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THE USE OF COOLING AND FREEZING FOR ANESTHESIA OF *Phrynops geoffroanus* TURTLES (PLEURODIRA: CHELIDAE) AND THE ESTABLISHMENT OF LOW INCIPIENT LETHAL TEMPERATURE

(Uso de resfriamento e congelamento para anestesia de cágados *Phrynops geoffroanus* (Pleurodira: Chelidae) e o estabelecimento da menor temperatura inicial letal)

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ABSTRACT - The use of hypothermia for anesthesia and euthanasia in ectothermic individuals has promoted many discussions, especially in the last four decades, and yet it continues today as a method that is prohibited or restricted to certain procedures. Despite this, some studies have shown satisfactory results in obtaining anesthetic plans, raising questions about the real possibility of using this method in research. The aim of the present experiment was to determine the time patterns to generate anesthesia and low incipient lethal temperature in *Phrynops geoffroanus* through cooling and freezing. Turtles reached the anesthesia grade nine within 30 minutes, making ease handling due to muscle relaxation, and, in 80 minutes, they reached anesthesia grade 11, becoming insensitive. The low incipient lethal temperature (LILT) shows that 1°C is the limit freezing from where the turtle can recover its motor activities while lower temperatures cause death. The low incipient lethal temperature was determined in a 300-minute period independently of the individual's size or weight.
Key words - anesthetic degree; ectotherm; humanized management; hypothermia.

RESUMO - O uso de hipotermia para anestesia e eutanásia em indivíduos ectotérmicos tem gerado muitas discussões, especialmente nas últimas quatro décadas, e apesar disso continua hoje como um método que é proibido ou restrito a certos procedimentos. Alguns estudos têm mostrado resultados satisfatórios na obtenção de planos anestésicos, levantando questionamentos sobre a real possibilidade do uso deste método em pesquisas. O objetivo do presente experimento foi determinar o padrão de tempo de anestesia e da menor temperatura inicial letal através do resfriamento e

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congelamento em cágados da espécie *Phrynops geoffroanus*. Os cágados chegaram ao grau de anestesia nove em 30 minutos, tornando fácil seu manuseio devido ao relaxamento muscular e, em 80 minutos, alcançaram o grau 11 de anestesia tornando-se insensíveis. A menor temperatura inicial letal demonstrou que 1°C é o congelamento limite a partir do qual o cágado pode retomar sua atividade motora, sendo que temperaturas inferiores levam à morte. A menor temperatura inicial letal foi determinada em 300 minutos independentemente do tamanho ou peso do indivíduo.

Palavras-chave - ectotermia; grau de anestesia; hipotermia; manejo humanizado.

INTRODUCTION

The growing concern with an ethical and humanized management of animals in the 21st century has raised specific demands within forensic veterinary medicine concerning domestic pets, production animals, and wild animals, as a result of the negligent treatment that society has shown towards these animals (Amaral et al., 2017).

Contemporary society has a moral wish to strongly disagree with any injurious practices that may cause irreparable harm, suffering, and death to animals (Miziara et al., 2012). In this context, and based on the terms of the Brazilian Law No. 11,794 of 2008, the National Council for Animal Control and Experimentation (CONCEA) has established a set of criteria for animal husbandry/breeding, animal keeping, and testing on animals, to supervise and guarantee appropriate conditions for humanized and rational handling; thereby, promoting animal welfare (MCTI, 2016).

The uses of general, inhalational, dissociative, and local anesthetic drugs are listed in all protocols involving species of the phylum Chordata. Each of these drugs having a wide range of administration routes, respecting the morphological particularities of the classes, aiming at promoting the animal’s loss of consciousness before procedures or leading to euthanasia through increased drug doses (MCTI, 2016). It is known, however, that these agents can have adverse side-effects and may accumulate in certain tissues and organs (Lewis et al., 2003; Menck et al., 2013). In these cases, alternative methods may be used, albeit with restrictions, provided that the methodology recommended – meaning the use of drugs – could have the risk of jeopardizing research results (MCTI, 2016).

