THE REPRODUCTIVE CYCLE OF MALE NEOTROPICAL RATTLESNAKES (*CROTALUS DURISSUS TERRIFICUS*)

MARIA DA GRAÇA SALOMÃO[1] AND SELMA MARIA ALMEIDA-SANTOS[1,2]

ABSTRACT: The reproductive cycle of male Neotropical Rattlesnakes (*Crotalus durissus terrificus*) from southeastern Brazil is described, with emphasis on seasonal cytological changes of the testis and anterior pituitary. Ninety-eight adult specimens from the State of São Paulo were examined from 1991 to 1993. The germinal epithelium of the testis was inactive (regressed) during austral winter (July to September), recrudescence was initiated in early spring (early October), and spermatogenesis peaked in summer (January to March) just prior to the mating period in autumn (April to June). This pattern suggests an austral (= postnuptial) (Type I) spermatogenic cycle. The pituitary, particularly the anterior (= distal) lobe, showed the presence of basophils, chromophobes, orange cells, and acidophils. The latter showed finer granules in the summer and closely packed granules in late autumn and winter, whereas basophils appeared rich in granules from early spring to summer. Based on testis activity, sexual maturity in male *C. d. terrificus* occurs at a snout-vent length (SVL) ca. 560 mm. Reproductive behavior of male *C. d. terrificus* is asynchronous with certain sexual activities of females (e.g., ovulation), and this pattern is similar to that found in species of *Crotalus* from temperate regions of North America. We propose that male *C. d. terrificus* show the potential to reproduce annually, whereas females reproduce biennially or less frequently.

INTRODUCTION

Studies on the reproductive cycles of male snakes have analyzed seasonal data on the testis (e.g., spermatogenesis), plasma and gonadal sex steroid levels, sexual segment of the kidney, and sexual behavior (e.g., courtship, mating, and male-male aggression) (Seigel and Ford, 1987; Schuett 1992, 1997; Aldridge, 1993; Duvall et al., 1993; Aldridge and Brown, 1995; Schuett et al., 1997, this volume; Goldberg and Rosen, 2000; Holycross and Goldberg, 2001; Bonnet et al., this volume). Although data are meagre, histological changes in the testis and sexual segment of the kidney, for example, are associated with seasonal differences in sex steroid levels which, in turn, modulate male sexual behavior and maintain secondary sexual characteristics (Weil and Aldridge, 1981; Moore and Lindzey, 1992; Bonnet et al., this volume; Schuett et al., this volume).

Seasonal histological changes of the vertebrate testis are typically associated with certain cells present in the anterior (= distal) lobe of the pituitary, and the primary types are acidophils, basophils (or cyanophils), chromophobes, and orange cells (Saint Girons, 1970 a, b; Bentley, 1998). Basophils are considered to be the source of gonadotropic hormones, e.g., follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in vertebrates (Doerr-Schott, 1976; Wurst et al., 1989). Acidophil activity, for example, has been associated with growth in snakes (Cieslak, 1945; Doerr-Schott, 1976). Our knowledge of male reproduction of snakes is growing (Fitch, 1970, 1982; Blem, 1982; Aldridge et al., 1995; Bonnet et al., this volume, Schuett et al., this volume), but, unfortunately, few detailed studies concern tropical species (Saint Girons, 1982; Seigel and Ford, 1987). One important and controversial issue in snake reproduction concerns the relationship of sexual behavior, gonadal activity, and levels of circulating sex steroid hormones (e.g., testosterone, 17β-estradiol, progestogens), which has led some authors to classify reproductive cycles as either associated or dissociated (Crews and Garstka, 1982; Crews, 1984; Krohmer et al., 1987; reviewed by Moore and Lindzey, 1992; for a criticism of this simplistic classification see Schuett, 1992; Saint Girons et al., 1993; Schuett et al., 1997, this volume).

