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Amphibia-Reptilia (2019) DOI:10.1163/15685381-20191189

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Reproductive cycle and sperm storage of female coral snakes, *Micrurus corallinus* and *Micrurus frontalis*

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Abstract. We analyzed the hypothesis that the lack of synchronization between the mating and ovulation period of *Micrurus frontalis* (BRT clade) is indicative of the capacity of females to store sperm. Conversely, since these reproductive events occur in the same season for *Micrurus corallinus* (BRM clade), sperm storage is not expected. Thus, we analyzed the reproductive cycle of female *M. corallinus* and *M. frontalis*, and investigated the occurrence of sperm storage. Our results showed that these two species of coral snakes (clades BRM and BRT) possess different reproductive cycles. *Micrurus frontalis* exhibits an extensive reproductive period encompassing three seasons (summer, autumn and winter), while *M. corallinus* directs secondary vitellogenesis and ovulation to the hottest period of the year (spring and summer). We confirm, for the first time, the strategy of sperm storage (SSr) in females of the genus *Micrurus*. We observed sperm storage receptacles located in the non-glandular uterus in all seasons of the year for *M. corallinus* and in spring, summer and autumn in *M. frontalis*. Furthermore, the presence of SSr in females in the non-reproductive (post-ovulatory) period, the verification of myoid cells around the receptacles and secretion of neutral carbohydrates in the lumina of SSrs may indicates a long-term storage. The posterior infundibulum is another possible region of sperm storage by the presence of tubular ciliated gland; however, reproductive studies with other species of the genus are necessary for a better understanding of the reproductive strategies of the BRT and BRM clades.

Keywords: Elapidae, long-term storage, *Micrurus*, reproduction.

Introduction

Reproductive strategies of snakes comprise a diversity of morpho-physiological and behavioral characteristics and mechanisms (e.g., sexual dimorphism, parental care, male-male combat, offspring size, and multiple paternity), that improve reproductive success (cf. Shine, 2003; Clark et al., 2014). The reproductive events (e.g., spermatogenesis, vitellogenesis, ovulation, mating season) may converge during certain periods of the year, and these events may be shortened or lengthened at the time of occurrence (Brown and Shine, 2002; Aldridge et al., 2009).

During the transition between reproductive and non-reproductive phases of the female life-cycle, cyclic morphological changes occur in the oviduct, such as changes in the conformation of the luminal epithelium (e.g., height and proportion of ciliated and non-ciliated cells), density and development of glands in the lamina propria, and quantity of secretion in the oviductal lumen (Perkins and Palmer, 1996; Girling, 2002; Siegel and Sever, 2008a; Loebens et al., 2017). These modifications are related to the functions of the oviduct in preparation for receiving the egg, provisioning of water and calcium, fertilization and shell formation until oviposition (Fox, 1977; Blackburn, 1998). Female snakes exhibit remarkable diversity of specialized structures related to reproduction, such as uterine muscular twisting (Almeida-Santos and Salomão, 1997, 2002; Barros, Rojas, and Almeida-Santos, 2014), mating plugs (Friesen et al., 2013; Barros, Rojas and Almeida-Santos,

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2017), sperm storage tubules (Fox, 1956; Sever and Hamlett, 2002; Siegel et al., 2011; Rojas, Barros and Almeida-Santos, 2015; Holt and Fazeli, 2016), and sperm storage receptacle (Rojas, Barros and Almeida-Santos, 2017), that have been investigated in different genera for the functional advantages that they provide.

The majority of previous work on snake reproduction has used macroscopic data for cycle analysis (e.g., Pizzatto and Marques, 2006; Marques, Pizzatto and Almeida-Santos, 2013). However, the inclusion of histological data permits a more detailed analysis of the reproductive cycle of females and provides a better anatomical-functional understanding of the oviduct (Mathies, 2011; Almeida-Santos et al., 2014; Barros, Rojas and Almeida-Santos, 2014; De Resende and Nascimento, 2015). According to Mathies (2011), the best metric for reproductive cycle analysis is data from specimens with eggs in the oviduct. However, finding specimens that exhibit this condition in biological collections is very difficult and rare. Thus, analysis of the morphological conditions of the oviduct is important for understanding and delimiting the reproductive period.

The strategy of female sperm storage has been documented in several species of different groups of vertebrates (Holt and Fazeli, 2016). Sperm storage allows a temporal dissociation of copulation and female ovulation, and several hypotheses have been presented regarding the function, advantages and evolutionary origin of this strategy among snakes (Birkhead and Møller, 1993; Siegel et al., 2011). In snakes, sperm storage can occur in two different regions of the oviduct: in the posterior infundibulum, usually in receptacle structures (Perkins and Palmer, 1996; Siegel et al., 2011; Rojas, Barros and Almeida-Santos, 2015); and in the posterior oviduct, in epithelial folds, crypts or in the lumen (Halpert, Garstka and Crews, 1982; Almeida-Santos and Salomão, 1997, 2002; Siegel et al., 2011). Accordingly, two types of sperm storage have been described for snakes: short-term storage, when the spermatozoa are stored in the region of the posterior infundibulum until ovulation; and long-term storage, in which sperm are first stored in the posterior oviduct and then, in a period close to ovulation, spermatozoa ascend the oviduct to the posterior infundibulum where they are re-stored until ovulation (Halpert, Garstka and Crews, 1982; Schuett, 1992; Siegel et al., 2011). Some authors have proposed hypotheses for the occurrence of sperm storage in certain species of coral snakes (cf. Quinn, 1979; Marques, Pizzatto and Almeida-Santos, 2013), but the mechanism of sperm storage by females has not been confirmed and data regarding this strategy for the family Elapidae (Reptilia: Squamata) are scarce.

The species *Micrurus corallinus* exhibits a wide distribution within the Atlantic Forest biome, from southern of Bahia to Rio Grande do Sul (Campbell and Lamar, 2004), while *M. frontalis* is distributed in the Cerrado biome, from southern Minas Gerais to Mato Grosso do Sul (Roze, 1996; Silva Jr. and Sites Jr., 1999). According to Köppen's climate classification, the climate in Southeast Brazil is classified as humid subtropical zone (cfa and cfb) while that of the central region is tropical zone (Am and Aw) (cf. Alvares et al., 2013).