Obtaining numbness by cooling reptiles and amphibians is not a well-accepted method for experimental purposes. The use of cooling is restricted to those procedures influenced by the drugs as recommended. Additionally, as was pointed out previously (Rose, 2002, 2007; Rosenberg, 1978) there’s an understanding that ice crystals are formed in the peripheral tissues and, in the thermal threshold of body freezing, the animal feels excruciating and gradual pain as the body temperature falls. This statement of excruciating and gradual pain during the formation of the crystals is not physically possible due to block diffusion of ions across membrane channels of nerves caused by freezing (Keifer and Zheng, 2017; Lillywhite et al., 2017).

According to Shine et al. (2015) animal ethics committees initially look into the use of this empirical technique, but they end up not using it in the research. However, in the research study that these authors carried out using the frog *Rhinella marina* (Linnaeus, 1758) specimens, results were satisfactory, with a slight decline in brain activity – showing that brain activity declines smoothly, without any indication of perceived pain. In this context, non-invasive and non-medical research are necessary, as they do not generate adverse reactions in the organisms (Shine et al., 2015). These findings are reinforced by the study by Keifer and Zheng (2017) that uses and electrophysiologically monitors the use of hypothermia in pond turtles (*Trachemys scripta elegans*).

There are opinions against the use of cold and freezing for anesthesia and euthanasia of ectothermic animals based on the precautionary principle (Leary et al., 2020; Warwick et al., 2018) that lead the ethics committees to prohibit worldwide this technique in research (Keifer and Zheng, 2017; Lillywhite et al., 2017). In concerning this matter, more research must seek to solve this conflict with empirical data that could support one of these points of view.

Therefore, considering the effectiveness of cooling followed by freezing to obtain anesthesia in turtles, what would be the time and temperature to guarantee an adequate anesthetic degree? Would the temperature be related to the mass or size of the specimen? What is the limit temperature at which the specimen could be submitted to hypothermia and safely return from trans-anesthetics? What would the lethal incipient low temperature be?

Hence, this paper examines the effects of cooling and freezing, aiming to define the time patterns to generate anesthesia and low incipient lethal temperature (LILT) in the turtle *Phrynops geoffroanus* (Schweigger, 1812). It was considered the timing of anesthesia (motor recovery and LILT) and cooling and freezing as being proportionally linked to the morphometric conditions of the individual, especially body mass.

MATERIALS AND METHODS

Focal species

The species *Phrynops geoffroanus* belongs to the Chelidae, a family with a complicated taxonomic status. Phylogeographic studies are required in order to address the allopathy and discernment of this complex *P. geoffroanus* (Carvalho et al., 2017; Rhodin et al., 2017).

Sample size and collection

Animals were randomly collected with a galvanized wire net, with a diameter of 45 cm and made of a 2 × 2 mm hexagonal multifilament mesh. The collection occurred in Leite creek (6° 25'S – 43° 00'W), a tributary of the Parnaíba river in the municipality of Floriano, State of Piauí, Brazil. Animals were then taken to laboratories and hand washed. The sample comprised 67 specimens in seven field incursions, with a collection of 10 specimens per raid, or a 60-minute waiting period.

Morphometrics

Measurement: calipers measuring to an accuracy of 0.01mm;

Carapace Length: aligned over vertebral plates, in a craniocaudal direction between the cervical plate and the marginal plate of the cloacal region.

Carapace width: aligned between the sixth marginal side plates, from side to side.

Plastron Length: aligned near the medial fissure of the plastron plates, between the rostral portion of the intergular plate and the anal plate.

Plastron Width: aligned in the fissure of the abdominal plates with the pectoral, in the right-to-left side.

Body Mass: animals were individually measured with a precision fishing gripper scale with a precision of 100 g. To avoid interference with movements, animals were placed in dark bags, making them remain motionless inside their shells (carapace/plastron).

