



Mating plugs and male sperm storage in *Bothrops cotiara* (Serpentes, Viperidae)

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We describe two new aspects of male reproduction in the pit viper *Bothrops cotiara*: the formation of mating plugs and epididymal sperm storage. Based on histological analyses of specimens (four males and three females), we propose that long-term sperm storage occurs in the ductus deferens, whereas macroscopic hypertrophy indicates that the epididymis may be a short-term sperm storage site. Secretions of the sexual segment of the kidneys probably form the mating plug that males deposit in the female vagina during copulation. A mating plug without sperm was observed in the spring. Future studies should address the functions and longevity of mating plugs and the role of the epididymis in *Bothrops* reproduction.

Key words: ductus deferens, ductus epididymis, neotropical region, reproduction, snakes, SSK

Male snakes have a complex reproductive system which includes sperm storage (Trauth & Sever, 2011) and a sexual segment of the kidney (SSK) that produces secretory granules in response to testosterone (Bishop, 1959; Aldridge et al., 2011). An increase in the production of secretory granules with a consequent hypertrophy of the tubules in the SSK during the mating season may have evolved to enhance male reproductive success after mating, including the formation of a mating plug which males of some species deposit in the female genital opening after copulation (Aldridge et al., 2011).

Mating plugs have evolved independently in a variety of taxa including invertebrates such as spiders (Uhl et al., 2010), scorpions (Althaus et al., 2010), crustaceans (Bauer & Min, 1993), nematodes (Cutter, 2008), primates (Parga et al., 2006), lizards (Bosch, 1994; Moreira et al., 2006, 2007) and snakes (Crews & Garstka, 1982; Shine et al., 2000; Friesen et al., 2013). In squamates, the functions of mating or copulatory plugs may include passive mate-guarding (Shine et al., 2000), the prevention of sperm leakage (Moreira & Birkhead, 2004) and the gradual release of spermatozoa (Friesen et al., 2013).

Sperm storage is also a component of the reproductive systems of snakes (Fox, 1956; Almeida-Santos et al., 2006; Trauth & Sever, 2011; Rojas et al., 2013; 2015). Sperm storage can be categorised as long-term (LTSS, sperm is stored in the oviducts for prolonged periods prior to ovulation) and short-term (STSS, sperm is stored for some weeks prior to ovulation, Schuett, 1992). The ductus deferens is the acknowledged sperm storage organ for male snakes (Fox, 1952; Almeida-Santos et al., 2004; Trauth & Sever, 2011). Sperm is produced in the seminiferous tubules in the testes and transported to the ductus deferens by the anterior testicular ducts (including the rete testis, ductuli efferentes and ductuli epididymis) (Trauth & Sever, 2011). While the epididymis is used for sperm storage, maturation and capacitation in turtles and lizards (Gist et al., 2002; Bian et al., 2013; Stuart-Smith et al., 2005), its function as a sperm storage organ in snakes is still unclear (Dufaure & Saint-Girons, 1984). Recently, Sever (2010) and Sever & Freeborn (2012) detected the existence of secretory activity in the ductus epididymis of snakes, which may be indicative of a sperm storage function. The aim of this paper is to describe the formation of mating plugs, and occurrence of sperm storage in the epididymis in the Neotropical pit viper, *Bothrops cotiara* (Gomes, 1913).

Bothrops cotiara belongs to the basal *B. alternatus* group (Wüster et al., 2002, Carrasco et al., 2012), and is endemic to the Araucaria forests in the Brazilian Meridional Plateau, ranging from South São Paulo to the North Rio Grande do Sul, extending into Argentina (Campbell & Lamar, 2004). Due to recent range contractions, *B. cotiara* is considered locally endangered (São Paulo, 2008; Mikich & Bérnils, 2004). Information on its biology is scarce, and more natural history information to support conservation efforts is required (Mikich & Bérnils, 2004; Bressan et al., 2009). *Bothrops cotiara* preys only on small mammals, whereas most other *Bothrops* are diet generalists (Martins et al., 2002); the clutch size varies from 4 to 12 new-borns (Mikich & Bérnils, 2004). We analysed four males and three females of *B. cotiara* from two scientific collections: Museu de Ciências e

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Tecnologia (MCT), Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) and Universidade Federal do Mato Grosso do Sul (UFMS). All individuals were sexually mature. Individuals were collected in Paraná and Santa Catarina States (Appendix).