The Neotropical Rattlesnake (*Crotalus durissus*) has a wide distribution, ranging from Mexico to South America where it reaches its southern limit in northern Argentina (Campbell and Lamar, 1989). In southeastern Brazil, *C. d. terrificus* is primarily active from late summer (March) to early winter (July), feeds mainly in summer and autumn (Salomão et al., 1995), and males shed during early spring (September) (Langlada, 1972). In autumn (April to June), the period of sexual activity, males engage in ritualized fights (combat) and winners presumably gain priority of access to females (Almeida-Santos et al., 1990). Based on multiple lines of evidence, Salomão et al. (1995) hypothesized that male *C. d. terrificus* from Brazil show the capacity for annual reproduction, different from females which show biennial, triennial, or even less frequent cycles (Langlada, 1972).

The main goal of this study was to determine certain aspects of seasonal reproduction of male *C. d. terrificus* in Brazil. Cytological changes in the testis were

[1]Laboratório de Herpetologia, Instituto Butantan, Av. Dr. Vital Brasil 1500, Butantã, 05503-900, São Paulo SP, Brazil
E-mail (MGS): mgsalomao@hotmail.com
[2]Departamento de Cirugia, Anatomia, FMVZUSP, São Paulo SP, Brazil

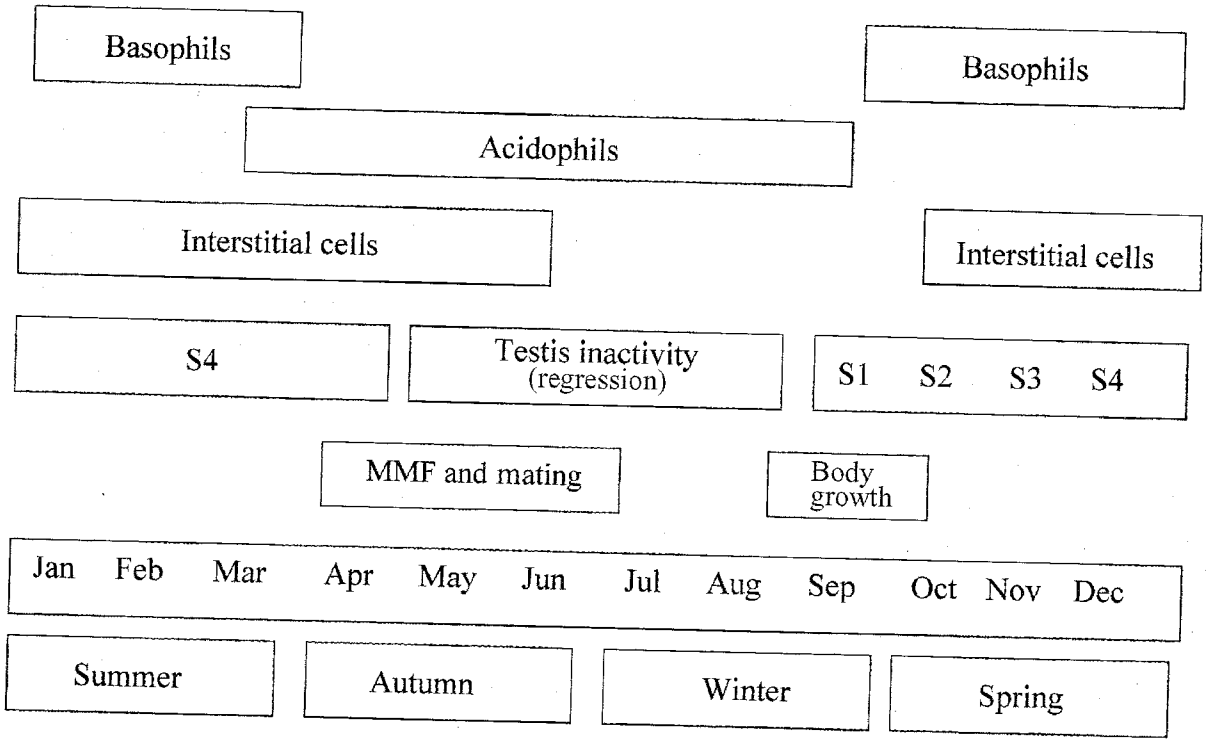

Fig. 1. Timing and duration of reproductive events in Brazilian *Crotalus durissus terrificus*. S = testicular activity. S1 = spermatogonia. S2 = spermatocytes. S3 = spermatids. S4 = spermatozoa. MMF = male-male fighting.