Cooling, freezing and degree of anesthesia

The specimens were cooled for ten minutes at 1° C in a refrigerator and then put in a -20°C freezer, until losing their reflexes. Reflexes were checked with obliteration forceps by stimuli every thirty minutes. When there were no more responses to the stimuli, the specimens were removed and the parameters of the degree of anesthesia were then appraised as according to Santos et al. (2011) being this procedure realized always by the same evaluator (Table 1).

Physical recovery

The animal was kept in dorsal recumbency inside plastic trays and stimulated every thirty minutes, to check its reflexes. When reflexes were present, corresponding to the end of anesthesia, the animal got up using its neck and claws. When recovering their movements, animals were released in the Meladão river (6° 47'S – 43° 2' W), another tributary of the Parnaíba river, in the same municipality, thus avoiding a duplicate experiment being performed with the same individuals (since they were not marked and did not suffer any harm that would allow individualization).

Six individuals, approximately 8%, of the specimens were used to test the Low Incipient Lethal Temperature (LILT) pattern been kept in the Coleção de História Natural da Universidade Federal do Piauí (vouchers CHNUFPI0038-043).

Anesthesia pattern

The assessed morphometric values were correlated to the anesthesia onset and end time, and to the post-anesthetic recovery time. The results were then analyzed using Spearman's rank correlation coefficient and, if there was statistical dependence between variables, we would perform a linear regression in order to obtain the time pattern. The adherence of the model was submitted to the χ^2 (chi-square) test, with n-1 degrees of freedom and 95% significance.

RESULTS AND DISCUSSION

The use of a scale for anesthetic monitoring was first proposed by Guedel (1937), with the purpose of characterizing the depth of the anesthetic plan by means of a set of physiological parameters and signs, thus helping to maintain the anesthetic procedure. The advent of the scale has paved the way for greater safety in anesthetic procedures and, to this day, the scale has ranked among the auxiliary monitoring methods recommended by the main international guidelines (Tranquilli, Thurmon and Grimm, 2013). However, this method applies mainly to mammals. According to Sneddon et al. (2017), regardless of the species, it is critical to the integrity of results and legitimacy of science that refined procedures be pursued, thereby ensuring animal health and welfare both before and during experiments. The scale for obtaining the scores of the anesthetic plan proposed in the present study scores was that drawn up by Santos et al. (2011), who, in their study, used turtle's specimens of *Podocnemis expansa* (Schwergger, 1812).

The specimens of the present study had a body mass of 1.422±0.72 and a caudal rostrum length of 24.375±4.81. Out of the 67 individuals submitted to the cooling anesthesia process, only one had no analgesia (Table 1). The other 66 individuals had locomotion, muscle relaxation, manipulation, and stimuli with an anesthetic degree 11, meaning that the animal became insensitive without any stimulus or expression of protective reflexes, with a mean time of 80 minutes (80 ± 6 minutes).

Using anesthetics in *Podocnemis expansa*, Santos et al. (2011) achieved grade 11 of numbness within 15 minutes after midazolam administration via the left forelimb and propofol via a cervical vertebral sinus.

The removal of a granuloma from the lateral cervical region of *Trachemys dorbigni* (Duméril & Bibron, 1835), case report, a 290-gram animal had pre-anesthetic medication (midazolam-associated ketamine), reaching a state of lethargy within 20 minutes, being later anaesthetized with isoflurane through a face mask, intubated with a Baraka circuit, receiving 100% oxygen (Flôres et al., 2008). On considering the pharmacological procedure and metabolic suppression by cooling in case of immediate veterinary intervention, the temperature reduction does not comply with the urgent clinical conditions, thus satisfying only the elective interventions. However, it can be used for retention and management, given that individuals are lethargic within 30 minutes (30 ± 10) with anesthesia grade 9, which means the absence of mobility plus muscle relaxation and easy handling.

Table 1 – Degrees of Anesthesia.

