An Acad Bras Cienc (2024) 96(2): e20230949 DOI 10.1590/0001-3765202420230949
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

ANIMAL SCIENCE

Corallus hortulanus testes histology: morphological and reproductive aspects

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Abstract: In general snakes show differentiate anatomical, biological and behavioral particularities compared to other species. Basic information about the snakes anatomy, physiology and reproductive biology is scarce in several species, making the reproduction a challenge. Thus, the present work aims to evaluate morphological aspects of the *Corallus hortulanus* testes, correlating these findings with environmental factors and reproductive aspects. The testes of three specimens of *Corallus hortulanus* were cut to a thickness of 3µm in microtome, stained with 1% toluidine blue, photo documented and described. Seasonality was observed in the sperm production of *Corallus hortulanus*, with the presence of mature spermatozoa in the wettest and hottest periods of the year, as well as the largest testicular volume in these periods.

Key words: snake, reproduction, spermatogenesis, testicular morphometry.

INTRODUCTION

Family Boidae snakes are found in the Americas, South Pacific Islands, India, Central Africa and South Asia. They are divided into two subfamilies: Boinae (genera *Boa*, *Candoia*, *Corallus*, *Epicrates*, *Eunectes* and *Sanzinia*) and Erycinae (genera *Charina* and *Eryx*) (Zug et al. 2001, Garcia et al. 2015).

There are various species belonging to the Boidae Family, among them the *Corallus hortulanus* have a wide distribution in South America, with nocturnal and arboreal habits, and feeds on birds and small rodents (Grego et al. 2014, Costa & Bérnils 2018). In general, snakes have anatomical, biological and behavioral particularities that differentiate them from other animal species, including species belonging to their own Squamata Order.

They have an elongated body, defined musculature, locomotor limbs absence and mandibular symphysis, braincase lateral closure and movable eyelids absence. Regarding the male reproductive system, snakes have elongated testicles located cranial to the kidneys and a paired of copulatory organs called hemipenis. The hemipenis are located in the tail and inverted when at rest, being a characteristic structure of the Squamata Order. The spermatozoa is filiform, with elongated head presenting conical acrosome and elongated intermediate piece (synapomorphy) (Olsson & Madsen 1998, Zacariotti & Guimarães 2010, Zug et al. 2001, Bento et al. 2022).

Anatomy, physiology and reproductive biology about snake's basic information is scarce in several species, making the natural or assisted reproduction a challenge. To understand morphological variations that occur in the testicles size and their relationship with seasonality in order to identify the different reproductive periods, histological and

ultrasound evaluation of the reproductive tract has been used (Hernández-Gallegos et al. 2002, Bertona & Chiaraviglio 2003, Ibargüengoytía et al. 2006, Garcia et al. 2015).

Thus, the present study aims to evaluate *Corallus hortulanus* testes morphological aspects, correlating these findings with environmental factors and reproductive aspects.

MATERIALS AND METHODS

This study was conducted with the Ethics Committee on the use of Animals authorization (CEUA/UFMT) n° 23108.050100/2019-01.

The testicles of three specimens of *Corallus hortulanus* kept in the Laboratory of Zoological Collections – Herpetology Sector - Institute of Biosciences of the Universidade Federal de Mato Grosso (IB/UFMT) where used. The animals came from different regions of the Mato Grosso state, Brazil, and named 7490, (captured at Tangará da Serra, on March 23, 2009), 9577 (captured at Aripuanã, on June 17, 2008), and 11123 (captured at Poconé on February 2011).

The testicles collected were measured with a caliper, and the testicular volume was calculated based on the ellipsoid volume according to Méndez & Villagrán (1998). The rostrocloacal length was measured with a tape measure.

Climate data were obtained from the Meteorological Database for Teaching and Research (BDMEP) of the Instituto Nacional de Meteorologia (INMET). Datas were from available weather stations based on the proximity or climatic similarity between the collection site according to Souza et al. (2013), in a period of approximately six months before and six months after the capture date.

The tissues were fragmented and packed in Eppendorf for histological processing. Initially, the dehydration process was performed, consisting in two stages: (1) the samples were kept in 70° ethyl alcohol, and refrigerated for a minimum period of 24 hours; (2) the ethyl alcohol 70° was removed and ethyl alcohol 97° was added for 4 hours, the samples were kept refrigerated.

After the dehydration procedure, the samples were incorporated into methacrylate glycol plastic resin according to the manufacturer's recommendations.

