs of the control treatment. The number of erythrocytes recovered in the animals evaluated at times12, 24, and 48 h after transport, not differing from the frogs of the control treatment (Fig. 4).

The mean corpuscula volume (MCV) values of the frogs evaluated at times 0 and 6 h after transport were lower in relation to the animals of the control treatment. The MCV re-established in the frogs evaluated at times 12, 24, and 48 h after transport, not differing from the animals of control treatment (Fig. 4).

There was a reduction in the hematocrit and hemoglobin of the frogs evaluated at time 48 h after transport in relation to the animals of the control treatment. The values of this variable in the animals of the other collection times did not differ from the frogs of control treatment (Fig. 4).

The mean corpuscular hemoglobin (MCH) of the animals evaluated at times 0, 6, and 12 h after transport showed lower values than that of the frogs of the control treatment. MCH recovered in the animals evaluated at times 24 and 48 h after transport, not differing from the values of the frogs of the control treatment (Fig. 4).

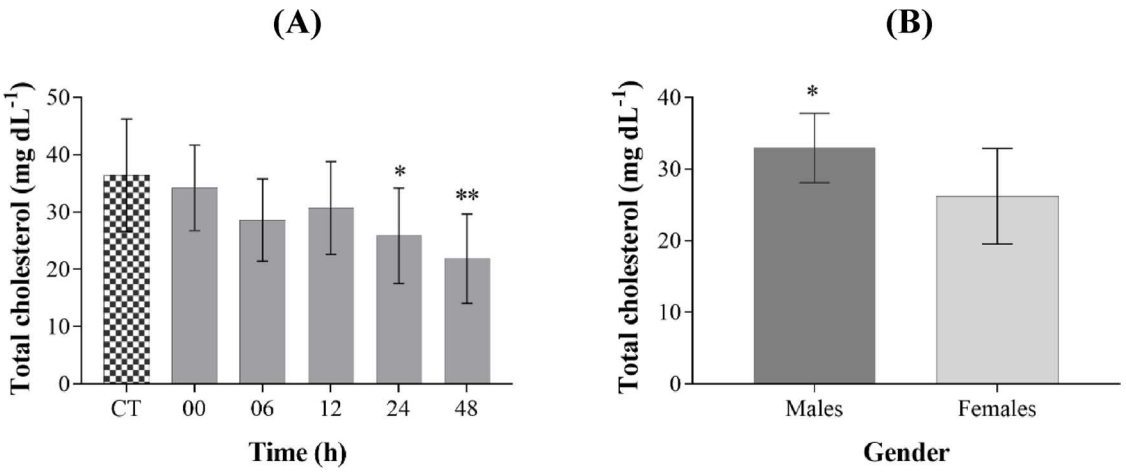

Fig. 2. (**A**) Bars represent mean ± standard deviation of the variable total cholesterol of bullfrogs before and after transport. CT – control treatment (animals in homeostasis sampled before transport); animals sampled immediately after transport; 06 – animals sampled 6 h after transport; 12 – animals sampled 12 h after transport; 24 – animals sampled 24 h after transport; 48 – animals sampled 48 h after transport. ** Significant difference ($P < 0.01$) in relation to the control treatment by the Dunnett's test. * Significant difference ($P < 0.05$) in relation to the control treatment by the Dunnett's test. (**B**) The bars represent mean ± standard deviation of the total cholesterol variable in bullfrogs males and females. * Significant difference ($P < 0.05$) by the ANOVA.

