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Hemoglobin protein profile as a parameter for taxonomic analysis in Brazilian Testudinidae

T.L. Silva[1], V.L.O. Cardoso[2], N.R.A. Costa[3], L.P.R. Venancio[4], L.R. Pereira[5] and C.R. Bonini-Domingos[5]

[1] Universidade Federal do Acre, Campus Floresta em Cruzeiro do Sul, Cruzeiro do Sul, AC, Brasil
[2] Instituto Nacional de Pesquisas da Amazônia, Departamento de Genética, Conservação e Biologia Evolutiva, Manaus, AM, Brasil
[3] Universidade de São Paulo, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, SP, Brasil
[4] Universidade Federal do Oeste da Bahia, Campus Reitor Edgard Santos - Centro de Ciências Biológicas e da Saúde, Barreiras, BA, Brasil
[5] Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências, Letras e Ciências Exatas, São José do Rio Preto, SP, Brasil

Corresponding author: C.R. Bonini-Domingos
E-mail: bonini.domingos@unesp.br

Genet. Mol. Res. 21 (3): gmr18977
Received January 31, 2022
Accepted March 18, 2022
Published July 31, 2022
DOI http://dx.doi.org/10.4238/gmr18977

ABSTRACT. The Brazilian Testudinidae family is widespread across South America. It includes *Chelonoidis denticulatus*, the largest tortoise in South America and *Chelonoidis carbonarius*, found mostly in the north and northwestern part of the continent. Using hemoglobin to identify species is cheaper than other methods such as DNA sequencing and can offer useful information, since the hemoglobin molecule is a well-preserved protein chain during the evolution of species. Thus, in order to establish a hemoglobin profile for the Brazilian Testudinidae *C. denticulatus*, *C. carbonarius* and morphotype 1, hemoglobin electrophoresis was performed at acid pH in phosphate agar and at alkaline pH in cellulose acetate, in order to visualize the specific fractions of each species. High performance liquid chromatography was used for the quantification of fractions.

For an in-depth analysis and better detailing of the hemoglobin profile of the species, polypeptide chain electrophoresis was performed at acid and alkaline pH. We observed differences in the hemoglobin profiles of *C. denticulatus* in relation to *C. carbonarius* and morphotype 1, which suggests that this methodology, not common in taxonomic studies, can help determine relationships between species, since hemoglobins are proteins with well-preserved genes. We found differences in hemoglobin mobility between *C. denticulatus*, *C. carbonarius* and morphotype 1 in electrophoresis at alkaline pH, however, the behavior of globin chains was similar between the three groups. High performance liquid chromatography showed different retention times in the globin fractions of *C. denticulatus* and *C. carbonarius*, but not between *C. carbonarius* and morphotype 1, indicating that, possibly, the divergence time between *C. carbonarius* and morphotype 1 is more recent than the divergence between *C. denticulatus* and *C. carbonarius*, due to the highly conserved character of this functional protein. Thus, considering the high degree of conservation of hemoglobins in vertebrates, and the differences observed in electrophoresis at alkaline pH and HPLC, we infer that *C. carbonarius* and morphotype 1 present a common branch.

Key words: Testudinidae; Hemoglobin; Electrophoresis; *Chelonoidis*

INTRODUCTION

The Testudinidae family, whose representatives are popularly known as tortoises, is an important group for genetic and taxonomic studies, mainly due to its phylogenetic history, which carries unique characteristics that were conserved during evolution. (Shafter, 2009). The species are terrestrial, including more than 200 fossils and nearly 40 living species. (Auffenberg, 1974; Pough et al., 2001). In Brazil there are currently two species, *Chelonoidis denticulatus* (Testudinidae), popularly known as "jabuti-tinga", and *Chelonoidis carbonarius* (Testudinidae), or "jabuti-piranga". These species seem alike regarding some aspects, as body size, diet and behavior. However, some authors have reported some distinct patterns for *C. carbonarius* as well as populations with divergent mitochondrial DNA haplotypes (Vargas-Ramírez et al., 2010; Silva et al., 2011).