Most coral snakes (genus *Micrurus*) in the new world belong either to the clade of species with patterns of black rings in monads (BRM) or species with the black rings in triads (BRT) (Roze, 1996; Campbell and Lamar, 2004). According to Marques et al. (2013), these two clades evolved several different reproductive strategies, such as male-male combat behavior (presence or absence), sexual dimorphism (males or females with greater snout-vent length) and reproductive cycle pattern (period). Females of the BRT clade possess an extended reproductive cycle, such as *M. frontalis*, and the period of copulation usually does not coincide with the period of ovulation (Marques, Pizzatto and Almeida-Santos, 2013). In contrast, females of the BRM clade have a short period

of secondary vitellogenesis and ovulation in the spring, in synchrony with the mating season, as occurs in *M. corallinus* (Marques, Pizzatto and Almeida-Santos, 2013). Thus, it is hypothesized that females of the BRT clade would perform the strategy of sperm storage because there is no synchronization between the copulation period and ovulation. On the other hand, the BRM clade is not expected to exhibit the strategy of sperm storage because these reproductive events occur during the same season. To assess sperm storage by these two species, histological analyses of the reproductive cycle are needed. Therefore, the present study intended to investigate the reproductive strategies of females of both the BRM and the BRT clades, as represented by *Micrurus corallinus* and *M. frontalis*, respectively.

Material and methods

Specimen sampling

We examined a total of 52 preserved female specimens of *Micrurus corallinus* (50 mature and 2 immature) and 24 of *M. frontalis* (22 mature and 2 immature) preserved in Brazilian herpetological collections (Appendix 1), immature specimens were used only as a parameter for the non-reproductive stage (qualitative analysis) and were not included in the results of the study. Specimens were considered adult females if they had oviducts exhibiting a pleated conformation (post-spawning period), follicles in secondary vitellogenesis, or eggs in the oviduct (cf. Shine, 1977; Mesquita et al., 2011; Loebens et al., 2017). Immature females (or juveniles) were consider those with a SVL of less than 400 mm, ovarian follicles > 5 mm (Marques, 1996) and oviducts with a smooth conformation (absent folded). The analyzed adult females were grouped among the four seasons in accordance with the date and place of collection: *Micrurus corallinus* (spring, n = 17; summer, n = 20; autumn, n = 7; winter, n = 6) and *M. frontalis* (spring, n = 6; summer, n = 6; autumn, n = 8; winter, n = 2).

The studied female specimens of *M. corallinus* were collected in the states of Espírito Santo, São Paulo, Rio de Janeiro, Paraná and Santa Catarina while those of *M. frontalis* were from the states of Minas Gerais, São Paulo, Goiás and Mato Grosso do Sul (supplementary map S1). These areas of the Atlantic Forest biome (ES, SP, RJ, PR and SC – coastal region) experience 1300-2200 mm of annual rainfall, and have an average temperature of 18-22°C, while the areas of the Cerrado (MG, SP, GO and MS) experience 1000-1600 mm of annual rainfall and have an average temperature of 20-24°C (cf. Alvares et al., 2013). Species of the genus *Micrurus* are semi-fossorial, secretive, and difficult to sight in the field. Thus, we analyzed specimens from different localities within the distribution area of each species (supplementary table S1A, B), which is a common methodology for research on morphology and reproductive cycle (cf. Quinn, 1979; Marques, 1996; Goldberg, 1997; Almeida-Santos, Pizzatto and Marques, 2006; Barros, Rojas and Almeida-Santos, 2014; De Resende and Nascimento, 2015; Loebens et al., 2017).

Reproductive cycle analysis is typically done according to the austral seasons in order to allow comparisons to be made among snake species. We measured snout-vent length (SVL) of each specimen and, after dissection, recorded the length of the largest vitellogenic follicle present in an ovary or of an egg in the oviduct, regardless of the side (right or left). To differentiate between vitellogenic follicles in primary (V1) and secondary vitellogenesis (V2) (cf. Aldridge, 1979; Bassi et al., 2018), we measured the length of ovarian follicles using a digital caliper, initially analyzed the follicle growth period according to the length of the follicles among seasons, and then classified follicles as in V1 or V2, as proposed by Almeida-Santos et al. (2014).

Histological and histochemical analyses

The anatomical nomenclature used for the oviduct followed the proposals of Siegel and Sever (2008b), and Siegel et al. (2011). For histological analysis, tissue samples from the right oviduct were collected from five to six randomly selected specimens from each season. Samples were taken from the following structures: infundibulum (anterior and posterior), glandular uterus, non-glandular uterus and vaginal pouch.

The organs were preserved in 70% alcohol and tissue samples were submitted to routine histological procedures for inclusion in paraffin. Sections 5-7 μm thick were cut and stained with hematoxylin and eosin (H/E) in paraffin (cf., Junqueira, Bignolas and Brentani, 1979; Junqueira, 1995). To assess variation in the secretory activity of the glands in the oviducts during the different phases of the reproductive cycle, as well as the occurrence of sperm storage, the sections were submitted to the following histochemical reactions: alcian blue (AB) pH 2.5 for carboxylated glycosaminoglycans (we adapted the protocol of Junqueira (1995) and counter-stained with hematoxylin); and periodic acid-Schiff (PAS) for the identification of neutral carbohydrates. For the identification of proteins produced by shell glands we used Coomassie brilliant blue R-250 (CB) (cf. Braz et al., 2018). The histological sections of the oviduct were analyzed using a Leica DM4 B microscope with a Leica DMC 4500 camera.

Specialized gland, called tubular ciliated gland (TCG) and sperm storage receptacle (SSr) have the same morphology, so we only use SSr to refer to structures that had spermatozoa in the lumen.

Qualitative analysis

For qualitative analysis of histological sections, we used the criterion of morphological condition of the oviduct of specimens in the reproductive and non-reproductive state. For this we used juvenile females (i.e., up to 400 mm

SVL and with all follicles in V1) as references for the non-reproductive state and females in advanced stage of secondary vitellogenesis (i.e., 30 mm of follicular length), as references for the reproductive state of females with the oviduct prepared for receiving the egg. The characteristics exhibited by the tissues collected from the three regions of the oviduct of these specimens allowed the determination of glandular quantity and state, and condition of the luminal epithelium and luminal secretions.

The morphological characteristics considered for the criterion of the shell gland were: **hypotrophied gland** = cuboid epithelium, absence or low presence of secretory granules in the glandular cytoplasm; **hypertrophied gland** = columnar epithelium and a large amount of secretory granules in the glandular cytoplasm (cf. Perkins and Palmer, 1996; Braz et al., 2018).

We adopted a blind-test for the analysis of oviduct sections, that is, without prior knowledge of which group (season) a particular slide belonged to. Thus, we examined the three regions of the oviduct of each specimen, assigning a score from 1 to 5, with the minimum value of 1 (one) referring to morphological characteristics close to the non-reproductive state and the maximum value of 5 (five) for the reproductive state (supplementary table S2A).

