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BIOLOGY

Anticoagulants and their effects on the hematological and biochemical parameters of yellow-spotted amazon river turtle

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ABSTRACT: *Knowledge of suitable methods and reagents for assessing the health condition of specimens of a given species is essential. The present study evaluated the efficacy of the heparin anticoagulants 5,000 I.U. mL^{-1}, Na_2EDTA, and K_3EDTA on the blood parameters of yellow-spotted amazon river turtle Podocnemis unifilis, employing different solutions for red blood cells count. The use of the various anticoagulants evaluated after 10 hours of storage efficiently inhibited coagulation in blood samples from P. unifilis. An increased number of erythrocytes was observed with the use of K_3EDTA 5% when compared with heparin. Statistically significant changes in the erythrocyte number were observed with the use of the different solutions. Solutions which featured sodium citrate and formaldehyde in their composition, allowed erythrocytes counting up to 120 hours after blood collection, without a change in values. The use of the heparin anticoagulants 5,000 I.U. mL^{-1}, Na_2EDTA 3%, Na_2EDTA 5%, K_3EDTA 3% was recommended in the hematological analysis of P. unifilis. Also recommended was the use of the formaldehyde-citrate solution containing 1.9 g of sodium citrate and 1.0 mL of formaldehyde (in 50 mL of distilled water) to perform red blood cells counts in yellow-spotted amazon river turtle.*
Key words: *blood, Chelonia, erythrocytes, Podocnemis unifilis, tracajá.*

Anticoagulantes e seus efeitos em parâmetros hematológicos e bioquímicos de tracajá

RESUMO: *O conhecimento sobre métodos e reagentes apropriados para as avaliações da condição de saúde de exemplares de determinada espécie é fundamental. Este estudo avaliou a eficácia dos anticoagulantes heparina 5.000 U.I. mL^{-1}, Na_2EDTA (3% e 5%), e K_3EDTA (3 e 5%) em parâmetros sanguíneos de tracajá (Podocnemis unifilis), bem como diferentes soluções para contagem de eritrócitos totais. A coagulação foi eficientemente inibida nas amostras de sangue de P. unifilis com o uso dos diferentes anticoagulantes avaliados após 10 horas de armazenamento. Maior número de eritrócitos com o uso de K_3EDTA 5% foi observado quando comparado com a coleta de sangue realizada com heparina. Diferenças, estatisticamente significativas, entre as contagens de eritrócitos com o uso das diferentes soluções de reagentes avaliadas foram verificadas. As soluções contendo citrato de sódio e formol na composição possibilitaram contagens de eritrócitos até 120 horas após a coleta, sem alteração em seus valores. Recomenda-se o uso dos anticoagulantes heparina 5000 U.I. mL^{-1}, Na_2EDTA 3%, Na_2EDTA 5%, K_3EDTA 3% nas análises hematológicas de tracajá. Assim como o uso da solução de formol-citrato contendo 1,9 g de citrato de sódio e 1,0 mL de formol (em 50 mL de água destilada) para realização das contagens de eritrócitos totais em tracajá.*
Palavras-chave: *sangue, Chelonia, eritrócitos, Podocnemis unifilis, tracajá.*

INTRODUCTION

Turtles are reptiles of the Chelonia order, with both sea and freshwater representatives. They are ectothermic, maintaining the balance of their body temperature by exchanging thermal energy with the environment (POUGH et al., 2008). They are of great medicinal, economic, and subsistence-related importance for the human populations of the Amazon. Among the most notable genus are *Podocnemis* and *Kinosternon* (ALHO, 1985). From Podocnemididae family, *Podocnemis* is the most representative genus,

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with six living species: *Podocnemis erythrocephala* Spix, 1824; *Podocnemis expansa* Schweigger, 1812; *Podocnemis lewyana* Duméril, 1852; *Podocnemis sextuberculata* Cornalia, 1849; *Podocnemis unifilis* Tröschel, 1848, and *Podocnemis vogli* Muller, 1935. Of these, yellow-spotted amazon river turtle, *P. unifilis*, is one of the most frequently caught species for human consumption, according to the International Union for the Conservation of Nature (IUCN). Additionally, the species adapts well to management conditions in cultivation (PEZZUTI et al., 2008). *Podocnemis unifilis* has great economic importance in the Brazilian Amazon, and its breeding is authorized through Normative Instruction No. 07/2015 (IBAMA, 2015), as it is widely consumed by the populations of the region, and for these reasons, this is one of animal species vulnerable to extinction, in the red list of threatened species announced by the IUCN.