Parameter for Evaluation	1	2	3
Locomotion	Speed (escape)	Difficulty (slow)	Absence
Muscle Relaxation	Manual traction, and retraction of the head	Head slightly relaxed	No resistance
Manipulation	Difficulty	Moderate ability	Easy handling
Stimuli	**0**	**1**	
Forelimb	Manifestation of pain	No manifestation of pain	--
Hind Limb	Manifestation of pain	No manifestation of pain	--

In a study with different doses of etomidate, Magalhães et al. (2011) used the anesthetic parameter of body mass (kg) per unit of anesthetic (mg), distributing the individuals (*P. geoffroanus*) into three groups (G): G1, 1.0 mg/kg; G2, 1.5 mg/kg and G3, 3.0 mg/kg. The success of anesthesia with grade 11 was only obtained in group G3. In Santos et al. (2011), subjects were divided into two groups: G1, 20 mg/kg/IM, and propofol 10 mg/kg/IV, having successfully achieved grade 11 analgesia, in both cases, within 15 minutes after drug administration.

In the present study, we have sought to establish a correlation between body mass, carapace length, carapace width, plastron length and plastron width, and the period of time for which the individual stayed in a state of grade 11 numbness, since all subjects underwent analgesia, on average, 80 minutes after cooling.

None of the morphometric parameters were correlated with the time of anesthesia. Body mass showed a weak correlation coefficient for Spearman ranks (r^2 = 0.26), followed by plastron width (r^2 = 0.14), while the other parameters showed no correlation at all (r^2 = 0.0). The linear regression equation for the morphological parameters is shown in Figure 2. When the pattern adherence was evaluated, χ^2 was higher than the tabulated value (n-1 = 65). This means that the differences were significant, it not being possible to establish a pattern (Table 2).

Table 2 – Chi-square significance.

Correlations	Linear Equation	Value of chi-square χ^2
AnT vs BM	y = 3.2641x + 0.862	1.72*
AnT vs CL	y = 0.2059x + 0.6737	3.18*
AnT vs CW	y = 0.2063x + 1.6136	2.66*
AnT vs PL	y = 0.2621x + 0.5756	3.11*
AnT vs PW	y = 0.4994x – 1.5285	2.72*

Analgesia Time (AnT), Body Mass (BM), Carapace Length (CL), Carapace Width (CW), Plastron Length (PL), Plastron Width (PW); * statistically significant differences for α = 5%.

As for the time of anesthesia in chelonians, we obtained results similar to those obtained by FLôres et al. (2008) with *Trachemys dorbigni*. In Flores' study, the animals were kept under anesthesia grade 11 for 15 minutes. In our experiment, the mean anesthetic period was 17 minutes (17 ± 5 minutes). In the experiment developed by Magalhães et al. (2011) on individuals of *P. geoffroanus,* the average anesthesia time was 22 minutes, while the maximum and minimum values were not shown. In contrast, 22 minutes corresponds to the maximum values obtained in our experiment.

The experiment conducted by Santos et al. (2011), using propofol in *Podocnemis expansa* showed an anesthesia time of 180 minutes, a higher value than found in the present study with *P. geoffroanus*. The maximum and minimum values were not informed. In this case, propofol causes hypotension for several minutes, with decreased systemic vascular resistance, myocardial contractility depression (Duke, 1995), and depressed ventilation and high apnea during induction, so endotracheal intubation is recommended (Pye and Carpenter, 1998; Sebel and Lowdon, 1989). In this regard, Santos et al. (2011) admit that prolonged apnea may result in a risk of death for the animal.

The use of propofol for surgical induction in chelonii should not exceed twenty minutes (Bouts and Gasthuys, 2002). In the case of larger turtles, these should undergo endotracheal intubation. Duke (1995) makes a similar observation; however, the experiment was performed on dogs, showing different results among breeds.

