http://dx.doi.org/10.1590/1678-4162-9984

(cc) BY

Arq. Bras. Med. Vet. Zootec., **v.71, n.3, p.782-790, 2019**

J. Morais
http://orcid.org/0000-0001-5258-4480
J. Volpato
http://orcid.org/0000-0003-1441-9649
A. Costa
http://orcid.org/0000-0003-4320-3310
C. Kolesnikovas
http://orcid.org/0000-0002-8048-6551
M. Souza
http://orcid.org/0000-0002-9539-5695
M. Saito
http://orcid.org/0000-0002-1232-4657

Clinical biochemistry of rescued Magellanic Penguins (*Spheniscus magellanicus*): serum or heparinized plasma?

[*Bioquímica clínica de pinguins-de-magalhães* (Spheniscus magellanicus) *de resgate: soro ou plasma heparinizado?*]

J. *Morais*[1], J. *Volpato*[1], A. *Costa*[1], C. *Kolesnikovas*[2], M. *Souza*[2], M. *Saito*[1]

[1]Universidade do Estado de Santa Catarina - Lages, SC
[2]CETAS - Parque Estadual do Rio Vermelho - Florianópolis, SC

ABSTRACT

In the last few years, an increasing number of debilitated Magellanic penguins (*Spheniscus magellanicus*) has been rescued and taken to rehabilitation centers on Brazil's southern coast to be clinically treated and evaluated for re-introduction. This work aims to compare the viability of heparinized plasma with the viability of serum for biochemistry analyses under rehabilitation conditions. Blood sampled from 31 physically healthy rescued penguins was processed into serum/plasma-paired samples and analyzed for 12 biochemical parameters: alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), cholesterol (CHOL), creatine kinase (CK), gamma-glutamyl transpeptidase (GGT), glucose, (GLU) lactate dehydrogenase (LDH), total proteins (TP), triglycerides (TG), urea (UR), and uric acid (UA).The results showed that six paired samples presented visual signs of hemolysis (visual hemolytic score≥1), four of which occurred exclusively in the serum counterpart. Significant differences (P≤ 0.5) between sample types were found for CHOL (3%), GLU (6%) and TG (52%). Only TG was considered clinically relevant (>10%). All mean/median results fell within the available reference intervals by the Association of Zoos and Aquariums (Penguin, 2014). In conclusion, we verified that heparinized plasma is a viable sample for the clinical biochemistry of rescued Magellanic penguins as it yields compatible results with serum, while providing practical benefits. The adoption of this practice favors a faster bird recovery, by minimizing blood sampling volume, and optimizes material resources, allowing use of the same collector tube as for hematology.

Keywords: penguin, rehabilitation, blood sampling, heparin, optimization of resources

RESUMO

Nos últimos anos, um número crescente de pinguins-de-magalhães (Spheniscus magellanicus) *debilitados vem sendo resgatado e encaminhado aos centros de reabilitação do litoral sul do Brasil para cuidados clínicos e posterior avaliação de reintrodução. Este trabalho teve como objetivo comparar a viabilidade do plasma heparinizado com a do soro para análises bioquímicas, em condições de reabilitação. Amostras de sangue de 31 pinguins de resgate fisicamente saudáveis foram processadas em amostras pareadas de soro e plasma heparinizado, e 12 parâmetros bioquímicos foram analisados: alanina aminotransferase (ALT), fosfatase alcalina (ALP), aspartato aminotransferase (AST), colesterol (CHOL), creatina quinase (CK), gamaglutamil transpeptidase (GGT), glicose (GLU), lactato desidrogenase (LDH), proteínas totais (TP), triglicérides (TG), ureia (UR) e ácido úrico (UA). Os resultados mostraram que seis amostras pareadas apresentaram sinais visuais de hemólise (escore hemolítico visual≥1), das quais quatro ocorreram exclusivamente no soro. Observaram-se diferenças significativas (P≤0,5) entre os tipos de amostra em CHOL (3%), GLU (6%) e TG (52%), sendo apenas TG considerado clinicamente relevante (>10%). Todos os resultados de médias e medianas situaram-se dentro dos intervalos de referência disponíveis fornecidos pela Associação de Zoológicos e Aquários (AZA). Como conclusão, verificou-se que o plasma heparinizado é uma amostra viável para a bioquímica clínica de pinguins-de-magalhães de resgate, produzindo resultados compatíveis com os do soro. Além disso, a adoção dessa prática favorece uma recuperação mais rápida dos animais, ao diminuir o volume de sangue amostrado, e otimiza os recursos materiais, ao permitir o aproveitamento do mesmo tubo de colheita de hematologia.*

Palavras-chave: pinguim, reabilitação, colheita de sangue, heparina, otimização de recursos

Recebido em 7 de fevereiro de 2018
Aceito em 12 de junho de 2018
E-mail: juliabragamorais@gmail.com

INTRODUCTION

The Magellanic penguin (*Spheniscus magellanicus*) is a native South American seabird and an annual visitor to Brazil's coastal waters in austral winter (Boersma, 2008). Due to the increasing anthropogenic threats that compromise winter migration, each year a significant number of debilitated juvenile individuals are being rescued on Southern Brazilian beaches and taken to rehabilitation centers to be clinically treated and evaluated for reintroduction (Mäder *et al*., 2010; Vanstreels *et al.*, 2015).