compared to those of the pituitary, particularly of the anterior (= distal) lobe, in an attempt to establish a relationship with the onset and offset of seasonal sexual activity. The existence of synchronized cycles between males and females will be discussed, and our results will be compared to those of *Crotalus* from North America.

MATERIALS AND METHODS

We studied a total of 98 male adult and subadult *C. d. terrificus* from the State of São Paulo in south-eastern Brazil [23°02' S, 45°33' W, elevation ca. 640 m, average annual temperature 22–25°C (summer) and 14–17°C (winter); Nimer (1989)]. From 1991 to 1993, seven adult *C. d. terrificus* were dissected every month of the year at the Instituto Butantan. The specimens were seriously injured, recently killed by people working in agricultural areas, or found as road-kills. Snout-vent length (SVL) and body mass (M) were collected from all specimens. Size (SVL) at maturation was determined through examination of the ductus deferens and histology of the testis. Testis and pituitary were preserved in Bouin's fluid and 10% neutral-buffered formalin, sectioned at 10 µm, and stained with Hematoxylin-Eosin (HE), Fucsin-Toluidin (FT), Mallory's trichrome, Mac Conaill's lead hematoxylin and Halmi's trichrome. In addition, histochemical analyses such as Periodic-Acid Schiff and Alcian Blue (pH 2.5) were performed.

Staging of seasonal spermatogenesis follows criteria suggested by Nilson (1980); spermatogenetic cycle classification criteria proposed by Schuett (1992) were also used. Activity of interstitial cells (Leydig) of the testis was inferred through increase of their size, as proposed by Manna and Sircar (1985). The criteria for identification and definition of the activity of basophils and acidophils of the pituitary were based on the type and abundance of granules in their cytoplasm according to Hartmann (1944), Saint Girons (1970 a, b), and Doerr-Schott (1976).

RESULTS

Spermatogenesis

The seasonal stages of spermatogenesis in *C. d. terrificus* are represented in Figure 1. From June to August (late autumn/winter), only one layer of cells was present in the testis, which included spermatogonia, few numbers of Sertoli cells, and few and small-sized interstitial cells (e.g., Leydig cells). The germinal epithelium was inactive. The lumen had

small numbers of degenerating spermatocytes or spermatids (Stages 8 and 1 as described by Nilson, 1980; Fig. 2a). Recrudescence was initiated in September (late winter/early spring) with spermatogonial divisions and the presence of few numbers of primary spermatocytes (Stage 2). From late October through November (spring), one or two layers of spermatogonia, numerous primary and secondary spermatocytes, and small numbers of differentiated primary spermatids were present (Fig. 2b). Also, an increase in size and number of interstitial cells was observed (Stage 3 and 4). In late November to early December (spring), several layers of spermatocytes, spermatids, and mature spermatozoa were present (Stage 5; Fig. 2c). From late December through January (summer), spermatogenesis reached its peak in activity; all stages could be observed, and spermatids predominated. Bundles of mature spermatozoa lined the lumen, and there were large numbers of free spermatozoa in the lumen. Sertoli syncytia were well formed and easily detected, and the epithelial height was maximum (Stage 6; Fig. 2d). From February through March (late summer), the seminiferous tubules looked similar to those in January, but their height had decreased slightly and interstitial cells were still evident. From April to August, spermatogenesis had terminated, and free spermatozoa were observed in the lumen, as well as a high degree of degeneration of the germinal epithelium. At this stage (Stage 7), interstitial cells with enlarged cytoplasmic areas were nearly absent (Fig. 2e). Enlarged interstitial cells were observed from October to May, a period ca. eight months, and thus were active in the production of sex steroids. Animals under 56 cm (SVL) showed only Sertoli cells in the seminiferous tubules, and the ductus deferens were empty and transparent (Fig. 2f).

