



## Reproductive ecology of the Amaral's Blind Snake *Trilepida koppersi* in an area of Cerrado in south-eastern Brazil

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Studies on reproductive biology have largely contributed to the understanding of snake ecology. However, detailed reproductive data are scarce for many groups, particularly blind snakes. Here, we describe the reproductive biology of *Trilepida koppersi* (Leptotyphlopidae), a widely distributed species in the savannas of south-central Brazil. We describe its macro- and microscopic reproductive anatomy, female reproductive cycle, potential clutch size, seasonal activity, and sexual dimorphism of a population from south-eastern Brazil. Males have plurilobulated testes. Spermiogenesis occurs in early spring (October), when gonads and kidneys show a textured surface, the sexual segment of the kidney is hypertrophied, and the *ductus deferentia* are opaque and packed with sperm. Females have only the right oviduct, which shows developed epithelium and uterine glands in spring. Mating likely occurs in spring (October–December), and females store sperm in infundibular receptacles until ovulation between late spring and early summer. Potential clutch size ranges from three to five eggs. Females grow larger than males. The synchrony between spermiogenesis and mating defines the male cycle as prenuptial, which is considered the ancestral state of Squamata. These results agree with the hypothesis of conservative parameters for the group.

**Keywords:** reproductive morphology, sexual dimorphism, Scolecophidia, clutch size, female sperm storage

### INTRODUCTION

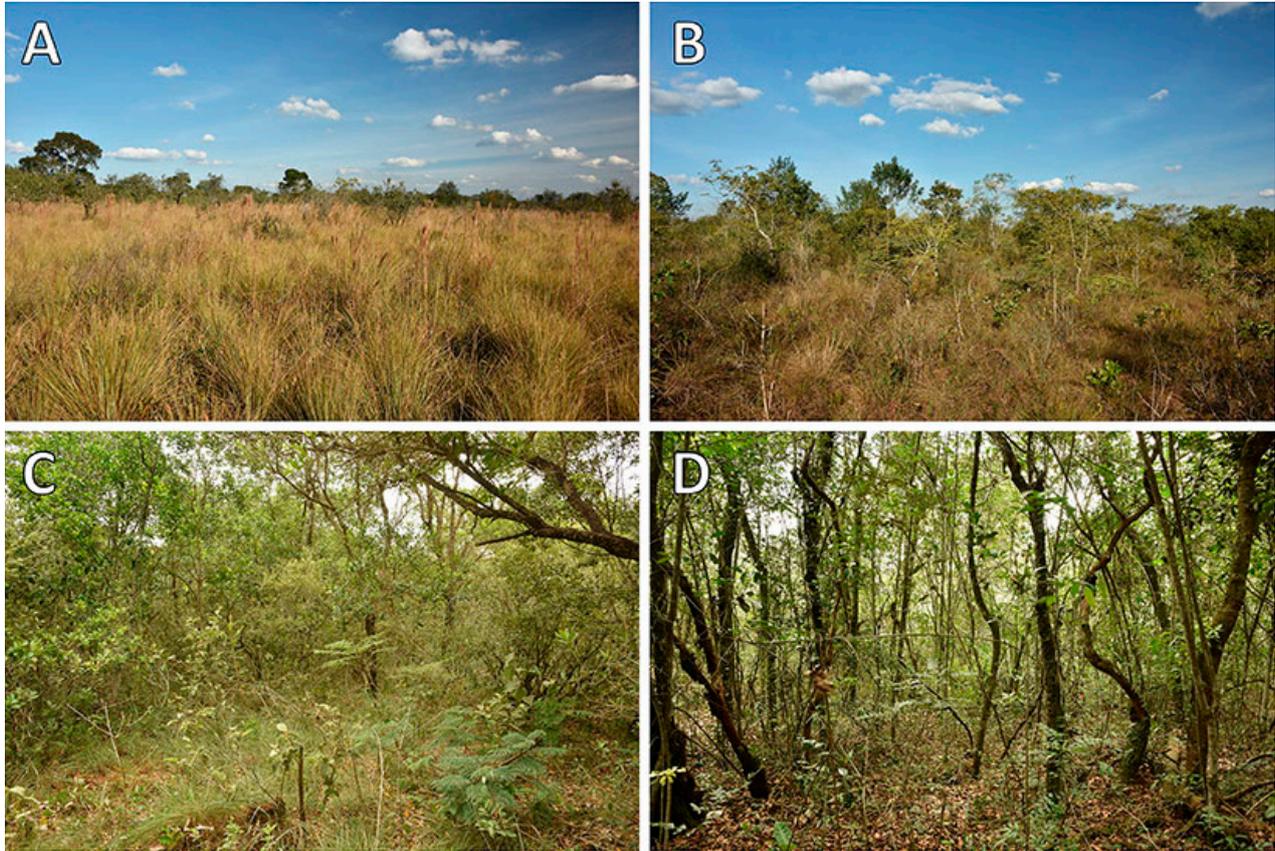
Studies on reproductive biology have largely contributed to the understanding of snake ecology, considering that a significant portion of snake activity is related to their reproductive cycle (Pizzatto et al., 2007a and 2007b; Nilson, 2011). However, anatomical and histological information about reproductive cycles remain scarce for several groups, particularly blind snakes (Scolecophidia) (Shea, 2001; Khouri et al., 2020). Few studies have addressed the reproductive anatomy of scolecophidians and how it is associated with their natural history (Khouri et al., 2020). Snakes usually exhibit a generalised anatomical pattern in reproductive organs, with a pair of oviducts and ovaries and a single or bifurcated vaginal pouch in females (Siegel et al., 2012), and a pair of testes, kidneys, and *ductus deferentia* in males (Trauth & Sever, 2011). However, some scolecophidians present important anatomical variations, such as the lack of the left oviduct (Fox & Dessauer, 1962; Khouri et al., 2020) and vaginal pouch (Siegel et al., 2011).