A ventral incision was made to analyse the reproductive tract. The convoluted aspect of the ductus epididymis and ductus deferens was used as a macroscopic indicator of sperm storage in males (Almeida-Santos et al., 2006). After macroscopic examination, the testes, kidneys and the caudal portion of the ductus deferens of males and the vaginas of females were collected for histological analysis. All organs were preserved in 70% alcohol and processed for light microscopy by the paraffin method. Sections were cut 5 µm thick, and stained for histology with hematoxylin and eosin (H/E, Spector & Goldman, 2006).

A mating plug was observed in one vitellogenic female (largest follicle: 13.76 mm diameter, MCT 17498) collected in spring. Macroscopically, we observed a

white to yellowish gelatinous mass occluding the female vagina. Microscopically, this mating plug was distributed throughout the lumen of the vagina (Fig. 1A). The epithelium of the vagina was composed of secretory and ciliated cells, and the lamina propria showed many mastocytes (Fig. 1B). Sperm was not observed in the mating plug or in the vagina of any sampled female. The SSK, which produces the mating plug substances, was present in the kidneys of males. The active condition of the SSK in summer and autumn was characterised by a high production of sexual granules with evident hypertrophy of the nephron distal tubules (Fig. 1C), differing from the inactive period in winter (Fig. 1D).

In males, after spermatozoa are produced in seminiferous tubules in summer, they pass through a system of ducts (Fig. 1E, F). The rete testis epithelium is simple squamous and cuboidal and non-ciliated, and it communicates with the ductuli efferentes, which are smaller in diameter and contain simple cuboidal epithelium with ciliated and non-ciliated cells (Fig. 1E,