After assigning scores they were averaged for each group and rounded to a final value for making comparisons among seasons. Thus, the seasons with higher scores had morphological conditions of the oviduct closer to the reproductive phase and those with lower scores were of the non-reproductive phase. Qualitative analysis was only performed in *M. corallinus* that reached the minimum sample size of 5 specimens per season.

Results

Analysis of the reproductive cycle

The smallest adult female analyzed with follicles in V1, presenting an oviduct with pleated conformation and non-translucent coloration, had a SVL of 480 mm. According to the graphic of seasonal variation in length of the largest ovarian follicle, the species *Micrurus corallinus* and *M. frontalis* exhibit secondary vitellogenic follicles with lengths above 13 mm (fig. 1).

Micrurus corallinus

Females of *Micrurus corallinus* exhibited follicles in V2 and eggs in the oviduct between October and March (spring-summer). Of the specimens with follicles in V2, 60% were observed in the spring and 26.7% in the summer, while for females with eggs in the oviduct, 40% were found in the spring and 60% in the summer (fig. 1).

In the spring, almost the entire extension of the oviduct exhibited columnar luminal epithelium with evident nuclei and a higher proportion of ciliated cells; the glandular uterus exhibited a high concentration of hypertrophied shell glands (fig. 2A, B, C), when compared to that

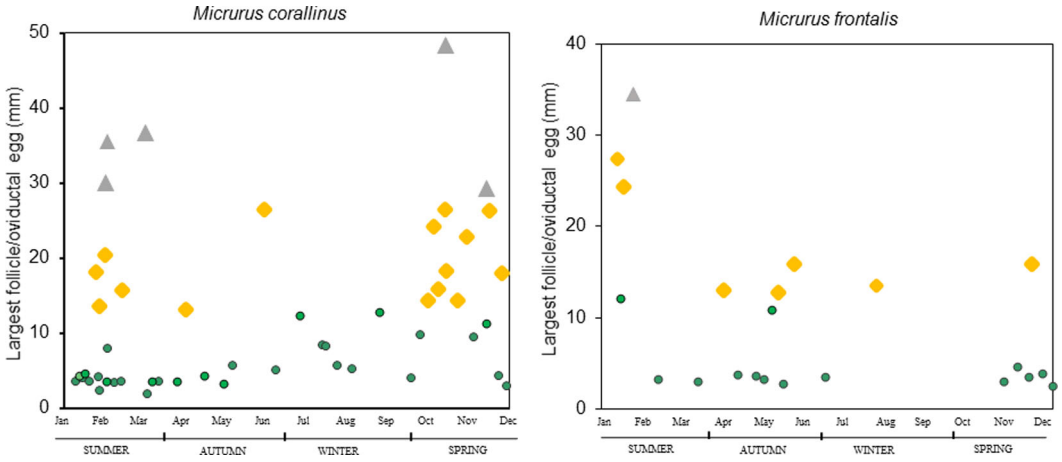

Figure 1. Female reproductive cycle: seasonal variation in length of largest ovarian follicle and occurrence of eggs in the oviduct of *Micrurus corallinus* (left) and *Micrurus frontalis* (right). Green circles = primary vitellogenic follicles; yellow diamonds = secondary vitellogenic follicles; gray triangles = eggs in the oviduct.