The penguins that come to shore display a variety of health problems, such as malnutrition and dehydration, intoxication and external contamination with oil, injuries, severe intestinal parasitosis, regurgitation secondary to ingestion of foreign bodies (hooks and plastics) and infections (Cranfield, 2003; Niemeyer *et al.*, 2010). Once held in captivity to be rehabilitated, penguins are particularly susceptible to infectious disease due to their debilitated state and confinement stress, mostly aspergillosis, malaria and bumblefoot (Teare *et al.*, 2013). As this species is considered Near Threatened on the IUCN red list, adequate rehabilitation efforts to ensure the opportunity of an innocuous return to the breeding colonies on the Argentinean Patagonian coastline are regarded as important conservation steps (Borboruglu & Boersma, 2015). Since wild birds often hide signs of illness until they reach very advanced stages, it is vital to invest in their laboratory evaluation (Villa, 2013). As preparation of biological samples is known to widely affect the interpretation of laboratory results, controlling pre-analytic factors is essential for accurate diagnosis and suitable treatment (Friedrichs *et al.*, 2012). A biochemical analysis is an important tool to assist in achieving a comprehensive clinical picture that is compatible with the animal's real capacity for future release. (Graczyk *et al.*, 1995; Harr, 2002; Campos *et al.*, 2014). A field manual compiled by Brazilian environmental and marine authorities proposed standardizing diagnostic methods during penguin rehabilitation. These guidelines recommend hematology be performed with the use of anticoagulants, such as heparin or EDTA, which yield plasma, and biochemistry analysis in samples without anticoagulant, yielding serum in lesser amounts due to coagulation phenomena (Vanstreels *et al.*, 2011). However, the small blood volume available for sampling in recovering birds frequently excludes blood chemistry tests from rehabilitation facilities' protocols (Rodrigues *et al.*, 2010).

A biochemistry literature review about *Spheniscus* spp clinical, compiled in Table 1, shows that both heparinized plasma and serum have been used from 1985 to 2016. It should be noted, however, that the present reference intervals provided by the Association of Zoos and Aquariums (AZA), published in Teare *et al.* (2013), as well as the most recent study by Mayorga *et al.* (2016), do not clearly specify the utilized sample type.

Table 1. Chronological review of Spheniscus spp clinical biochemistry literature, describing each study's year of publication, utilized sample type, sampled species and number of different sampled animals (n)

Reference	SampleType	Species	n
Ghebremeskel *et al.* (1989)	Plasma /Heparin	*S. magellanicus*	9
Graczyk *et al.* (1995)	Serum	*S. demersus*	9
Wallace (1995)	Plasma /Heparin	*S. humboldti*	51
Villouta *et al.* (1997)	Plasma /Heparin	*S. humboldti*	15
Travis *et al.* (2006)	Serum	*S. mendiculus*	83
Smith *et al.* (2008)	Plasma /Heparin	*S. humboldti*	83
Bechert *et al* (2010)	Plasma /Heparin	*S. demersus*	18
Cray *et al.* (2010)	Plasma /Heparin	*S.demersus*	20
Coraiola. (2012)	Serum	*S.magellanicus*	29
Mazzaro *et al.* (2013)	Plasma /Heparin	*S. demersus*	21
Campos *et al.*(2014)	Serum	*S. magellanicus*	75
Moreno-Salas *et al.* (2014)	Plasma /Heparin	*S.humboldti*	42
Teare *et al.* (2013)	Indiscriminate	AllSpecies	≈800
Parsons *et al.* (2015)	Serum	*S. demersus*	108
Mayorga *et al.* (2016)	Indiscriminate	*S. m*agellanicus	34

This highlights the importance of comparing results between serum and plasma in order to obtain greater interpretation security. In this work we tested the viability of heparinized plasma (vs serum), as this practice allows minimizing sampling volume, which favors a faster bird recovery, and optimizes material resources, by allowing the same collector tube for other laboratory analyses to be used.

MATERIAL AND METHODS

The procedures described in this study were approved by the Ethics Committee of CAV-UDESC and Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), according to Protocol 1.08.15 and Protocol 48654-1, respectively.

Of the 34 Magellanic penguins housed at Centro de Triagem de Animais Silvestres (CETAS) of Florianopolis in August 2015, 31 were selected for re-introduction evaluation blood tests after excluding those with visible disabling lesions (bumblefoot and abscesses). Animals were admitted into rehabilitation, due mainly to severe dehydration and malnourishment, and remained in captivity for a mean 30-day period before blood sampling began. After subjecting them to a 24-hour fast, a fixed 5mL blood volume was collected from each animal via jugular venipuncture with physical restraint using 5mL syringes and 24G needles.