In addition to morphological traits, histological data

are also helpful for understanding key traits in snake reproductive biology (Almeida-Santos et al., 2014). For instance, female sperm storage is a major feature in snake reproduction, as it allows the temporal dissociation between copulation and ovulation and, consequently, the evolution of different reproductive phenologies (Sever & Hamlett, 2002; Siegel et al., 2011). Female sperm storage has been suggested to occur in many snake species (Birkhead & Møller, 1993). However, some of these suggestions (particularly those implying long-term sperm storage) may alternatively be cases of parthenogenesis (Booth & Schuett, 2011). True female sperm storage can be reliably demonstrated using standard histological techniques. Sperm storage in infundibular receptacles has been suggested to occur in some scolecophidians (Fox & Dessauer, 1962; Siegel et al., 2011), but it has only recently been confirmed (Khouri et al., 2020).

The leptotyphlopoid *Trilepida koppersi* (Amaral, 1955) is a typical species of the savannas of south-central South America (the Cerrado), where it is widely distributed (Passos et al., 2006; Nogueira et al., 2010; Nogueira et al., 2019). Although the species is known in several

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**Figure 1.** Natural vegetation types of Santa Bárbara Ecological Station where *T. koppesi* was sampled. **A)** *Campo sujo* (grassy scrubland). **B)** *Campo cerrado* (grassy scrubland with scattered trees). **C)** *Cerrado sensu stricto* (dense savanna). **D)** *Cerradão* (cerrado woodland).

localities (there are 41 occurrence records in the snake distribution database of Nogueira et al., 2019), its natural history remains poorly known (Sawaya et al., 2008). This species is believed to be oviparous, with females producing a relatively small clutch size (five to seven eggs) from spring (Sawaya et al., 2008). However, there is no detailed information about its reproductive anatomy and cycle. Furthermore, the habitat of *T. koppesi* (the open vegetation types of the Cerrado) has been increasingly degraded throughout its range (Sano et al., 2010; Bonanomi et al., 2019). Thus, knowing the reproductive biology of this species may also help in planning conservation actions. Anatomical descriptions of scolecophidians are still needed (Martins et al., 2019), and this knowledge may be helpful to group or separate evolutionary lineages (Passos et al., 2006; Pinto et al., 2015). Reproductive data also show both specific and general patterns that may contribute to our understanding of the evolution of snake reproduction in general (Nilson, 2011; Almeida-Santos et al., 2014).

We hypothesise that *T. koppesi* shares traits with other scolecophidian species, such as female-biased sexual size dimorphism and seasonal reproduction. As a member of a basal clade, we also hypothesise that *T. koppesi* shows particularities that might be useful to understand the evolution of the reproductive strategies of the ancestor of snakes. To explore our hypotheses, we provide novel information on the reproductive biology of a population

of *T. koppesi* in an area of Cerrado, in south-eastern Brazil. Specifically, we describe reproductive anatomy, female reproductive cycle, potential clutch size, seasonal activity, and sexual dimorphism.

## MATERIALS & METHODS

### Study Site

Snakes were collected at the Santa Bárbara Ecological Station (SBES), municipality of Águas de Santa Bárbara, state of São Paulo, south-eastern Brazil (approximate coordinates: 22°48' S, 49°13' W; 600–680 m a.s.l.). The SBES has a total area of 3,223 ha (Melo & Durigan, 2011) and contains different Cerrado vegetation types, from *campo sujo* (grassy scrubland) to *cerradão* (cerrado woodland; Fig. 1), and some small areas of semideciduous forests. The climate type is humid subtropical (Cwa in the Köppen's classification; Peel et al., 2007). Temperature averages 17 °C in the coldest months and 24 °C in the warmest months (range: 3.4 to 35.2 °C), with occasional frosts in autumn and winter. Annual rainfall varies from 1010 to 2051 mm (mean = 1454.2 mm), with marked dry and rainy seasons from autumn to winter (April to September; monthly mean = 70.2 mm) and from spring to summer (October to March; monthly mean = 172.1 mm), respectively (data for 1995-2014 at Manduri, SP, 20.3 km from our study area; CIIAGRO, <http://www.ciiagro.sp.gov.br/>).