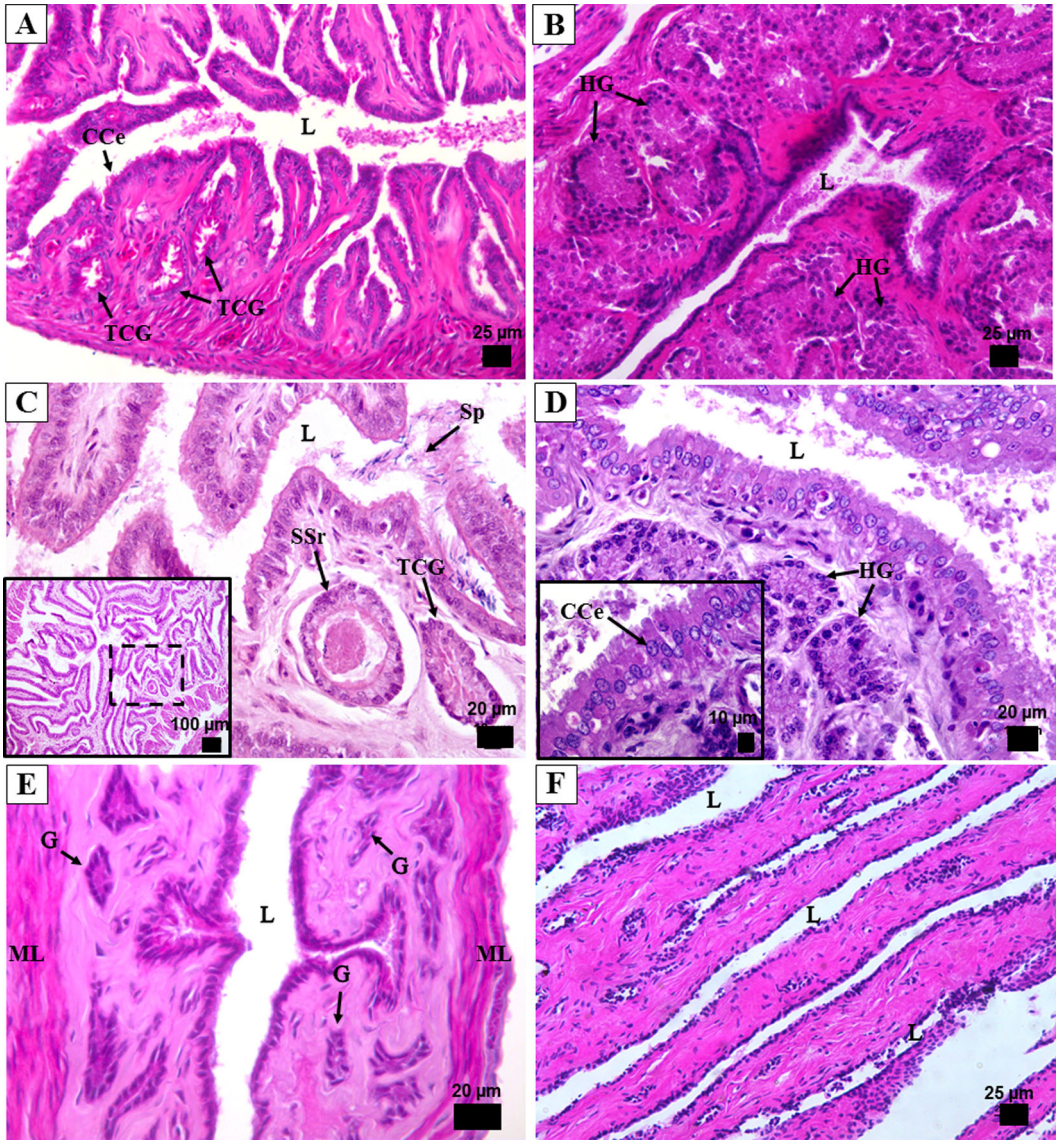

Figure 2. Longitudinal section of female reproductive tract (stained H/E). (A) *Micrurus corallinus* (spring): posterior infundibulum showing ciliated columnar epithelium and the presence of tubular ciliated gland. (B) *Micrurus corallinus* (spring): glandular uterus with hypertrophied shell glands in the lamina propria. (C) *Micrurus corallinus* (spring): region of the non-glandular uterus (represented in the rectangle – insert) with ciliated columnar epithelium, presence of tubular ciliated glands and sperm storage receptacle. Insert: photomicrograph of non-glandular uterus with low magnification of the same area of the dashed rectangle. (D) *Micrurus corallinus* (summer): glandular uterus exhibiting reproductive stage with ciliated columnar epithelium, hypertrophied shell glands and luminal secretion in the oviduct. Insert: higher magnification of ciliated columnar epithelium. (E-F) *Micrurus corallinus* (summer): glandular and non-glandular uterus exhibiting decreased activity with hypotrophied shell glands and cubic epithelium. **CCe**, ciliated columnar epithelium; **G**, hypotrophied gland; **HG**, hypertrophied gland; **L**, lumen; **ML**, muscle layer; **SSr**, sperm storage receptacle; **TCG**, tubular ciliated gland.

of the other seasons. In the summer, approximately 66.7% of females still exhibited characteristics related to the reproductive phase that was observed in the spring (fig. 2D), indicating that they were still suitable for receiving eggs (fig. 2A, B, C, D). However, one of the specimens (summer group) already exhibited a regression of the reproductive stage (ovary only

with follicles in V1), presenting hypotrophied (or atrophied) shell glands and a smaller proportion of ciliated cells in both portions of the uterus (fig. 2E, F).

Qualitative analysis of our data revealed that the highest scores were in the spring and summer seasons (table 1), with a reduction in the scores of all analyzed characteristics of the oviduct in autumn and winter. One of the autumn specimens (with follicles in V2) still maintained the reproductive conditions of the oviduct, including shell glands showing hypertrophy, columnar epithelial cells and the presence of luminal secretion.

Nevertheless, females of autumn and winter exhibited a cuboidal epithelium with few ciliated regions (fig. 3A), and a low concentration of hypotrophied shell glands in the lamina propria (table 1), corresponding to the non-reproductive stage. Specimens collected in winter exhibited a non-glandular uterus with a large number of tubular ciliated glands, most of which had granules in their interior being released into the oviductal lumen (fig. 3B).

Micrurus frontalis

A total of 71.42% of the follicles in V2 occurring from the end of December to May (summer-autumn), while the only female with eggs in the oviduct was observed in January (fig. 1). In the spring, females possessed morphological characteristics of the non-reproductive phase, with only one female (follicles in V2) having shell glands in process of hypertrophy on the lamina propria (posterior infundibulum and glandular uterus). The other females of spring (follicles in V1) possessed cuboidal epithelium and hypotrophied glands throughout practically the entire oviduct (fig. 3C, D); the non-glandular uterus exhibited ciliated cells and TCGs in specific regions. At the beginning of the summer, the analyzed specimens exhibited an increased concentration of shell glands, which continued throughout the season (fig. 3E), while other specimens (with follicles in V2) had these exocrine glands already in hypertrophied state. In addition, an increased proportion of ciliated cells were present throughout the oviduct (fig. 3F). In autumn, the specimens with follicles in V2 exhibited a ciliated columnar conformation of the oviduct epithelium, hypertrophied shell glands of the posterior infundibulum and glandular uterus, a greater amount of luminal secretion (fig. 4A, B), and an increase in ciliated tubular glands in the non-glandular uterus. In winter, the luminal epithelium of the oviduct still remained columnar and ciliated, and the shell glands were hypertrophied but in a reduced concentration on the lamina propria (fig. 4C).

Histochemical analysis revealed that in both species the glands present in the connective tissue did not react to AB and PAS in the posterior infundibulum and glandular uterus (fig. 4D, E). However, the shell glands exhibited a positive reaction to CB, indicating protein (fig. 4F), in *Micrurus corallinus* in the spring and summer, and in *M. frontalis* in summer and autumn. The luminal epithelial layer and its secreted material of the three portions of the oviduct (posterior infundibulum, glandular uterus and non-glandular uterus) had low reactivity for CB, but exhibited reactivity to AB, indicating the presence of glycosaminoglycans and acid glycoproteins (fig. 4D), and PAS, indicating the presence of acid and neutral glycoproteins (fig. 5A).

Strategy of sperm storage

Sperm storage receptacles were found in the lamina propria of the non-glandular uterus (or utero-vaginal junction) in both *Micrurus corallinus* and *M. frontalis*. The SSrs were usually interspersed with TCGs, around which were capillary vessels. The TCGs and SSrs possessed epithelium composed of ciliated and non-ciliated cells, and myoid cells involved the entire receptacle (fig. 5B); the lumen sometimes exhibited the presence of small granules or a single, dense, compact granule (fig. 5C, D, E, F). We found that approximately 36%

Table 1. Qualitative analysis of morphological characters of the female reproductive tract of *Micrurus corallinus*. Data are expressed as mean ± standard deviation, (min-max) of scores. **Lum.**, luminal.