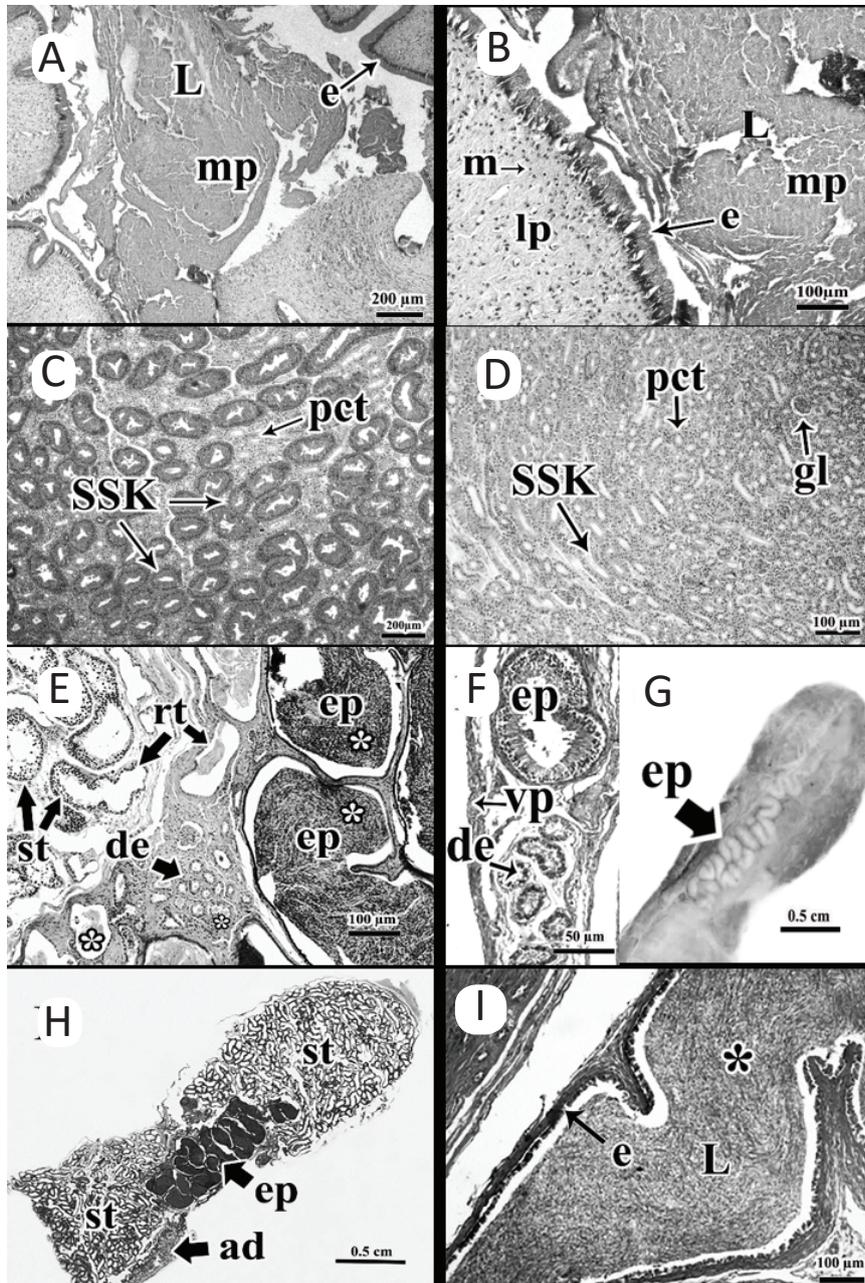


Fig. 1. A) Mating plug in female vagina during spring. B) Higher magnification of A showing the epithelium of the vagina and the presence of mastocytes. C) Active SSK in summer and autumn. D) Inactive SSK in winter. E) Anterior testicular ducts with epididymal hypertrophy in autumn. F) Anterior testicular ducts with empty ductus epididymis in summer. G) Macroscopic aspect of the testes with the epididymal hypertrophy. H) Microscopic view of the testes showing the hypertrophied ductus epididymis and the position of the adrenal. I) Sperm storage in the ductus deferens. ad: adrenal; de: ductuli efferentes; e: epithelium; ep: ductus epididymis; gl: glomerulus; m: mastocytes; mp: mating plug; lp: lamina propria; L: lumen; pct: proximal convoluted tubule st: seminiferous tubules; rt: rete testis; *: sperm; vp: visceral pleuroperitonium; SSK: sexual segment of the kidneys.

F). Sperm was observed in the lumen of the rete testis and ductuli efferentes in every male sampled. During spermatogenesis in summer, the ductus epididymis was empty and invisible to the naked eye (Fig. 1F), whereas in autumn, when it contained a large amount of sperm, the ductus epididymis was macroscopically visible and showed a convoluted aspect (Figs. 1E, G, H). The epithelial cells of the ductus epididymis were high with basal nuclei in the summer (Fig. 1F), whereas the epithelium of the hypertrophied ductus epididymis was low in autumn (Fig. 1E). In winter, the ductus epididymis was not macroscopically visible, and only a few spermatozoa could be observed. Sperm was stored throughout the seasons in the ductus deferens (Fig. 1I). The distal portion of the ductus deferens contained many spermatozoa in all sampled individuals regardless of the testes condition (whereas spermatogenesis occurred in the summer, regression of the seminiferous epithelium occurred in autumn and winter). The epithelium of the ductus deferens was low and pseudostratified, and the spermatozoa were surrounded by a mass (Fig. 1G).