### Fieldwork

We conducted fieldwork monthly from August 2016 to July 2018. Snakes were collected through time-constrained searches (Campbell & Christman, 1982; Martins & Oliveira, 1998), pitfall traps with drift fences (Greenberg et al., 1994; Cechin & Martins, 2000; Mendes et al., 2015), and opportunistic encounters (i.e., all snakes found in situations other than searching activities). Time-constrained searches occurred mainly at night (18.00–23.00) in open vegetation types. We performed 1,248 person-hours of searches in different vegetation types. We installed three units of pitfall traps with drift fences in four vegetation types: *campo cerrado*, *campo sujo*, *cerrado sensu stricto* and *cerradão* (Fig. 1). Each unit of pitfall traps corresponded to two 40 m trap lines, 60 m apart. Each trap line had four 100 L plastic buckets every 10 m, connected by a 60 cm-high plastic fence. The buckets were perforated at the bottom to avoid the accumulation of rainwater. The fence was buried 10 cm into the soil and attached to wooden stakes (see Cechin & Martins, 2000; Sawaya et al., 2008). To prevent dehydration of the captured animals, we placed a styrofoam (20 × 20 × 5 cm) supported by wooden sticks and a small water plate inside each bucket to provide shelter and moisture (Sawaya et al., 2008). We installed 12 trap units (24 lines, 96 buckets, and 960 m of fences) and kept them open for ten consecutive days every month. Thus, sampling by pitfall traps occurred during 240 non-consecutive days, corresponding to 23,040 bucket-days. Snakes were collected under SISBIO (50658-1) and COTEC (SMA nº 260108–011.518/2015) scientific collection permits. Specimens were euthanised by intracelomic injection of lidocaine, fixed in 10 % formaldehyde, and preserved in 70 % ethanol for later examination. Specimens were deposited in the Museu de Zoologia da Universidade de São Paulo and the Instituto Butantan (Appendix A).

### Macroscopic data

To obtain reproductive information, we dissected 24 specimens (10 females and 14 males) collected during fieldwork. The dissected females were collected in October 2016 (n = 5), December 2016 (n = 3), and February 2017 (n = 1). All dissected males were collected in October 2016. We sexed individuals by direct observation of the reproductive tract. Males were considered adults if they had enlarged testes, convoluted *ductus deferentia*, or sperm in the reproductive tract (Shine, 1977a). Females were considered adults if they had pleated oviducts, vitellogenic follicles, oviductal eggs, or sperm in the reproductive tract (Shine, 1977b). In males, we measured (1) the length, width, and thickness of the testes and the major lobule, (2) the length of the kidneys, and (3) the width of the *ductus deferentia* in the distal portion (close to the cloaca) using a Mitutoyo analogical caliper. We calculated testis volume per individual using the ellipsoid volume formula:  $V = (2/3)\pi abc$ , where a = length, b = width, and c = thickness (Pleguezuelos & Feriche, 1999). In females, we recorded (1) the gross morphology of the right infundibulum, glandular uterus, and nonglandular uterus, (2) the number of ovarian follicles, and (3) the diameter (at the longitudinal axis) of the largest follicle

(using a Mitutoyo manual caliper; to the nearest 0.02 mm). Because we found no gravid females (see Results), we estimated clutch size by counting the number of ovarian vitellogenic follicles (Almeida-Santos et al., 2014; Braz et al., 2019). Thus, we treated this estimate as potential clutch size. To test for sexual dimorphism, we measured snout-vent length (SVL), tail length, body mass, body width, head length (from the base of the maxilla to the tip of the snout), head height (from the base of the maxilla to the top of the head). We measured SVL using a ruler (to the nearest 1 mm), body mass using a Pesola spring scale (to the nearest 0.1 g), and tail length, head length, and head height using a Mitutoyo digital caliper (to the nearest 0.01 mm).

### Histological analyses

Reproductive structures were described following the nomenclature proposed by Siegel et al. (2011) and Trauth & Sever (2011). We collected samples of the right side of the reproductive tract for histological analysis. In males, we collected samples of the kidney, testis, and distal portion of the *ductus deferens*. The stage of the seminiferous tubules was classified according to Goldberg & Parker (1975). In females, we collected the oviduct and two different sized follicles to determine the vitellogenic stage. Tissue samples were processed for paraffin's standard method (Junqueira et al., 1979). Histological sections were cut at 5 µm using a Leica microtome, mounted on glass slides, and stained with hematoxylin and eosin. Histological sections were photographed and measured using Olympus Cell Sens Standard software and an Olympus BX51 microscope with a DP73 lens (Olympus Corporation, Japan). We estimated the seminiferous epithelial height, seminiferous tubule diameter, sexual segment of the kidney (SSK) epithelial height, and SSK diameter by taking five random measurements for each individual. Values for all measured variables were averaged to obtain a mean value per individual. We characterised female reproductive cycles (Mathies, 2011) and described male traits for the season in which they were collected.

