

Reproductive strategies of the semifossorial snake *Atractus zebrinus* from the Atlantic Forest

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ABSTRACT

Secretive snakes (*e.g.*, fossorial or semifossorial) show morphological adaptations that may constrain potential reproductive output. However, females can store sperm in the reproductive tract, increasing their reproductive potential. Thus, studies on the reproduction of fossorial and semifossorial species are of great interest for a complete understanding of snake reproductive strategies. Here, we investigate the reproductive strategies of the semifossorial snake *Atractus zebrinus* through macroscopic and histological analyzes of the male and female reproductive tracts. Females were larger and had more ventral scales than males, the most common pattern in snakes, and apparently conserved in the genera. The female reproductive cycle was seasonal, with gravid females occurring in spring and summer. Rainfall seems to be the major factor driving female reproductive seasonality, and sperm storage in the infundibular receptacles may ensure fertilization and multiple paternity. The male reproductive cycle was aseasonal. Spermiation, hypertrophy of the sexual segment of the kidney, and sperm storage in the ductus deferentia occurred over several months. This aseasonal reproduction in males may enhance paternity by increasing the species mating chances.

Key words: Female sperm storage; Reproductive cycle; Secretive snakes; Sexual dimorphism; Spermiogenesis.

RESUMO

Las serpientes fosoriales y semifosoriales presentan adaptaciones morfológicas que pueden limitar su reproducción. Sin embargo, las hembras pueden almacenar espermatozoides en el tracto reproductivo aumentando su potencial reproductivo. Por lo tanto, estudios sobre la reproducción de especies fosoriales y semifosoriales son de gran interés para la comprensión de las estrategias reproductivas de las serpientes. En este trabajo, investigamos las estrategias reproductivas de la serpiente semifosorial *Atractus zebrinus* a través de análisis macroscópicos y cortes histológicos de los tractos reproductivos de machos y hembras. Como resultado, encontramos que las hembras fueron más grandes y tenían más escamas ventrales que los machos. Por otro lado, el ciclo reproductivo de las hembras fue estacional, con hembras grávidas en primavera y verano. La lluvia parece ser el factor principal que influencia la estacionalidad reproductiva de las hembras, y el almacenamiento de espermatozoide en los receptáculos infundibulares podría asegurar la fertilización y la paternidad múltiple. Además, el ciclo reproductivo de los machos no fue estacional. En varios meses, se encontraron procesos de espermiación, hipertrofia del segmento sexual del riñón y almacenamiento de semen en el conducto deferente. Esta reproducción no estacional en los machos puede aumentar las posibilidades de apareamiento de la especie.

Palavras-chave: Almacenamiento de espermatozoide femenino; Ciclo reproductivo; Serpientes secretivas; Dimorfismo sexual; Espermiogénesis.

Introduction

Reproductive traits may vary substantially in closely related snake species (Vitt, 1992; Pizzatto *et al.*,

2008). To achieve reproductive success, the timing of mating and gametogenesis can occur in diffe-

rent periods in congeneric species (Aldridge and Duvall, 2002; Pizzatto and Marques, 2006; Barros *et al.*, 2012). Temperature and rainfall are the main abiotic factors influencing the reproductive cycles in snakes and, therefore, responsible for modulating the seasonality of cycles (Aldridge and Duvall, 2002; Lutterschmidt and Mason, 2009). Thus, reproductive variation between congeneric species is associated with geographical distribution and climate (Aldridge and Duvall, 2002, Pizzatto and Marques, 2006; Barros *et al.*, 2012). Generally, temperate-zone snakes have strictly seasonal reproductive cycles, while tropical snakes have extended reproductive cycles (Saint-Girons, 1982; Bizerra *et al.*, 2005; Mathies, 2011; Silva *et al.*, 2019a).

Most Dipsadidae (*sensu* Zaher *et al.*, 2019) reproduce seasonally, with males presenting spermatogenesis peaks in spring and summer (*e. g.* rainy season; Rojas *et al.*, 2013; Braz *et al.* 2014; Braz *et al.*, 2019), and females showing gravidity/pregnancy and oviposition/parturition mainly in the rainy season (summer) (Pizzatto and Marques, 2002; Pizzatto *et al.*, 2008; Mathies, 2011; Bellini *et al.*, 2013; Braz *et al.* 2014; De Resende and Nascimento, 2015; Loebens *et al.*, 2016; Teixeira *et al.*, 2020). However, relatively few dipsadids have been studied, particularly those with fossorial habits (Braz *et al.*, 2014; Braz *et al.*, 2019; Gualdrón-Durán *et al.*, 2019). This paucity of information probably reflects their secretive nature and the scarcity in zoological collections, even highly biodiverse regions (Martins and Oliveira, 1993; Böhm *et al.*, 2013).

Adaptations to underground life may increase a species' vulnerability to environmental fluctuations and extinction (Cyriac and Kodandaramaiah, 2018; Braz *et al.*, 2019). Evolutionary adaptations to fossorial/semifossorial habitats may require a series of physiological and morphological modifications, such as changes in thermal ecology, head shape, and body shape (Pough, 1980; Navas *et al.*, 2004; Barros *et al.*, 2011; Abegg *et al.*, 2020; Khouri *et al.*, 2020). Consequently, the physiological and morphological constraints imposed by fossoriality may influence a range of life-history traits, such as clutch size and egg size (Marques, 1996; Marques and Puerto, 1998; Balestrin and Di-Bernardo, 2005; Braz *et al.*, 2014; De Resende and Nascimento, 2015; Braz *et al.*, 2019). Thus, studies on the reproduction of fossorial or semifossorial snakes are especially relevant for understand possible reproductive adaptations in species with these habits.