Blood was immediately divided into previously prepared containers: a 1.5mL round- bottomed microtube with 10µL sodium heparin (5000UI/mL) and a 2mL Vacutainer®tube with clot activator and no serum separation gel. The remaining volume (1.5mL) was used for other evaluations. About 2h after collection (minimum 1h, maximum 4h), samples were centrifuged at 2000g for 10 minutes on site or at the Carijós Ecological Station (Estação Ecológica dos Carijós/ICMBio) located 30 minutes away from CETAS to ensure that coagulation was finalized upon centrifuging the tubes with no anticoagulant. Serum and plasma samples were then separated into conic-bottomed microtubes and transported in dry ice to the Veterinary Clinical Pathology CAV-UDESC Laboratory, where they were stored at -20°C until processed. After thawing, a visual evaluation of the degree of hemolysis was made based on a gradual score, as shown in Figure 1 (A, B and C).

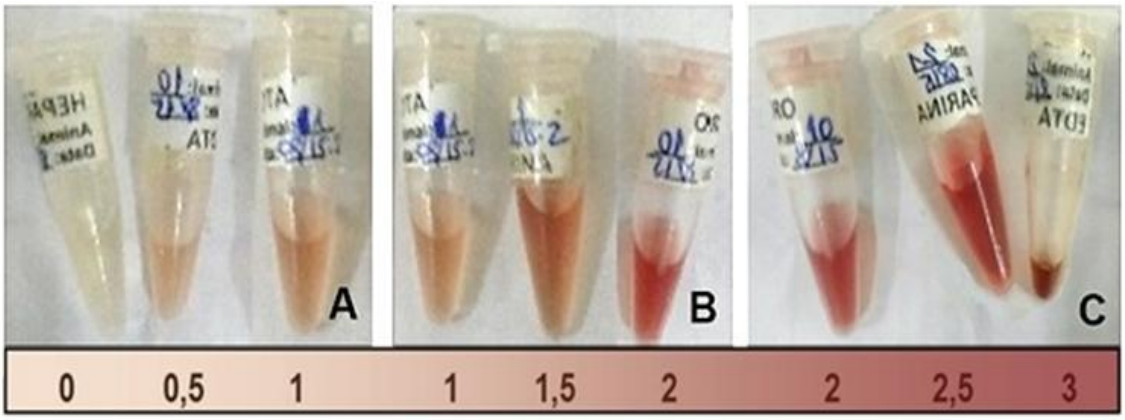

Figure 1. Adopted visual hemolytic score: (A) sample with no visual hemolysis (<1); (B) mild to moderate hemolysis (1≤x<2) or (C) moderate to severe hemolysis (≥2).The selected images are merely illustrative.

All the samples with a visual hemolytic score that equaled or went above 1 were excluded from the study. Biochemistry analyses were performed in duplicate for both sample types by spectrophotometry in an automatic wet chemistry analyzer (LabMaxPlenno, Labtest®, Lagoa Santa - Brazil) by the test methods referred to in Table 2. The albumin measurements taken by the green bromocresol method were not included, in agreement with Cray *et al.* (2011).

Table 2. Summary of the test methods employed for analyte measurements, as described in the reagent kit