### Data analysis

The annual activity pattern of *T. koppesi* was inferred from the frequency of adult males, adult females, and juveniles recorded monthly. A Sexual Size Dimorphism (SSD) index was calculated as the mean SVL of the larger sex divided by the mean SVL of the smaller sex minus one. This index is conventionally expressed as positive when females are the larger sex and negative when males are larger (Lovich & Gibbons, 1992). We tested for intersexual differences in mean adult SVL with a Student's t-test. We tested for sexual dimorphism in head length, head height, tail length, body width and body mass using analysis of covariance (ANCOVA), with SVL as the covariate. For these analyses, we used only the measures from dissected individuals to avoid bias due to sexing errors in non-dissected specimens. Data are provided in supporting information Data 1, and the R script used in the analysis is provided in supporting information Script 1.

## RESULTS

### Seasonal activity

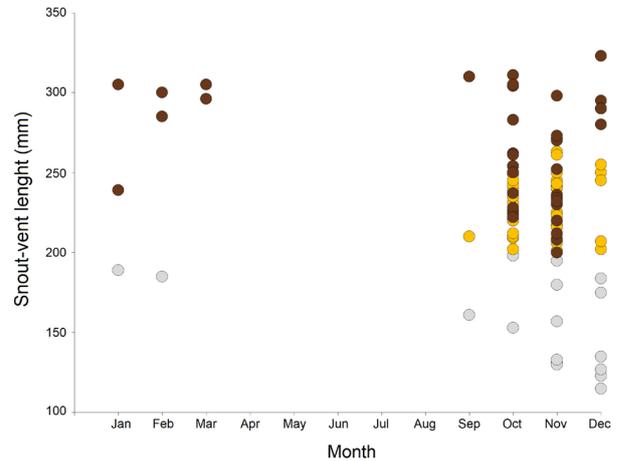
We found 121 individuals of *T. koppesi*. Most snakes (91 %) were found from late winter to late spring (September to December), and no snake was found between autumn and mid-winter (April to August; Fig. 2). Adult males were collected between late winter and late spring (September to December) but were more abundant in spring (97.67 %, October–December; Fig. 2). Adult females were collected between late winter and late summer (September to March) but were more abundant in spring (82.5 %, October–December; Fig. 2). Juveniles were collected between late winter and mid-summer (September–February) but were more abundant in spring (88.23 %, Fig. 2).

### Male reproductive tract

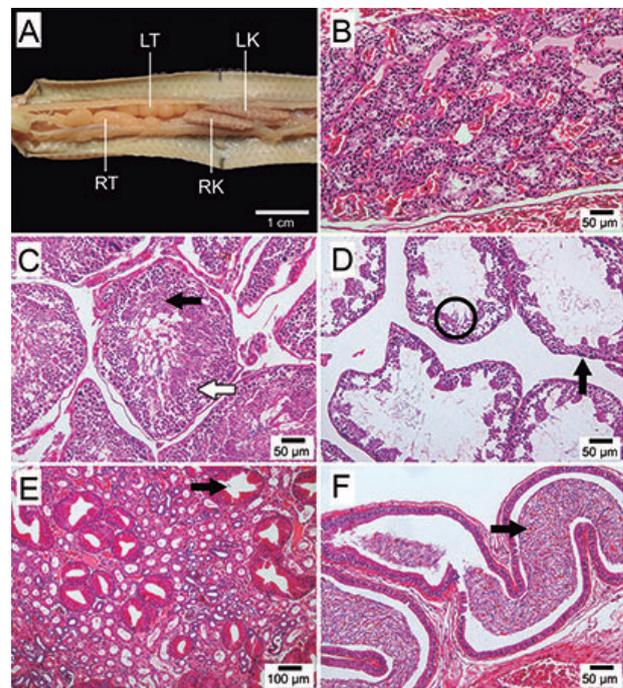
All dissected males were collected in October (early spring). Thirteen out of fourteen dissected males were sexually mature. All males had paired reproductive structures, with the right organs located more cranially than the left ones. The single juvenile male examined (SVL = 153 mm) had undeveloped gonads and smooth kidneys. All adult males had elongated, plurilobulated testes (with 3–7 lobes, supporting information Table S1) with slightly textured surfaces (Fig. 3A). The right testis volume in adults averaged  $40.53 \pm 10.25 \text{ mm}^3$  (range = 26.89–63.47  $\text{mm}^3$ ), and the left testis averaged  $40.23 \pm 14.13 \text{ mm}^3$  (range = 4.59–71.78  $\text{mm}^3$ ). The seminiferous tubules were either in spermiogenesis ( $n = 3$ , Fig. 3C) or early regression ( $n = 5$ , Fig. 3D). Almost all individuals had testicular lobules with seminiferous tubules at the same spermatogenic stage. The exceptions were two males that showed one of the lobules smaller than the others. In these cases, the larger lobules had seminiferous tubules in early regression, while the smaller lobules had regressed seminiferous tubules (Fig. 3B). The kidneys were highly textured in ten adult males (SVL = 154–198 mm), slightly textured in two males (SVL = 231–238 mm), and smooth in one male (SVL = 254 mm). All adult males had hypertrophied SSKs with acidophilic and basophilic secretions in the lumen (Fig. 3E). The width of the right *ductus deferens* averaged  $0.61 \pm 0.11 \text{ mm}$  (range = 0.48–0.90 mm). The *ductus deferentia* of all adult males were packed with sperm, which contained secretion granules with an unidentified function (Fig. 3F). The seminiferous tubule diameter, SSK diameter, and epithelial thickness were slightly larger in individuals in spermiogenesis than in early regression (supp. information Table S2). However, the diameter and epithelial thickness of the *ductus deferentia* were larger in individuals with testes in early regression (supp. information Table S2).

