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Testosterone Level, Testicular Histology And Reproductive Events Of Caucasian Pit Viper, Gloydius Halys Caucasicus (Serpentes: Viperidae)

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ABSTRACT

The steroid hormones regulate the reproductive physiology of animals and may influence by habitat and seasonal conditions. In this study, body weight and length, testosterone concentration and testicular histology were measured in Caucasian pit viper from Lar region, Tehran province, during summer and autumn seasons in year 2014. Serum samples were collected from 24 anesthetized snakes and testicles were removed and fixed for histological study. Enzyme immunoassay (ELISA) was conducted on serum samples to determine concentrations of testosterone (T) and standard histological procedure was performed on gonads. Results indicated that body weights and lengths in summer and fall seasons were respectively 49.88±9.6 gr, 50.25±7.4 cm and 42.75±4.5gr, 43.25±2.3 cm, serum testosterone level in summer, 29.3± 5.12 ng/ml was more than fall, 13.75±2.80 ng/ml at $P\leq0.05$.The numbers of spermatogonias, spermatocytes and spermatids in summer were more than fall and statistically different at $P\leq0.05$. The leydig cells number and diameter in fall were more than summer but differences were not significant.There were significant differences between seminiferous tubules diameter, germinal epithelial height and tunica albuginea thickness in summer as compared to fall at $P\leq0.05$. The data suggest male Caucasian pit-viper of Lar region of Tehran displaying estival spermatogenesis and some relationship was revealed between T level and snake's body weight, and high level of T coincide with the mating period.

KEYWORDS: Testosterone, Testis histology, Snake, Body weight, Reproduction event

1. INTRODUCTION

The timing of reproductive events and testicular functions of animals regulated by endogenous factors, seasonal variations and (Jallageas et al., 1978; Maurel and Boissin, 1981; Saboureau and Boissin, 1978) and varying in different species.There are correlation between reproductive activities ie. spermatogenesis, vitellogenesis, mating, breeding season and gonadal cycle and hormonal concentration. These interrelations are little known for snakes.Reports show reproduction, in the European Viperidaeis mainly in spring, follows a period of hibernation of varying length according to the species and their geographical distribution (Prestt, 1971; Niison, 1980).The timing of the sexual cycle is partially dependent on annual period of inactivity.The V. aspis has two mating periods in flat country, but only one in mountains where hibernation is longer. North American pitvipers have been characterizedas exhibiting two major types of mating seasons, with courtship,copulation, and male–male combat restricted to either: (i) latesummer/fall, or (ii) late summer/fall and spring (Schuett, 1992;Aldridge and Duvall, 2002; Schuett et al., 2002, 2006). A mating season restricted to spring is reported in the taxon, Crotalus ruber (Aldridge and Duvall, 2002). Theannual cycle of plasma testosterone of North American pit-vipers exhibits either a unimodal or bimodal patterns, and is associated with and considered a robust predictor of the mating season (Aldridge and Duvall, 2002; Schuett et al., 2002, 2005).These unimodal and bimodal patterns of sex steroidsecretion, secondary sex characteristics (e.g., the kidney sexual segment, SSK), and reproductive behavior,persist despite a conserved sequence of spermatogenesis, whichpeaks during the late summer/fall in all North American pit-vipers(the aestival, or Type I pattern; Saint Girons, 1982; Schuett, 1992; Aldridge and Duvall, 2002).Although the mating patterns of pit vipers are complex andmay vary within family,genus, and even species (Aldridge and Duvall, 2002). In many studies the gonadal cycles have still been used to describe the mating systems of many pit viper species (*Agkistrodon piscivorus*: Johnson et al., 1982; *C. atrox*: Tinkle, 1962; *C. helleri*: Aldridge, 2002; *C. horridus*: Gibbons, 1972: *C. lepidus*: Goldberg, 2000a; *C. mitchellii*: Goldberg, 2000b; *Crotalus molossus*: Goldberg, 1999a; *C. oreganus*: Aldridge, 2002; *C. ruber*: Goldberg, 1999b; *C. scutulatus*: Goldberg and Rosen, 2000; *C. viridis*: Aldridge, 1979; *C.willardi*: Holycross and Goldberg, 2001). Although four types of male reproduction in snakes is defined by Saint Girons (1982), the distinctly seasonal (displaying estival spermatogenesis) to aseasonal (displaying continuous spermatogenesis), and exceptions certainly exist. Snakes display considerable variability in reproductive traits among species (Hartmann ,et al., 2004) exhibiting a wide range of mating systems and male and male reproductive behaviors (Tourmente, et al., 2009).Anatomical and physiological indicators of the mating season in male pit- vipers have been identified, as patterns of annual testosterone (T) and kidney sexual segment (SSK) hypertrophy (Aldridge and Duvall, 2002; Schuett et al., 2002, 2005).In the Trapelus lessonae from the Western plateau of Iran, Central Zagros, three spermatogenesis phases are reported;active phase, in months where spermatogenesis occurs and primary and secondary spermatocytes and also spermatozoa can be seen in luminal seminiferous or lumen epididymis, the resting phase, in which, spermatocytes and spermatozoids cannot be observed, and in the transitional phase, some specimens have spermatozoa and spermatocytes in seminiferous but others lack spermatozoa and spermatocytes in the seminiferous tubules().