Analyte / Acronym	Labtest® Reference	*Method name* and summary
Alanine aminotransferase/ALT	108	*UV-IFCC*:ALT specifically catalyzes the transfer of the amine group from alanine to ketoglutarate, with glutamate and pyruvate formation. Pyruvate is reduced to lactate by lactate dehydrogenase (LDH), while coenzyme NADH is oxidized to NAD. The absorbance reduction at 340nm or 365nm, as a result of NADH oxidation, is monitored photometrically and directly proportional to the ALT activity in the sample.
Aspartate aminotransferase / AST	109-2	*Kinetic UV*: AST specifically catalyzes the transfer of the amine group from aspartic acid to ketoglutarate with glutamate and oxaloacetate formation. Oxalacetate is reduced to malate through the action of malate dehydrogenase (MDH), while the NADH coenzyme is oxidized to NAD. The reduction of the absorbance at 340 or 365nm, due to NADH oxidation, is monitored photometrically and directly proportional to the AST activity in the sample.
Alkaline phosphatase/ALP	40	*Modified Roy:* Serum alkaline phosphatase hydrolyzes thymolphthalein monophosphate by releasing thymolphthalein, which is blue in an alkaline medium. The formed color, which is directly proportional to enzymatic activity, is measured at 590nm. The final reaction product consists of a mixture of blue and the substrate's color itself.
Cholesterol/CHOL	76-2	*Enzymatic hydrolysis /oxidation (Trinder)*: Cholesterol esters are hydrolyzed by cholesterol esterase to free cholesterol and fatty acids. Cholesterol free is oxidized by cholesterol oxidation to cholest-4-em-one and hydrogen peroxide. In the presence of peroxidase and hydrogen peroxide, phenol and 4-aminoantipyrine are oxidized to form antipyrilquinonimine with maximum absorbance at 500nm.
Creatine kinase/CK	117	*UV-IFCC*: CK catalyzes the dephosphorylation of creatine phosphate to produce adenosine triphosphate (ATP), which reacts with glucose in the presence of hexokinase (HK) to form glucose-6-phosphate which, in the presence of glucose-6-phosphate dehydrogenase (G-6- PDH), is oxidized to 6-phosphogluconate (6-PG) and reduces NAD to NADH. The rate of increased absorbance at 340nm is proportional to the CK activity in the sample.
γ-glutamyl transferase/GGT	105-2	*Modified Szasz*: GGT catalyzes the transfer of the glutamyl group of L-γ-glutamyl-3-carboxy-4-nitroanilide to glycylglycine by forming L-γ-glutamylglycylglycine and p-nitroaniline. The amount of p-nitroaniline, which has a high absorbance at 405nm, is directly proportional to the GGT activity in the sample.
Glucose/GLU	1012	*Glucose oxidase (GOD Trinder)*: Glucose oxidase catalyzes the oxidation of glucose by forming gluconic acid and hydrogen peroxide. The formed hydrogen peroxide reacts with 4-aminoantipyrine and phenol under peroxidase catalyzing action through an oxidative coupling reaction to form a red antipyrilquinonimine whose color intensity is proportional to the glucose concentration in the sample
Lactate dehydrogenase/LDH	86	*UV piruvate to lactate*: LDH catalyzes the conversion of pyruvate into lactate in the presence of NADH. The drop-in absorbance at 340nm due to NADH oxidation is proportional to the LDH activity in the sample
Total protein/TP	99	*Biuret*: Copper ions (Cu^{2+}) in the alkaline medium (Biuret Reactant) react with the peptide bonds of the serum proteins to form a purple color, which has a maximum absorbance at 545nm that is proportional to the concentration of the proteins in the sample.
Uric acid/UA	140-1	*Uricase-peroxidase*: Uric acid is oxidized by uricase to allantoin and hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase reacts with DHBS and 4-aminoantipyrine to form the chromogen antipyrilquinonimine. The intensity of the red color formed is directly proportional to the uric acid concentration in the sample.
Urea/BUN	104-2	*UV enzyme*: urease-GLDH: Urea is hydrolyzed by urease to produce ammonia and carbon dioxide. Ammonia reacts with 2-ketoglutarate and NADH in a reaction catalyzed by glutamate dehydrogenase (GLDH) by promoting the oxidation of NADH to NAD. The resulting drop in absorbance measured at 340nm is proportional to the urea concentration in the sample.
Triglycerides/TG	87-2	*Glycerolperoxidase*: Lipoprotein lipase promotes the hydrolysis of triglycerides by releasing glycerol, which is converted by glycerol kinase into glycerol-3-phosphate. This is oxidized to dihydroxyacetone and hydrogen peroxide in the presence of glycerol phosphate oxidase. Then a coupling reaction occurs between hydrogen peroxide, 4-aminoantipyrine and 4-chlorophenol that is catalyzed by peroxidase to produce a quinoneimine with a maximum absorbance at 505nm. The intensity of the formed red color is directly proportional to the concentration of the triglycerides in the sample.

All the data were evaluated for normality of distribution and outliers by the D'Agostino-Pearson test and the Tukey's method, respectively. Mean and standard deviation were calculated for the variables with normal distributions in both sample types, while median and median absolute deviation (MAD) were employed to describe the non-parametric sets. Following the American Society for Veterinary Clinical Pathology guidelines (ASCVP), minimum and maximum values were also included. A comparison of the means was performed by the paired Student's t-test, and paired medians were compared by the Wilcoxon Signed Rank test (Friedrichs *et al.*, 2012). Statistical significance was set at P≤ 0.05. Analyses were conducted with the Excel RealStats computer program (2010).

The percentage of change (% Change) between the values measured in serum to plasma was calculated according to Hrubec *et al.* (2002) by the following formula: ([Serum] - [plasma] / [serum]) x100, where [Serum] was the mean or median concentration of the analyte measured in the serum, and [plasma] was the mean or median measured in plasma. Clinically relevant differences between sample types were considered for the analytes with a % change exceeding 10% (Andreasen *et al*, 1996; Hawkins *et al.*, 2006).

RESULTS

Of the thirty one paired serum-plasma samples, six presented visual signs of hemolysis, and scored equal to or above 1, by the adopted method demonstrated in Figure 1, and were thus rejected. These hemolytic alterations occurred simultaneously in serum and plasma in only two pairs, while the other four showed hemolysis exclusively in the serum counterpart. The scoring of the samples with no visual signs of hemolysis (n= 25) showed that the mean hemolytic score for serum was higher than it was for heparinized plasma (0.23>0.17), although no statistically significant differences were found.