The genus *Atractus* is highly diverse, grouping about 150 species distributed from the eastern region of the Isthmus of Panama to northern Argentina (Fernandes and Puerto, 1993; Girauo and Scrocchi, 2000; Myers, 2003; Passos *et al.*, 2018). Of these, 39 species occur in Brazil (Costa and Bérnils, 2018). *Atractus zebrinus* (Jan, 1862) inhabits the Atlantic Forest, with some records at high elevations in the Araucaria Forest (Passos *et al.*, 2010a; Barbo *et al.*, 2011; Nogueira *et al.*, 2019). There are few ecological data available for this species in the literature (Marques *et al.*, 2019). Like other congeners, *A. zebrinus* exhibits semifossorial habits, probably nocturnal activity, and females are bigger than males (Fernandes *et al.*, 2000; Marques *et al.*, 2019). However, there is virtually no reproductive data of the species. Herein, we use macroscopic and histological data of male and female reproductive tracts of *A. zebrinus*, to describe the species reproductive cycle. We additionally provide detailed information about the sexual segment of the kidney (SSK), sexual maturity, sexual dimorphism, and clutch size. We also discuss our results for *A. zebrinus* by comparing its reproductive pattern to that reported for another dipsadid species of *Atractus* genus.

Materials and methods

Specimens and area

We examined 38 preserved specimens of *A. zebrinus* housed in museums (Appendix 1). Our sample consisted of 20 females (14 adult and six subadult specimens) and 18 males (13 adults and five subadults) collected in the Atlantic Forest areas of the states of Espírito Santo, Minas Gerais, São Paulo, and Paraná (Southeastern and Southern Brazil; between S 27°22'23", W 51°54'14" and S 22°7' 3", W 45°3'6"; 440-1029 m above sea level). The climate in this region is seasonal, with warmer temperatures occurring from spring (October–December) to summer (January–March) and are associated with higher rainfall, whereas lower temperatures occur from autumn (April–June) to winter (July–September) and are generally associated with lower rainfall (Mendonça and Danni-Oliveira, 2007).

Morphological data

We collected morphological data of adult individuals to test for sexual dimorphism. Before performing the dissections, we measured snout-vent length (SVL) and tail length and counted the number of ventral

and subcaudal scales of each specimen following Dowling *et al.*, (1951). Females were considered sexually mature (adult) if they had spermatozoa in the reproductive tract, however indirect evidences were also used, as ovarian follicles bigger than 5 mm length (De Resende and Nascimento, 2015), oviductal eggs, corpora lutea, or folded oviducts (indicative of recent egg-laying; Almeida-Santos *et al.*, 2014; Silva *et al.*, 2020). Males were considered adults if they had spermatozoa in the reproductive tract (Almeida-Santos *et al.*, 2014; Silva *et al.*, 2020). In adult females, we recorded the number of ovarian follicles and oviductal eggs and the diameter of the largest ovarian follicle and egg (Almeida-Santos *et al.*, 2014). In adult males, we recorded the diameter of the right distal ductus deferentia (between the kidney and the cloaca) and the length, width, and thickness of the right testis. The organs were measured using a digital caliper (± 0.1 mm).

Histology

For histological data, only adults were used. For females ($N = 14$), we excised the posterior portions of the right nonglandular uterus, right posterior infundibulum, and pouch (nomenclature of Siegel *et al.*, 2011) to check for the presence of spermatozoa. We also collected ovarian follicles from each individual to identify highly vacuolated ooplasm (vitellogenic follicle), the last stages that precedes the ovulation (Manes *et al.*, 2007; Vieira *et al.*, 2010).

For males ($N = 13$), we collected the mid-region of the testes, the distal ductus deferentia (the portion between the kidney and the cloaca), and the proximal region of the kidneys (Rojas *et al.*, 2013). Tissue samples were stored in 70% ethanol, dehydrated, and embedded in paraffin. We used a rotary microtome to produce transverse and longitudinal sections ($5 \mu\text{m}$) and stained the slides with hematoxylin-eosin (Junqueira and Carneiro, 2013). Testis and ductus deferens sections were assessed for sperm production and presence, respectively. Kidney sections were examined to identify SSK hypertrophy. We classified spermatogenesis according to the six cell stages presented by Silva *et al.*, (2019a). However, we only observed the following three stages: spermiogenesis (metamorphosing spermatids), spermiation (mature spermatozoa in the lumen) and early regression (reduced germinal epithelium, with few spermatogonia, spermatocytes, and spermatids). For each specimen, we measured the height of the seminiferous epithelium height, seminiferous tubule

diameter, SSK epithelium height, and SSK tubule diameter (Rojas *et al.*, 2013) using ImageJ software v1.46 (Abramoff *et al.*, 2004).

Data analysis

A sexual size dimorphism (SSD) index was calculated as: (mean SVL of females/ mean SVL of males)-1. This index is arbitrarily expressed as positive if females are the larger sex and negative if males are the larger (Lovich and Gibbons, 1992). Morphological sexual dimorphism was tested only in adults. Before the analysis all variable was tested for normality of distribution using the Shapiro-Wilk's test. The only variable that showed deviation for normal distribution were SVL on females ($W = 0.71$, $p < 0.005$), and in this sense we accessed sexual variation in this trait using a Mann-Whitney test. Differences in the number of ventral and subcaudal scales was tested in using a Welch t-test. A previous Analysis of Covariance (ANCOVA) showed that TL does not increase with SVL ($F = 2.68$, $p = 0.11$, $N = 30$), therefore sexual variation in TL were also tested using a Welch t-test. We determined clutch size by counting the number of enlarged ovarian follicle at the beginning of the vitellogenesis process (>5 mm; potential clutch size) or oviductal eggs in preserved specimens. We investigated the relationship between clutch size and maternal SVL using Spearman's rank correlation test. We calculated testis volume (TV) in each season using the ellipsoid formula: $TV = (4/3)\pi(abc/2)$, where $a =$ length, $b =$ width, and $c =$ thickness (Pleguezuelos and Feriche, 1999). Also, a previous test showed that testis volume was not correlated with male SVL ($r = 0.31$, $p = 0.38$, $N = 10$), in this sense we present the raw data of the mean testicular volume. All analyses were performed using R Statistical Software version 4.0.2 (R Core Team, 2021).