It is generally accepted that adult's testis size is correlated with capacity to produce sperm and that total sertoli cell numbers determine mature testis size in males of various mammalian species [In temperate zone pit vipers, spermatogenesis takes place in the late summer and fall before over-wintering (Aldridge and Duvall, 2002). Therefore, male snakes that breed in the spring must store sperm in the vas deferens over winter until the spring breeding season (Schuett, 1992). Spring unimodal species are the only pit vipers where spermatogenesis is completely uncoupled from mating. Altogether, reports reveal reproductive events are affected by eco-geographical and hormonal status and may vary within different snake's species. This study is conducted, to understand the interrelation of the testosterone concentration, testicular histology and reproductive events in the Caucasian pit viper of Lar mount of Tehran-Iran.

2. MATERIALS AND METHODS

Twenty four male Caucasian pit-viper were collected from Lar mountain (N35, 54, E51, 33) in the Tehran province, Iran during fall and summer seasons year 2014. Snakes were weighed, serum samples were drawn off from blood of anesthetized snakes and placed at -20 ◦C until testosterone concentration, assayed by Enzyme immunoassay (ELISA).

The testis was removed, fixed in 10% formalin, the tissue was further processed and embedded in paraffin, sectioned at 5µm and stained with Erlich's Hematoxylin and Eosin. For each sample, 5 measurements of seminiferous tubules diameter, leydig cell's number and diameter, germinal epithelial height and tunica albuginea thickness were taken under observation, using Olympus (BX51TRF, Japan) for light microscopy. The mean of values was determined and analyzed statistically by t-test, using Graph Pads Quicq Calcs software.

3. RESULTS

3.1. Vipers body weight

Data in figure 1 show, there is an increase of body weight of Caucasian viper at the end of summer (from July to August P <0.001) and then decreased during autumn (October-November, P <0.001). Overall, body weight of vipers in summer is significantly different from autumn (P≤ 0.05).

Table1. Body weight, SVL and testosterone concentrationin the male Caucasian pit viper

Months / Seasons	BW (gr)	SVL (cm)	T conc.(ng/ml)
July	48±9.7 n=10	50.4±6.6 n=10	31.12±5.7 n=10
August	53±9.2 n=6	46.67±5.0 n=6	28.15±6 n=6
Summer	49.88±9.6 n=16	50.25±7.4 n=16	29.3±5.1 n=16
October	44±2.58 n= 4	43.25±1.2 n=4	15.09±2 n=4
November	41.50±6.3 n=4	43.25±3.3 n=4	12.4±2.8 n=4
Autumn	42.75±4.5 n=8	43.25±2.3 n=8	13.75±2.8 n=8