Fibrin clot formation occurred in 20% of the serum samples, which corresponded to 5 samples of 25. The means/medians of the biochemical analytes measured in heparinized plasma, compared with the results from serum and the AZA Reference Intervals, are shown in Table 3.

Table 3. Magellanic penguin clinical biochemistry results in Serum and Heparinized plasma: number of samples (n); Mean/Median; Standard deviation (Std)/Median Absolute Deviation (MAD); Minimum and Maximum values; % Change between sample type and AZA reference intervals for the species

Analyte	n	Serum Mean±Std (Min.-Max.)	Heparinized plasma Mean±Std(Min.-Max.)	% Change	Reference Interval (AZA)
ALP (U/L)	23	78.30±6.22 (36-137)	74,93±5,88 (37-128)	-	29-388
CHOL (mg/dL)	25	217.76±8.11 (128.5-273)	210.72*±8.29 (123-239)	3.23%	165-463
GGT (U/L)	16	12.63±1.77 (2-30.5)	14.94±2.06 (2-33)	-	-
GLU (mg/dl)	24	185.85±5.02 (139.5-231)	176.29*±4.49 (122.5-216.5)	5.53%	149-283
LDH (U/L)	23	662.2±44.32 (249-973)	594.7±38.34 (294-977)	-	65-1033
TG (mg/dL)	25	42.88±3.27 (19-82)	65.06*±3.63 (22-96.5)	-51.70%	-
TP (g/dL)	25	7.68±0.21 (6-9,94)	7.69±0.20 (6.32-10.21)	-	3.7-8.4
UA (mg/dL)	23	9,64±0.33 (6.57-12.74)	9.32±0.4 (5.97-13.56)	-	1.9-26.1
UR (mg/dL)	22	5.05±0.41 (1-8)	4.50±0.54 (1-10)	-	0-9
	n	Serum Median±MAD(Min.-Max.)	Heparinized plasma Median±MAD(Min.-Max.)	% Change	
ALT(U/L)	20	27.0±7.5 (5-80)	20±9 (5-72)	-	0-191
AST(U/L)	22	137±24.25 (76-252)	147.5± 29 (73--279)	-	59-538
CK(U/L)	23	209±56 (65-545)	207±44 (87-396)	-	56-1121

*Values with significant statistical differences (P≤ 0.05) according to the Student's t-test for paired samples.

The n for some analytes varied from 16-25 due to some analytical problems arising in at least one counterpart of the paired samples, which rendered the pair(s) unfit for comparison purposes. We acknowledge the main problem as the impossibility of measurement from a small available sample volume due to fibrin clots, reagent replacement or the low detection limit of the employed method (GGT).

The statistical differences between heparinized plasma and serum were essentially the means of GLU (overestimated), CHOL (underestimated) and TG (overestimated). The mean CHOL and mean GLU dosed in heparinized plasma did not clinically differ from the means dosed in serum, and only one analyte (TG) presented pertinent differences between sample types.

By comparing our results from the physically healthy animals with the reference values suggested by AZA, we found that all mean/median values measured in heparinized plasma and serum fell within the reference range, and only GLU with minimum and TP with maximum values fell beyond this reference range.

DISCUSSION

In this study, serum biochemical measurements were considered the gold standard, and were used as a reference to compare and discuss the results from plasma with an anticoagulant such as heparin. This choice was supported partly by the studies of Parsons *et al.* (2015) in African penguins (*S. demersus*), wherein the authors gave preference to serum samples over plasma when a rapid separation between the blood cell fraction and liquid fraction was not possible. They argued that the natural separation of blood fractions through coagulation was associated with fewer artificial changes, so the results were presumably more reliable. In the Clinical Pathology Laboratory of CAV-UDESC, rapid separation is considered up to 1h. In this work it was not possible to standardize the exact time from sampling to separation, which often occurs in wildlife sampling procedures (Low *et al.*, 2006). Nevertheless, centrifugation/parting occurred within 4h for all the samples, with an average of about 2h when prioritizing the samples with an anticoagulant. The reason for establishing this order was to provide ample time for coagulation to take place in the tubes with no anticoagulants, while still reducing the contact time between the two blood fractions in whole blood, either with or without an anticoagulant, as much as possible. According to the studies of Eisenhawer *et al.* (2008) in loggerhead sea turtles (*Caretta caretta*), whole blood samples with an anticoagulant were stored in thermal transport containers for up to 24h and no significant change were noted in most biochemical analytes.

The presence of fibrin clots in 20% of the serum samples agreed with the studies of Harr (2002), which described this phenomenon in as many as 25% serum samples taken from birds. Unfortunately, we only recorded cases of intense and clearly visible fibrin clots, which implies limitations in the available serum volume. Therefore, no comparison was made between the samples with and without fibrin clots as there could have been other cases of softer fibrin clots which were not accounted for. Further studies are recommended to investigate the potential alterations in the interpretation of the biochemical results.