To establish the hematological reference values of each species, it is essential that the most suitable reagents used in evaluations are known, as changes caused by the use of certain anticoagulants have been reported (HATTINGH, 1975; MAINWARING & ROWLEY, 1985; WALENCIK & WITESKA, 2007; ISHIKAWA et al., 2010), including those used to determine erythrocyte parameters. Among the anticoagulants used in clinical hematology, potassium (K_3EDTA) and sodium (Na_2EDTA) ethylenediaminetetraacetic acids (EDTA) are more commonly used, in addition to sodium heparin (HARR et al., 2005). Heparin is the most commonly used anticoagulant during blood tests in humans, fish, reptiles, and birds (HATTINGH & SMITH, 1976; WALENCIK & WITESKA, 2007, PERPIÑÁN et al. 2010; FAZIO, 2019). Its anticoagulant activity is caused by the acceleration of antithrombin III activity, which in turn inhibits the action of thrombin and other proteases responsible for the coagulation cascade (HARR et al., 2005). Heparin has been used as an anticoagulant of choice for turtles, due to the observation of hemolysis caused by EDTA (JACOBSON, 1987; MURO et al., 1998).

The anticoagulant effect of EDTA is due to its action in the chelation of factor IV (Ca^{2+}) in the coagulation cascade, acting as a mediator, as well as in the cell-to-cell relationship during coagulation reactions (HARR et al., 2005; TAVARES-DIAS & OLIVEIRA, 2009). However, EDTA is recommended as an anticoagulant, preferably for the total and differential counting of chelonian leukocytes, due to the preservation properties of leukocyte blood cells (NCCLS, 1990; OVIEDO & RODRÍGUEZ, 2019; BURTIS & BURNS, 2016). Nevertheless, these anticoagulants have not been evaluated for blood collection specifically in *P. unifilis*. The present study compared the most suitable methods and reagents for assessing the qualitative and quantitative characteristics of the blood cells of yellow-spotted amazon river turtle, using sodium heparin, sodium ethylenediaminetetraacetic acid (Na_2EDTA 3 and 5%) and potassium ethylenediaminetetraacetic acid (K_3EDTA 3 and 5%) as anticoagulants, in addition to different solutions for total erythrocyte counting.

MATERIALS AND METHODS

Anticoagulant evaluation based on hematological analysis

Podocnemis unifilis (n=10), belonging to the vivarium of Embrapa Amapá, captured with the aid of a dip net and transported to the Laboratory of Nutrition of Aquatic Organisms, Embrapa Amapá, Macapá, AP, were individually weighed (2.1 ± 0.3 kg), measured (carapace length, 25.2 ± 1.2 cm) and identified. The animals were manually restrained with the aid of a damp cloth and the use of rubber gloves for safe handling, and blood samples were collected by puncturing the caudal vessel.

Disposable syringes (capacity of 3.0 mL) and hypodermic needles (25 x 7 mm), without the use of anticoagulants, were used to collect blood samples for the evaluation of anticoagulants. The blood sample (≈ 2.5 mL) was quickly distributed into five polyethylene microtubes (capacity of 1.5 mL), 500 µL in each, to which 12.5 µL of anticoagulant was added as follows: in microtube 1, sodium heparin 5,000 I.U. mL^{-1}; in microtube 2, Na_2EDTA 3%; in microtube 3, Na_2EDTA 5%; in microtube 4, K_3 EDTA 3%; and, in microtube 5, K_3EDTA 5%; all homogenized by inversion. After aliquoting the blood in polyethylene tubes, 10 µL of each aliquot was placed in polyethylene microtubes (capacity of 1.5 mL), kept under refrigeration for a period of 10 hours, and visually (with naked eyes) evaluated every 30 minutes for the occurrence of coagulation and/or hemolysis (HATTINGH & SMITH, 1976).