### Female reproductive tract

All females ( $n = 10$ ) dissected were sexually mature; these females were collected in spring (October,  $n = 5$ ; December,  $n = 3$ ) and summer (February,  $n = 1$ ). Data on the collection season of a vitellogenic female was missing, so the characteristics of this individual are not

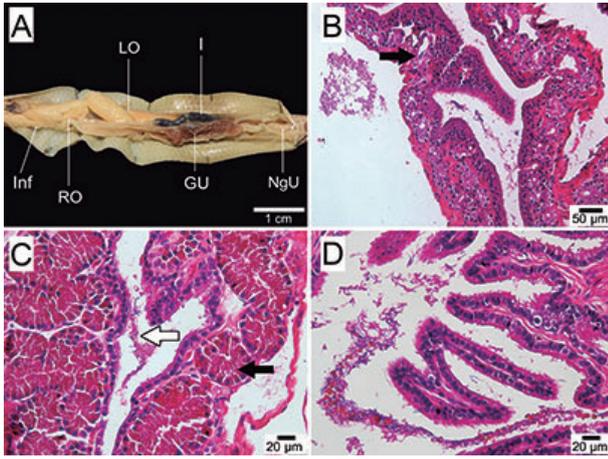


**Figure 2.** Temporal variation of snout-vent length of individuals of *T. koppesi* observed at the Santa Bárbara Ecological Station. Juveniles: grey circles; Females: dark brown; Males: yellow circles.



**Figure 3.** Reproductive anatomy and histology of male *T. koppesi* from Santa Bárbara Ecological Station. **A)** Anterior reproductive system, with plurilobulated testes; LK: left kidney, RK: right kidney, LT: left testis; RT: right testis. **B)** Regressed seminiferous tubules. **C)** Seminiferous tubules in spermiogenesis; white arrow: spermatocyte I cells; black arrow: spermatocyte II cells. **D)** Seminiferous tubules in early regression; black arrow: Sertoli cells, black circle: sperm. **E)** Hypertrophied SSK; black arrow: basophilic secretion. **F)** *Ductus deferens* with sperm; black arrow: unknown substance among the sperm.

detailed below, although it was analysed anatomically. The female reproductive tract consisted of a pair of ovaries and a single oviduct, present only at the right side (Fig. 4A). The infundibulum was pleated and opaque



**Figure 4.** Reproductive anatomy and histology of female *T. koppesi* from Santa Bárbara Ecological Station. **A)** Overview of the reproductive system of a vitellogenic female; I: intestine, Inf: infundibulum, GU: glandular uterus, LO: left ovary, NgU: non-glandular uterus, RO: right ovary. **B)** Infundibulum epithelium and receptacles; black arrow: sperm stored in the receptacle. **C)** Glandular uterus; black arrow: uterine glands, white arrow: sperm. **D)** Nonglandular uterus.

in most females ( $n = 9$ ) but pleated and translucent in one vitellogenic female from spring. All females ( $n = 8$ ) collected in spring had infundibular sperm receptacles with highly ciliated epithelial cells (Fig. 4B). Three of these females had sperm in the receptacles; in all cases, sperm were parallelly aligned (Fig. 4B). The female collected in summer lacked infundibular sperm receptacles. The glandular uterus was pleated in the posterior region in three females from spring (December) and smooth in the remaining ones ( $n = 6$ ). All females had some secretion in their glandular uterus, four of them (October = 2, December = 2) with sperm (Fig. 4C). Uterine glands were recrudescing in early spring (October, ranging from 77–118  $\mu\text{m}$ ), larger in late spring (December, 116–180  $\mu\text{m}$ ), and regressed in summer (February) (supp. information Table S3). The gross morphology and epithelium of the nonglandular uterus showed no seasonal variation, with ciliated cells in all individuals (Fig. 4D). Five females (October = 2, December = 3) had sperm in the lumen of the nonglandular uterus, and three of them with granules with similar histological staining to those found in the male's *ductus deferentia*.