The incorporated samples were cut in a microtome at a thickness of 3µm, fixed on a histological slide and stained with 1% Toluidine Blue, aqueous solution, slightly acidic pH (pH ≅ 6.0). The dye was dripped onto the histological sections using a Pasteur pipette, waiting three minutes, after that the excess dye was removed and the slides were dried on a heating plate. Subsequently the slides were prepared, and photo documented.

RESULTS

Specimen 7490 was captured in March, a period characterized by higher temperatures compared to other periods of the year and considerable rainfall levels (Figure 1). Presented 93cm in a rostro-cloacal length, 2.35cm³ in a right testicle volume and a 3.24cm³ in a left testicle volume. Histological analysis show testes presenting evident light in the seminiferous tubules with spermatozoa inside, germinal epithelium cells in different phases of gametogenesis, and less evident interstitial tissue with interstitial Leydig cells (Figure 2a). In the morphometric evaluation the seminiferous tubules diameter was 201.75µm ± 46.37µm, and a germinal epithelium height was 89.73µm ± 18.72µm (Figure 3).

Specimen 11123 was captured in February; this period was characterized by high rainfall and temperature rates (Figure 1). Presented 111cm in a rostro-cloacal length, 1,86cm³ in a right testicle volume and a 3.31cm³ in a left