Right after blood samples were mixed with anticoagulant, hematocrit (Ht) was determined using the microhematocrit technique (GOLDENFARB et al., 1971), with a reading of the red cell percentage on standardized cards. The hemoglobin concentration (Hb) was determined by the cyanmethemoglobin method (COLLIER, 1944), with the absorbance reading taken in a spectrophotometer (Biospectro, SP-220, Curitiba, PR, Brazil) at 540 nm. The red blood cells count (RBC) was performed using

formaldehyde-citrate solution and a Neubauer chamber (RANZANI-PAIVA et al. 2013), under a light microscope (Boeco, model BOE-01, Germany). These data (Ht, Hb and RBC) were used to determine the hematimetric indices (WINTROBE, 1934): mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC). The remaining blood was centrifuged at 75 G (Centrifuge, model MCD-2000, MRC Lab, United Kingdom), during 10 min to obtain plasma. The plasma samples were kept at -18ºC until the analysis to determine glucose, total protein and albumin levels using specific colorimetric kits (Ebram®, São Paulo, SP, Brazil), for each metabolite, with absorbance readings from a spectrophotometer (Biospectro, SP-220, Curitiba, PR, Brazil).

Blood evaluation based on different solutions used to total erythrocyte count

The following solutions were evaluated for the total erythrocyte count (red blood cells count, RBC), for which a total volume of 50 mL of each solution was prepared, made up with distilled water: Solution A: Sodium chloride solution 0.9 %; Solution B: 1.45 g sodium citrate and 1.5 ml formaldehyde; Solution C: 1.9 g sodium citrate, 1.0 ml formalin and 0.01 g toluidine blue; Solution D: 1.9 g sodium citrate and 1.0 mL formaldehyde; Solution E: 1.9 g sodium citrate and 0.01 g toluidine blue; Solution F: 1.0 mL formaldehyde and 0.01 g toluidine blue. Blood samples in a sodium chloride solution (Solution A) were kept in the refrigerator, as this solution does not include any preservative-containing reagents.

Another *P. unifilis* (n=6) were captured with a dip net and transported to the Laboratory of Nutrition of Aquatic Organisms, Embrapa Amapá (Macapá, AP) and individually weighed (2.2 ± 0.1 kg) and measured (carapace length, 25.6 ± 1.0 cm). Blood samples were taken from those animals manually restrained by puncturing the caudal vessel. Disposable syringes (capacity of 3.0 mL) and hypodermic needles (25 x 7 mm) with sodium heparin as anticoagulant, were used to collect blood samples for the evaluation of solutions for erythrocyte counting. and a Neubauer chamber (RANZANI-PAIVA et al. 2013), under a light microscope (Boeco, model BOE-01, Germany).

The red blood cells counts (RBC) were performed in each of six different solutions (A to F) in a Neubauer chamber (RANZANI-PAIVA et al. 2013) under a light microscope (Boeco, model BOE-01, Germany), at the moment of blood samples collection (0h) until 120h, every 24h, as indicated: the first, at the time of blood collection (0h); the second, 24 hours (1 day) after blood collection; the third, 48 hours (2 days) after collection; the fourth, 72 hours (3 days) after collection; the fifth, 96 hours (4 days) after collection and the sixth, 120 hours (5 days) after collection.

Data analysis

After tests of normality (Shapiro-Wilk) and homogeneity (Levene), the data were subjected to one-way analysis (ANOVA) of variance with the use of parametric and nonparametric multiple comparison tests (ZAR, 2010). The analyses were performed using the GraphPad Instat® Statistical Program, version 3.01 (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

Different anticoagulants effects on P. unifilis blood sample

This is the first study about the effects of heparin sodium and EDTA (sodium and potassium) as anticoagulants on the hematological parameters of *P. unifilis*. The use of sodium heparin in the hematological evaluation of turtles and other reptiles has been widely described in several studies (AGUIRRE et al., 1995; MAFUVADZE & ERLWANGER, 2007; SANTOS et al., 2009; SABINO et al., 2010, ISHIKAWA et al., 2010, COSTA et al., 2020), and has been found to be an effective anticoagulant. Blood clotting inhibition was efficient in all the tests with the anticoagulants evaluated. The hematological and biochemical values of apparently healthy *P. unifilis* using different anticoagulants are shown in table 1. However, in the samples collected with heparin, the mean RBC was lower ($P < 0.05$) than with the use of K_3EDTA 5%, showing that hemolysis or blood clot could be happened with that anticoagulant. A significantly lower MCV was observed with the use of the Na_2EDTA 3% anticoagulant than with the use of sodium heparin, Na_2EDTA 5% and K_3EDTA 5%. Differences were not observed ($P > 0.05$) in glucose, total protein, and albumin plasma levels, or in Ht, Hb, and MCHC, with the use of the different anticoagulants.