C. denticulatus is considered the largest chelonian in South America; it has yellow scales in the cephalic region and locomotive limbs. *C. carbonarius* is commonly found in Savanna regions in Venezuela, Colombia, Suriname, Guianas, French Guiana, Bolivia, Paraguay, Argentina, Caribe, Venezuela, and Brazil. Their most common color pattern in the head and locomotive limbs is red. There are also some differences related to the shape of the scales in the cephalic region. (Pritchard, 1979; Pritchard and Trebbau, 1984)

Based in morphological and cytogenetic analysis from animals found captive on the countryside of São Paulo state, Silva (2011) observed a tortoise morphotype, which diverged from usual patterns of *C. denticulatus* and *C. carbonarius*. While *C. denticulatus* has yellow scales in the cephalic region and locomotive limbs This morphotype, firstly

denominated morphotype 1, differentiates by the nasal scales pattern, color of the cephalic region and dermal scales from locomotive limbs, whose color is orange and have intermediate size between *C. denticulatus* and *C. carbonarius*. Those reports question the current idea that the morphotypes are considered as *C. carbonarius* and they correspond to a single taxonomic unity.

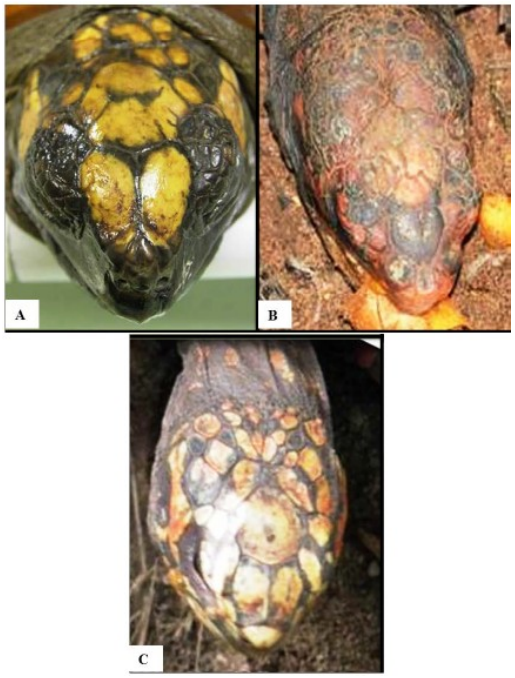

Figure 1. Patterns and colors of the cephalic region scales. A – *Chelonoidis denticulatus,* B. *Chelonoidis carbonarius*. C – Morphotype 1.

In order to elucidate taxonomic aspects, researchers constantly perform studies with morphological and molecular data. An example of a taxonomical study that is not mainly performed consists in the establishment of a hemoglobin profile (Hb) as a taxonomical parameter, due to the conservation degree of the globin in vertebrates. The used methodology to establish this profile represents an economic option to taxonomic studies and DNA analysis, as any alterations in the run pattern and profile of the hemoglobin fractions can represent significant changes in the molecule, which can be later compared to other species individuals and help us trace its phylogenetic relationship. (Dessauer et al., 1957; Bonini-Domingos et al., 2007; Bonini-Domingos; Oliveira, 2017). Hemoglobins are homologous proteins with marked heterogeneity, which makes evolutionarily close species show similarities in the profile, what can be used as an indicator of phylogenetic relationship. (Dessauer et al., 1957).

This work aimed to evaluate the profile of hemoglobin fractions in *C. denticulatus, C. carbonarius* and morphotype 1 by determining the pattern of electrophoretic and globin migration in alkaline and acid pH. We also quantified the fractions using high performance liquid chromatography (HPLC) in order to explore the discriminative potential of these techniques as a method of taxonomic study, which is not common in literature.

MATERIAL AND METHODS

Sample collection

The animals in this study were collected from the City Zoo of São José do Rio Preto and City Zoo "Dr. Flávio Leite Ribeiro" of Araçatuba, both in Sao Paulo state. The species were distinguished based on morphological characteristics found in the literature (Pritchard; Trebbau, 1984; Silva, 2011). Whole blood samples from 30 animals (1mL) were collected from the occipital vein according to Silva et al. (2012), this being 5 male and 5 female of each group (*C. carbonarius, C. denticulatus* and the morphotype) which were identified by using morphological patterns according to the literature. The collected samples were stored in tubes containing heparin as anticoagulant and put into cold temperature (approximately 5ºC) until use in order to avoid any degradation. Due to its fast anticoagulant action, and its high speed of dimerization, which enables degradation, all analysis were performed less than 24 hours after sampling. The sampling and the study are in accordance with the ethical standards in animal experimentation approved by the CEEA-Ibilce/UNESP (number 037/11) and also was approved by the Brazilian Institute of Environment and Renewable Natural Resources (Ibama/SISBIO – Number 19514-1).