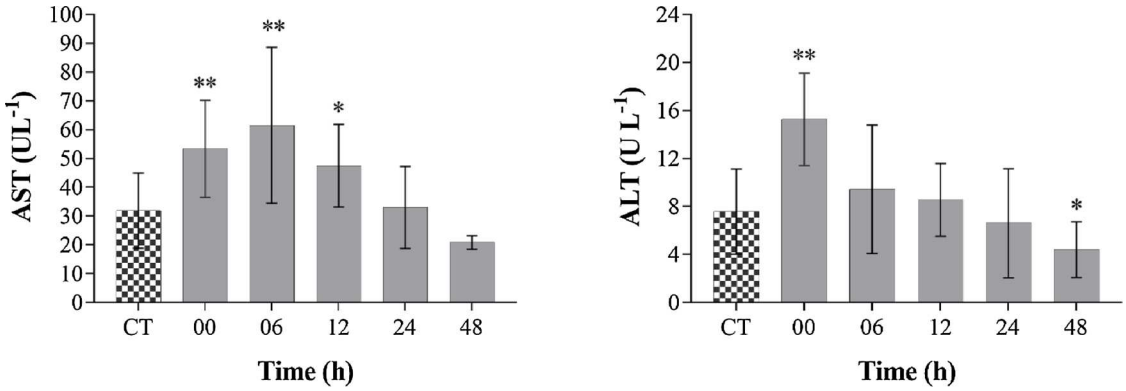

Fig. 3. Bars represent mean ± standard deviation of the plasma variables aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of bullfrogs before and after transport. CT – control treatment (animals in homeostasis sampled before transport); animals sampled immediately after transport; 06 – animals sampled 6 h after transport; 12 – animals sampled 12 h after transport; 24 – animals sampled 24 h after transport; 48 – animals sampled 48 h after transport. ** Significant difference ($P < 0.01$) in relation to the control treatment by the Dunnett's test. * Significant difference ($P < 0.05$) in relation to the control treatment by the Dunnett's test.

4. Discussion

A long time ago glucose has been an excellent indicator of stress in studies with amphibians, showing an increase in plasma levels after the action of a stressor agent. (Herman, 1977; Broughton and deRoss, 1984; Rosenthal and deRoos, 1985). This increase is one of the secondary responses to stress and is necessary to meet the body's energy demand in these circumstances. According to Herman (1977), in addition to the probable role of adrenaline in the fast increase in plasma glucose levels in response to the stressor agent, hormones of the pituitary-adrenocortical axis may also be involved. Thus, both adrenocorticotropic hormone (ACTH) as well as glucocorticoids cause an increase in plasma glucose levels in bullfrogs as result of the breakdown of hepatic glycogen (Broughton and deRoss, 1984; Rosenthal and deRoos, 1985). In the present study, a situation of hyperglycemia was observed in the animals evaluated immediately after stress, returning to baseline levels 24 h after the end of transport, indicating a rapid recovery of homeostasis in relation to this variable. The increase in glycemia was also verified by Sounderraj et al. (2011) when individuals from *Rana tigrina* were subjected to poisoning by the organophosphate insecticide phosphamidone; by Mbangkollo and deRoos (1983) when bullfrog specimens were subjected to different dosages of adrenaline and noradrenaline and by Harri (1981) when specimens of *Rana temporaria* were subjected to a stirring process, using an automatic stirrer (60 cps min^{-1}) for 1 h.

Lactate is also an important physiological indicator for evaluation stress in amphibians (Bennett and Licht, 1974; Christiansen and Penney, 1973; Fournier and Guderley, 1992; Harri, 1981; Mbangkollo and deRoos, 1983; Fournier and Guderley, 1992). "Vigorous muscle activity" (Fournier and Guderley, 1992) and stress, such as hypoxia and low temperatures (Christiansen and Penney, 1973), have been shown to promote glycogen mobilization through anaerobe glycolysis, with lactate dehydrogenase (LDH) being the final enzyme that catalyses the reduction of pyruvate to lactate, promoting a rapid increase in lactate concentrations. Therefore, probably the densification associated with the stuffy container in which the frogs were transported induced the performance of anaerobic glycolysis by the muscles, resulting in elevated plasmatic lactate concentrations. Elevation of lactate concentrations was also observed 15 min after the brief manipulation of bullfrog adults for two minutes (Mbangkollo and deRoos, 1983). Increased lactate levels were also observed in *Rana temporaria* specimens recently captured and transported by car 200 km away and when these animals were subjected to a stirring process in the laboratory, using an automatic stirrer (60 cps min^{-1}) for 1 h (Harri, 1981).

In the present study, 6 h after transport, the frogs had already restored baseline plasma lactate standards. According to Hutchison and Turney (1975), lactate levels return to baseline levels when oxygen consumption returns to normal. According to the same authors, specimens of *Rana pipiens* re-established the baseline lactate pattern 4 h after the electric shock stimulus. The decrease in plasma lactate in frogs 48 h after transport was probably due to energy demand since these animals were fasting for a long period. However, it is also important for anurans to recover glycogen stores after a period of activity to serve as a substrate for a future need for anaerobic energy production (Withers et al., 1988). A study with *Rana pipiens*, hepatectomized and normal, showed that muscle is the main tissue responsible for recycling lactate after performing intense exercise (Fournier and Guderley, 1992). According to these authors, the liver plays an insignificant role in the reuse of this metabolite, not having the capacity to transform lactate into glucose. According to the authors, this inability to transform is not related to the absence of enzymes active in gluconeogenesis, but due to the liver's limitations in metabolizing lactate and/or its limited permeability in this organ. This ability to recycle lactate promotes a muscle glycogen sparing effect that may be related to the evolution of the species and its survival in dangerous circumstances.