Some studies have reported that heparin, despite being considered a natural anticoagulant, can interfere with the staining of leukocyte blood cells, in addition to being expensive in comparison with other anticoagulants (GILOR & GILOR, 2011). Whereas EDTA is a more widely used anticoagulant than heparin, as it reduces the agglutination processes, preserving blood cell morphology and staining (GILOR & GILOR, 2011). Nevertheless, several studies have reported that EDTA, when used as an anticoagulant in chelonian, can cause hemolysis (MURO et al., 1998, PERPIÑÁN

Table 1 - Hematological variables and plasma concentrations (glucose, total proteins and albumin) of *Podocnemis unifilis* by the use of different anticoagulants: sodium heparin (5000 U.I. mL^{-1}), Na_2EDTA (3 and 5%) and K_3EDTA (3 and 5%).

Parameters	Sodium heparin	Na_2EDTA 3%	Na_2EDTA 5%	K_3EDTA 3%	K_3EDTA 5%
Ht (%)	22.69±2.07[a]	24.25±4.43[a]	25.06±3.17[a]	25.50±4.21[a]	26.00±3.53[a]
Hb (g dL^{-1})	7.13±0.73[a]	7.19±1.47[a]	7.53±0.79[a]	7.35±1.38[a]	7.23±1.19[a]
RBC (x 10^6 μL^{-1})	0.35±0.07[b]	0.43±0.11[ab]	0.38±0.04[ab]	0.44±0.09[ab]	0.49±0.09[a]
MCV (fL)	632.87±84.70[a]	424.21±111.15[b]	631.84±78.20[a]	633.60±106.55[a]	495.45±114.07[ab]
MCHC (g dL^{-1})	32.23±2.69[a]	32.47±7.45[a]	31.16±5.81[a]	32.01±6.30[a]	31.52±3.51[a]
Glucose (mg dL^{-1})	37.95±7.31[a]	42.76±9.42[a]	35.59±7.94[a]	36.00±7.69[a]	39.70±8.80[a]
Protein (g dL^{-1})	3.54±0.35[a]	3.69±0.55[a]	3.57±0.44[a]	3.85±0.29[a]	3.69±0.34[a]
Albumin (g dL^{-1})	1.31±0.26[a]	1.33±0.20[a]	1.48±0.13[a]	1.46±0.12[a]	1.49±0.19[a]

et al., 2008; OLIVEIRA-JUNIOR et al. 2009; TAVARES-DIAS et al., 2009; PERPIÑÁN et al., 2010).

There was a trend towards higher hematocrit values with the use of EDTA in relation to heparin, indicating a desired cell preservation. It was reported that the use of different anticoagulants did not alter most blood parameters. The hematocrit values of *P. unifilis* in the present study using sodium and potassium EDTA were similar to those reported by BOGAN et al. (2020) for the eastern indigo snake *Drymarchon couperi*, using K_3EDTA. However, it was lower than that reported by ANDRADE (2008) for *P. unifilis* in captivity, and by TAVARES-DIAS et al. (2012) for *P. unifilis* from the Abufari biological reserve (Amazon State, Brazil). In addition, both studies used heparin as an anticoagulant.

The hemoglobin concentration of *P. unifilis* did not differ with the use of different anticoagulants in the present study, corroborating previous studies with animals of the same species kept in captivity and under adequate cultivation conditions (TAVARES-DIAS et al, 2009). However, the number of total erythrocytes was lower with the use of heparin than with the use of K_3EDTA 5%. In addition, MURO et al. (1998) observed a reduction in the number of erythrocytes in *Testudo hermanni,* also with the use of K_3EDTA 5%, in comparison with the use of lithium heparin. In contrast, such differences were not observed in studies with macaws and pythons (HARR et al., 2005). ISHIKAWA et al. (2010) showed the efficiency of the anticoagulant Na_2EDTA 3% in hybrid catfish, known as surubim (*P. reticulatum* x *P. corruscans*). Thus, such results demonstrated the importance of knowledge about the most suitable anticoagulant for use in each species, due to their peculiarities and specificities.