Hemoglobin profile

The whole blood samples were washed and centrifuged with saline solution, for plasma removal and obtaining red blood cells. This concentrate was then lysed with distilled water and the total product purified with chloroform to obtain hemoglobin solution (Hb). With the hemoglobin solution, electrophoresis were performed at acid pH (6,2) in phosphate agar gel (Vella, 1968) and at alkaline pH (8,6) in cellulose acetate (Marengo; Rowe, 1965). For the polypeptide chain analysis, we used the globin electrophoresis, after preparation with urea and 2-mercaptoethanol, at acid pH in polyacrylamide gel (Alter et al., 1980, modified by Bonini-Domingos, 2006) and at alkaline pH in cellulose acetate (Schneider, 1974), modified by Bonini-Domingos, (2006) and Bonini-Domingos and Oliveira (2017).

In the execution of electrophoresis, the use of a known pattern is common, allowing the comparison of the samples under study, however, the migration pattern of tortoise hemoglobin is unknown. Thus, a human blood sample with Hb AS pattern was used. The choice of the Hb AS pattern was based in the already known standardization and isoeletric point from this hemoglobin common in humans. An aliquot (5µL) of the total blood samples was submitted to high performance liquid chromatography for quantification of globin fractions with the equipment VARIANT from BIO-RAD with an specific kit for hemoglobin analysis. The use of this kit was due to the lack of specific reagents for tortoise hemoglobins and ease in obtaining a qualitative and quantitative profile of hemoglobins.

RESULTS

The Hb profile obtained by electrophoresis at alkaline pH shows the presence of majority and minority fractions. In the electrophoresis at alkaline pH in cellulose acetate (Figure 2) for Samples of *C. denticulatus*, we found four globin fractions, two of which were the majority (F_1 and F_2). The F_1 fraction was found in the lower part of the cellulose

acetate tape, below the fraction corresponding to the Hb S of the pattern, while the F_2 fraction was visualized at the top of the cellulose acetate tape, above the fraction corresponding to Hb A.

In *C. carbonarius*, we observed the formation of five different fractions, two of them predominant, named F_1 and F_2 (Figure 2). The F_1 fraction appears below the Hb S position, while the F_2 fraction is above the position of Hb A, a pattern similar to that visualized for morphotype 1 corresponding to Hb A.

For *C. denticulatus*, the electrophoresis at acid pH in agar-phosphate gel showed two major fractions, both above the Hb S position, one of which (F_1) was located in the Hb A position and the other above this fraction (F_2). In *C. carbonarius*, we also observed two major fractions, and the Fraction F_1 is located similar to the position of Hb S and fraction F_2, slightly above the position of Hb A. For morphotype 1, we noticed the presence of two majority fractions, similar to the profile of *C. carbonarius* (Figure 3).

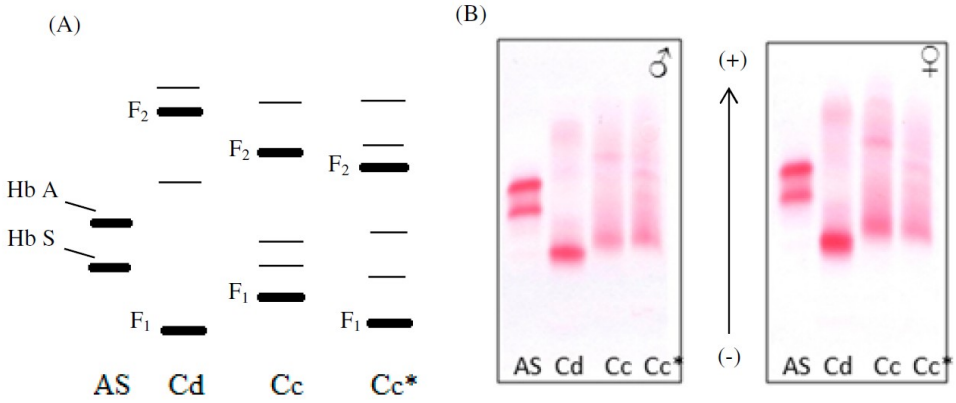

Figure 2. Hemoglobin electrophoresis at alkaline pH in cellulose acetate. (A) Model with the representation of electrophoretic running patterns for human standard Hb AS, *Chelonoidis denticulatus*, *Chelonoidis carbonarius* and morphotype 1. (B) Cellulose acetate tapes showing electrophoretic running patterns for human standard Hb AS and male and female specimens of *C. denticulatus*, *C. carbonarius* and morphotype 1. AS - Heterozygous pattern for sickle cell anemia; Cd - *C. denticulatus*; Cc - *C. carbonarius*; Cc* - Morphotype 1.