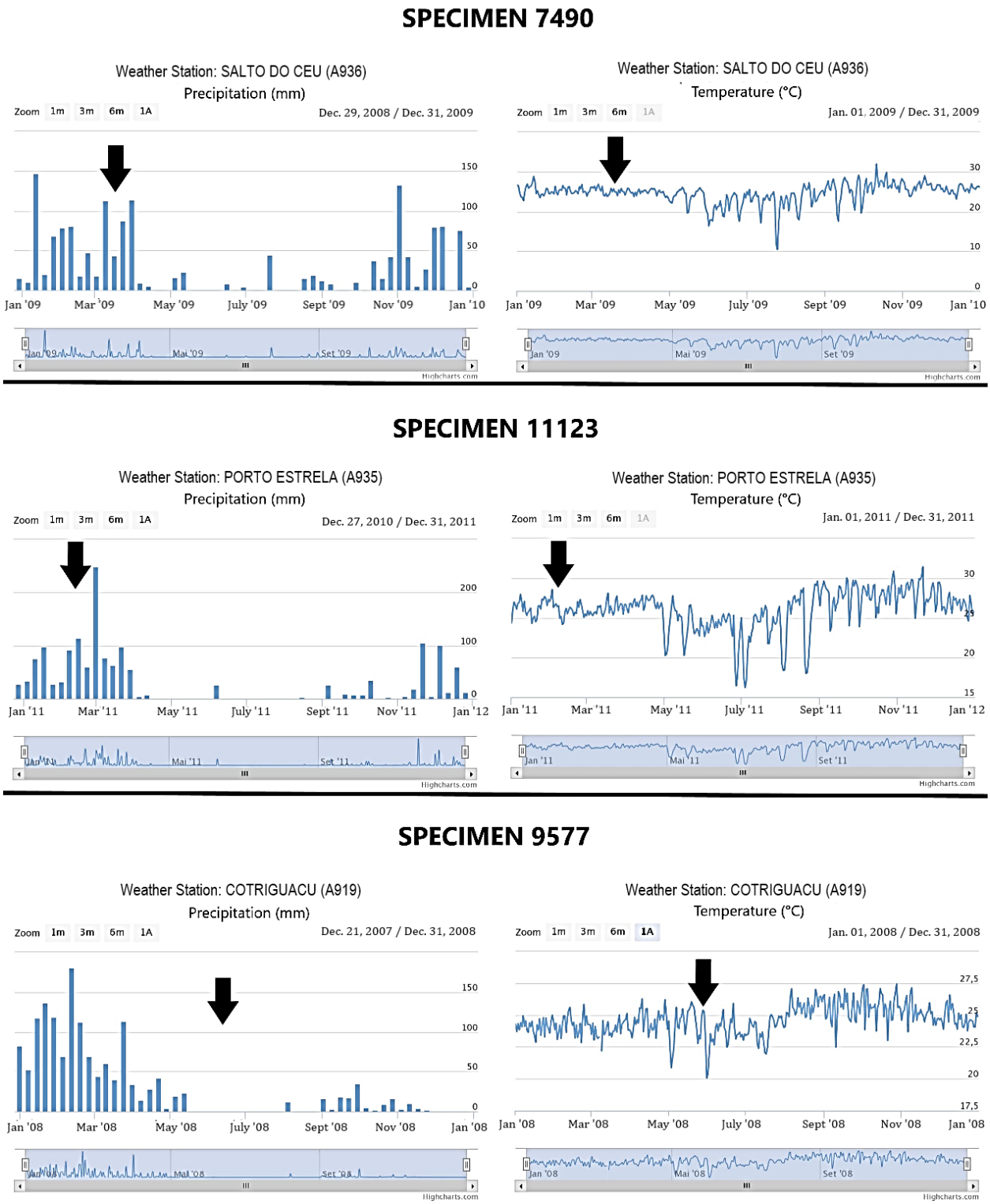

Figure 1. Rainfall (mm) and temperature (°C) average of capture period in Weather Station Salto do Céu/A936 (specimens 7490), Weather Station Porto Estrela/A935 (specimens 11123) and Weather Station Cotriguaçu/A919 (specimen 9577). The arrow indicates the capture moment. (Source: BDMEP-INMET).

testicle volume. Histological analysis shows similar testes that described in specimen 7490, however, in this individual there was abundant cellular remains and a less sperm cells when compared to specimen 7490 (Figure 2b). In the morphometric evaluation the seminiferous tubules diameter was 202,22µm ± 35,45µm and a germinal epithelium height was 83,48µm ± 15,16µm (Figure 3).

Specimen 9577 was captured in June, characterized by lower rainfall and temperature (Figure 1). Presented 121 cm in a rostro-cloacal length, 0.53 cm^3 in a right testicle volume and a 0.44 cm^3 in a left testicle volume. In the