The hematological and biochemical parameters of *P. unifilis* revealed few variations, with mainly minimal differences observed in the blood count. The samples collected with K_3EDTA 5% exhibited higher number of RBC, while the MCV differed between the samples with the anticoagulants evaluated. In *P. unifilis*, MCV was lower with the use of Na_2EDTA 3% than with the use of heparin, Na_2EDTA 5% and K_3EDTA 3%. However, the MCV values of this study, with these different anticoagulants, were lower than those reported for wild yellow spotted amazon river turtle obtained with heparin (OLIVEIRA-JÚNIOR et al., 2009; TAVARES-DIAS et al., 2012). This reduction in MCV may be due to osmotic crenation in erythrocytes when blood is collected with high concentrations (7.2 mg mL^{-1} e 14.4 mg mL^{-1}) of EDTA (OLIVEIRA et al., 2010), even without Ht alterations observed.

Total plasma protein concentrations were not influenced using different anticoagulants in *P. unifilis*. Similarly, the levels of total plasma proteins of hybrid surubim were not influenced by heparin or Na_2EDTA, even in different concentrations of 3%, 5% and 10% (ISHIKAWA et al., 2010). However, OLIVEIRA et al. (2015) reported that EDTA 5% and 10% were not efficient, and caused a reduction in total plasma proteins, as the coagulation process includes protein retention, reducing its amount in plasma. The use of EDTA, in the concentrations assessed in the present study, allowed hematological and biochemical values to be obtained, corroborating the results of studies carried out in yellow-blotched map turtles (*Graptemys flavimaculata*) (MARTINEZ-JIMENEZ et al., 2007). Other studies have reinforced the applicability of the use of EDTA for several species, in particular reptiles (HATTINGH, 1975;

MARKS & CITINO, 1990; SALAKIJ et al., 2002; BOGAN et al., 2020).

Different solutions effects on P. unifilis total erythrocyte count

Hemolysis was detected with the use of the reagent containing only 0.9% sodium chloride solution, 72 hours after collection. Solutions B and D, both composed of sodium citrate and formaldehyde, but in different concentrations, demonstrated less variation in the erythrocyte count, which could be performed up to 120 hours after the collection of the blood samples. Solution C, with sodium citrate and toluidine blue dye, revealed a lower number of erythrocytes, demonstrating problems with carrying out the count, and allowing cell visualization up to 72 hours after the collection of blood samples. Differences in the total erythrocyte counts of solution E, with sodium citrate and toluidine blue dye, and solution F, with formaldehyde and the addition of toluidine blue dye, were observed at the time of collection, meaning that the blood cell preservation potential of the use of these solutions was not observed (Table 2). All the reagent solutions evaluated exhibited statistically significant differences in the total erythrocyte count.

The use of solution A (sodium chloride), with samples preserved under refrigeration, revealed that the *P. unifilis* erythrocytes can be counted up to a maximum of 48 hours without significant changes in numbers, and after this period there is a considerable reduction, with secure determination not guaranteed. RADISIC et al. (2020) observed greater osmotic fragility in sodium chloride concentrates of under 0.38%, causing hemolysis in 50% of samples from sea turtles, *Caretta caretta* e *Chelonia mydas*. The use of the crystal violet method for counting erythrocytes with dilution in 0.45% sodium chloride solution enabled the erythrocyte nuclei of slider turtles *Trachemys scripta* to be stained, allowing the differentiation and proper distinguishing of erythrocytes from other cells, such as lymphocytes, thrombocytes, and granulocytes (TSAI et al., 2014).

Solution C revealed problems in the counts performed after 96 hours of collection, although with less damage than was observed with the use of solutions E and F. Thus, solutions B and D proved more suitable for counting the erythrocytes of *P. unifilis*. It is notable that both solutions B and D were prepared without the use of toluidine blue dye. With solutions E and F it was only possible to visualize the erythrocytes at the time of blood collection, and they could not be seen 24 hours after blood collection due to the large number of lysed cells. Of these two solutions, solution F exhibited a very low erythrocyte count number, the lowest among all the solutions evaluated. The possible cause of the hemolysis that occurred in a short period of time with the use of solution F is presumed to be due to the absence of sodium citrate in the composition, even considering the fact that citrate is widely used in blood clotting time tests. When comparing the counts using evaluated solutions, it is possible to observe that the counts performed with solution D had a lower coefficient of variation and can be recommended as the first choice in hematological studies of this species of chelonian, *P. unifilis*. This solution was prepared only with sodium citrate and formaldehyde, without toluidine blue dye.