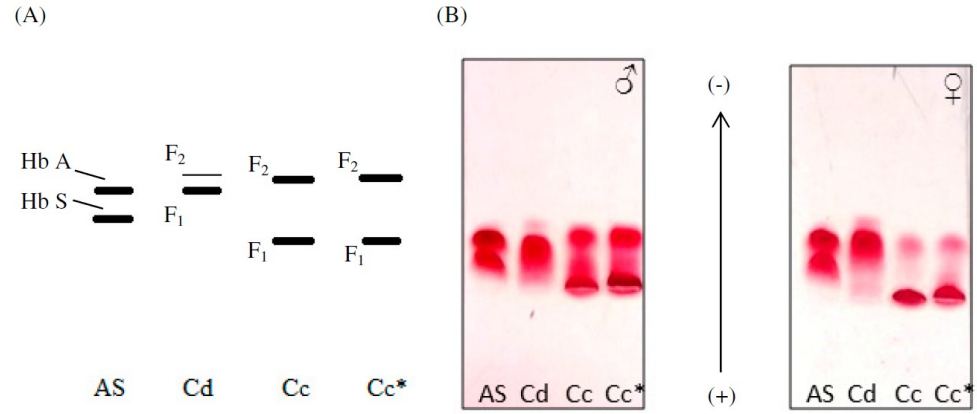

Figure 3. Hemoglobin electrophoresis at acid pH in Agar-phosphate gel. (A) Model with the representation of electrophoretic running patterns for human standard Hb AS, *Chelonoidis denticulatus*, *C. carbonarius* and Morphotype 1(B) Electrophoretic running patterns for human standard Hb AS and male and female specimens of *C. denticulatus*, *C. carbonarius* and morphotype 1. Cd – *C. denticulatus*; Cc – *C. carbonarius*; Cc* - Morphotype 1. Source: Prepared by the author.

The chromatographic profile of *C. denticulatus* presented four peaks, one majority peak and three smaller peaks, while for *C. carbonarius* and morphotype 1, we observed similar chromatographic profiles, composed of five peaks, one majority and four minority. It can be verified that the majority fraction of *C. denticulatus*, *C. carbonarius* and morphotype 1 have retention time interval of 4.5 to 5.5 minutes and percentage value of 68.4%, 60.4% and 54.2%, respectively.

The chromatographic profile shown different graphics as show in Figure 4.