We also consider that the anesthetic effects over time were similar to those obtained in our experiment, except when propofol was used; in this specific case, greater care is required concerning anesthesia time, twenty minutes is recommended, together with endotracheal intubation. Thus, the use of cooling and freezing for turtles (chelonii) provides satisfactory results for management and rapid surgical interventions.

The recovery time of chelonii after an anesthesia period may vary and, in the case of benzodiazepines, may take from 24 to 72 hours (Tracchia, 2018). In the case report of surgical intervention by Flôres et al. (2008) on specimens of *T. dorbigni,* the effects ceased and a full recovery occurred within 40 minutes, unlike *P. geoffroanus*, which took 192 minutes to recover, as shown in the experiment performed by Magalhães et al. (2011). In our experiment, recovery occurred in 34 minutes, reaching up to 66 minutes. These values are within the framework of recovery of turtles under the influence of the environment. According to Tracchia (2018), this is due to their ectothermic metabolism, with thermotolerant pecylothermic body temperature and regulating compensatory physiological response.

The morphometric values in correlation with the recovery time, showed no correlation when subjected to Spearman's rank coefficient (r^2 = 0.0), except for plastron width, which showed weak correlation (r^2 = 0.14).

From the 67 chelonii, 61 were released back into the Meladão stream after recovering their movement. The other six specimens were used to evaluate the LILT through the freezing process.

We constructed a temperature polygon (Moyes and Schulte, 2009) identifying that the average body temperature of *P. geoffroanus* was 22.87° C (OST = 22.87 ± 0.23). Temperatures of species of freshwater reptiles vary between 25 and 35°C (Tracchia, 2018). Pessoa et al. (2008) found temperatures of 23°C in *Trachemys scripta elegans* (Wied, 1838). We understand that this variation we have identified must be related to environmental influence, the place of collection and temperature measurement, and the local population.

As for the low incipient lethal temperature - LILT (Moyes and Schulte, 2009), we found values below 1°C. This means that, until this limit, the specimen recovers its motor activities and can be released. Lower temperatures cause the animal to die (Fig. 1).

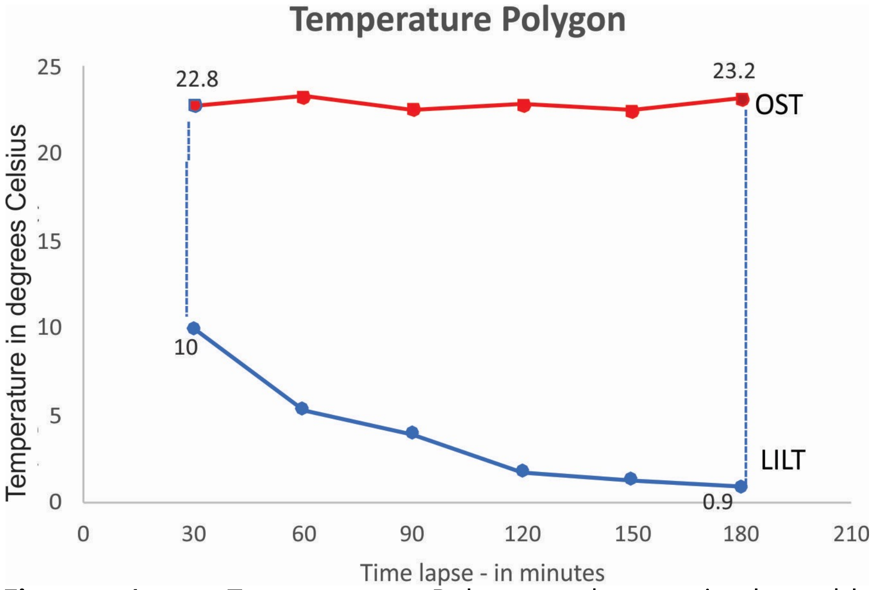

Figure 1 – Temperature Polygon, the optimal stable temperature (OST) is highlighted in red, the low incipient lethal temperature (LILT) is highlighted in blue.