	Posterior infundibulum			Glandular uterus			Non-glandular Uterus	
Season	Lum. epithelium	Glands shell	Lum. secretions	Lum. epithelium	Glands shell	Lum. secretions	Lum. epithelium	Lum. secretions
Spring (n = 6)	4.9 ± 0.2	4.3 ± 0.5	2.5 ± 0.5	4.8 ± 0.2	4.5 ± 0.5	2.3 ± 0.7	5.0 ± 0.0	3.1 ± 0.9
	(4.5–5.0)	(4.0–5.0)	(2.0–3.0)	(4.5–5.0)	(4.2–5.0)	(1.0–3.0)	(5.0–5.0)	(2.0–5.0)
Summer (n = 6)	4.5 ± 0.7	4.6 ± 0.6	2.6 ± 0.5	4.2 ± 0.7	4.8 ± 0.2	2.5 ± 1.5	4.3 ± 0.8	2.0 ± 0.8
	(3.0–5.0)	(3.5–5.0)	(2.0–3.0)	(3.0–5.0)	(4.0–5.0)	(1.0–4.5)	(3.0–5.0)	(1.0–3.0)
Autumn (n = 5)	2.8 ± 0.8	2.7 ± 0.4	2.0 ± 0.9	2.5 ± 0.4	2.9 ± 0.2	2.1 ± 0.7	3.3 ± 0.6	2.8 ± 0.6
	(2.0–4.0)	(2.0–3.0)	(1.0–3.0)	(2.0–3.0)	(2.5–3.0)	(1.0–3.0)	(2.5–4.0)	(2.0–3.5)
Winter (n = 5)	2.9 ± 1.0	2.4 ± 0.6	2.1 ± 0.7	2.8 ± 0.2	2.3 ± 0.4	1.8 ± 0.6	3.7 ± 0.6	1.9 ± 0.8
	(1.0–3.5)	(1.5–3.0)	(1.0–3.0)	(2.5–3.0)	(2.0–3.0)	(1.0–2.4)	(3.0–4.8)	(1.0–2.8)

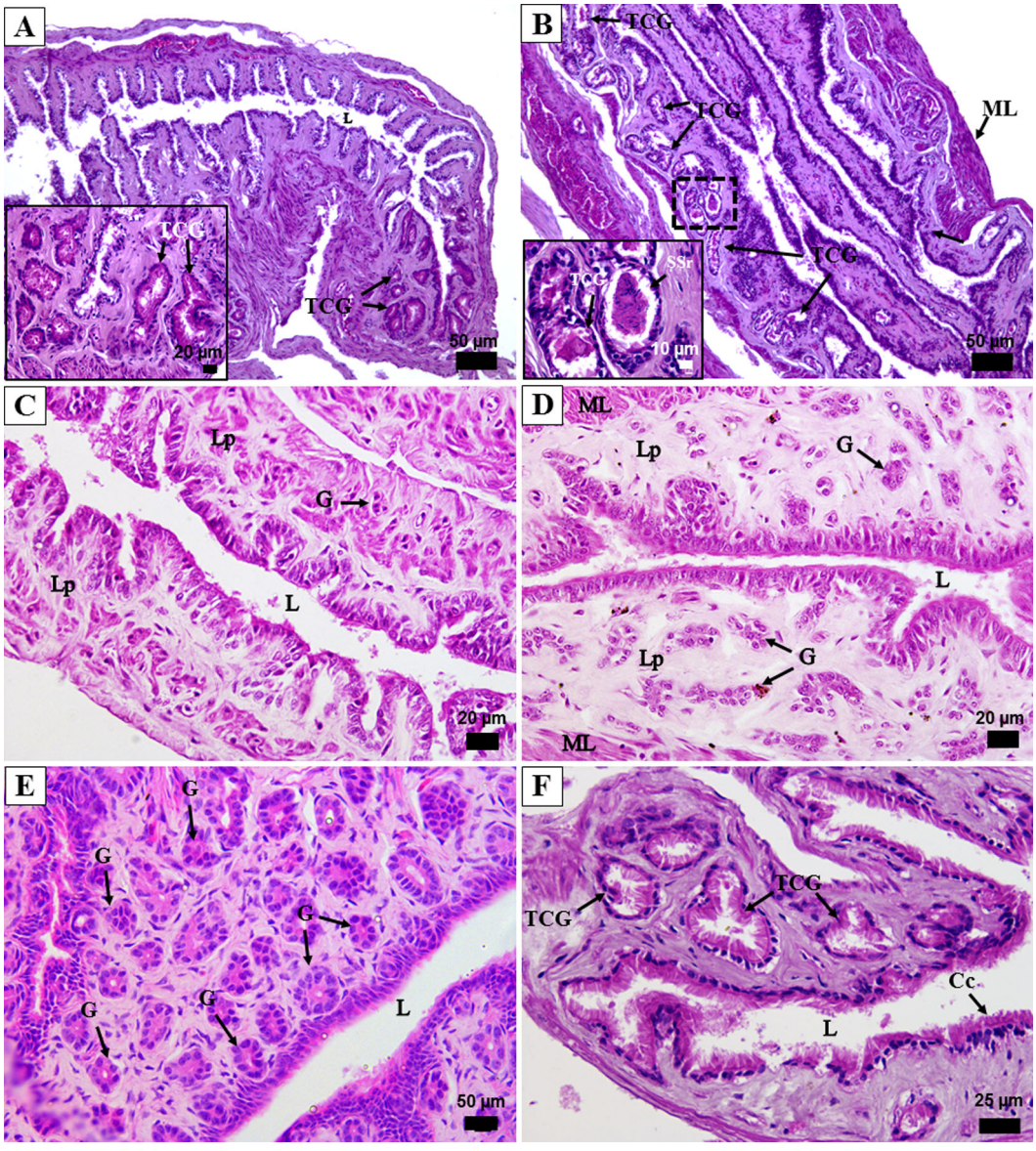

Figure 3. Longitudinal sections of female reproductive tract (stained H/E). (A) *Micrurus corallinus* (autumn): posterior infundibulum showing cuboidal epithelium. Insert: presence of tubular ciliated glands in posterior infundibulum. (B) *Micrurus corallinus* (winter): non-glandular uterus with cuboidal epithelium and the presence of a large quantity of tubular ciliated glands. Insert: higher magnification with the dashed rectangle showing the presence of a sperm storage receptacle. (C-D) *Micrurus frontalis* (spring): posterior infundibulum and glandular uterus with cuboidal epithelium, low proportion of non-ciliated cells and hypotrophied shell glands. (E) *Micrurus frontalis* (summer): glandular uterus with an increased quantity of shell glands. (F) Posterior infundibulum with an increased proportion of ciliated cells in the epithelium. **CC**, ciliated cell; **G**, hypotrophied gland; **L** lumen; **Lp**, lamina propria; **ML**, muscle layer; **TCG**, tubular ciliated gland; **SSr**, sperm storage receptacle.

of the specimens with SSr were in the non-reproductive period (supplementary table S3A).