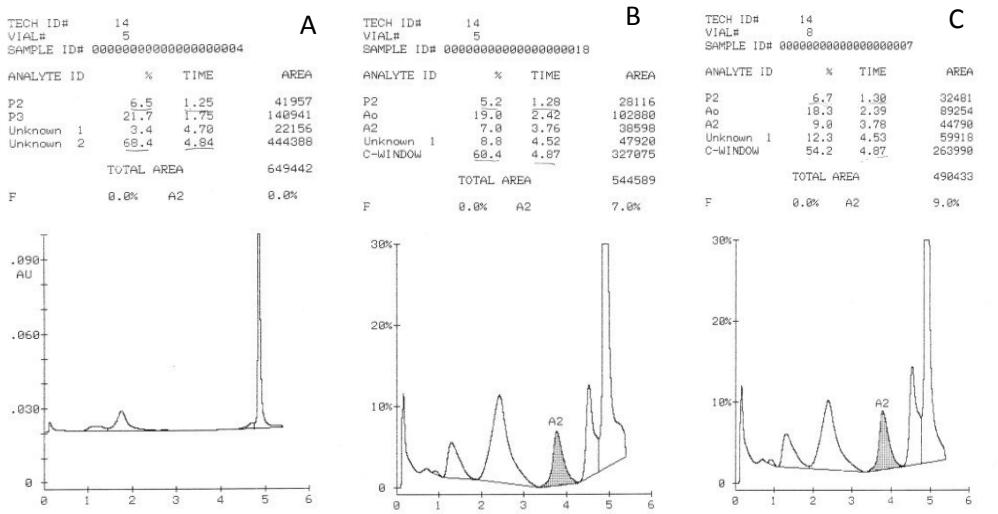

Figure 4. Chromatogram with Hb profile, obtained from whole blood samples. A) *Chelonoidis denticulatus* B) *Chelonoidis carbonarius* and C) Morphotype 1, showing the peaks patterns of each group. Equipment used - HPLC - VARIANT (BIORAD), with ion exchange columns and phosphate buffer system.

In order to verify which globin components are involved in the formation of functional molecules, we performed electrophoresis of polypeptide chains in acid pH, and the electrophoretic profile of the three groups presented several globin patterns, three major fractions (identified as F_1, F_2 and F_3) and three smaller subfractions (Figure 5). The two subfractions, located in the upper part of the polyacrylamide gel, were more evident in *C. carbonarius* and morphotype 1 when compared to the profile of *C. denticulatus*. Regarding the profile for the pattern (Hb AS), the fractions and subfractions of the three groups migrated above the position of the αA human chain, and the fraction was identified by F_3, with migration similar to that of the betaA human chain. We did not observe a distinction in the hemoglobin profile between males and females of *C. carbonarius* and morphotype 1, only for *C. denticulatus*, however, as there is no reference in the literature that discusses the factors associated with this difference found, these results should be better investigated.

In the electrophoretic profile at alkaline pH, for both species and for morphotype, the globinic chains migrated forming two bands (Figure 6), with the upper band positioned above the position of the betaA human chain of the control, and the lower band positioned between alfaA and betaA of the control. The bands of *C. denticulatus* were more defined in comparison with the other groups; the lower band of *C. carbonarius* showed to be the majority fraction, while for the morphotype, the upper band presented as the majority band.

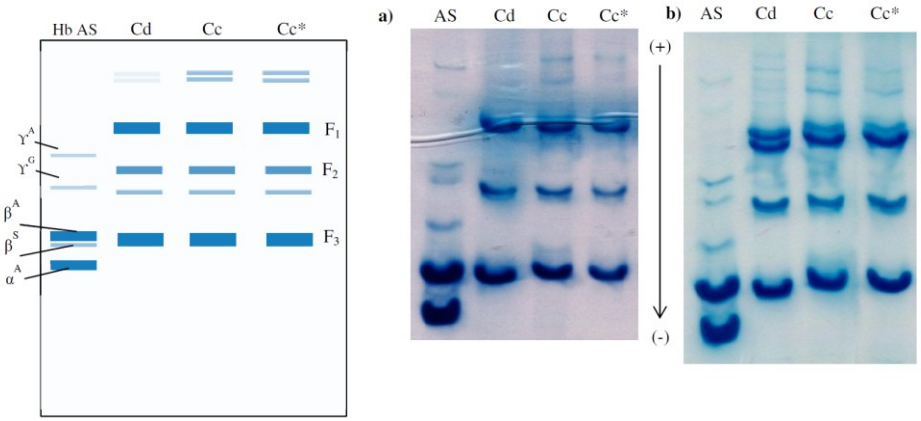

Figure 5. Schematic representation and photos of electrophoresis gels of polypeptide chains in acid pH. a) Females, b) Males. Human Hb AS Standard. Cd - *Chelonoidis denticulatus*; Cc - *Chelonoidis carbonarius*; Cc* - Morphotype 1.

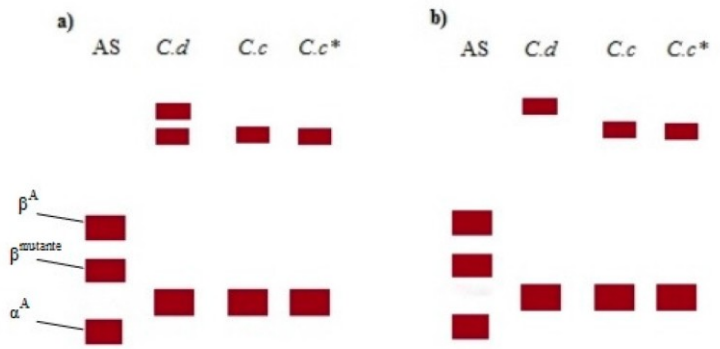

Figure 6. Scheme and photos of electrophoresis tapes of polypeptide chains at alkaline pH. a) Males, b) Females. Human Hb AS Standard. Cd - *Chelonoidis denticulatus*; Cc - *Chelonoidis carbonarius*; Cc* - Morphotype 1.