Results

Sexual maturity, sexual dimorphism, and clutch size

Our smallest adult female was 423 mm SVL, while the smallest adult male was 315 mm SVL (Table 1). Adult females were larger ($U = 181$, $df = 28$, $p < 0.005$) and had more ventral scales ($t = 9.02$, $df = 24.8$, $p < 0.005$) than adult males (Table 1). However, males had relatively larger tails ($t = -2.86$, $df = 28$, $p = 0.008$) and more subcaudal scales ($t = -5.69$, $df = 19.2$, $p = 1.67e-5$) than females (Table 1). The SSD

Table 1. Variation in snout-vent length (mm), tail length (mm), and number of ventral scales and subcaudal scales in male and female adults of *Atractus zebrinus*. The results are expressed as the mean \pm standard deviation (SD) of the total sampling number and range of distribution.

Variable	Male			Female		
	Mean \pm SD	Range	N	Mean \pm SD	Range	N
Snout-Vent Length	386 \pm 58	315-487	13	463 \pm 55	423-621	14
Tail Length	49 \pm 9	40-68	13	41 \pm 8	31-54	14
Ventral Scales	146 \pm 4	140-153	13	159 \pm 4	153-166	13
Subcaudal Scales	26 \pm 4	21-34	13	19 \pm 2	16-23	13

Note: Due to the process of tissue autolysis, there was only one individual for histological measurements.

was 0.20. Clutch size averaged 6 ± 3 eggs/vitellogenic follicles (range = 3-11, N = 8) and was not correlated with female SVL ($r = 0.48$, $p = 0.22$, Fig. 1A).

Female reproductive cycle and sperm storage

Females in early vitellogenesis (follicles ranging from 5.0-8.5 mm) were observed from October to March, except in November (Figs. 1B, and Fig. 2A-B). We did not find ovarian follicle in the last stages of follicle maturation before ovulation. Gravid females were observed in October (N = 1), November (N = 2), December (N = 1) and January (N = 1) (Figs. 1B, 3). In July 1992, a clutch of six eggs (ZUEC-REP 1407) was found buried (~10 cm depth) in a pile of pine splinters (*Pinus elliotti*) at Parque Estadual de Campos do Jordão, state of São Paulo (southeastern Brazil). Eggs were collected and fixed and housed in zoological collection. Dissections revealed fully formed *A. zebrinus*, which were close to hatching. One of the individuals was measured (140 mm SVL and 10 mm tail length) and weighed (3 g body mass). Recruitment period (hatchlings with 140-168 mm SVL; N = 5) occurred in October, November, December, and January (Fig. 3).

A female in early vitellogenesis (follicle size = 5 mm) collected in late summer (March) exhibited sperm stored inside the alveolar glands of the posterior infundibulum (Figs. 4A, B). No female had sperm in the nonglandular uterus (Fig. 4C) and the pouch (Fig. 4D). The epithelium lining the posterior infundibulum had ciliated and secretory cells, which were cuboidal to columnar. The outer wall of the nonglandular uterus and the pouch consisted of two layers of smooth muscle: an inner circular layer and an outer longitudinal layer (Fig. 4D). The epithelium lining the nonglandular uterus was pseudostratified, and presented many ciliated cells (Fig. 4C). The pouch had a thick lamina propria with folds, and the epithelium was pseudostratified columnar (Fig. 4D).

Male reproductive cycle

Adult males were collected in February (N = 3), March (N = 2), April (N = 1), May (N = 1), July (N = 1), September (N = 1), and November (N = 4). Males had testes in spermiogenesis (Fig. 5a) or spermiation (Fig. 5b) in all seasons (Fig. 3). In au-

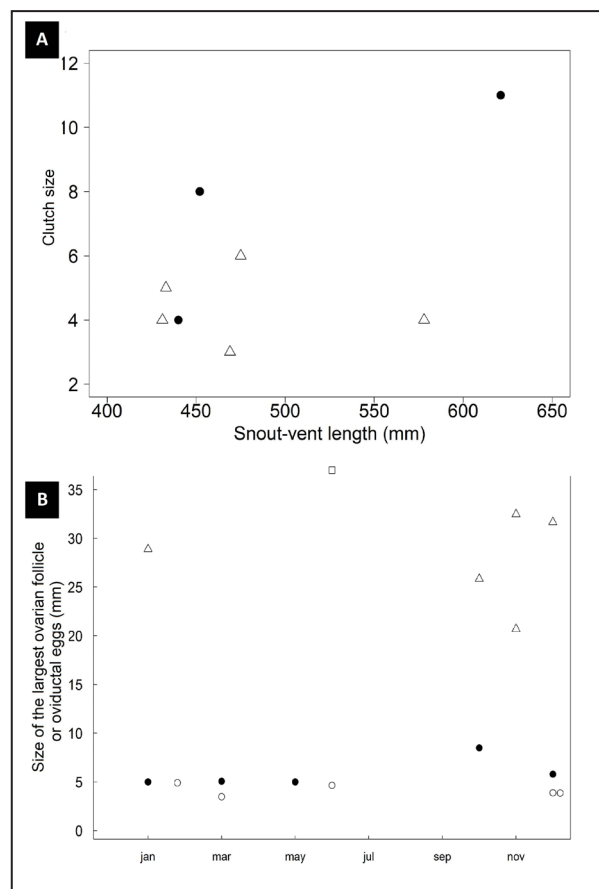


Figure 1. Reproductive traits of female *Atractus zebrinus*. (a) Relationship between maternal snout-vent length and clutch size. (b) Seasonal variation in the size of the largest ovarian follicles/oviductal eggs and timing of eggs found in nature. Note: Black circles = follicles in early vitellogenesis; White triangles = oviductal eggs; White circles = previtellogenic follicles; White square = eggs in nature.