The granules present in the cytoplasm of the epithelium and in the lumina of the SSrs and TCGs reacted positively to PAS (fig. 5A), indicating that the receptacles themselves secrete the granules. These structures also exhibited granules reactive to AB and CB, indicating the

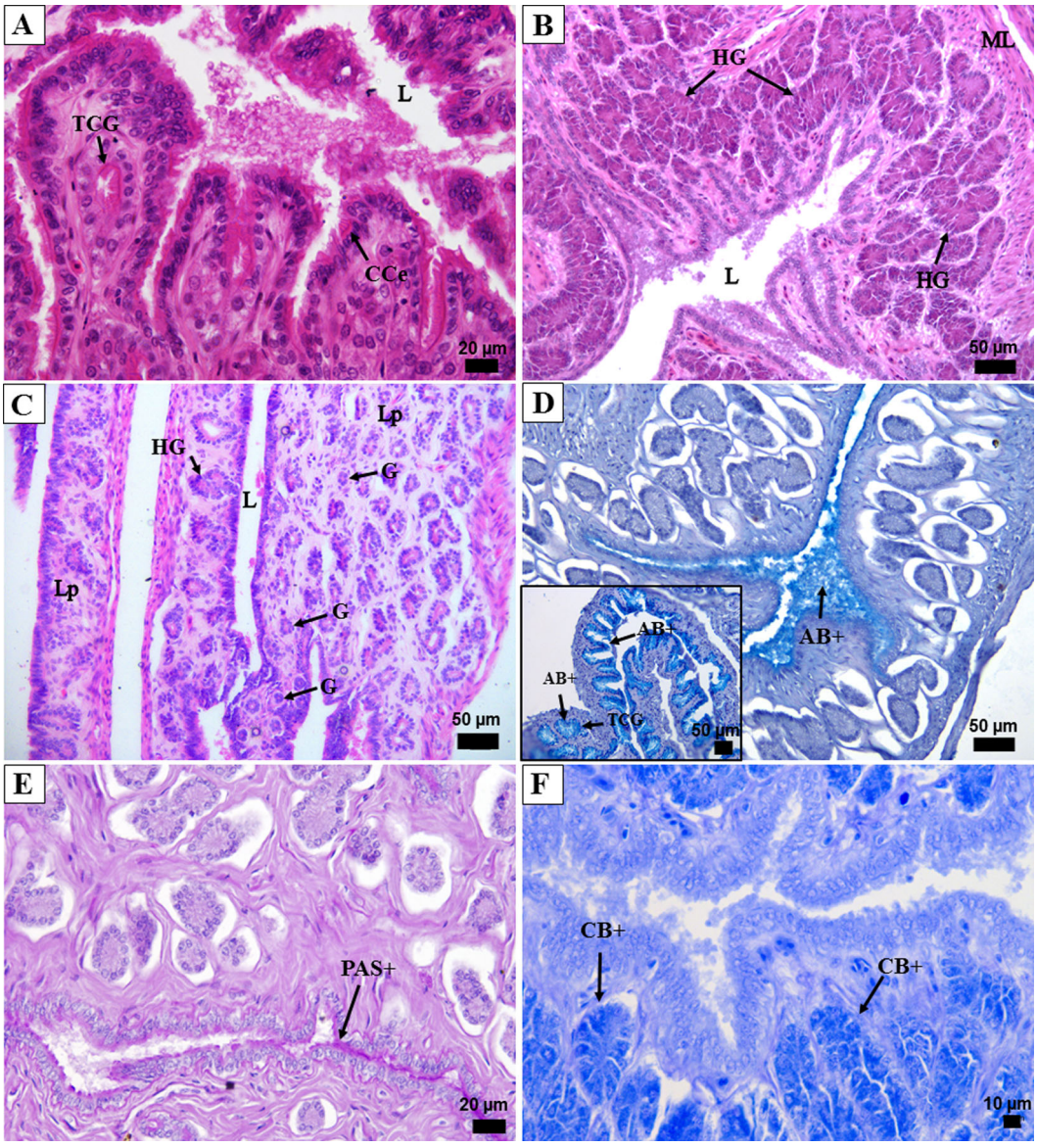

Figure 4. Longitudinal histological (stained H/E) and histochemical sections of female reproductive tract. (A) *Micrurus frontalis* (autumn): posterior infundibulum with ciliated columnar epithelium, presence of tubular ciliated glands and luminal secretion. (B) *Micrurus frontalis* (autumn): glandular uterus showing hypertrophied glands. (C) *Micrurus frontalis* (winter): posterior infundibulum exhibiting hypertrophied and hypotrophied shell glands. (D) *Micrurus frontalis* (autumn): glandular uterus exhibiting the luminal secretion with positive reaction for AB (pH 2.5). Insert: luminal epithelium with positive reaction for AB (pH 2.5). (E) *Micrurus corallinus* (summer): glandular uterus showing the luminal epithelium with positive reaction for PAS. (F) *Micrurus corallinus* (autumn): glandular uterus exhibiting shell glands with positive reaction for CB. **AB**+, positive reaction to alcian blue; **CB**+, positive reaction to Coomassie blue; **CCe**, ciliated columnar epithelium; **G**, hypotrophied gland; **HG**, hypertrophied gland; **L**, lumen; **Lp**, lamina propria; **ML**, muscle layer; **PAS**+, positive reaction to periodic acid-Schiff; **TCG**, tubular ciliated gland.