We propose that, after a peak of sperm production in summer, *B. cotiara* males release spermatozoa to the ductus epididymis for a period of STSS, resulting in macroscopic hypertrophy before the sperm is released to the ductus deferens for LTSS, where sperm is stored throughout the year (e.g., Rojas et al., 2013; Barros et al., 2014). Sperm storage in the epididymis has been reported for other reptiles (e.g., turtles and lizards) with macroscopically visible epididymal hypertrophy (Bian et al., 2013; Stuart-Smith et al., 2005). The existence of a macroscopically convoluted and hypertrophied epididymis has been previously demonstrated for other snakes (*Vipera berus*: Volsøe, 1944; *Zaocys dumnades*: Gang et al., 2011; *Naja kaouthia*: Tumkiratiwong et al., 2012). Recent analyses using electron microscopy techniques indicate that secretory activity occurs in the epididymis of *Seminatrix pygaea* and *Pelamis platurus* (Sever, 2010; Sever & Freeborn, 2012; but see also Dufaure and Saint-Girons, 1984). Thus, the function of the epididymis in snakes may vary among species, and may be more similar to other amniotes than previously thought (Trauth & Sever, 2011).

The sperm storage period may be linked to seasonal hypertrophy of the SSK (Saint-Girons & Pfeffer, 1971). In *B. cotiara*, sperm storage in the epididymis was indeed synchronous to SSK hypertrophy. Mating is usually linked to SSK hypertrophy (Krohmer, 2004; Rojas et al., 2013). Mating in autumn has been observed for several *Bothrops* (Almeida-Santos & Salomão, 2002), and the peak in the production of sexual granules in the SSK corroborates the hypothesis of autumnal mating for *B. cotiara*. Although we do not have male samples from spring to verify the SSK condition during this season, we infer that SSK plays a role in the formation of the mating plug in *B. cotiara*. The presence of a mating plug in a female in spring indicates that *B. cotiara* may reproduce in autumn as well as in spring, depending on how long the mating plug remains inside the vagina. In *Thamnophis sirtalis*, the mating plug only lasts for 2–3 days and releases spermatozoa while dissolving, thereby serving as a spermatophore and as a

chastity belt (Friesen et al., 2013; Shine et al., 2000). No sperm was found, however, in the copulatory plug of *B. cotiara*. Shine et al. (2000) proposed that the formation of mating plugs in *T. sirtalis* may be linked to the brief duration of copulation (on average, 17 minutes, King et al., 2009). For bothropoids, copulation usually lasts between 3 to 5 hours (Silva et al., 2013), but the precise copulation duration of *B. cotiara* is as yet unknown. Species of the genus *Bothrops* may show both passive and active mate guarding tactics. Male-male combat, a form of active mate guarding, occurs in the species of the *B. atrox* group (Almeida-Santos & Salomão, 2002), whereas mating plugs may be a form of passive mate guarding. Bosch (1994) for the first time reported mating plugs for lizards, which neither appeared to act as a chastity belt nor to decrease female attractiveness and receptivity (Moreira & Birkhead, 2003). It did not assure higher rates of fertilisation, and its function was assumed to prevent sperm leakage (Moreira et al., 2007). Future histochemical analysis of both mating plugs and SSK secretions may confirm the role of the kidneys in producing mating plug substances and provide information on the composition of plugs.

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APPENDIX

Appendix 1. Voucher number and provenance of *Bothrops cotiara* specimens examined.

Males: MCT 17299, Cruz Machado, Paraná (26°01'03''S and 51°20'48''W); MCT 17339, União da Vitória, Paraná (26°13'48''S and 51°05'11''W); MCT 17304, Palmas, Paraná (26°29'03''S and 51°59'26''W); UFMS 1644, Curitiba, Paraná (25°25'40''S and 49°16'23''W).

Females: MCT 17335, União da Vitória, Paraná (26°13'48''S and 51°05'11''W); MCT 17497, Lebon Regis, Santa Catarina (26°55'44''S and 50°41'43''W); MCT 17498, Lebon Regis, Santa Catarina (26°55'44''S and 50°41'43''W).