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# Comparison of the effects of anticoagulants used in blood collection to determine blood parameters of free-living stingrays from the *Potamotrygon genus* (Elasmobranchii: Potamotrygonidae)

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**ABSTRACT:** This study assessed the effectiveness of three anticoagulants in blood parameters of "cururu" stingrays *Potamotrygon* cf. *histrix*. Blood from ten individuals were collected and diluted with anticoagulants EDTA 5% and 10%, heparin 2500 and 5000 UI and sodium citrate 3.2%. A blood sample without anticoagulant was also evaluated. The blood of samples without anticoagulant and with sodium citrate 3.2% coagulated in 20% and 30% of the cases, respectively. Clumps of cells were observed during erythrocyte counting in 30% of samples with EDTA 10%, 40% of samples with heparin 2500 IU and 60% of samples with heparin 5000 IU. No alterations were observed on the erythrogram of "cururu" stingrays with different anticoagulants, the values of plasma glucose were similar in all groups and total protein levels were lower in the samples with EDTA 5% and 10%. The use of sodium citrate 3.2% is not recommended for blood sample conservation of *Potamotrygon* cf. *histrix*. stingrays, but anticoagulants did not affect the parameters analyzed in the determination of plasma glucose and erythrogram. Therefore, these results indicate that in order to blood coagulation the samples should be collected directly with any of these anticoagulants concentrations.

**Keywords:** potamotrygonid, anticoagulants, blood, hematology.

## Comparação dos efeitos de anticoagulantes usados na colheita sanguínea para a determinação dos parâmetros do sangue em arraias de vida livre do gênero Potamotrygon (Elasmobranchii: Potamotrygonidae)

**RESUMO:** Este estudo avaliou a eficácia de três anticoagulantes em parâmetros sanguíneos de arraias cururu *Potamotrygon* sp. O sangue de dez animais foi coletado e diluído nos anticoagulantes: EDTA 5% e 10%, heparina 2.500 e 5.000 UI e citrato de sódio 3,2%, outra parte do sangue também foi analisada sem anticoagulante. O sangue de 20% e 30% das amostras de sangue que não continha anticoagulante e citrato de sódio (3,2%), respectivamente, coagularam. Grumos de células foram observados durante a contagem de eritrócitos em 30% das amostras com EDTA 10%, 40% das amostras com heparina 2.500 UI e 60% das amostras com heparina 5.000 UI. Não foram observadas alterações no eritrograma da arraia cururu com diferentes anticoagulantes, os valores da glicose plasmática foram semelhantes em todos os grupos, enquanto os níveis de proteínas totais foram menores nas amostras coletadas com EDTA 5% e 10%. Em arraias *Potamotrygon* sp., para a conservação de amostras de sangue não é recomendado o uso de citrato de sódio 3.2%, porém, para a determinação da glicose plasmática e do eritrograma os anticoagulantes usados não influenciaram os parâmetros analisados. Portanto, esses resultados indicam que para evitar qualquer sinal de coagulação do sangue deve ser coletadas amostras diretamente com qualquer uma dessas concentrações de anticoagulantes.

**Palavras-chave:** potamotrigonídeos, anticoagulantes, sangue, hematologia

## 1. Introduction

Blood is a liquid tissue distributed to the organs by the circulatory system and transports nutrients, hormones, electrolytes, water, residues from cellular metabolism and several other substances (VERRASTRO et al. 1998). The study of blood parameters have been used as an important tool for monitoring the health status of elasmobranch (WALSH; LUER, 2004; STOSKOPF, 2010) and teleost fish (TAVARES-DIAS; MORAES, 2010).

For the determination of blood parameters is essential to obtain the blood, even if an anticoagulant has been added (HATTINGH, 1975). Some anticoagulants used in teleost fish may have limitations during the processing of blood samples, causing alterations mainly in the erythrogram (HATTINGH, 1975; TAVARES-DIAS; SANDRIM, 1998; WALENCIK; WITESKA, 2007). The main changes caused by anticoagulants are hemolysis (HATTINGH, 1975; WALENCIK; WITESKA, 2007), coagulation (HATTINGH, 1975), increase of erythrocyte number (HATTINGH, 1975; MAFUVADZE; ERLWANGER, 2007), changes in the morphology of blood cells (WALENCIK; WITESKA, 2007) and leukopenia (MAINWARING; ROWLEY, 1985).