Within 30 minutes, the temperature of 22.87°C had plummeted to 5°C, corresponding to a fall of 17.87°C. Within 180 minutes, the body temperature had bottomed out at 0.9°C, meaning that the animal shall no longer recover. When removing the animal from the freezer, it will have nasal bleeding, closed eyes, and reflex in the hindlimb, without achieving motor recovery. Our experiment found that a 300-minute period, regardless of weight, is sufficient to reach the LILT in the specimen without causing any bodily agony such as bleeding or protective reflexes (Fig. 2).

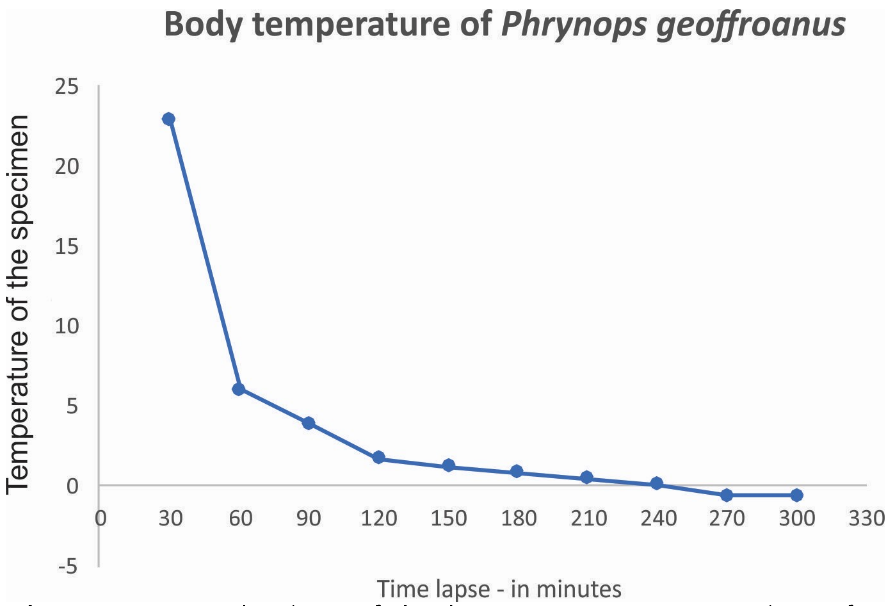

Figure 2 – Evaluation of body temperature over time, for *Phrynops geoffroanus,* and its LILT.

According to Tracchia (2018), the reptilian metabolism decreases from two to three times for every 10°C of reduction, meaning that would have a complete reduction of *P. geoffroanus* metabolism within 180 minutes, when the specimen does not present temperature and has a decrease of three times its metabolism, considering the OST of 22.87°C.

None of the morphometric parameters were correlated to the time required for LILT. The body mass showed a poor correlation coefficient for Spearman ranks (r^2 = 0.45), followed by plastron width (r^2 = 0.41), while other parameters showed no correlation (r^2 = 0.0). These results are similar to those obtained for anesthesia. It was not possible to establish an individual standard for specimens, and anesthesia and LILT results had overall parameters for the cooling time to reach the respective anesthesia grade 11 (80 minutes) and LILT (180 minutes), with a safety time of 300 minutes to avoid any bodily agony response.

When assessing the symptoms of anesthesia and LILT, we found that, in anesthesia, the animal keeps its eyes open, while it closes its eyes when in LILT (Fig. 3).