In the present study, one cannot fail to take into account the possible interference of the circadian cycle on the biochemical and hematological variables analyzed. Hutchison and Turney (1975) were the first to demonstrate the presence of a daily rhythm in plasma lactate levels in amphibians. According to the authors, there is an increase in the concentration of this variable in *Rana pipens* at the beginning of the night, reaching its maximum level at 19:00, at which time the species has greater activity. Then, there is a decrease in plasma lactate concentrations until the end of the night period. Thus, the increase in lactate levels in bullfrog samples evaluated immediately after transport (time 0 h) could also be attributed to this daily rhythmicity, since these frogs were evaluated at 20:00 and the treatment frogs control at 08:00. However, when comparing the control (evaluation at 08:00) with times 6 (evaluation at 02:00), 12 (evaluation at 08:00) and 24 h after transportation (evaluation at 20:00) it was found there were no differences in plasma lactate levels, even though the collection occurred at different times. In addition, there was a significant difference in the lactate concentration in the animals evaluated immediately after transport in relation to the control group, and this difference was not observed in the animals evaluated 24 h after transport, and the collections of times 0 and 24 were performed at the same time (at 20:00). Hutchison and Turney (1975) also demonstrated that there is no daily glycemic rhythm in *Rana pipens*. Therefore, it is very likely that the elevated glucose levels in the present study with bullfrogs were influenced by transport stress. In addition, for lactate and glucose levels, the action of the stressor is likely to have