Heparin is the anticoagulant most commonly used in clinical hematology of teleosts (80%), followed by EDTA (12%), citrate (4%) and acid citrate dextrose (ACD) which is rarely used (4%) (WALENCIK; WITESKA, 2007; PÁDUA et al., 2010). The use of dipotassium ethylenediaminetetraacetic acid (EDTA) for the preservation of blood components for a prolonged time has been recommended (BRAXHALL; DAISLEY, 1972). On the other hand, the use of natural anticoagulants such as heparin has also been indicated, although do not preserve the blood components for a long time (SVOBODOVA et al., 1991). Therefore, there is not a pattern for the use of anticoagulant in elasmobranchs and there are particularities in the use of these substances in the species.

In shark (*Carcharhinus plumbeus*), blood collection has been accomplished using EDTA (ARNOLD, 2005), such as in the freshwater stingray *Potamotrygon motoro* (PÁDUA et al., 2010). On the other hand, in the marine ray *Dasyatis americana* (CAIN et al., 2004), in the shark *Heterodontus portusjacksoni* (COOPER; MORRIS, 1998) and in the freshwater stingray *Paratrygon aiereba* (DUNCAN et al., 2009) the anticoagulant heparin has been used. Oliveira et al., (2012) recommended the use of EDTA 10% or heparin 5000 UI, despite the need to preserve the blood, there are no studies showing the effects of anticoagulants in the blood parameters of freshwater stingrays. This study aim to determine

the appropriate anticoagulant for blood conservation of *Potamotrygon* cf. *histrix*. stingray, popularly known as "cururu" stingray.

## 2. Material and methods

The stingrays were captured with hand net ("rapiché") in flooded areas of the Daracuá community, Barcelos, Amazonas, Brazil (Figure 1, licence: 15116-1 IBAMA). Handling procedures and withdrawal of blood samples followed the recommendations of Oliveira et al. (2012), posteriorly the animals were maintained in net-tanks and the blood of 10 specimens was collected with disposable syringes previously stored at 4°C, without use anticoagulants. No anesthetic was used because it causes hematological alterations in teleost fish (INOUE et al., 2005). Using microtubes, part of the blood was homogenized and diluted 20 times (10 µl anticoagulant to 200 µl of blood) with the anticoagulants EDTA 5%, EDTA 10%, heparin 2500 IU, heparin 5000 IU and sodium citrate 3.2%. A portion of the blood was maintained without anticoagulant for analysis of blood parameters, and total time not exceeded 30 seconds, including blood collection and anticoagulant dilution.

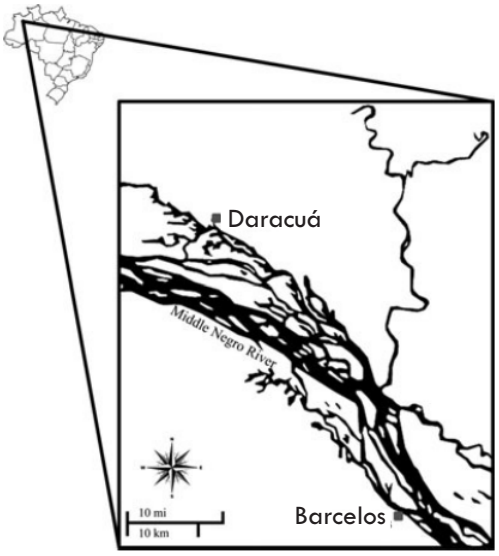


**Figure 1.** Map of Daracuá community (0°30'44,1 "S; 63°12'37,2" W), located in the middle Rio Negro, Barcelos, Amazonas.

The blood collected was divided in two aliquots, one for determining the erythrogram and another to obtain plasma. Erythrocyte (RBC) count was performed in a Neubauer chamber after dilution in formalin-citrate solution, hematocrit (Ht) was measured by the microhematocrit method and hemoglobin (Hb) concentration by the cianometahemoglobin method. Based on these data, the hematimetric parameters were calculated: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).