Pituitary Histology

Histological analysis of the anterior (distal) lobe of the pituitary showed the presence of acidophils, orange cells, basophils, and chromophobes; and seasonal changes of these cells were detected (e.g., presence of granules in the cytoplasm). Acidophils had cytoplasm rich in secreting granules from March (late summer/early autumn) to early September (late winter), and showed finer granulation in April (early autumn) (Fig. 3a); their cytoplasm had closely packed granules from July to September (winter) (Fig. 3b). Basophils had their cytoplasm full of granules from late September to March (Fig. 3c). All of the above cell types occurred in mixed groups. The pituitary of immature snakes showed the same overall structure, but only acidophilic cells appeared rich in granules (Fig. 3d). The timing and duration of the above events are summarized in Figure 1.

DISCUSSION

Spermatogenic Cycle

Spermatogenesis in *C. d. terrificus* from southeastern Brazil was observed from September (late winter) to March (late summer), with peak activity in January (summer). The fact that recrudescence of the germinal epithelium (September) followed male combat and mating (March to May) (Almeida-Santos et al., 1990; Langlada et al., 1993), suggests a postnuptial cycle according to the definitions of Volsøe (1944) and Saint Girons (1982); summarized by Schuett (1992) as Type I. This assumption is corroborated by seasonal variation in the testicular mass, which exhibits a peak in summer (January to March) (S. Almeida-Santos and M. Salomão, unpublished). Further evidence that supports the view of a postnuptial cycle in *C. d. terrificus* is that histological analyses showed mature spermatozoa in the seminiferous tubules only from late November to early December (spring), indicating that spermiogenesis occurs in summer, just before mating in autumn. Histological analyses also showed testicular regression in winter, when testicular mass is lowest (S. Almeida-Santos and M. Salomão, unpublished).

Over a period of three years it was possible to determine with some level of confidence that the pattern of spermatogenesis in *C. d. terrificus* from Brazil is postnuptial, and its frequency is annual. This has been demonstrated in other species of *Crotalus*, primarily those that occur in the United States (Aldridge, 1975, 1979; Johnson et al., 1982; Saint Girons, 1982; Jacob et al., 1987; Schuett et al., this volume). Snake species from temperate zones that show postnuptial spermatogenic cycles have been reported to mate in late summer and autumn, with a second mating period occurring in spring (Schuett, 1992); peak testicular mass is exhibited during the mating period (Seigel and Ford, 1987). In *C. d. terrificus* we studied, copulation is restricted to autumn (S. Almeida-Santos et al., unpublished) like several of its congeners from the United States and Mexico, such as *C. adamanteus*, *C. horridus*, *C. lepidus*, *C. molossus*, *C. pricei*, *C. viridis*, and *C. willardi* (Ludwig and Rahn, 1943; Klauber, 1972; Armstrong and Murphy, 1979; Diller and Wallace, 1984; Macartney et al., 1990; Schuett, 1992; Duvall et al., 1993).