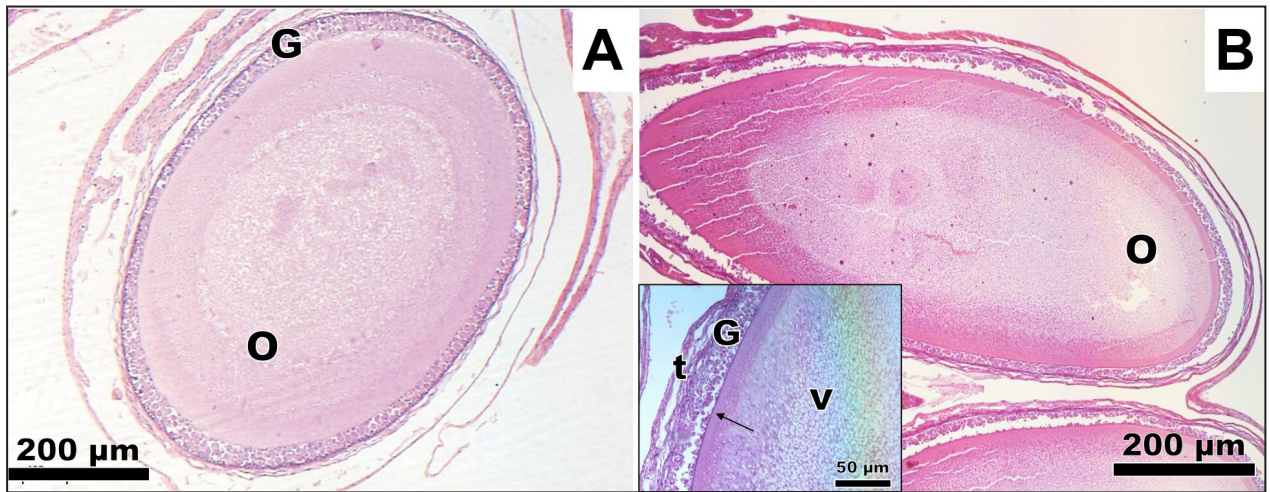


Figure 2. Histology of the ovarian follicles of *Atractus zebrinus* at the onset of vitellogenesis. (a) 5 mm diameter follicle. (b) 8.5 mm diameter follicle. Note the vacuolated ooplasm: G = Granulosa layer; V = vesicles; O = oocyte; t = theca. The arrow indicates the zona radiata. Hematoxylin-eosin.

tumn, males had testes in spermiogenesis in April (N = 1) and spermiation in May (N = 1). In late winter (September), the only individual examined had testes in spermiation. In spring (November), males (N = 4) had testes in spermiogenesis or spermiation; only one male had regressed testes, although some spermatids were still present. In summer (February and March), all males (N = 4) had testes in spermiation. All males showed hypertrophied SSKs (Figs. 3, 5C) and ductus deferentia packed with sperm (Figs. 3, 5D). Table 2 shows the variation in testicular volume, seminiferous epithelial height, seminiferous tubule diameter, SSK diameter, and SSK epithelial height. Unfortunately, the sampling per season was too small to test for seasonal differences statistically.

Discussion

Sexual dimorphism, sexual maturity, and clutch size

Our finding of female-biased SSD agrees with previous results reported in this species (Fernandes *et al.*, 2000). Female-biased SSD is consistent with size-related fecundity selection and the absence of male-male combat (Shine, 1994). This SSD pattern and maturity at larger sizes in females are common in *Atractus* (Balestrin and Di-Bernardo, 2005; De Resende and Nascimento, 2015; Zanella and D'Agostini, 2018; Ferreira-Silva *et al.*, 2019; Abegg *et al.*, 2020). In all species studied so far, females are larger than males, and male-male combat is absent, suggesting that the female-biased SSD is phylogenetically conserved. Additionally, female *Atractus* have more

ventral scales and fewer subcaudal scales than males (Passos *et al.*, 2010b). In snakes, the number of ventral and subcaudal scales is often correlated with the number of vertebrae and body size (Arnold, 1988; Lindell *et al.*, 1993). Because female snakes commonly have more body vertebrae and fewer caudal vertebrae than males (Shine, 1993), our results on sexual dimorphism in scalation may be a function of sexual variation in the number of vertebrae.

Male *A. zebrinus* show relatively larger tails than females, a pattern similar to that observed in other *Atractus*, except *A. paraguayensis* (Balestrin and Di-Bernardo, 2005; Zanella and D'Agostini, 2018; Ferreira-Silva *et al.*, 2019; Abegg *et al.*, 2020). The morphological constraint hypothesis states that the male tails are longer to accommodate the hemipenis and associated muscles. King, (1989)

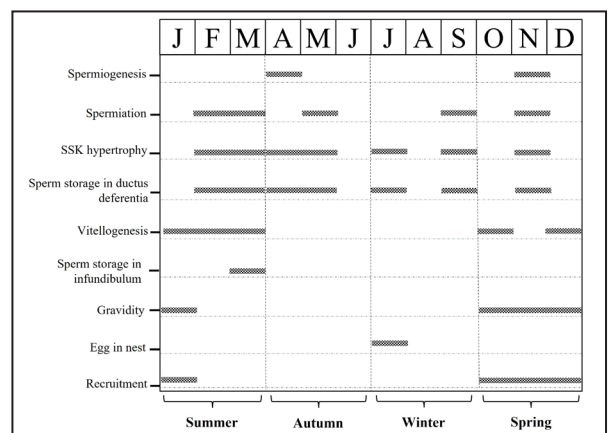


Figure 3. Overview of the reproductive events in male and female *Atractus zebrinus*.