The second blood aliquot was centrifuged at 75G for 3 minutes to obtain plasma and determine the concentration of glucose and total protein, by enzymatic-colorimetric methods quantified by commercial kits (Doles-GO).

A single-factor analysis of variance and Tukey test were used to evaluate the differences between means of results obtained in the groups evaluated (without anticoagulant, EDTA 5%, EDTA 10%, heparin 2500 IU, heparin 5000 IU and sodium citrate 3.2%). Alfa error less than 5% was accepted to reject the null hypothesis.

## 3. Results

The animals presented the following body measurements (mean ± SD) for total length, width of the disc, and body weight: 21.1 ± 1.6 cm, 10.7 ± 3.8 cm and 89.2 ± 6.6 g, respectively. Only 20% of the blood samples collected without anticoagulant and 30% of the samples with sodium citrate (3.2%) coagulated, which was not observed for blood samples containing EDTA 5% and 10%, heparin 2500 and 5000 IU.

Clumps of cells were observed during microscopic counting of erythrocytes (RBC) in Neubauer chamber in 30% of samples with EDTA 10%, 40% of samples with heparin 2500 IU and 60% of samples with heparin 5000 IU. The use of these kind of anticoagulants can affect the RBC counting and, consequently, other erythrogram parameters of "cururu" stingray. Samples without anticoagulant and containing EDTA 5% and sodium citrate did not present clumps of cells during RBC counting. However, no significant changes were observed in the erythrogram of "cururu" stingray with different anticoagulants (Table 1). Values of plasma glucose were similar in all groups and total protein levels were lower in samples with EDTA 5% and 10% (Table 2).

**Table 1.** Erythrogram values of "cururu" stingray with different anticoagulants.

| Parameters / Group | Ht (%) | Hb (g dL$^{-1}$) | RBC (x 10$^6$ µL$^{-1}$) | MVC (fL) | MCH (g dL$^{-1}$) | MCHC (pg) |
|---|---|---|---|---|---|---|
| Without anticoagulant | 19.0 ± 2.6$^a$ | 3.9 ± 1.2$^a$ | 0.47 ± 0.09$^a$ | 367.1 ± 18.8$^a$ | 72.1 ± 7.3$^a$ | 22.3 ± 6.2$^a$ |
| EDTA 5% | 17.8 ± 2.0$^a$ | 3.5 ± 1.0$^a$ | 0.47 ± 0.02$^a$ | 364.7 ± 29.9$^a$ | 71.9 ± 17.3$^a$ | 23.9 ± 2.6$^a$ |
| EDTA 10% | 17.8 ± 2.6$^a$ | 4.7 ± 1.4$^a$ | 0.43 ± 0.03$^a$ | 403.1 ± 63.5$^a$ | 114.9 ± 33.9$^a$ | 30.3 ± 6.7$^a$ |
| Heparin 2.500 IU | 17.8 ± 2.6$^a$ | 4.8 ± 0.2$^a$ | 0.49 ± 0.09$^a$ | 376.2 ± 103.8$^a$ | 97.3 ± 18.5$^a$ | 28.4 ± 6.0$^a$ |
| Heparin 5.000 IU | 18.7 ± 5.7$^a$ | 3.9 ± 0.4$^a$ | 0.43 ± 0.10$^a$ | 376.7 ± 55.2$^a$ | 89.2 ± 22.7$^a$ | 19.9 ± 4.8$^a$ |
| Sodium citrate 3.2% | 16.2 ± 4.1$^a$ | 3.6 ± 0.7$^a$ | 0.45 ± 0.12$^a$ | 346.2 ± 21.9$^a$ | 67.4 ± 4.4$^a$ | 19.9 ± 1.4$^a$ |

Similar letters represent no significant statistical differences

**Table 2.** Values of glucose and total protein of "cururu" stingray with different anticoagulants.

| Parameters / Groups | Glucose (mmol L$^{-1}$) | Total protein (g L$^{-1}$) |
|---|---|---|
| Without anticoagulant | 0.8 ± 0.1$^a$ | 10.0 ± 2.0$^a$ |
| EDTA 5% | 0.9 ± 0.3$^a$ | 3.0 ± 2.0$^b$ |
| EDTA 10% | 1.0 ± 0.4$^a$ | 4.0 ± 2.0$^b$ |
| Heparin 2.500 UI | 0.8 ± 0.2$^a$ | 6.5 ± 1.0$^a$ |
| Heparin 5.000 UI | 0.9 ± 0.1$^a$ | 6.5 ± 1.0$^a$ |
| Sodium citrate 3.2% | 0.9 ± 0.1$^a$ | 7.0 ± 3.0$^a$ |