The smallest follicle showing vitellogenic granules (determined by histology) measured 6.7 mm (Fig. 4A). Therefore, we considered all follicles larger than 6 mm as vitellogenic and follicles smaller than 6 mm as non-vitellogenic. Vitellogenic females collected in October (early spring) had follicles with 6.4–10.2 mm diameter, and vitellogenic females in December (late spring) had follicles with 16.5–21.6 mm diameter. Non-vitellogenic females were collected in October (follicle diameter = 4.1 and 4.9 mm;  $n = 1$ ) and February (follicle diameter = 3.1–3.5 mm;  $n = 1$ ). We found no gravid female. Potential clutch size (estimated by the number of vitellogenic follicles) ranged from one to five follicles (mean =  $3.9 \pm$

**Table 1.** Sexual dimorphism in morphological traits of *T. koppesi*. Values in bold indicate significant differences at  $P < 0.05$ .

Trait	Effect	t/F	df	P
SVL (mm)	sex	5.852	21	<b>&lt; 0.0001</b>
Tail length (mm)	slopes	0.098	1, 19	0.757
	sex	24.114	1, 20	<b>&lt; 0.0001</b>
Head length (mm)	slopes	0.048	1, 19	0.828
	sex	0.302	1, 20	0.589
Head height (mm)	slopes	1.422	1, 19	0.248
	sex	0.014	1, 20	0.908
Body width (mm)	slopes	1.821	1, 12	0.202
	sex	1.609	1, 13	0.227
Body mass (g)	slopes	1.914	1, 19	0.183
	sex	7.256	1, 20	<b>0.014</b>

1.4 follicles; supp. information Table S1). However, most (6 out of 8) females had either four or five vitellogenic follicles.

#### Sexual dimorphism

Adult females ( $n = 10$ ) ranged from 225–311 mm SVL ( $285.0 \pm 25.4$  mm), and adult males ( $n = 13$ ) ranged from 198–254 mm SVL ( $232.6 \pm 17.6$  mm). Adult females were significantly larger and heavier than adult males, but adult males had relatively longer tails than adult females (Table 1). The SSD index was 0.225. We found no sexual dimorphism in head length, head height, and body width (Table 1).

## DISCUSSION

#### Seasonal activity

*T. koppesi* is endemic to the Cerrado and typical of open habitats (see Passos et al., 2006; Nogueira et al., 2010; Pinto & Curcio, 2011; Pinto & Fernandes 2012). Scolecophidian snakes are usually small and slender, which may result in low thermal inertia (Christian et al., 2006). Parpinelli & Marques (2008) suggested that the lower temperatures in autumn and winter may explain the lower detectability of *L. beui* on the surface during this period. This hypothesis may also explain the surface inactivity we observed in *T. koppesi* between autumn and winter. The higher surface activity observed in spring coincides with the timing of mating in *T. koppesi*. In many snake species, the encounter rate of individuals increases during the mating season (Pizzatto et al., 2007).

#### Reproductive anatomy and cycles

Our results show that male *T. koppesi* are sexually active at least in spring (October), as evidenced by

sperm production, sperm in the *ductus deferentia*, and hypertrophied SSKs. The occurrence of individuals with regressed seminiferous tubules in spring suggests that individual males show a discontinuous cycle (Mathies, 2011). Discontinuous sperm production has also been observed in at least two scolecophidians: the typhlopids *Anilios nigrescens* from Australia (Shea, 2001) and *Amerotyphlops brongersmianus* from Brazil (Khouri et al., 2020). Unfortunately, the lack of males and histological data from months other than October prevents us from inferring reproductive seasonality at the population level. Nevertheless, given the apparent inactivity on the surface and the low temperatures between mid-autumn and mid-winter (no specimen was collected between May and August), testes are likely inactive during this period. Spermatogenesis is seasonal in the typhlopids *A. nigrescens* and *A. brongersmianus*; however, sperm production peaks in summer – autumn in *A. nigrescens* and winter in *A. brongersmianus* (Shea, 2001; Khouri et al., 2020). In a Brazilian anomalepidid (*Liotyphlops beui*), Parpinelli & Marques (2015) suggested that spermatogenesis is continuous, but their suggestion lacks microscopic confirmation. Our data for *T. koppesi* suggest that the timing of spermatogenesis varies among the scolecophidians studied so far.