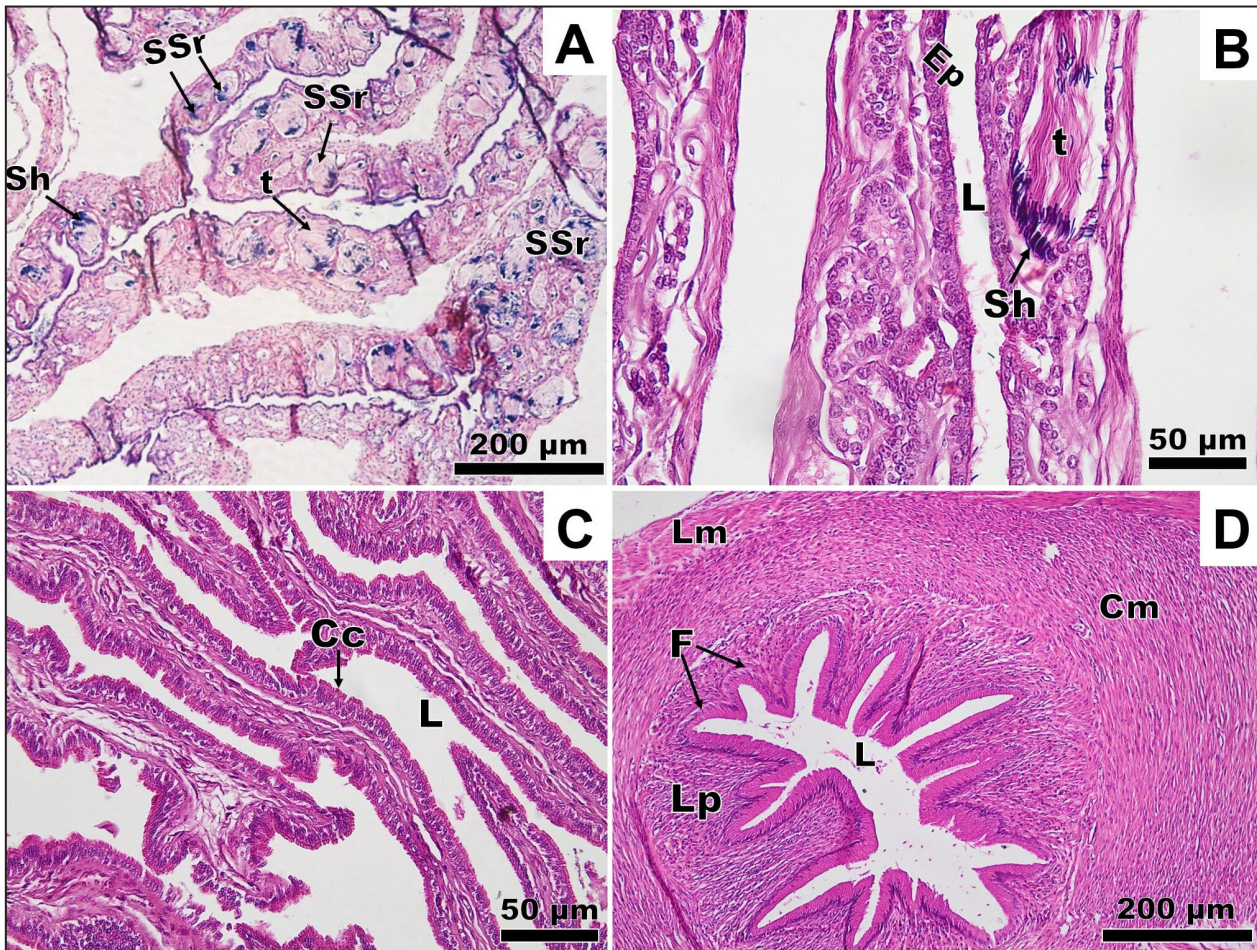


Figure 4. Histology of the reproductive tract of female *Atractus zebrinus*. (a) Posterior infundibulum showing various alveolar glands with sperm. (b) Higher magnification of sperm receptacles. (c) Nonglandular uterus. (d) Pouch showing thick epithelium. *Note:* SSr = Sperm storage receptacles; Sh = sperm head; t = sperm tail; L = lumen; Ep = luminal epithelium; Cc = ciliated cells; F = folds; Lp = lamina propria; Cm = circular muscle; Lm = longitudinal muscle. Hematoxylin-eosin.

found strong support for this hypothesis after testing it using 104 colubrid species. On the other hand, Shine *et al.* (1999) provide evidence that sexual selection may also occur in species that form mating aggregations, as males with intact tails may be until three times more successful in mating. Because mating aggregation has been observed in at least one *Atractus* species (*A. marthae*: Meneses-Pelayo and Passos, 2019), sexual selection could also explain the sexual dimorphism in the tail length of *A. zebrinus*, although, field observations are needed in order to provide evidence for this hypothesis.