Similar letters represent no significant statistical differences

## 4. Discussion

The process of coagulation in blood samples of the "cururu" stingray without anticoagulants presented similar results to the found with sodium citrate, demonstrating that this anticoagulant should not be used to collect blood of freshwater stingrays. In the teleosts *Labeo umbratus* and *Labeo capensis*, EDTA (from 0.03 to 5.0%) was inappropriate for conservation of blood samples because coagulation occurred in 100% of samples (HATTINGH, 1975). The study of Clarke et al. (1979) reported for *Micropterus salmonides* that heparin did not prevent blood coagulation as efficiently as EDTA. In *Potamotrygon* cf. *histrix*, no blood clotting occurred when used EDTA at 5% or 10%, heparin 2500 IU or 5000 IU. The occurrence of clumps of cells were detected in 30, 40 and 60% of the blood samples containing EDTA 10%, heparin 2500 and 5000 IU, respectively, since blood was diluted with these anticoagulants after collecting. The presence of clumps of cells were also reported for the teleost *Blennius pholis* when heparin 50 IU was used (MAINWARING; ROMLEY, 1985), due to the reduced activity of heparin diluted at this concentration (OKUNO; NELSON, 1975).

In *Potamotrygon* cf. *histrix* stingrays no significant change in the erythrogram occurred when used EDTA 5% or 10%, heparin 2500 or 5000 IU and sodium citrate. Similar results were reported for erythrogram parameters of *Cyprinus carpio* (WALENCIK; WITESKA, 2007) and hematocrit (Ht) of the hybrid "surubim" *Pseudoplatystoma reticulatum* x *Pseudoplatystoma corruscans* (ISHIKAWA et al., 2010). Nevertheless, studies demonstrated the occurrence of changes in Ht values caused by the anticoagulant in *Colossoma macropomum* (TAVARES-DIAS; SANDRIM, 1998), *Oreochronis aureus* (ALLEN, 1993) and *Clarias gariepinus* (ADEYEMO et al., 2009).

The effects of blood collection on biochemical parameters using different anticoagulants is poorly described in the literature for elasmobranchs and teleosts. Glucose plasma levels did not present alterations caused by the anticoagulant used in "cururu" stingray. On the other hand, total protein plasma levels were lower when used EDTA 5% and 10%; in addition, a not significant upward trend of total protein levels was observed, proportional to the increase of the concentration of EDTA, which was also observed for the hybrid catfish *P. reticulatum* x *P. corruscans* (ISHIKAWA et al., 2010). These reductions may be associated to the coagulation process on which several plasma components, referred as coagulation factors, respond in a complex cascade, starting with the conversion of thrombin into fibrinogen in the blood plasma, and then the clot formation (TAVARES-DIAS; OLIVEIRA, 2009). The beginning of the coagulation process could be observed by the presence of clumps during erythrocytes counting in the blood of "cururu" stingray containing EDTA 5% and 10%; the protein plasma levels in this case were lower compared to blood without anticoagulant due to the initial clot formation that retains proteins, consequently reducing the total levels of plasma proteins.

## 5. Conclusion

In *Potamotrygon* cf. *histrix* stingrays, the use of sodium citrate 3.2% is not recommended for conservation of blood samples, because this anticoagulant was little effective. However, anticoagulants did not affect the parameters evaluated in the determination of plasma glucose and erythrogram, although evidence of clotting was observed at erythrocytes counting when used EDTA 10%, heparin 2500 and 5000 IU. Nevertheless, no changes in RBC, MCV and MCHC were observed. For the determination of total protein in blood plasma, heparin 2500 and 5000 IU is the ideal anticoagulant. Therefore, the results indicate that samples should be collected directly with any of these anticoagulant concentrations in order to avoid blood coagulation.

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