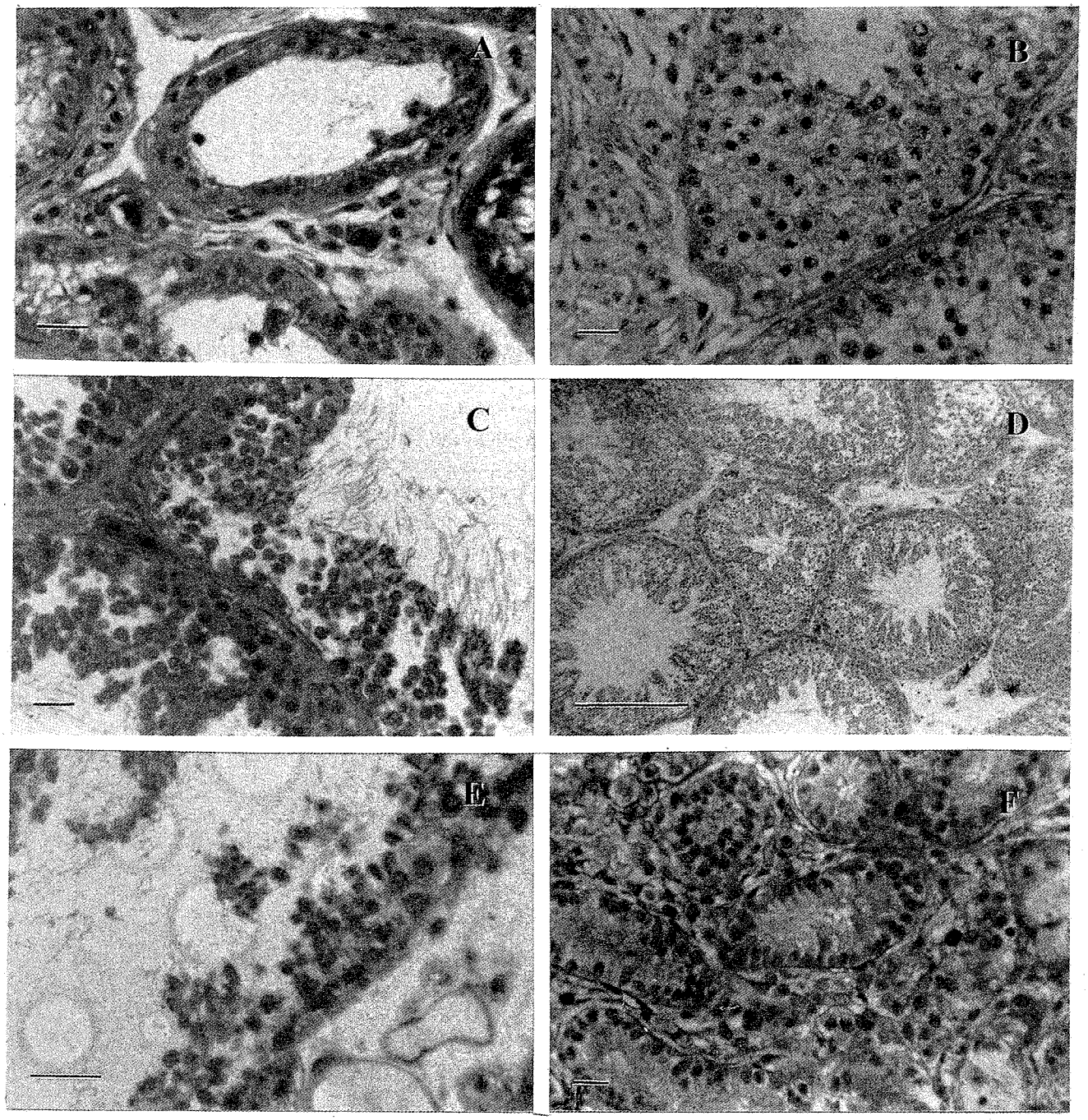

Fig. 2 Transverse sections of the testis in *Crotalus durissus terrificus* at FT. 500x except where noted. (A) Inactive period (winter). (B) Increased epithelium (spring). (C) Spermatids and the first spermatozoa (spring). (D) Maximal height (summer) showing interstitial cells (L). FT. 130 x; (E) Degeneration of the germinal epithelium (autumn). (F) Immature condition. Horizontal bar = 10 μm except for D (= 1μm).