Sexual maturation at smaller body sizes in males is also common in snakes (Shine, 1994). Larger females are often more fecund, mainly because a larger celomic cavity allows for larger clutches/litters and offspring (Rivas and Burghardt, 2001). Thus, it is advantageous for females to reach sexual maturity at larger body sizes than males (Shine, 1978). However,

the lack of correlation of body size and clutch size suggests that fossorial habits probably constrain clutch size, reducing clutch size variance (Braz *et al.*, 2014; Braz *et al.*, 2019). Alternatively, the small sampling of gravid females may have affected the power of the analysis.

Clutch size of *Atractus* may be more strongly influenced by other factors, such as abiotic/biotic factors (*e.g.*, climate or prey availability, Seigel and Fitch, 1985, Barros *et al.*, 2014), life-history traits (*e.g.*, habitat use), and phylogeny (Zanella and D'Agostini, 2018). Although clutch size increases with maternal body size in some species (*A. pantostictus*: De Resende and Nascimento, 2015), this relationship does not occur in others (*e.g.*, *A. paraguayensis*: Zanella and D'Agostini, 2018; this study). The clutch size of *A. zebrinus* (3-6, considering only eggs) is similar to that of various similar-sized congeners such as *A. pantostictus* (2-4 eggs),

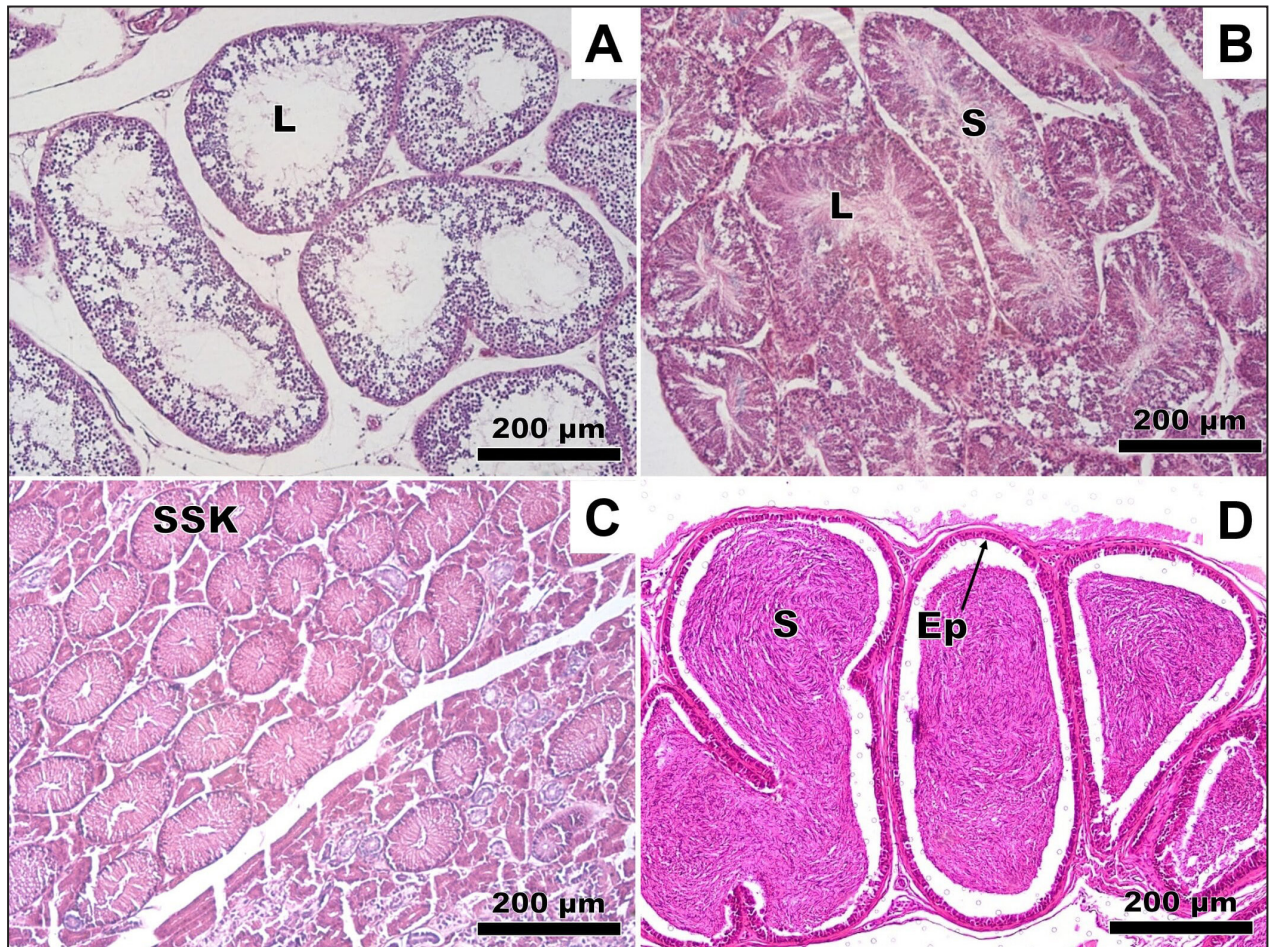


Figure 5. Histology of the reproductive tract of male *Atractus zebrinus*. (a) Seminiferous tubules in spermiogenesis. (b) Seminiferous tubules in spermiation, peak of cell division. (c) Hypertrophy of the sexual segment of the kidney. (d) Ductus deferens with the lumen packed with sperm. Note: L = lumen; S = spermatozoa; SSK = sexual segment of the kidney; Ep = epithelium. Hematoxylin-eosin.

A. reticulatus (1-3 eggs), *A. carrioni* (3-5 eggs), *A. paraguayensis* (3-8 eggs), *A. schack* (8 eggs), and *A. francoi* (6 eggs), but it is smaller than the larger ones (e.g., *A. gigas*; 12 eggs) (Martins and Oliveira, 1993; Balestrin and Di-Bernardo, 2005; Passos *et al.*, 2010; Barbo *et al.*, 2011; Passos *et al.*, 2013; De Resende and Nascimento, 2015; Zanella and D'Agostini, 2018; Marques *et al.*, 2019).