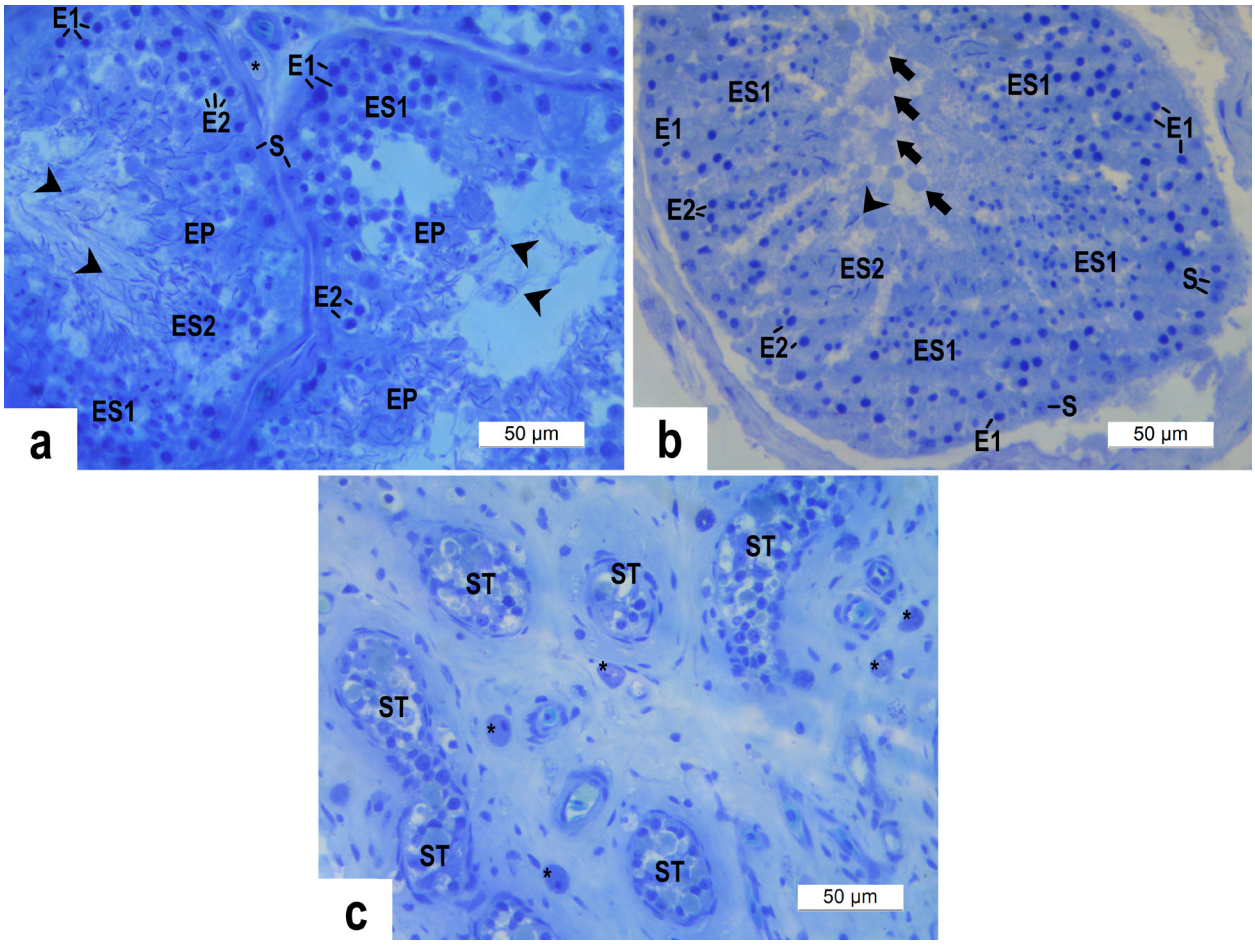

Figure 2. ***Corallus hortulanus*** **testicle histology (a) - Specimen 7490 (stage 6), captured in high temperatures and rainfall period. Seminiferous tubules with sperm (arrowhead), and absence of cellular remains inside. Cells in different phases of gametogenesis in the germ epithelium. Toluidine blue 1%, obj. 40x. (b) - Specimen 11123 (stage 6), captured in high rainfall and temperatures. Seminiferous tubules with evident lumen, with sperm (arrowhead) and cellular debris (arrow) inside. The germ epithelium presents cells in different phases of gametogenesis, and evident interstitial tissue with interstitial Leydig cells. Toluidine blue 1%, obj. 40x. (c) - Specimen 9577 (stage 8), captured in a lower rainfall and temperature period. Testicles with few seminiferous tubules, lumen absence, no sperm and cellular remains inside. Germ cells in apoptosis process after the reproductive period is observed in the seminiferous tubules, which are characterized by homogeneous aspect, with condensed chromatin and cytoplasm in fragmentation and collapse, no evident Sertoli cells. Interstitial tissue is evident, with interstitial Leydig cells grouped. Toluidine blue 1%, obj. 40x. Legend: (S) Sertoli cells, (E1) spermatogonia type A, (E2) spermatogonia type B, (ES1) primary spermatocyte, (ES2) secondary spermatocyte, (EP) spermatid, (ST) seminiferous tubule, (*) Leydig interstitial cells.**

histological analysis the testicles presented less seminiferous tubules with little evident lumen, and spermatozoa and cellular remains absent (Figure 2c). Sertoli cells not evident and cells in apoptosis process was observed in the seminiferous tubules after the reproductive period. The apoptosis cells were characterized by a homogeneous aspect, with condensed chromatin and cytoplasm in fragmentation and collapse. The interstitial tissue was apparent, with Leydig interstitial cells grouped. In the morphometric evaluation the seminiferous tubules diameter was 41.08µm ± 7.12µm. It was not possible to measure the germinal epithelium height due to the apoptosis process. (Figure 3).

DISCUSSION

The *Corallus hortulanus* presents reproductive seasonality according the histological evaluation. Correlating the histological findings with environmental data, we observed that in this species the gametes production (spermatozoa) occurs in the hottest and rainiest periods of the year, while in the milder temperatures and lower rainfall a quiescence condition is observed similarly reported in other snakes of the Boidae Family, such as *Boa c. occidentalis* (Bertona & Chiaraviglio 2003, Ibargüengoytía et al. 2006), *Boa c. constrictor* (Bento et al. 2019), and *Epicrates cenchria* (Bento et al. 2022).

According to Ballinger & Nietfeldt (1989), the spermatogenic cycle can be expressed in eight distinct stages: stage 0 (juvenile) non-apparent germ cells are observed; stage 1 (crescent) germ cell division is observed without luminal development; stage 2 (early spermatogenesis) primary spermatocytes are observed in the luminal margin; stage 3 (spermatogenesis) secondary spermatocytes are observed in the luminal margin; stage 4 (spermiogenesis) undifferentiated spermatids are observed in the luminal margin; stage 5 (mature) spermatozoa are observed in metamorphosis in the luminal margin; stage 6 (reproductive) mature sperm are observed in the lumen of the seminiferous tubules; stage 7 (post-reproductive) there is early regression and presence of cellular remains in the lumen; and stage 8 (inactive) complete regression, without cell division and without lumen of the seminiferous tubules.