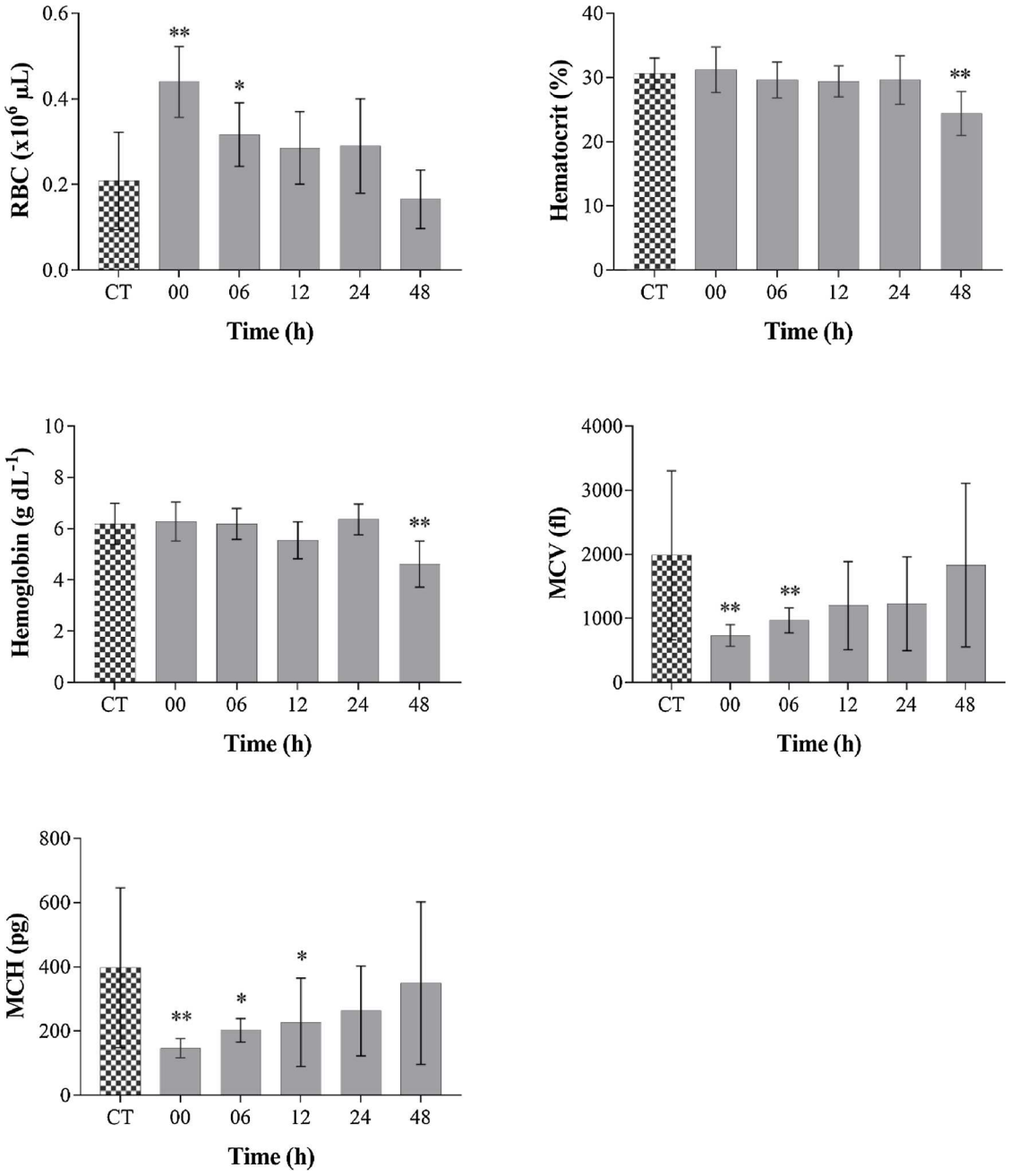

Fig. 4. Bars represent mean ± standard deviation of the variables number of erythrocytes (RBC), hematocrit, hemoglobin, medium corpuscular volume (MCV), and medium corpuscular hemoglobin (MCH) of bullfrogs before and after transport. CT – control treatment (animals in homeostasis sampled before transport); animals sampled immediately after transport; 06 – animals sampled 6 h after transport; 12 – animals sampled 12 h after transport; 24 – animals sampled 24 h after transport; 48 – animals sampled 48 h after transport. ** Significant difference ($P < 0.01$) in relation to the control treatment by the Dunnett's test. * Significant difference ($P < 0.05$) in relation to the control treatment by the Dunnett's test.

greater interference than the circadian cycle, requiring a study with a bullfrog to assess the interference of the circadian cycle and the seasons on the other variables hematological and biochemical.

According to Dornelles and Oliveira (2014), the decrease in proteins may indicate a physiological adaptation to compensate for the high energy demand caused by stress. Therefore, the decrease in total proteins, albumin and globulins of animals evaluated 48 h after transport may be related to a physiological regulation due to high energy demands, which stimulates the mobilization of amino acid reserves for gluconeogenesis. The decrease in the levels of this variable can also be related to the long period of fasting that these animals were submitted before transport until the end of blood collections, 99 h (42 h of fasting before transport plus, 9 h of transport and 48 h referring to the time of the last collection after transport). According to Coppo et al. (2005) albumin is an excellent indicator of protein biosynthesis, in addition to functioning as a reserve of amino acids. The albumin/ globulin ratio (A/ G) of the frogs did not change in any evaluation time after transport. According to Gras (1983), the maintenance of the A/ G ratio indicates a balance between plasma protein fractions, and this balance is maintained until the compensation mechanisms of stressor agent fail. The decrease in temperature also did not influence the A/ G ratio of adults of the salamander *Batrachupems tibetanus* (Xia and Li, 2010).

Triglycerides represent most body lipids in anurans (Brown, 1964; Ryuzaki and Oonuki, 1990), with fatty bodies being the most abundant

structure in this type of lipid (Brown, 1964). In the present study, the animals evaluated 24 and 48 h after transport showed triglyceride values below half in relation to the frogs evaluated in the control treatment. During prolonged fasting, lower insulin levels and, consequently, there is a lower insulin/ glucagon ratio. McGarry et al. (1973) showed that liver isolates, when exposed to increasing concentrations of glucagon, increase ketogenesis and triglycerides production decreases. Therefore, the higher the rate of hepatic ketogenesis, the lower the proportion of free fatty acids available to be esterified in triglycerides.