Reproductive cycles

The reproductive cycle of female *A. zebrinus* is seasonal, with vitellogenesis, sperm storage, and gravidity occurring in the rainiest seasons (spring and summer). Gravidity is the best metric to classify reproductive cycles, as the completion of reproduction (oviposition) is relatively imminent, and the length of time a female is ovigerous may not be as variable as the time it is vitellogenic, which may vary among individuals due to many factors, such as hormonal levels or body condition (Mathies, 2011).

Oviductal eggs, oviposition and recruitment occur mainly in spring and summer in *Atractus carrioni* (Passos *et al.*, 2013), *A. reticulatus* (Fernandes and Puerto, 1993; Balestrin and Di-Bernardo, 2005), *A. paraguayensis* (Zanella and D'Agostini, 2018), *A. pantostictus* (Travaglia-Cardoso and Maia, 2012; De Resende and Nascimento, 2015), and *A. ronnie* (Ferreira-Silva *et al.*, 2019). Therefore, the reproductive cycle of female *Atractus* is completed within the period of high rainfall. However, gravidity appears to occur during the months of lower rainfall in several Amazonian *Atractus* (Martins and Oliveira, 1993). Further studies are needed to understand the environmental influence on the type of reproductive cycle in *Atractus*.

The reproductive cycle of female oviparous snakes is often heavily influenced by rainfall, with oviposition peaking in the months of lower rainfall to ensure hatching success (Brown and Shine, 2006). In contrast, oviposition in *Atractus* occurs during

Table 2. Variation in testicular volume (mm³) and microscopic measurements (µm) of the urogenital structures of *Atractus zebrinus*. The results are expressed as the mean ± standard deviation (SD).

	Summer (N = 5)	Autumn (N = 2)	Winter (N = 2)*	Spring (N = 4)
Volume testicular	72 ± 14 (61-87)	126 ± 114 (45-207)	130 ± 32 (107-153)	198 ± 112 (116-326)
Seminiferous tubule diameter	201 ± 22 (187-233)	252 ± 57 (212-292)	115	192 ± 86 (91-267)
Seminiferous epithelial height	58 ± 14 (39-72)	66 ± 3 (64-68)	28	53 ± 16 (28-64)
Sexual segment of kidney (SSK) diameter	142 ± 36 (83-177)	98 ± 3 (97-101)	82	104 ± 51 (65-162)
SSK epithelial height	64 ± 15 (38-76)	41 ± 4 (37-43)	34	43 ± 21 (25-66)

Note: Due to the process of tissue autolysis, there was only one individual for histological measurements.

the rainiest months (December–March; Fernandes and Puerto, 1993; Martins and Oliveira, 1993; Sandoval *et al.*, 2009; Travaglia-Cardoso and Maia, 2012; Ferreira-Silva *et al.*, 2019). For *Atractus zebrinus*, although the finding of eggs in nest (July) and neonates (October-January), it was not possible to identify the oviposition period. This is because, the incubation period of *Atractus* eggs is quite variable (2 to 8 months - Balestrin and Di-Bernardo, 2005; Travaglia-Cardoso and Maia, 2012; Fernandes and Puerto, 1993) and come from captivity, in which they may not reflect the climatic conditions in nature.

In *A. pantostictus*, mating occurs in the rainy season – spring and summer (De Resende and Nascimento, 2015). In *A. marthae*, a mating aggregation was recorded in April, when rainfall peaks (Meneses-Pelayo and Passos, 2019). The mating season of *A. zebrinus* remains unknown, but it probably occurs in the rainy season (spring and summer), when we found receptive vitellogenic females (estrus, DeNardo and Taylor, 2011) and sperm storage (in late summer). Sperm storage until the follicles reach ovulatory size is crucial to ensure fertilization. In addition, estradiol and testosterone levels are elevated in females during vitellogenesis (DeNardo and Taylor, 2011; Taylor *et al.*, 2004). While estradiol stimulates vitellogenesis, testosterone can stimulate receptivity in female (DeNardo and Taylor, 2011).

To ensure fertilization and, in some cases, multiple paternity (Friesen *et al.*, 2020), females of *A. zebrinus* store sperm in the posterior infundibulum in the beginning of the vitellogenesis, a strategy recently reported in the genus (Gualdrón-Durán *et al.*, 2019). Sperm has also been reported in the nonglandular uterus of *Atractus pantostictus* as a result of

recent mating (De Resende and Nascimento, 2015). In snakes, female sperm storage occurs mainly in the uterus and infundibulum (Siegel *et al.*, 2011). As in other snakes (Siegel *et al.*, 2011), the spermatozoa aggregated in the alveolar glands are positioned in a parallel alignment, with the acrosomes orientated towards the epithelium of the lamina propria of the posterior infundibulum. This arrangement is often suggested to increase sperm survival (Siegel *et al.*, 2011; Rojas *et al.*, 2015; Rojas *et al.*, 2017).

The morphology of the infundibular glands (sperm receptacles) seems to be phylogenetically conserved among snake families, consisting of alveolar glands in dipsadids (Perkins and Palmer, 1996; Rojas *et al.*, 2015) and tubular glands in viperids (Saint-Girons, 1957; Siegel and Sever, 2008; Silva *et al.*, 2019b). Sperm storage may be advantageous for reproductive success by allowing sperm competition and multiple paternity (Uller and Olsson, 2008; Friesen *et al.*, 2020). In the dipsadid *P. patagoniensis*, gravid and postpartum females remain with sperm stored in the nonglandular uterus, suggesting the ability to produce several clutches from a single mating (Rojas *et al.*, 2015; Loebens *et al.*, 2016). If mating ball (Crews and Garstka, 1982) is a common reproductive strategy in *Atractus*, sperm storage in the female reproductive tract would be extremely advantageous for sperm competition and multiple paternity. However, mating order effects and other mechanisms that might confer cryptic female choice in snakes with long-term sperm storage (up to 6 years) remains unknown (Levine *et al.*, 2021).