BW= Body weight, SVL=Snout vent length, T= Testosterone

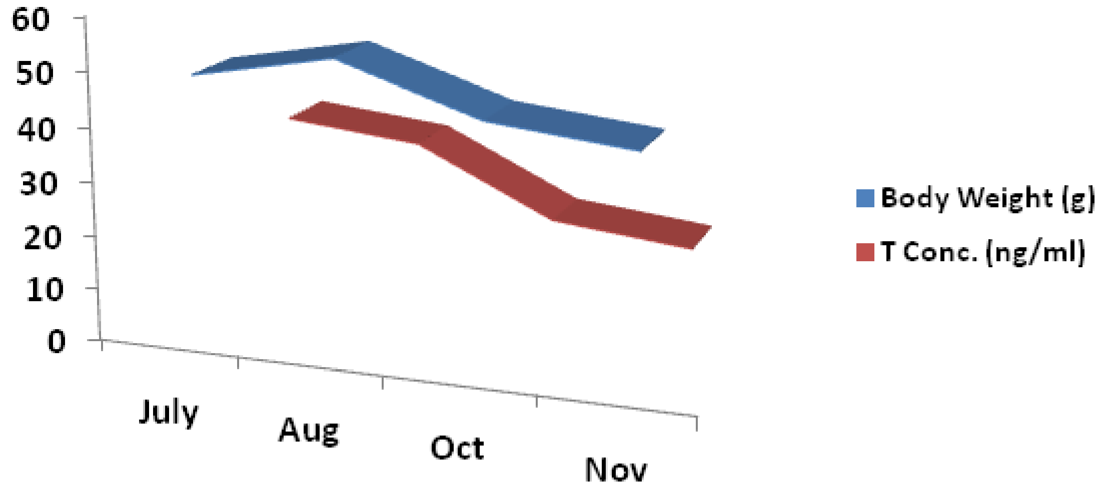


Figure 1. Relationship between serum testosterone levelsand body weight in Caucasian viper

3.2. Serum testosterone concentration

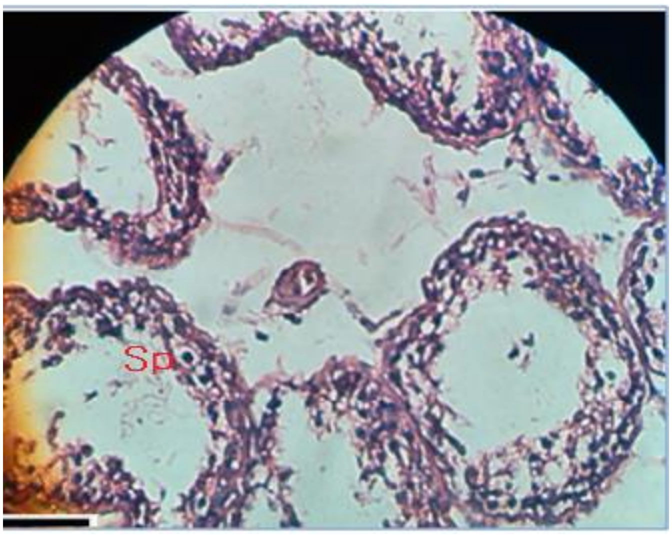
The serum testosterone (T) level was different from one season to the next P≤ 0.05 (Figure 1). High values of serum testosterone were observed during summer, between July and August (Table 1). The one peak of T was observed in July, then concentration decreased and reached to its half in the October and lowest in the November.

3.3. Testicular histology

Germ cells develop spermatogenically in close association with the Sertoli cells of seminiferous epithelium. The seminiferous epithelium contains spermatogonia cells (figure 1A& B) during all months of the study duration but more abundant in the July. Spermatogonia undergo mitotic divisions and enter prophase of meiosis in August to form spermatocytes. These cells contain a nucleus with a well-defined dark staining nucleolus.(Figure 2 A, Sp) Further cell division and chromosomal aggregation and chromatin condensation form the spermatids that are large number in July toAugust (figure 2 B, SpT). The acrosomal formation, nuclear elongation, and chromosomal condensation of germ cells mark the beginning of spermiogeneses in September. Once spermiogenesis is completed, the mature spermatozoa (Figure 2C, MS) are released into the lumen of the seminiferous tubules where they will be transported to the ducts of the male reproductive system.

This annual spermatogenesis is supported by seminiferous tubule diameter andgerminal epithelial height data. Seasonal variation in seminiferous tubule diameter and germinal epithelial height is shown in Figure 3. The increase in mitosis and meiosis leads to larger seminiferous tubule diameters in July and August.

A



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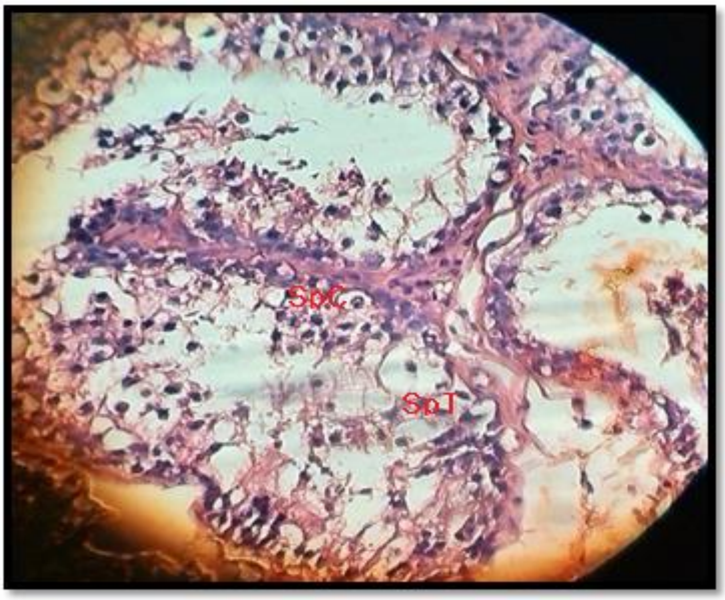