Pituitary Histology and Seasonal Changes

In *C. d. terrificus*, two conspicuous stages in the pituitary could be observed: one was from September (late winter) to March (late summer), where basophils appeared rich in granules; the other stage occurred from April (early autumn) to August (winter), when acidophils were rich in granules. These periods coincided with testicular recrudescence and inactivity (regression), respectively. This association between testicular activity and number of basophils was also reported by Cieslak (1945) in the natricine snake *Thamnophis radix* (Colubridae). Basophils are considered a source of gonadotropin releasing hormones (GnRH) and prostaglandins (Saint Girons, 1970 a, b; Doerr-Schott, 1976; Wurst et al., 1989; Whittier and Tokarz, 1992), and their presence is associated with the period of spermatogenesis (spring and summer).

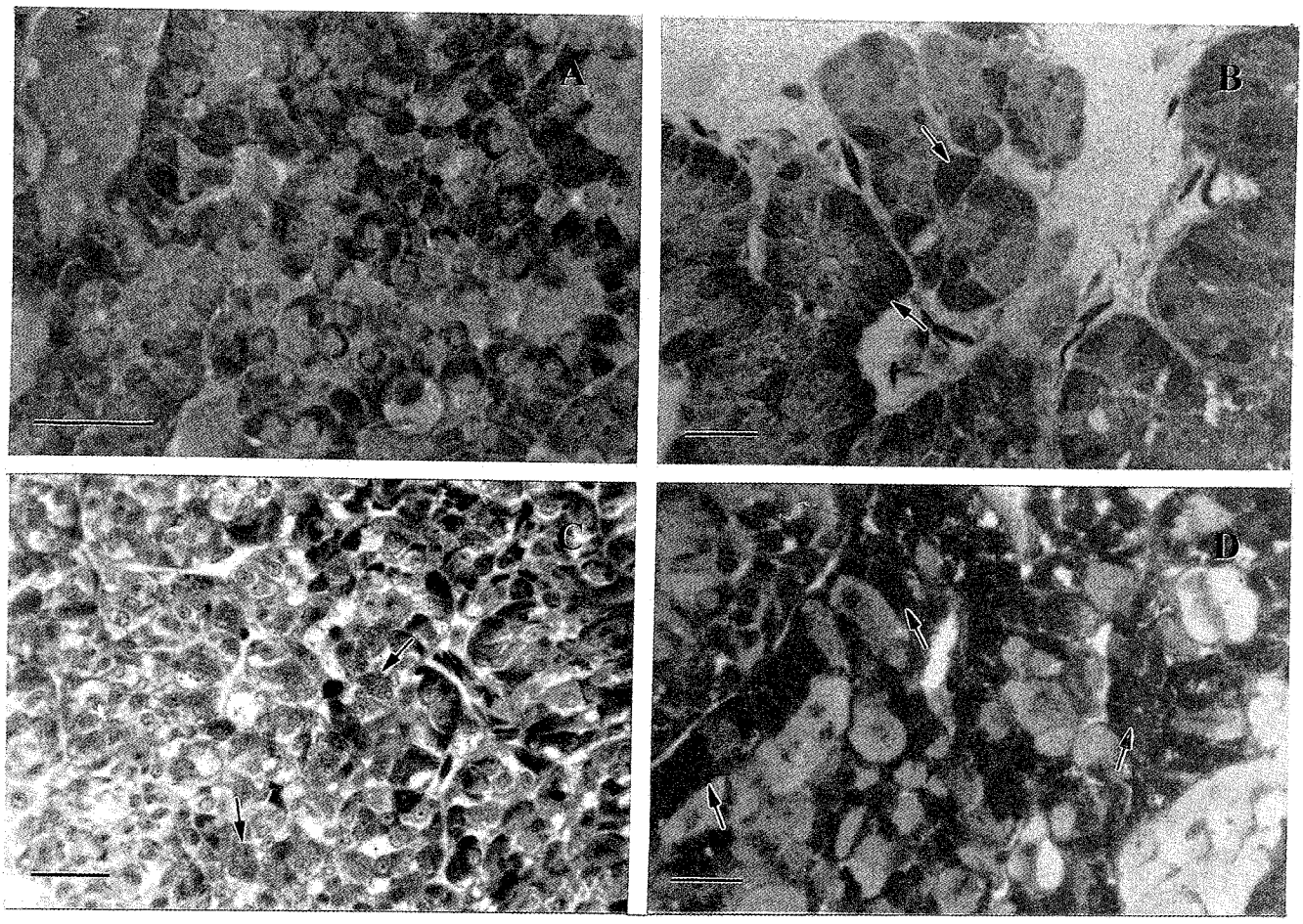

Fig. 3 Transverse sections of the pituitary in *Crotalus durissus terrificus* at FT. 500x: (A) Acidophils with finer granulation in their cytoplasm (summer). (B) Closely packed granules in the acidophils (winter, arrows); (C) A basophilic cell (spring, arrows). (D) Acidophils with granules (arrows). Horizontal bar = 10 μm.

The presence of acidophils rich in packed granules was associated with snake activity in *T. radix* (Cieslak, 1945). In *C. d. terrificus,* a peak of movement activity and feeding occurs during autumn and winter (Salomão et al., 1995), and a shed cycle (ecdysis) often occurs in September (late winter) (Langlada, 1972). High levels of feeding and frequent ecdysis indicate that growth likely occurs during this period, probably under the influence of growth hormone. The hypothesis that acidophils are related to growth in snakes is supported here by the fact that immature *C. d. terrificus* showed only acidophils rich in granules in their pituitary. Since immature snakes did not show active basophils in their pituitary, we assume that substances produced by these cells are related to growth and sexual maturation.

Control of Male Reproduction