The presence of sperm in the lumen of the vagina and nonglandular uterus suggests recent mating (Siegel et al., 2011). Thus, our finding of sperm in these regions suggests that *T. koppesi* copulates at least in spring. The synchrony between spermiogenesis and mating defines male spermatogenesis as prenuptial (Saint-Girons, 1982; Aldridge et al., 2020), which seems to be a basal characteristic in squamates (Aldridge et al., 2020). Mating in *T. koppesi* occurs with females either in early or late vitellogenesis. Consequently, females must store sperm in their reproductive tract for a short time until ovulation, which likely occurs in late spring-early summer. In snakes, female sperm storage occurs in crypts in the nonglandular uterus, infundibular glands, or both (Siegel et al., 2011). In *T. koppesi*, we found no crypts that could serve as sperm storage receptacles. Thus, we hypothesise that the nonglandular uterus does not function for sperm storage. In contrast, we found sperm stored in infundibular glands (sperm receptacles) of various females collected in spring. Infundibular glands have been reported in leptotyphlopids and typhlopids, but their function as sperm receptacles in these scolecophidian families has been questioned because no sperm had been observed in them (Fox & Dessauer, 1962; Siegel et al., 2011). However, sperm storage in the infundibular glands was recently confirmed in the typhloid *A. brongersmianus* (Khouri et al., 2020). Our finding of infundibular sperm storage in another scolecophidian family (Leptotyphlopidae) suggests that this feature appeared early in snake evolution.

We suggest that female reproduction in this population is seasonal, with vitellogenesis and egg-laying occurring in spring–early summer. In a geographically close population (Itirapina, ~160 km), female *T. koppesi* were hypothesised to lay eggs in late spring (December) (Sawaya et al., 2008). We found no gravid female or egg-laying to confirm this

hypothesis, but we suspect that female *T. koppesi* oviposit mostly in summer (January–February). Female squamates typically retain eggs in the oviducts after ovulation and lay them with partially developed embryos (mainly at limb bud stages; Shine, 1983; Blackburn, 1995). The duration of retention varies interspecifically but may last  $\geq$  two weeks (Andrews & Mathies, 2000). We lack data on the embryo stage at oviposition for *T. koppesi*, but other scolecophidians appear to retain eggs longer than other snakes (Erasmus & Branch, 1983; Shine & Webb, 1990; Kamosawa & Ota 1996; Sandoval et al., 2020; see also Blackburn, 1995 to compare with other snakes). In the typhloid *Indotyphlops braminus* from Japan, oviposition occurs about a month after ovulation (Kamosawa & Ota, 1996). Since preovulatory females in our study occurred only in December (late spring), we suggest that female *T. koppesi* likely lay eggs from early to mid-summer (January – February). Female reproductive seasonality (with egg-laying concentrated in summer) also occurs in many other scolecophidians and snake groups, including tropical species (Shine & Webb, 1990; Kamosawa & Ota, 1996; Webb et al., 2000; Webb et al., 2001; Ávila et al., 2006; Mathies, 2011; Parpinelli & Marques, 2015). By laying eggs in early to mid-summer, hatchings are likely to occur between mid-summer and early autumn, based on the incubation period (30–70 days) in other scolecophidians (Sandoval et al., 2020).

We suggest that the textured kidney found in *T. koppesi* reflects SSK development (Figs. 3A and 3E) and can be considered an indicator of sexual maturity. This is because the SSK development is related to male sexual activity and mating (Aldridge et al., 2011). The relationship between the SSK development and the textured aspect of the kidneys was also observed in the typhloid *A. brongersmianus* (Khouri et al., 2020). The semen shows granules both in the *ductus deferentia* and the oviducts. These granules exhibit an acidophilic aspect in other squamates (Burtner et al., 1965; Sever & Hopkins, 2005). However, histochemical studies have yet to be conducted to clarify the function of these secretions in scolecophidians, although they have a glycoprotein and mucoprotein nature in other snake groups (e.g., Rojas et al., 2013; Silva et al., 2020). Our finding that female *T. koppesi* show only the right oviduct agrees with previous observations in other scolecophidians (Fox & Dessauer, 1962).

Individuals of *T. koppesi* were found mostly in spring (Fig. 2), when most individuals are sexually active. In this period, there is a higher search activity for mates by males, which would increase the encounter rate (Pizzatto et al., 2007b). In spring, male and female *T. koppesi* also have more food content (M. Martins & B.F. Fiorillo, unpublished data), which may indicate that the snakes are optimising their foraging to store body fat and minimising exposure to predators, as seen in other Scolecophidians (Saint-Girons, 1982; Webb et al., 2000). The seasonal activity pattern observed here (Fig. 2) is similar to that observed in another conspecific population (Sawaya et al., 2008), where the species was most active on the surface in spring.

### Sexual maturity, sexual dimorphism, and clutch size

As in other scolecophidians, males reach sexual maturity at smaller body sizes than females (Shine & Webb, 1990; Webb et al., 2000; Webb et al., 2001; Parpinelli & Marques, 2015). Scolecophidian species with mean SVL similar to *T. koppesi* (males = 220 mm, females = 250 mm) achieve sexual maturity at smaller SVLs than we found for our study species (smallest adult male = 198 mm, smallest adult female = 225 mm). Shine & Webb (1990) found that males and females of *Anilius affinis* reach sexual maturity with 172 and 206 mm SVL, respectively (average SVL in males = 206 mm; females = 252 mm), and Ávila et al. (2006) found that male and female *A. brongersmianus* reach sexual maturity with 180 and 211 mm SVL, respectively (average SVL in males = 227.2 mm; females = 241.9 mm). This may indicate that *T. koppesi* mature at larger SVLs than other scolecophidians or that our sampling was insufficient to capture the variation in body size at maturity in this population.