Table 2 - Different solutions evaluation to red blood cells counts (x 10^3 μL^{-1}) in *Podocnemis unifilis* blood samples, performed at the time of blood collection and after 24, 48, 72, 96 and 120 hours.

	Solution A	Solution B	Solution C	Solution D	Solution E	Solution F
0h	100.00±48.99^{aAB}	112.00±64.19^{aAB}	62.00±16.43^{aAB}	130.00±23.45^{aAB}	138.00±73.96^{aA}	38.00±40.25^{aB}
24h	136.00±31.30^{aA}	128.00±58.05^{aA}	74.00±81.42^{aA}	120.00±43.01^{aA}	0.00±0.00^{bB}	0.00±0.00^{bB}
48h	158.00±57.62^{aA}	132.00±60.99^{aAB}	54.00±58.99^{aBC}	138.00±32.71^{aAB}	0.00±0.00^{bC}	0.00±0.00^{bC}
72h	82.00±45.50^{abAB}	92.00±32.71^{aAB}	38.00±41.47^{aBC}	112.00±31.14^{aA}	0.00±0.00^{bC}	0.00±0.00^{bC}
96h	16.00±35.78^{bB}	126.00±11.40^{aA}	0.00±0.00^{aB}	132.00±34.20^{aA}	0.00±0.00^{bB}	0.00±0.00^{bB}
120h	0.00±0.00^{bB}	140.00±14.14^{aA}	0.00±0.00^{aB}	120.00±42.43^{aA}	0.00±0.00^{bB}	0.00±0.00^{bB}

Solution A: Sodium chloride solution 0.9 %; Solution B: 1.45 g sodium citrate and 1.5 ml formaldehyde; Solution C: 1.9 g sodium citrate, 1.0 ml formalin and 0.01 g toluidine blue; Solution D: 1.9 g sodium citrate and 1.0 mL formaldehyde; Solution E: 1.9 g sodium citrate and 0.01 g toluidine blue; Solution F: 1.0 mL formaldehyde and 0.01 g toluidine blue.
Different lowercase letters in the same column mean statistical differences between counts ($P < 0.05$). Different capital letters in the same line mean statistical differences between solutions ($P < 0.05$). ANOVA, Tukey test.

The total erythrocyte count in *P. unifilis,* using methodologies applied to fish (TAVARES-DIAS et al., 2002; WALENCIK & WITESKA, 2007; RANZANI-PAIVA et al., 2013), makes the determination of this parameter for this species of chelonian imprecise. However, such evaluations are necessary to investigate whether the use of the formaldehyde-citrate reagent with toluidine blue dye could be the cause of the considerable reduction in these cells number during counting. We sought to evaluate the disassociation of some reagents from the complete solution, as a means of identifying which component was causing damage to the cells and making them difficult to determine. The lack of information on this theme makes the present study necessary, as it elucidated which methodology and reactive solutions are suitable for the yellow-spotted amazon river turtle.

CONCLUSION

Blood samples from *P. unifilis* using EDTA (Na_2EDTA 3%, Na_2EDTA 5%, K_3EDTA 3%, K_3EDTA 5%) and sodium heparin (5000 U.I. mL^{-1}) prevented clotting within 10 hours of storage. However, a higher number of erythrocytes was observed with the use of K_3EDTA 5%. Therefore, we recommend that the heparin anticoagulants 5,000 U.I. mL^{-1}, Na_2EDTA 3%, Na_2EDTA 5%, K_3EDTA 3% are used in these hematological analyzes. We also recommend the use of a formaldehyde-citrate solution composed of 1.9 g of sodium citrate and 1.0 mL of formaldehyde in 50 mL of distilled water, when performing total erythrocyte counts in *P. unifilis*.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This experiment is authorized by Ethics Committee for Animal Use (CEUA) of Embrapa Amapá, under procedural nr 010/2017-CEUA/CPAFAP.

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