Physiological regulation of male reproduction in reptiles and other vertebrates has been discussed with respect to dependence on and control of the neuroendocrine system, particularly the hypothalamic-pituitary-gonadal (HPG) axis. The HPG-axis is important in the seasonal expression of sexual behavior and gonadal activity (Hartmann, 1944; Foreman and Moss, 1977; Moore and Lindzey, 1992; Whittier and Tokarz, 1992; Schuett et al., 1997, this volume; Bonnet et al., this volume).

In *C. d. terrificus*, basophils were rich in secretions (gonadotropins) in spring and summer, just prior to mating and MMF (autumn). This period of high activity of the basophils coincides with the high activity of interstitial cells (synthesis of sex steroids). Pituitary GnRH stimulates the germinal epithelium (spermatogenesis) and interstitial cells increasing the levels of sex steroids (e.g., androgens), which influence sexual behavior in autumn for more details, (see Almeida-Santos and Salomão, 1997; Almeida-Santos et al., 1998). As expected, immature snakes did not show basophils rich in granules and active interstitial cells.

Male reproductive cycles of temperate and tropical species of rattlesnakes appear to be very similar,

despite the fact that these taxa are exposed to widely varying ecological conditions (Aldridge, 1979, 1993; Aldridge and Brown, 1995; Schuett et al., this volume). Accordingly, phylogeny appears to have an important influence in the maintenance of sexually related behaviors and their mechanisms. Similar to their northern congeners, male *C. d. terrificus* locate and mate with females in autumn, females show obligatory sperm storage in winter, and vitellogenesis, ovulation, and fertilization occur in spring. Finally, parturition occurs in summer (Almeida-Santos and Salomão, 1997).

Our findings herein clarify specific aspects of the male reproductive cycle in *C. d. terrificus*. In the future, we will continue to investigate reproduction in *C. d. terrificus* in the field and laboratory. In particular, we will examine the seasonal relationships of sexual and male agonistic behavior and circulating sex steroid hormones.

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