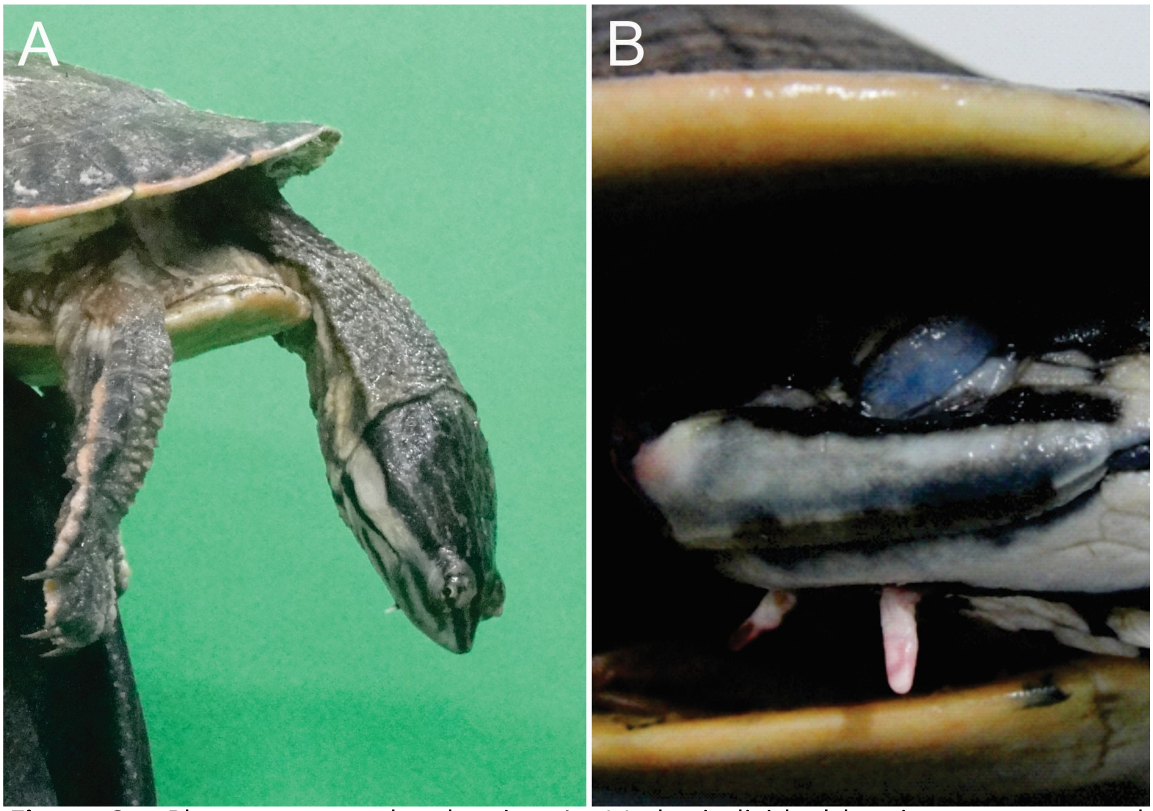

Figure 3 – Photomacrography showing: In (a) the individual has its eyes open and is anesthetised; in (b) it reached the LILT.

Considering that the morphometric variables were not significant when correlated with anesthesia time, body recovery time, and to reach the LILT time, that is, they are contrary to the expected results for the proportionality of cooling time depending on the morphometric condition, including body mass, we understand that there is a need for studies to evaluate the physiology of the species, such as hormone levels, heart rate, and bioelectric conduction. However, in the present study, we found satisfactory results of animal recovery and release, management under conditions of lethargy, anesthesia for swift action (15 - 22 minutes), besides establishing the global risk pattern for death by cooling, for *Phrynops geoffroanus*. These findings are in agreement with the results obtained in the studies by Shine et al. (2015) and Keifer and Zheng (2017), and those raised by Lillywhite et al. (2016) on the use of cooling and freezing (hypothermia) as an anesthetic in amphibians and reptiles. Despite demonstrating a satisfactory result that makes it possible to carry out containment and rapid clinical and surgical interventions, further studies are needed jointly with a reassessment by the main guidelines for the consolidation of this method.

Authorization and license

The research related to animals' use has complied with all the relevant national regulations and institutional policies for the care and use of animals (Collection and transport authorization SISBIO 61118-1; Animal Use Ethics Committee, process 23107.018093 / 2018-73 - protocol 30/2018 -UFAC – Universidade Federal do Acre / Federal University of Acre).

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