Male specimens of *C. denticulatus* presented an additional band when compared to females of the same species, located below the upper band. This difference between males and females for the migration pattern of a specific globin should be further investigated to reinforce the hypothesis of a possible sexual dimorphism for globin chains in this species.

DISCUSSION

According to Melo et al. (2003), *C. denticulatus* and *C. carbonarius* present two hemoglobin components, Hb A and Hb D, and in chromatographic studies conducted with *Geochelone gigantea*, two peaks were detected, with the majority component represented by Hb A, and minority by Hb D (Shishikura and Takami, 2001). We believe that the majority and minority components correspond to the profile observed by the researchers, however, with greater resolution, due to the refinement of the methodologies applied in the present study.

Although the literature shows that *C. denticulatus* and *C. carbonarius* have two hemoglobin components, it is evident the difference in the chromatographic profile of the two species (Figure 3A and B), and this difference may be due to some characteristics of the hemoglobin molecule, such as electrical load, size, and affinity of protein binding, resulting in a different chromatographic behavior for the species (Nelson; Cox, 2011).

The similarity of the chromatographic profiles of *C. carbonarius* and morphotype 1 suggests a high degree of conservation of this functional protein, and this may be due to the high degree of conservation of hemoglobin in vertebrates, evidenced by its use as an object of evolutionary studies in vertebrates (Petruzzelli et al., 1996).

The differentiation obtained through the chromatographic profile between *C. carbonarius* and morphotype 1 in relation to *C. denticulatus* validates the technique as an additional method for elucidating taxonomic issues in Testudinidae. The use of additional tools to the taxonomy of the group allows better characterization of species and populations, enabling the identification of possible units of evolutionary significance. In addition to the advantages mentioned above, we highlight the resolution for differentiation of hemoglobin profile in tortoise species, in addition to the low cost and speed, when compared with molecular biology techniques.

Reptiles have two or more major components of Hb and other smaller components with different mobility. In *C. carbonarius* and *C. denticulatus*, Hb A, composed of two αA chains and two β chains, and Hb D, consisting of two αD and two β chains, which exhibit intrinsic affinity to oxygen and low sensitivity for alllosteric effects, are most often modulated by the decrease in pH. AD chains, also found in birds, snakes and lizards, are considered embryonic Hb that persist in adulthood, giving these animals a high oxygen uptake capacity (Rücknagel et al., 1984; Rücknagel and Braunitzer, 1988; Fushitani et al., 1996; Melo et al., 2003).

The establishment of the hemoglobin profile of the species *C. denticulatus*, *C. carbonarius*, and morphotype 1, allowed the observation of differences in hemoglobin motility between the species *C. denticulatus* and *C. carbonarius*, two species known to be distinct, which demonstrates that the technique used in this study can be used for phylogeny studies, together with other methodologies. The results demonstrated here are unpublished in the literature, and therefore contribute to greater knowledge about the species addressed.

CONCLUSIONS

The chromatographic and electrophoretic profile of *C. carbonarius* and morphotype 1 were similar, indicating that, in relation to the hemoglobin profile, morphotype 1 presents a common ancestor to *C. carbonarius*. The chromatographic profile of *C. denticulatus* showed to be different from the other groups, indicating that this methodology has potential as a tool in taxonomic studies. The migration of *C. denticulatus* globins differs between males and females, indicating a possible sexual dimorphism. High performance liquid chromatography showed different retention times in the globin fractions of *C. denticulatus* and *C. carbonarius*, but not between *C. carbonarius* and morphotype 1.

ACKNOWLEDGMENTS

The authors are indebted to the Universidade Estadual Paulista “Julio de Mesquita Filho” (UNESP, Brazil), the Foundation for the Support of the Scientific and Technological

Research of São Paulo (FAPESP), the Brazilian Council for Scientific and Technological Development (CNPq), and the Foundation for the Support of the Scientific and Technological Research of Acre (FAPAC) for funding and scholarships.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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