The comparison of paired blood samples for serum and heparinized plasma, collected and stored at the same ambient temperature, gave overestimated GLU and underestimated CHOL values in plasma without considering clinically relevant differences. Although these findings were not consistent with those of Hrubec *et al.* (2002) when comparing serum and plasma in chickens, we encountered similar changes to the studies of Hrubec *et al.* (1999) in fish species, where the overestimated difference (GLU) most likely represented the metabolic utilization of blood constituents while blood clotted (*in vitro* glycolysis). This suggests that the analyte levels determined from serum may not accurately reflect those found in circulating blood. Alternatively, the underestimated results in heparinized plasma (CHOL) could be due to the inhibition of nonspecific chromogenic reagents. By eliminating those samples with visible signs of hemolysis, we attempted to exclude hemolysis interference as a possible cause of the measurements altered by the release of erythrocyte constituents (Hawkins *et al.*, 2006).

In this study, clinically significant higher TG values were obtained in heparinized plasma compared to serum. Heparin is known to activate the lipoprotein lipase enzyme and to hydrolyze TG by increasing free glycerol and lowering TG values. However, if the employed method did not correctly discount the excess free glycerol, it could account for the increase in TG (Harr, 2006). Additionally, Alves (2013) offers TG results of in Blue-throatedmacaws (*Ara ararauna*) with coefficients of variation above 90% in all the sample types, which proved to be a very unstable variable for this species. The literature review and AZA reference intervals for

the TG values in Magellanic penguins showed that there are no biochemical data available for this analyte in this species. As far as we know, TG values are published only for penguins of other species, namely African penguins (*Spheniscus demersus*): mean 56mg/dL measured in serum (Graczyk *et al.*, 1995) and median 82mg/dL in heparinized plasma (Cray*et al.*, 2010), *Pygoscelis* spp- mean 75mg/dL in heparin plasma (Aguilera *et al.*, 1993) and chinstrap penguins (*Pygoscelis antartica*), and approximately 80mg/dL measured in heparin plasma (Ferrer *et al.*, 1994). In these species, values were similar to those in the present study, which suggests that ours could be considered a preliminary reference for the Magellanic penguin, although its diagnostic importance remains unstudied.

The fact that the maximum PT values in both sample types were above the AZA reference intervals could reflect the dehydration, stress or subclinical inflammation of the most recently admitted animals. According to the studies of Villouta *et al.* (1997) in Humboldt penguins (*S. humboldti*), a confinement period that lasts more than 3 weeks can increase total proteins and globulin concentrations, which are most likely stress-related. Fibrinogen measurement is recommended to better assess the possible cause of these changes (Cray *et al.*, 2010). We suspect that the minimum GLU values beyond the AZA reference range in both serum and plasma could be due to individual variability. In previous penguin studies, the GLU value showed no significant difference due to feeding (Cray *et al.*, 2010) or post capture (Villouta *et al.*, 1997). Based on Hochleithner's (1994) avian medicine experience, GLU was maintained at adequate levels in plasma and pathological changes are rarely detected.

Parsons *et al.* (2015) worked with African penguins (*S. demersus*) and stated the preference of serum over plasma when immediate centrifugation was not possible. These authors referred to lack of the knowledge about the effect of anticoagulants in biochemical analyses.

Although not statistically significant, the differences found for the visual hemolytic score between sample types in this work may be related to the lower volume yield of serum samples, which hinders separation and increases the likelihood of accidentally pipetting the cell fraction. According to Blank *et al.* (1985), anticoagulants may minimize *in vitro* hemolysis. In the present study, the heparin results followed this hypothesis, but the preservative effect of heparin in relation to the samples with no anticoagulants requires confirmatory tests.

CONCLUSION

Based on the results obtained with the adopted methodology, we conclude that heparinized plasma is a viable sample for the biochemistry analysis of most of the metabolites and enzymes evaluated in rescued Magellanic penguins. The heparin samples were superior to the serum samples: no fibrin clot formation, larger available volume, and fewer presented signs of hemolysis. Adopting this practice in rehabilitation centers would favor faster bird recovery, by minimizing the blood sampling volume, and optimize material resources, by allowing the same collector tube for hematology to be used.

REFERENCES

AGUILERA, E.; MORENO, J.; FERRER, M. Blood chemistry values in three Pygoscelis penguins. *Comp. Biochem. Physiol. Part A Physiol.*, v.105, p.471-473, 1993.

ALVES G.M. *Efeitos dos anticoagulantes – citrato de sódio, EDTA-K3e heparina sódica nas análises hematimétricas e bioquímicas em Araras Canindé (Ara ararauna), Tigre d'água (Trachemysscripta) e Pacamãs* (*Lophiosilurusalexandri*). 2013, 85f. Dissertação (Mestrado em Medicina Veterinaria) – Escola de Medicina Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, MG.

ANDREASEN, J.R.; ANDREASEN, C.B.; SONN, A.B. ROBESON, D.C. The effects of haemolysis on serum chemistry measurements in poultry. *Vet. Pathol*., v.25, p.519-536, 1996.