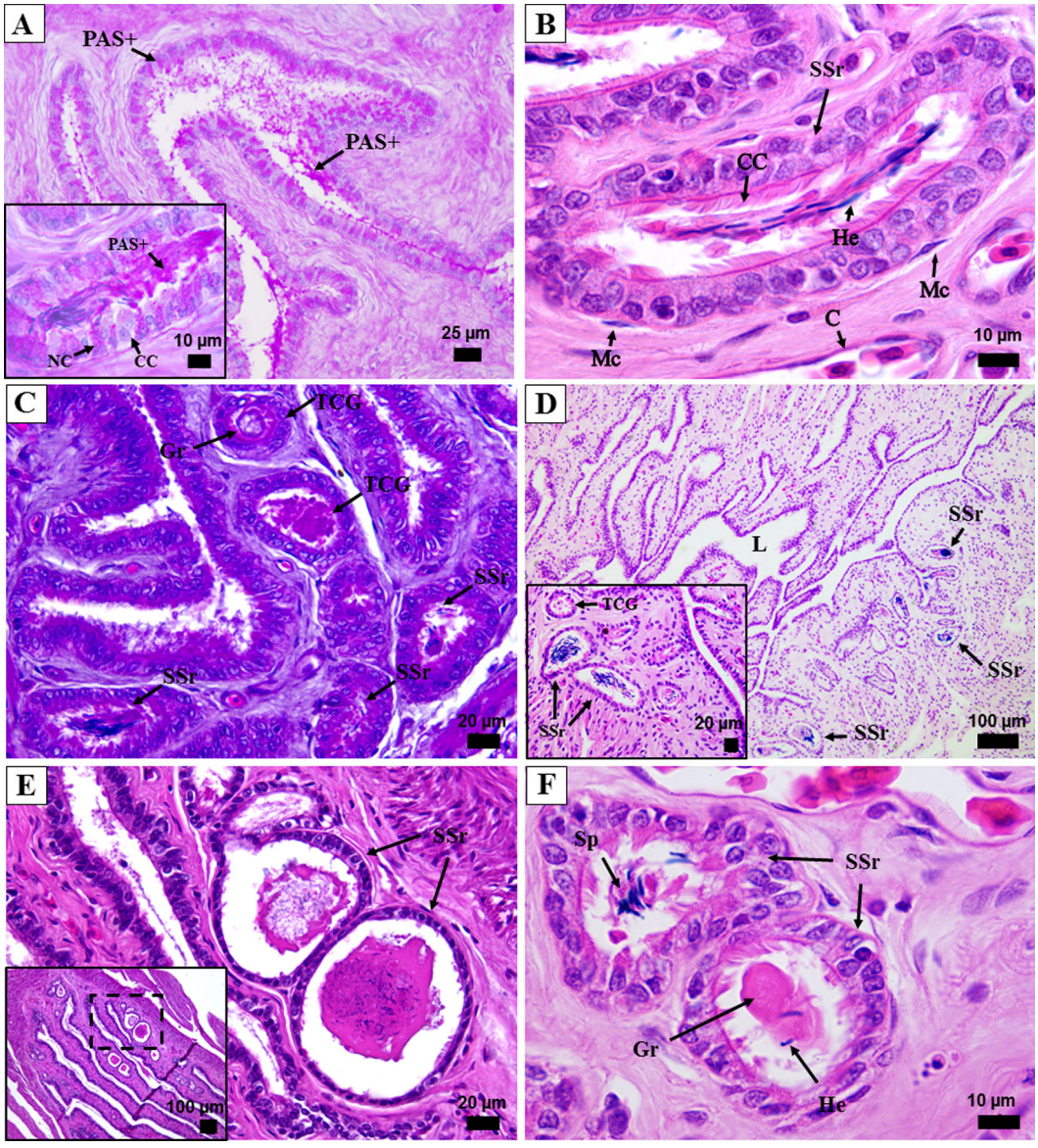

Figure 5. Longitudinal histological (stained H/E) and histochemical sections of the non-glandular uterus. (A) *Micrurus corallinus* (spring): luminal secretion and columnar epithelium with positive reaction for PAS. Insert: higher magnification of sperm storage receptacle showing granules in the lumen with positive reaction for PAS. (B) *Micrurus corallinus* (summer): detail of non-glandular uterus with at higher magnification, showing the sperm storage receptacle containing spermatozoa and the presence of myodes cells involving the structure of SSr. (C) *Micrurus frontalis* (autumn): sperm storage receptacles interspersed with tubular ciliated glands and, in detail, granules in the lumen of a tubular ciliated glands. (D) *Micrurus frontalis* (spring): presence of sperm storage receptacle. Insert: Sperm storage receptacle and tubular ciliated gland in the non-glandular uterus (the same specimen). (E) *Micrurus corallinus* (summer): photomicrograph with the dashed rectangle (from insert) showing a sperm storage receptacle with a single dense and compact granule in the lumen. Insert: Photomicrograph at low magnification of the same area of the dashed rectangle. (F) *Micrurus corallinus*: two sperm storage receptacles with granules and spermatozoa. **C**, capillary; **CC**, ciliated cell; **Gr**, granule; **He**, spermatozoa head; **L**, lumen; **Mc**, myoid cell; **NC**, non-ciliated cell; **PAS**+, positive reaction to periodic acid-Schiff; **Sp**, spermatozoa; **SSr**, sperm storage receptacle; **TCG**, tubular ciliated gland.

presence of acid glycosaminoglycans and proteins, respectively.

Discussion

The period of vitellogenesis of the reproductive cycle of females of *Micrurus corallinus* is restricted to the spring, which coincides with the onset of the rainy season (cf. Marques, 1996). We observed that for *Micrurus corallinus* the expression of reproductive characters in the oviduct occur during spring, but continue until the summer, as also evidenced by specimens exhibiting peak secondary vitellogenesis and gravid female specimens in both seasons.

Our qualitative analysis of the morphological characters of the oviduct indicated that the epithelial tissue and the shell glands gradually undergo a decrease in activity in autumn and winter, regressing to the non-reproductive state as clearly demonstrated by the lower scores in these months, but especially in winter. This decrease coincides exactly with the period of low activity pattern recorded for *M. corallinus* (cf. Marques, Almeida-Santos and Rodrigues, 2006). Therefore, the results presented here differ slightly from the data of Marques (1996), who analyzed specimens of *M. corallinus* collected previous to the material of the present study. This difference relates mainly to the summer season, during which we verified that females are in the reproductive stage. Almeida-Santos et al. (2017) recently described the first photographic record of copulation for *M. corallinus*, which occurred in the summer, thus reinforcing our results because females are usually more receptive to copulation within the reproductive period.

Analysis of the reproductive cycle of *Micrurus frontalis* found eggs and follicles in an advanced stage of secondary vitellogenesis in the summer. Marques et al. (2013) reported eggs in the oviduct in summer and winter for this species. Our histological analysis revealed that almost all specimens possessed the morphological characters of the non-reproductive stage in spring. In summer the oviduct was found to progressively exhibit increased activity (e.g., increased concentration and hypertrophy of the shell glands, modification of the epithelium to columnar ciliated and increased luminal secretion), with the peak occurring in autumn and some signs of regression of oviduct activity in the winter. Therefore, our results show that reproductive season of *M. frontalis* includes autumn. The summer and fall seasons also correspond to the period of highest activity for *M. frontalis* (cf. Marques, Almeida-Santos and Rodrigues, 2006). Thus, the reproductive phase begins in the summer and ends in mid-winter (i.e., a reproductive period of three seasons), corroborating the classification of the reproductive cycle as extended for this species.