In specimens 11123 and 7490 were observed mature spermatozoa in the lumen of the tubule, indicating the reproductive period – stage 6. In

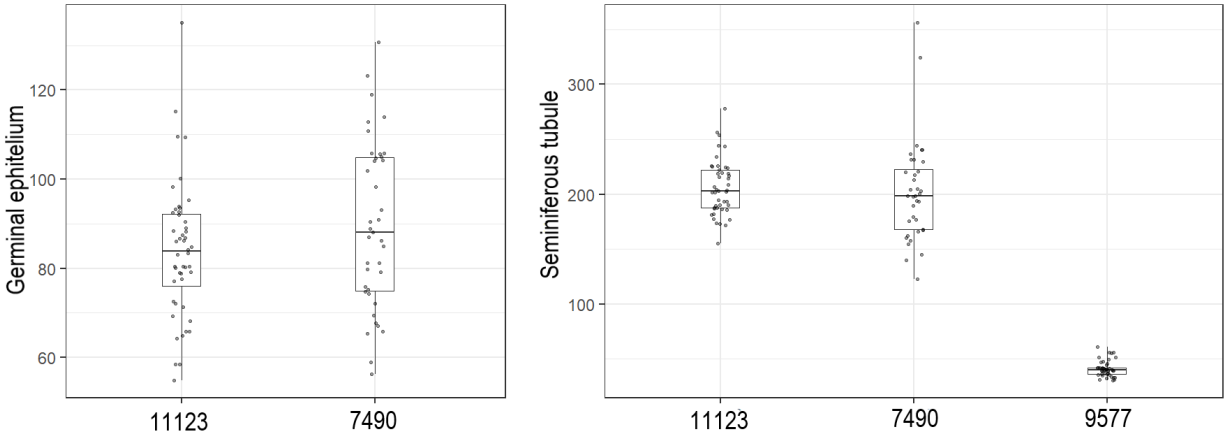

Figure 3. ***Corallus hortulanus*** **morphometric analysis in different reproductive periods: germinal epithelium height and seminiferous tubules diameter. The boxes represent the mean values (middle line) and standard deviation, and the lines above and below represent the upper and lower bounds, respectively.**

specimen 11123, presence of cellular remains associated with the few sperm cells indicates reproductive stage, however, there are two possibilities: (1) This animal is in advanced phase of the reproductive stage; or (2) in the regression period (post-reproductive period). Specimen 9577 is inactive (stage 8) due to the cell division absence and the cells in the apoptosis process.

The beginning of spermatogenesis stage can occur earlier or later, consequently the end of this stage as well, this condition was observed in *E. cenchria* (Bento et al. 2022) and also observed in the specimens 11123 and 7490 in this study. The food availability is directly related to the energy accumulation (fat) in the period, especially in the period preceding the snakes reproduction (Pizzatto et al. 2006). We should consider the availability of food as an important factor to justify the initiation of spermatogenesis.

Several species with seasonality or reproductive cyclicity present apoptosis process such a condition as part of the process responsible for maintaining the ideal ratio between germ cells and Sertoli cells, and thus ensuring the quality of sperm produced (Young & Nelson 2001, Gribbins et al. 2005, Zhang et al. 2008, Mahfouz et al. 2009, Shaha et al. 2010, Liu et al. 2017). The apoptosis process was observed in specimen 9577 (in inactive stage) and such condition was also observed in *Epicrates cenchria* who was in inactive stage (Bento et al. 2022).

The seminiferous tubules diameter increases in reproductive stage (gametogenesis) as a result of successive mitoses and meiosis that occur in this process and can also observed in other snakes (Goldeberg & Parker 1975, Gribbins et al. 2008, Bento et al. 2019, 2022).

The testes are larger during gametogenesis in *C. hortulanus*, this condition was observed in other Boidae, such as *E. cechria*, *Boa c. constrictor*, *Boa c. occidentalis* and *Eunectes murinus* (Bertona & Chiaraviglio 2003, Ibargüengoytía et al. 2006, Garcia et al. 2015, Bento et al. 2019, 2022). Although this condition is observed in these Boidae species, it should be considered that snakes may present maximum testicles volume in the regression period, like the *Crotalus scutulatus* for example (Schuett et al. 2002).

CONCLUSIONS

The *Corallus hortulanus* presents reproductive seasonality with an increase in testicular volume and gametogenesis occurring in the hottest and rainiest periods of the year. According to the reproductive stages the seminiferous tubules diameter increase, and germinal epithelium is evident in this period.

Acknowledgments

The authors thank the Laboratory of Zoological Collections – Herpetology Sector of the Institute of Biosciences of Universidade Federal de Mato Grosso (UFMT), for making available the specimens used in this study; and to the Laboratory of Morphological and Morphometric Analysis of the Institute of Biosciences of UFMT, for providing the structure and intellectual support for the development of this research.

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How to cite
BENTO HJ, FERREIRA A, IGLESIAS GA, ARAÚJO TG, CURCIO FF & PAZ RCR. 2024. *Corallus hortulanus* testes histology: morphological and reproductive aspects. An Acad Bras Cienc 96: e20230949. 10.1590/0001-3765202420230949.

Manuscript received on August 29, 2023;
accepted for publication on February 04, 2024

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