In situations of increased energy demand, animals can mobilize energy through these lipids stored as triglycerides (Byrne and White, 1975). Stored triglycerides can be rapidly mobilized by various hormonal changes. Pituitary hormones, such as prolactin and ACTH, as well as those produced by the adrenal cortex, cortisol and corticosterone, are generally lipolytic in anurans (Sheridan and Kao, 1998). On the other hand, in these animals, catecholamines have not been shown to affect lipolysis, these hormones being responsible only for decreasing the levels of circulating fatty acids (Farkas, 1969; Harri and Puuska, 1973; Migliorini et al., 1992). Under stress, the liver increases triglyceride production, making this metabolite available in the bloodstream. Cortisol, catecholamines and glucagon stimulate phosphatidate-phospho-hydrolase, leading to increased hepatic triglyceride synthesis. In order to be transported in the blood to the target tissues, triglycerides are transported by the very-low-density lipoprotein (VLDL). When VLDL-linked triglycerides are released into the circulation, they undergo the action of lipoprotein lipase present in blood capillaries (Brindley et al., 1993). Norepinephrine also decreases hepatic lipase activity, which could increase plasma levels of VLDL and low-density-lipoprotein (LDL) (Niaura et al., 1992), triglyceride and cholesterol transporters. In addition, cortisol, norepinephrine and fatty acids can reduce insulin sensitivity (Niaura et al., 1992; Brindley et al., 1993), which is advantageous for the organism during stress, since it favors the use of glucose by the central nervous system. Consequently, insulin resistance causes less activity of lipoprotein lipase. The reduction in the activity of lipoprotein lipase causes less uptake of lipoproteins in peripheral tissues, retaining lipoproteins in the blood, which increases triglyceride and cholesterol levels (Rizza et al., 1982). This increase in triglyceride levels was probably not seen right after transport since the transport was long (approximately 10 h) and the animals were already on a long period of fasting. An increase in triglycerides levels was observed in adults of the frog *Pelophylax ridibundus* exposed to the herbicide glyphosate (Păunescu and Ponepal, 2011), in adults of the salamander *Batrachupems tibetanus* exposed to low temperatures (Xia and Li, 2010). However, in the present study there was no increase in plasma triglyceride levels after transport of the frogs.

In the present study, cholesterol levels decreased in the 24 and 48 h after transport. In stressful situations, the reduction of cholesterol may be due to an inhibition of the biosynthesis of this compound by the liver or using fatty acid reserves as an energy source under these circumstances (Ganeshwade, 2012). It is likely that the reduction is also due to the prolonged fasting that the frogs were subjected to, before and after transport. On the other hand, as with triglycerides, the increase in cholesterol levels cannot be seen in the first evaluations after transport due to the long time that these animals were transported. Therefore, most likely the peak of glucocorticoids and their effects on triglyceride and cholesterol levels occurred at the beginning of the trip. Normally, under stress, cholesterol levels can increase. According to Cavenee and MeInykovych (1979), glucocorticoids and the presence of free fatty acids in the circulation stimulate the activity of the enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMG-CoA reductase) in the liver, increasing the synthesis of cholesterol. In addition to its importance in energy metabolism, lipid is involved in gametogenesis and in the formation of cell membranes (Bruscalupi et al., 1989). According to Alyousif (1991), the amount of cholesterol present in the cell membrane is related to the ability of water to diffuse through it, and cholesterol is, therefore, very important for waterproofing tissues that are in direct contact with body fluids. The same author demonstrated in a study with *Rana ridibunda* that the tissues responsible for the loss of water (kidney, lung, skin, ileum, and rectum) contain a large amount of cholesterol, while the tissues that are not responsible for the loss of water present low levels of this compound. In the present study, no increase in plasma cholesterol levels was observed immediately after transport, even though the animals remained approximately nine hours with water restriction. However, the amount of cholesterol in tissues responsible for water control was not measured.

In the present study bullfrog males had a higher concentration of total cholesterol. Bruscalupi et al. (1989), demonstrated in a study with *Rana esculenta* that the levels of total lipids and total cholesterol are also influenced by the time of year and the gender of the animals. According to the same authors, females of *Rana esculenta* had higher levels of total lipid and total cholesterol during the reproductive period. The cholesterol levels of the females in the present study were probably lower, since the transport was carried out after the reproductive period of the animals, in autumn. Therefore, in this circumstance, it is likely that a large part of the female cholesterol may have been used as a precursor to the steroid hormones involved in reproduction and another part directed to the formation of the oocyte.