BECHERT, U.; CHRISTENSEN, J.M.; POPPENGA, R. *et al*. Pharmacokinetics of orally administered terbinafine in African penguins (*Spheniscusdemersus*) for potential treatment of aspergillosis. *J. Zoo Wildl. Med*., v.41, p.263-274, 2010.

BLANK, D.; KROLL, M.H.; RUDDEL, M.E.; ELIN, R.J. Hemoglobin interference from in vivo hemolysis. *Clin. Chem*., v.31, p.1566-1569, 1985.

BOERSMA, P.D. Penguins as marine sentinels. *BioScience*, v.58, p.597-607, 2008.

BORBOROGLU, P.G.; BOERSMA, P.D. *Penguins:* natural history and conservation. Washington: University of Washington Press, 2015. 328p.

CAMPOS, S.D.; PIRES, J.R.; NASCIMENTO, C.L. *et al*. Analysis of hematologic and serum chemistry values of *Spheniscusmagellanicus* with molecular detection of avian malarial parasites (Plasmodium spp.). *Pesqui. Vet. Bras*., v.34, p.1236-1242, 2014.

CORAIOLA, A.M. *Indicadores clínicos, hematológicos, bioquímicos e toxicológicos na pré e pós-reabilitação de Pinguins-de-Magalhães (SpheniscusMagellanicus) no Sul do Brasil*. 2012. 98f. Dissertação (Mestrado em Medicina Veterinaria) - Universidade Federal do Paraná, Curitiba, PR.

CRANFIELD, M.R. Sphenisciformes (Penguins), In: FOWLER, M.E.; MILLER, R.E. (Eds.). *Zoo and wild animal medicine*. 5.ed. Philadelphia: W.B. Saunders Company, 2003. p.103-110.

CRAY, C.; STREMME, D.W.; ARHEART, K.L. Postprandial biochemistry changes in penguins (*Spheniscusdemersus*) including hyperuricemia. *J. Zoo Wildl. Med*., v.41, p.325-326, 2010.

CRAY, C.; WACK, A.; ARHEART, K.L. Invalid measurement of plasma albumin using bromcresol green methodology in penguins (*Spheniscus species*). *J. Avian Med. Surg*., v.25, p.14-22, 2011.

EISENHAWER, E.; COURTNEY, C.H.; RASKIN, R.E.; JACOBSON, E. Relationship between separation time of plasma from heparinized whole blood on plasma biochemical analytes of loggerhead sea turtles (*Carettacaretta*). *J. Zoo Wildl. Med.*, v.39, p.208-215, 2008.

FERRER, M.L.; AMAT, J.A.; VIÑUELA, J. Daily variations of blood chemistry values in the Chinstrap Penguin (*Pygoscelisantarctica*) during the Antarctic summer. *Comp. Biochem. Physiol. Part D Physiol.*, v.107, p.81-84, 1994.

FRIEDRICHS, K.R.; HARR, K.E.; FREEMAN, K.P. *et al*. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. *Vet. Clin. Pathol.*, v.41, p.441-453, 2012.

GHEBREMESKEL, K.; WILLIAMS, G.; KEYMER, I.F. *et al*. Plasma chemistry of Rockhopper (*Eudyptescrestatus*), Magellanic (S*pheniscusmagellanicus*) and Gentoo (*Pygoscelispapua*) wild penguins in relation to moult. *Comp. Biochem. Physiol. Part A Physiol*., v.92, p.43-47, 1989.

GRACZYK, T.K.; CRANFIELD, M.R.; BICKNESE, E.J. Evaluation of serum chemistry values associated with avian malaria infections in African black-footed penguins (*Spheniscusdemersus*). *J. Parasitol. Res.*, v.81, p.316-319, 1995.

HARR, K.E. Clinical chemistry of companion avian species: a review. *Vet. Clin. Pathol*., v.31, p.140-151, 2002.

HARR, K.E. Diagnostic value of biochemistry. *Clin. Avian Med*., v.2, p.611-630, 2006.

HAWKINS, M.G.; KASS, P.H.; ZINKL, J.G.; TELL, L.A. Comparison of biochemical values in serum and plasma, fresh and frozen plasma, and hemolyzed samples from orange-winged Amazon parrots (*Amazonaamazonica*). *Vet. Clin. Pathol*., v.35, p.219-225, 2006.

HOCHLEITNER, M. Biochemistries. In: RITCHIE, B.W., HARRISON, G.J., HARRISON, L.R. *Avian medicine*: principles and application. Florida: Wingers Publishing, 1994. p.223-245.

HRUBEC, T.C.; SMITH, S.A. Differences between plasma and serum samples for the evaluation of blood chemistry values in rainbow trout, channel catfish, hybrid tilapias, and hybrid striped bass. *J. Aquat. Anim. Health*, v.11, p.116-122, 1999.