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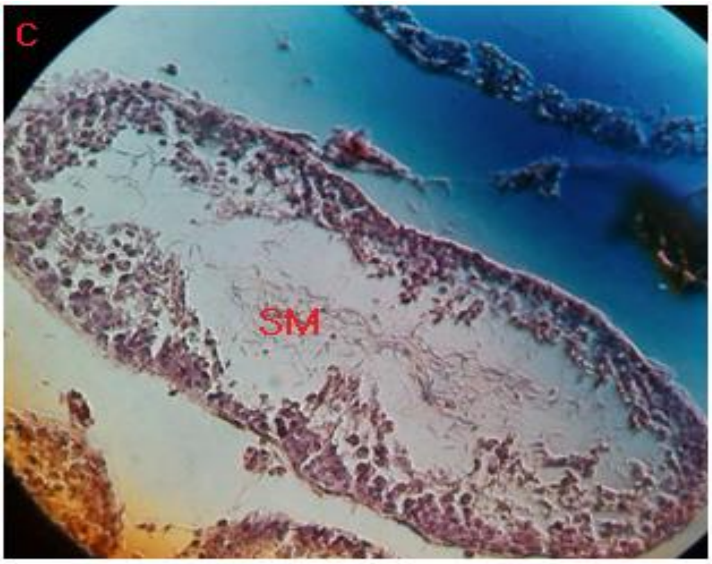

Figure 2. (A) Sagittal section (40 x) of a July seminiferous tubule. Bar = 100 mm. The cell types represented within the germinal epithelium Labeled cell type Sp, spermatogonia. (B) The cell types represented within the August germinal epithelium Labeled cell types:SpC, spermatocytes and SpT spermatid. (C) Cross-section (40X) of a September seminiferous tubule. Note, mature spermatozoa (SM) in the lumen suggest spermiation has started.