Female *T. koppesi* were larger and heavier than males, as in other scolecophidians (Webb et al., 2001; Cox et al., 2007) and alethinophidians (Shine, 1994). Sexual size dimorphism is often attributed to sexual and natural selection (Shine, 1994). The female-biased SSD index of *T. koppesi* (0.225) resembles those reported in many snake species lacking male combat. Indeed, such behaviour has never been reported in scolecophidians (Shine, 1978, 1994; Shine & Webb, 1990; Webb et al., 2000; Parpinelli & Marques, 2015). In the absence of male combat, female-biased SSD is attributed to selection for increased fecundity because larger females tend to produce larger clutches (Shine, 1994). Unfortunately, we could not test for such a relationship in this population of *T. koppesi*, but clutch size increases with maternal body size in many scolecophidians with female-biased SSD (Shine & Webb, 1990; Webb et al., 2001).

Longer tails in males are a common feature in snakes, including scolecophidians (Shine & Webb, 1990; Webb et al., 2000; Parpinelli & Marques, 2015), and are usually attributed to the presence of hemipenes and associated muscles (King, 1989; Shine et al., 1999). However, this condition may also be advantageous in tail wrestling during courtship (King, 1989; Shine et al., 1999). There is no record of reproductive aggregation in *T. koppesi*, but such behaviour has been reported in some scolecophidians (McCoy, 1960; Shine & Webb, 1990; S. M. Almeida-Santos, unpublished data). Thus, male scolecophidians may also benefit from larger tails in reproductive aggregations. The lack of sexual dimorphism in head size agrees with results for other scolecophidians (Webb et al., 2000). In snakes, sexual dimorphism in head size may reflect sex divergences in dietary niche (Shine, 1993). Thus, similarity in head size between the sexes may reflect the consumption of similar-sized food items.

Clutch size of *T. koppesi* was estimated by counting vitellogenic follicles (3-5) and is smaller than what was reported for a nearby conspecific population (5-7 eggs; Sawaya et al., 2008) and other similar-sized scolecophidians such as the typhlopids *A. affinis* (mean SVL = 252 mm) and *A. brongersmianus* (mean SVL = 241

mm) (3 and 4-5 eggs, respectively; Shine & Webb, 1990; Ávila et al., 2006). Inferring clutch size from vitellogenic follicles may sometimes overestimate clutch size because not all the vitellogenic follicles may be ovulated (Almeida-Santos et al., 2014). Nevertheless, our clutch size estimate is still smaller than that reported by Sawaya et al. (2008). Small clutches are common in small-sized snakes (including scolecophidians) and likely reflect space constraints within the female's body since clutch size tends to be correlated with body size (Shine & Webb, 1990; Webb et al., 2000). Small clutches also seem to be characteristic of several unrelated fossorial snakes (e.g., Marques & Puerto, 1998; Balestrin & Di-Bernardo, 2005; Braz et al., 2014; Braz et al., 2019).

Here, we show that female *T. koppesi* reproduce seasonally, with ovulation and egg-laying occurring in the warmer seasons, as observed in other basally split snakes. We also show that females lack the left oviduct, like other scolecophidians. The patterns of sexual dimorphism observed here may help to understand other aspects of scolecophidian reproductive ecology, such as breeding aggregations. Although male and female *T. koppesi* are reproductive in spring (when most individuals are active on the surface), we found some reproductive asynchrony between the sexes. Therefore, females store sperm in infundibular glands, a strategy rarely reported in scolecophidians. Thus, our study shows how scolecophidians still need to be studied, and the novelties we found can help to understand several other aspects of the biology and evolution of this group.

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### Authors' contribution

Marcio Martins (MM) and Bruno Ferreto Fiorillo (BFF) planned the study; BFF and Jorge Henry Maciel collected the specimens and field data, BFF and Henrique Bartolomeu Braz (HBB) analysed the data; Rebeca Stella Khouri (RSK) collected reproductive data, interpreted histological slides, led the writing of the manuscript. BFF, HBB, MM, RSK, and Selma Maria Almeida-Santos contributed writing to the manuscript. All authors approved the final version of this manuscript for publication.

**Ethical statement**

Snakes were collected under SISBIO (50658-1) and COTEC (SMA nº 260108–011.518/2015) scientific collection permits.

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## APPENDIX I

Voucher specimens of *Trilepida koppesi* analysed in this study (n = 24; field numbers): MRCM290, MRCM292, MRCM294, MRCM307, MRCM308, MRCM309, MRCM310, MRCM311, MRCM312, MRCM313, MRCM314, MRCM318, MRCM320, MRCM321, MRCM323, MRCM327, MRCM328, MRCM340, MRCM355, MRCM381, MRCM382, MRCM383, MRCM457 and MRCM546.

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