Although our sampling of males may be considered small for statistical analyses (N=13), all individuals except one showed seminiferous tubules

with epithelium filled with spermatids and/or lumen filled with sperm. These records were registered between the months of February and November, and when considering the seasons, they can be classified as happening during: mid to late summer, early to mid-autumn, late winter, mid-spring. This pattern can be characterized as aseasonal at the population level (Mathies, 2011). The one individual that was not in the spermatogenesis peak showed a reduction in activity in November (mid-spring), which may indicate that the cycles of individual males are continuous cyclical (Mathies, 2011). Thus, our results indicated sperm was produced over a long period, what apparently also occurs in *Atractus marthae* (Gualdrón-Durán *et al.*, 2019).

The reproductive cycle of male *Atractus* has been studied histologically only in *A. marthae* (Gualdrón-Durán *et al.*, 2019). In this species, males have an aseasonal reproductive cycle, with sperm production year-round (Gualdrón-Durán *et al.*, 2019). *Atractus* is taxonomically classified in the subfamily Dipsadinae (Grazziotin *et al.*, 2012), and other studies have already reported dipsadines presenting an aseasonal reproductive cycle for males (Angarita-Sierra and López-Hurtado, 2020; Alves *et al.*, 2005). The arboreal *Dipsas neivai* and *D. catesbyi*, although not confirmed by histological analyses, present constant testes length throughout the year (Alves *et al.*, 2005). Similarly, Angarita-Sierra and López-Hurtado (2020) and Goldberg (2004) suggested that the semifossorial *Ninia atrata* and *Ninia maculata* shows an aseasonal spermatogenesis or a prolonged reproductive cycle. Finally, the aquatic snake (*Helicops pastazae*), males have an aseasonal cycle at the population level (García-Cobos *et al.*, 2021). These findings indicate that aseasonal reproduction is a fairly common strategy among dipsadids.

Among dipsadids with terrestrial habits, the male reproductive cycle has been studied in detail in *Dipsas mikanii* (Rojas *et al.*, 2013), *Philodryas patagoniensis* (Loebens *et al.*, 2017), and *Tomodon dorsatus* (Loebens *et al.*, 2020). In these species, the cycle of individual males is strictly seasonal, with spermatogenesis and SSK hypertrophy peaking in summer (Rojas *et al.*, 2013) or spring and summer (Loebens *et al.*, 2017). Thus, semifossorial snakes such as *A. zebrinus* and *A. marthae* (Gualdrón-Durán *et al.*, 2019) may have more plastic or asynchronous reproductive cycles to ensure paternity, when meeting a receptive female. However, individual

males of the fossorial snake *Phalotris lativittatus* exhibit a discontinuous cyclical reproductive cycle, and a spermatogenesis peak in spring (Braz *et al.*, 2014), indicating that abiotic (*e.g.*, climate) or biotic (*e.g.*, diet) factors may modulate the reproductive cycles of dipsadids. While *P. lativittatus* occurs in the Brazilian Cerrado and feeds on amphisbaenids (Braz *et al.*, 2014), *A. zebrinus* occurs in the Atlantic Forest and feeds on annelids (Marques *et al.*, 2019). Biotic factors (*e.g.*, predation risk and food supply) may influence the extension of the reproductive season (Crews and Moore, 1986).

In squamates, SSK hypertrophy has been associated with mating or spermatogenesis (Schuett *et al.*, 1997; Krohmer, 2004; Sever and Hopkins, 2005; DeNardo and Taylor, 2011). The SSK secretory granules are deposited in the female reproductive tract during mating and may contribute to the seminal composition and formation of a copulatory plug (Aldridge *et al.*, 2011; Friesen *et al.*, 2013). In all male *A. zebrinus*, the SSK hypertrophy, spermiation, and the presence of sperm in ductus deferentia were synchronous and occurred in several months of the year. Therefore, male *A. zebrinus* could mate at various times of the year, depending on the signaling of the female (as observed in *A. marthae*, Gualdrón-Durán *et al.*, 2019).

In sum, the reproductive cycle of female *A. zebrinus* is seasonal, and females may potentially enhance their fertility and fitness through oviductal sperm storage and sperm competition. Moreover, male *A. zebrinus* may ensure their paternity due to their prolonged reproductive activity, increasing this way the species mating chances.

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Author Contributions

LHCS – Data collection, analysis and the manuscript writing; JLS - Data collection, analysis and the manuscript writing and KMPS - Data collection, analysis and the manuscript writing.

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Appendix 1– Voucher specimens of *Atractus zebrinus* analyzed in this study:

Museu Nacional (MNRJ): 18684; 18717. **Coleção Herpetológica Richard Alphonse Hoge, Instituto Butantan (IBSP):** 32367; 44049; 62537; 71747; 71952; 72872; 72904; 72905; 73735; 78563; 81405; 80487; 82070; 83995; 84373; 84374;

84982; 84983; 84984; 84985; 84986; 84987; 84988; 86384; 86462; 87639; 88762; 88763; 88764; 88765; 88766; 88767; 88769; 88768; 89731; 89799; 89896. **Museu de Zoologia da Universidade Estadual de Campinas (ZUEC):** REP 1407. **Museu de História Natural Capão da Imbuia (MNHCI):** 8331.

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