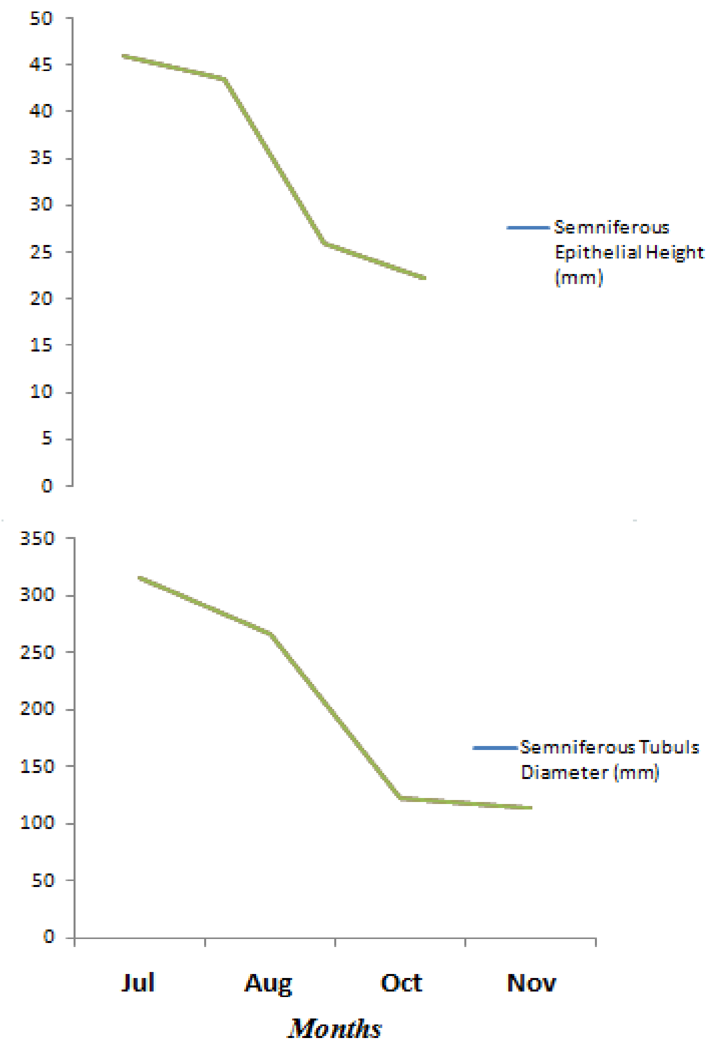

Figure 3.Top: variation in seminiferous tubule diameter and bottom: variation in germinal epithelial height during the Summer and Autumn seasons in *Glydius halys* caucasicus

4. DISSCUSION

Here, we report the profile of circulating (serum) steroid hormone, testosterone (T), and testicular histology during active season of summer and pre- hibernation period of autumn in wild-collected *Glydius halys* caucasicus from the Lar from Tehran province.

Our previous studies revealed in this viper, there is a mating season restricted to late spring; the females show ovulation and fertilization in late spring (Bahri etal, 20016), and births occur from mid to end of September (Shakoori etal, 20015). Males displaying, estival spermatogenesis (Mozafari et al., 2012).

The present data show that, in V. caucasicus, sera testosterone has one peak in the August. Testosterone and DHT are elevated at the time of both breeding and

spermatogenesis in male vipers studied to date (*A. contortrix*: Schuett et al., 1997; *A.piscivorous*: Graham et al., 2008; *C. atrox*: Taylor et al., 2004; *C. molossus*: Schuett etal., 2005; *C. scutulatus*: Schuett et al., 2002; *V. aspis*: Saint Girons et al., 1993). Of these, only *C. molossus* and *A. piscivorous* breed unimodally in the late summer and fall. The androgen profile for these species has a single peak in late summer coincident with spermatogenesis and mating (Graham et al., 2008; Johnson et al., 1982; Schuett et al.,2005). All other pit vipers studied exhibit the bimodal pattern, with two androgen peaks, one in spring (dissociated from spermatogenesis) and one in the late summer/fall (associated with spermatogenesis). In *C. atrox*, T is at peak concentrations early in the spring breeding season (March) and during an additional breeding season and peak spermatogenesis (August) (Taylor et al., 2004). However, most matings occur in April, when T concentrations, while elevated, were lower than in March. It is unclear why T concentrations are higher in March than in April, when the breeding season is at its peak. This trend has been observed in temperate garter snakes as well (Moore et al., 2000). This supports the organizational hypothesis, under which androgens, condition male snakes for reproduction at a slightly later date (Crews, 1991, Saint Girons, 1993). Basal androgen levels are recorded in May and June in all bimodal pit vipers studied. Schuett et al. (2006) quantified plasma T concentrations in *C. atrox* in the Sonoran Desert basking on warm winter days. They found that plasma T remains above baseline levels throughout the winter. Report indicate that *C. oreganus* in central California utilizes a bimodal pattern of breeding, with mating and agonistic behavior occurring in the spring and the late summer/fall(Craig M. Lind; June 2009).It seems each breeding season corresponds with elevated or highly variable androgen (T and DHT) levels.

In strong contrast to what has been documented in endotherms (Bronsson, 1998), a high body condition can be sufficient to initiate and fuel reproductive effort in snakes (Naulleau and Bonnet, 1996). The influence of body condition on reproduction has been demonstrated in many species (Albon, Mitchell, Huby, and Brown, 1986; Morisson, et al., 1994; Festa-Bianchet, Gaillard, and Jorgenson, 1998), we also found a positive correlation between early body condition and testosterone levels in males sampled from field that is similar to Bonnet et al., (2000) reports on asp. Viper.

Body condition is correlated with muscle mass in male snakes (Bonnet, 1996), and Shine et al., (2000) work shows that heavier bodied males obtain more matings. In strong contrast to females, a high-body-condition threshold is not necessary for the induction of male reproductive activity in V. aspis. However, high levels of testosterone are found only in males with abundant body reserves. A rapid elevation of plasma testosterone levels stimulates sexual activity after winter emergence in male asp vipers (Fleury and Naulleau, 1987; Naulleau and Fleury, 1987; Naulleau et al., 1987; Saint Girons et al., 1993), as in many other snake species (Bona-Gallo, Licht, Mackenzie, and Lofts, 1980; Krohmer, Grassman, and Crews, 1987; Aldridge, Greenhaw, and Plummer, 1990; Bonnet and Naulleau, 1996). In addition, high levels of testosterone provoke a strong anorexia in the asp viper, under both natural and experimental conditions. The cessation of feeding results in a marked decrease of body reserves during the mating period (Bonnet, 1996;). Males in high body condition mate-search more intensively than emaciated males, and can locate more reproductive females (Aubret et al., 2002).