The evaluation of liver enzymes in the plasma of animals is used to demonstrate hepatocellular damage (Peng et al., 2016) and stress has shown an intimate relationship with these enzymes. In the present study, aspartate-aminotransferase (AST) levels of bullfrogs increased immediately after transport and were restored to baseline levels within 24 h. According to Coppo et al. (2005), the elevation of plasma AST levels is related to necrosis of liver cells and skeletal or cardiac muscle, lack of vitamin E and hunger. In relation to the alanine-aminotransferase (ALT) assessed in bullfrogs after transport, the levels of this enzyme increased only immediately after transport. The increase in the concentration of ALT in the plasma is already well established as a marker of acute liver injury (Coppo et al., 2005). Janssens (1967) demonstrated that cortisol administered to *Xenopus laevis* increased the rate of urea excretion and the activity of the liver enzymes ALT and AST. Bullfrog adults demonstrated an increase in ALT levels during the hibernation period, which is considered a stressful situation, however the submission of frogs to low temperatures did not influence AST levels (Peng et al., 2016). On the other hand, in the present study there was a decrease in the ALT levels of the frogs evaluated 48 h after transport. It is widely known that during the fasting period, animals use amino acids to carry out gluconeogenesis. Alanine, which comes from muscle, is the main amino acid used, and when it reaches the liver is deaminated to the formation of pyruvate. However, in prolonged fasting the brain decreases the use of glucose, reducing gluconeogenesis in the liver, which stimulates muscle and other peripheral tissues to use energy sources from lipids. Therefore, as the frogs evaluated 48 h after transport were fasting for 99 h, they probably started using lipids as an energy source, as evidenced by the decrease in triglyceride and cholesterol levels at this time. Concomitantly, there was a decrease in the transamination process by ALT, reducing the release of gluconeogenic amino acids by the muscle to save proteins.

Despite the increase in the number of erythrocytes in the first 24 h after transport, this increase was not evident in the hematocrit of the frogs evaluated in the same interval. Under stress conditions, there is a greater recruitment of young erythrocytes from erythropoietic organs, due to the increased demand for oxygen by the tissues (Boutilier and Shelton, 1986). According to the same authors, the increase in erythrocytes can also be caused by dehydration, which makes the blood more viscous. Therefore, because it causes an increase in blood viscosity, the increase in the number of erythrocytes to improve oxygen transport is limited (Carvalho et al., 2017). Immature erythrocytes have a circular shape, high nucleus/ cytoplasm ratio, and distinctly smaller size (Broyles et al., 1981; Allender and Fry, 2008). In this way, the stability demonstrated by the hematocrit can be explained, since the frogs evaluated immediately after transport presented young erythrocytes and of

smaller volume, that is, lower mean corpuscular volume (MCV). According to Peng et al. (2016) the reduced size of erythrocytes during the active period may be a necessary physiological adaptation for the blood to circulate faster due to the increased physical activity of the animal. The increase in the number of erythrocytes at 0, 6, and 12 h after transport is also accompanied by a decrease in mean corpuscular hemoglobin (MCH), demonstrating a direct relationship between the size of the erythrocyte (MCV) and the amount of hemoglobin present in it. An increase in the number of erythrocytes and a decrease in MCV has also been demonstrated in bullfrog froglets submitted to management stress (Teixeira et al., 2012) and in adults of the same species submitted to low temperatures (Palenske and Saunders, 2003). On the other hand, 48 h after transport there was a decrease in the number of erythrocytes, hematocrit and hemoglobin, compared to the control treatment. The decrease in these red series variables may be related to a condition of hemodilution promoted by the rehydration of these animals after transport, or due to a possible condition of anaemia, since the total fasting time was very prolonged. The loss of water and the decrease in weight gain, as well as the weight gain due to the reintroduction of the frog in the water after transport, could elucidate the hydration status of the animals and a possible hemoconcentration and hemodilution, respectively. However, weighing the animals, especially after transport, could be an additional stress effect on the animals and interfere with the variables evaluated.

5. Conclusions

Transport promotes stress in adult bullfrogs. However, bullfrogs have shown to be very resistant to transport and have demonstrated a rapid recovery of homeostasis after transport, between 6 and 24 h depending on the variable evaluated. Between 24 and 48 h after transport, a decrease in energy reserves and red blood series is observed, probably caused by prolonged fasting.

CRediT authorship contribution statement

Bruno Dias dos Santos: Investigation, Formal analysis, Resources, Writing - original draft, Visualization. **Adriana Xavier Alves:** Formal analysis, Writing - review & editing, Visualization. **Nayara Netto dos Santos:** Investigation, Resources, Visualization. **Mariele Lana:** Investigation, Resources, Visualization. **Victor Ramos Pawlowski:** Investigation, Resources, Visualization. **Renan Rosa Paulino:** Investigation, Writing - original draft, Writing - review & editing, Visualization. **Fabíola de Oliveira Paes Leme:** Investigation, Resources, Visualization. **Frederico Augusto de Alcântara Costa:** Investigation, Visualization. **Marcos Ferreira Brabo:** Writing - original draft, Writing - review & editing, Visualization. **Daniel Abreu Vasconcelos Campelo:** Writing - original draft, Writing - review & editing, Visualization. **Galileu Crovatto Veras:** Conceptualization, Methodology, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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