HRUBEC, T.C.; WHICHARD, J.M.; LARSEN, C.T.; PIERSON, F.W. Plasma versus serum: specific differences in biochemical analyte values. *J. Avian Med. Surg*., v.16, p.101-105, 2002.

LOW, M.; EASON, D.; ELLIOTT, G. *et al*. Hematologic and biochemical reference ranges for the Kakapo (*Strigopshabroptilus*): generation and interpretation in a field-based wildlife recovery program. *J. Avian Med. Surg.*, v.20, p.80-88, 2006.

MÄDER, A.; SANDER, M.; CASAJR, G. Ciclo sazonal de mortalidade do pinguim de Magalhães, *Spheniscus magellanicus* influenciado por fatores antrópicos e climáticos na costa do Rio Grande do Sul, Brasil. *Rev. Bras. Ornitol*., v.18, p.228-233, 2010.

MAYORGA, L.F.; BHERING, R.C.; MEDEIROS, L.C. *et al*. Valores hematológicos e bioquímicos de pinguins-de-Magalhães em reabilitação no Espírito Santo, sudeste do Brasil. *Pesqui. Vet. Bras*., v.36, p.65-70, 2016.

MAZZARO, L.M.; MEEGAN, J.; SARRAN, D. *et al*. Molt-associated changes in hematologic and plasma biochemical values and stress hormone levels in African penguins (*Spheniscusdemersus*). *J. Avian Med. Surg.*, v.27, p.285-293, 2013.

MORENO-SALAS, L.; NILSSON-SAÉZ, L.; CORVALÁN, F. *et al*. Comparación de losvaloreshematológicos y bioquímicosem pinguinhos *Spheniscushumboldti* (Sphenisciformes: Spheniscidae) selvagens y em cautiverio. *Rev. Cient. Fac. Cienc*., v.24, p.267-272, 2014.

NIEMEYER, C.; VANSTREELS, R.; RUOPPOLO, V. *et al*. Resultados patológicos preliminares sobre a morte de pinguins de Magalhães (Spheniscus magellanicus) no litoral do estado de São Paulo. SEMANA CIENTÍFICA BENJAMIN EURICO MALUCELLI, 4., 2010, São Paulo. *Anais…* São Paulo: USP, 2010. p.32-33.

PARSONS, N.J.; SCHAEFER, A.M.; VAN DER SPUY, S.D.; GOUS, T.A. Establishment of baseline haematology and biochemistry parameters in wild adult African penguins (*Spheniscusdemersus*). *J. S. Afr. Vet. Assoc*., v.86, p.1-8, 2015.

PENGUIN (*Spheniscidae*) care manual. Silver Spring: Association of Zoos and Aquariums, 2014. (Penguin Taxon Advisory Group).

RODRIGUES, S.C.; ADORNES, A.C.; SANTOS-FILHO, E.A.D.; SILVA FILHO, R.P.; COLARES, E.P. Surviving probability indicators of landing juvenile Magellanic penguins arriving along the southern Brazilian coast. *Braz. Arch. Biol. Technol*., v.53, p.419-424, 2010.

SMITH, K.M.; KARESH, W.B.; MAJLU, P. Health evaluation of free-ranging Humboldt penguins (*Spheniscus humboldti*) in Peru. *Avian Dis*., v.52, p.130-135, 2008.

TEARE J.A. Spheniscus magellanicus No selection by gender all ages combined Conventional American units 2013. In*:* ISIS PHYSIOLOGICAL REFERENCE INTERVALS FOR CAPTIVE WILDLIFE, 2013, Eagan. *Procedings…* Eagan: International Species Information System, 2013. CD-ROM

VANSTREELS, R.E.T.; ADORNES A.C.; CABANA, A.L. *et al*. Manual de campo para colheita e armazenamento de informações e amostras biológicas provenientes de pinguins-de-Magalhães (*Spheniscus magellanicus*). São Paulo: Centro Nacional de Pesquisa e Conservação de Aves Silvestres, 2011. p.62.

VANSTREELS, R.E.T.; SILVA-FILHO, R.P.; KOLESNIKOVAS, C.K.M. Epidemiology and pathology of avian malaria in penguins undergoing rehabilitation in Brazil. *Vet. Res.*, v.46, p.30, 2015.

VILLA, L.G. *Hematologia em aves:* revisão de literatura. 2013. 46f. Dissertação (Mestrado em Ciência Animal) – Escola de Veterinária e Zootecnia, Universidade Federal de Goiás, Goiânia, GO.

VILLOUTA, G.; HARGREAVES, R.; RTVEROS, V. Haematological and clinical biochemistry findings in captive Humboldt penguins (*Spheniscus humboldti*). *Avian Pathol*., v.26, p.851-858, 1997.

WALLACE, R.S.; TEARE, J.A.; DIEBOLD, E.; MICHAELS, M.; WILLIS, M.J. Hematology and plasma chemistry values in free-ranging Humboldt penguins (*Spheniscus humboldti*) in Chile. *Zoo. Biol*., v.14, p.311-316, 1995.