The Gloydius halys caucasicus testis contains seminiferous tubules lined with seminiferous epithelia consisting of Sertoli cells and developing germ cells. Germ cells develop spermatogenically in close association with the Sertoli cells of this epithelium. Histological examination shows that testes of this population of pitvipers are spermatogenically active during the months of July – August with the proliferation of spermatogonia near the basal lamina (basement membrane) of the seminiferous epithelia and leads to larger seminiferous tubule diameters (315.1mm in July and 265mm in August)) and germinal epithelial heights (July, 46 mm; August, 43.6 mm). By November, spermiation is complete and the seminiferous tubules have entered their phase of quiescence, which leads to a dramatic decrease in seminiferous tubule diameter (113 mm) and germinal epithelial height (22.3 mm). Large numbers of immature spermatids have been sloughed into the seminiferous tubules in the July- October. Many of the developing spermatids have completed spermiogenesis and are being shed to the lumina of the seminiferous tubules as mature spermatozoa. The leydig cells are active during the sexual periods and hormones are suspected to be stored in the cytoplasmic lipids droplets in the leydig cells. Although these cells are active throughout the year contrast to the activity of the sertoli cells which are confined to the period of active spermetatogenesis.

Histological studies in temperate pit vipers (Aldridge and Duvall, 2002) have confirmed that spermatogenesis is restricted to the summer and early fall. Several studies have used testis mass and/or length to assess reproductive condition in pit vipers (Graham et al., 2008; Johnson et al., 1982; Schuett et al., 2002). Of these studies, all but one was conducted on snakes with a unimodal summer/fall breeding cycle (e.g., *A. piscivorus*, Graham et al., 2008; Johnson et al., 1982). In this species, testis mass and length were lowest in early spring and increase throughout spermatogenesis (Graham et al, 2008, Johnson et al., 1982). The only study measuring testis mass and or length in a bimodal pit viper was conducted on *C. scutulatus* (Schuett et al., 2002). This study found that testis length and mass were significantly elevated in the spring breeding season, coincident with mating and elevated T concentration, and in the late summer and fall, coincident with spermatogenesis, mating, and elevated T concentrations. Studies on other reptiles including snakes, turtles, and alligators, have shown that elevated androgen concentrations and breeding behavior do not always coincide with testis recrudescence.

In seasonal breeders, such as those reptiles found in temperate environments, once this single major spermiation event occurs in the summer or late fall, the testis enters a quiescent period where spermatogenesis slows or ceases altogether. The histology of the seminiferous tubules at this time shows reduced epithelia with typically only spermatogonia present. Within the lumina of the seminiferous tubules are pieces of the Sertoli cells with various generations of germ cells that have been sloughed from the epithelia and are thought to be recycled and reabsorbed along the length of the reproductive tract.

In continuous breeding reptiles, such as *Sceloporus bicanthalis* and *Anolis lineatopus.* Once a cycle of spermatogenesis is complete the basal compartment shows an increased rate of mitosis and meiosis, which amplifies the seminiferous epithelial height. These new generations of germ cells then enter spermiogenesis and spermiation again as a single wave or cohort. These steps are repeated over and over again leading to bimonthly sequential increases and decreases of the seminiferous epithelium and tubule diameter. Notice from that when the diameter is the widest in this bimonthly cycle the epithelium height is the lowest. Thus, when the seminiferous tubule diameter width is high then spermiation has occurred increasing the size of the lumen and tubule overall. In contrast, the spermiation event has caused a loss of germ cells and Sertoli cell columns therefore decreasing the overall seminiferous epithelial height (Gribbins, et al., 2011).However the highest peak of plasma testosterone precedes the relatively short spermiogenetic waves in summer. However, the much smaller autumnal concentration of plasma testosterone occurs during the largest spermiogenic wave (from the end of summer to the beginning of autumn).

However, further broad range of investigation of hormones in different seasons is required if an understanding of the relationship between steroid hormones and the reproductive cycle is ever to be achieved.

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