The oviductal epithelium exhibited an increase of secretions of neutral carbohydrates (PAS+) and carboxilated glycosaminoglycans (AB+) in the reproductive period of both *Micrurus corallinus* (spring-summer) and *M. frontalis* (summer-autumn). Furthermore, the shell glands exhibited an intense reaction for the presence of protein (CB+) during the same period. The increased luminal secretion is directly related to a morpho-physiological response in preparation for egg uptake and the execution of different functions after ovulation, such as improved lubrication of the oviduct (by the release of mucus), protection against microorganism (Rojas, Barros and Almeida-Santos, 2015); synthesis and provisioning albumen (Fox, 1977; Girling, 2002), extrusion of protein fibers that will make up the shell membrane, and the deposition of calcium in the egg (Palmer, Demarco and Guillette, 1993; Gist, 2011).

Our results confirm the occurrence of the strategy of sperm storage in the representatives of the BRM and BRT clades (*Micrurus corallinus* and *M. frontalis*, respectively). The granules produced by SSrs and TCGs (in the posterior infundibulum and non-glandular uterus) were reactive to the presence of proteins (CB+), neutral carbohydrates (PAS+) and carboxylated glycosaminoglycans (AB+) in both coral snake

species. As described for other snakes, it is possible that these granules have a dual function: to attract the spermatozoids into the storage receptacles in the oviductal lumen and, serve as a source of energy for sustaining sperm once the spermatozoa are stored (Hoffman and Wimsatt, 1972; Siegel and Sever, 2008b).

Sperm storage receptacles (SSrs) were found in all seasons for *Micrurus corallinus*, and in spring, summer and autumn for *M. frontalis*. In both species they were located in the non-glandular uterus, indicating long-term storage. The long-term storage hypothesis is due to the fact that SSrs were found in females with follicles in V1, especially in the post ovulatory period in *M. corallinus* (autumn-winter) and *M. frontalis* (spring), that is, females that present sperm storage outside the reproductive period. It is possible that even with the passage of the egg through the oviduct, some SSrs are not opened, and thus remain until the next cycle. This can be explained by the different thickness of the oviduct in the storage regions, with the muscular layer and lamina propria of the uterus being thicker than that of the infundibulum (Fox, 1977; Perkins and Palmer, 1996; Girling, 2002), which would facilitate the non-opening of receptacles, even with the pressure exerted by the passage of an egg. Thus, the myoepithelial cells observed in both species may act in the contraction of the receptacle structure, thereby aiding the shedding of sperm into the oviductal lumen, as suggested for *Philodryas patagoniensis* (cf. Rojas, Barros and Almeida-Santos, 2015). Myoid cells possess actin filaments that provide contractility and help to maintain the integrity of structures involved in the arrangement (Maekawa et al., 1996). Thereby, the presence of the remaining receptacles may contribute to sperm competition by generating a single clutch with multiple paternities (Siegel et al., 2011; Holt and Fazeli, 2016; Loebens et al., 2017), and to oocyte fertilization in the following cycle in the case that the female does not copulate.

The issue involved with the receptacles located in the posterior oviduct is that fertilization would have to occur prior to the formation of the shell's membrane (Blackburn, 1998), otherwise, the membrane could be a physical barrier to fertilization (Rojas, Barros and Almeida-Santos, 2015). Thus, sperm stored in furrows or the lumen migrate from the posterior oviduct to the infundibulum before the occurrence of ovulation (cf. Halpert, Garstka and Crews, 1982; Siegel et al., 2011). If both species adopted the strategy of long-term storage, we hypothesize that the posterior infundibulum may also be a region of sperm storage due to the presence of TCG in this portion of the oviduct.

The present study showed that the two studied species of the clades BRM and BRT possess different reproductive cycles. *Micrurus frontalis* exhibits an extensive reproductive period of three seasons, while *M. corallinus* directs secondary vitellogenesis and ovulation to the hottest period of the year (spring and summer). This represents the first confirmation of the strategy of sperm storage in females of the genus *Micrurus*. Furthermore, receptacles located in the non-glandular uterus, and the presence of neutral carbohydrates (granules) in the lumina of SSrs, indicate long-term sperm storage. In addition, the posterior infundibulum may also be a region of sperm storage; however, reproductive studies with other species of the genus are necessary for a better understanding of sperm storage strategies in the BRT and BRM clades.

Acknowledgements. The authors thank Julio C. Moura-Leite, Paulo R. Manzani, Paulo G. H. Passos, Paulo C. A. Garcia, Gláucia M. Funk Pontes, Selvino N. de Oliveira, Giuseppe Puorto, Maria R. S. Pires, Luciana B. Nascimento, Vanda L. Ferreira, and Natan M. Maciel for allowing access to preserved specimens. Special thanks to Prof. Dr. Sebastião Roberto Taboga for the help and availability of the Multiuser Center for Microscopy and Microanalysis (CMMicro) of IBILCE/UNESP, and Prof. Dr. Classius de Oliveira (Laboratory of Comparative Anatomy) for all support and collaboration with this research. E.A.B. was granted scholarships by Fundação de Amparo à Pesquisa do

Estado de São Paulo (FAPESP; 2014 /12813-9) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Supplementary material. Supplementary material is available online at:
https://doi.org/10.6084/m9.figshare.9374792

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Submitted: December 29, 2018. Final revision received: July 5, 2019. Accepted: August 2, 2019.
Associate Editor: Jonathon C. Marshall.

Appendix 1

Voucher specimens of *Micrurus corallinus* analyzed in this study (n = 52): Museu de Zoologia da Universidade Estadual de Campinas (ZUEC; n = 7). Museu de História Natural Capão da Imbuia (MHNCI; n = 6): Museu Nacional/UFRJ (MNRJ; n = 18), Coleção Herpetológica da Universidade Federal de Minas Gerais (CHUFMG; n = 3), Coleção Herpetológica da Universidade Federal de Santa Catarina (CHUFSC; n = 4), Museu de Ciências e Tecnologia da Pontifícia Universidade Católica do Rio Grande do Sul (MCT-PUCRS; n = 14). *Micrurus frontalis* analyzed in this study (n = 24). Universidade federal de Goiás (UFG; n = 1), Coleção Herpetológica da Universidade Federal de Minas Gerais (CHUFMG; n = 8), Universidade Federal de Ouro Preto (UFOP; n = 1), Coleção Herpetológica do Instituto Butantan (IB; n = 2), Museu de Zoologia da Universidade Estadual de Campinas (ZUEC; n = 7), Pontifícia Universidade Católica de Minas Gerais (PUC-MG; n = 3), Universidade Federal do Mato Grosso do Sul (UFMS; n = 2).