Chapter 3

Hormonal regulation of testicular functions in reptiles☆

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1 INTRODUCTION

Reptiles represent an important phylogenic link between the anamniotes and amniote vertebrates. Despite that all the extant reptiles are ectotherms such as fishes and amphibians, they are grouped with birds and mammals due to the presence of an amniotic membrane during development. Moreover, they are ancestral to both birds and mammals. The living reptiles can be classified into four orders: lizards and snakes in Squamata; *Sphenodon punctatus* (living fossil) in Sphenodonta; turtles and tortoises in Chelonia; and alligators and crocodiles in Crocodilia. The basic components of reptiles testis are quite like those of mammals, implying conserved development and cellular differentiation. The genital ridge develops as a thickening of coelomic epithelium on the ventromedial surface of the mesonephric kidney. Initially, gonads are bipotential and indistinguishable in either sex. A detailed study of gonadal development has been carried out in turtles (Pieau et al., 1999) in which germ cells migrate from the posterior germinal crescent to the genital ridge at the end of gastrulation. This migration is assumed to occur through the vascular system as in avian species since germ cells lie at the surface of the coelomic epithelium in both groups. During the development of gonadal primordia, the medullary region of the developing gonad is invaded by epithelial cells from (a) the external epithelium of Bowman's capsule, (b) the coelomic epithelium bordering mesonephric kidneys on the lateral side of each gonad, and (c) the germinal epithelium of the gonad. Cells from the Malpighian corpuscles and lateral coelomic epithelium become organized in the dorsal part of the gonadal primordium to form the rete cords. Germinal epithelial cells give rise to thin sex cords that penetrate the underlying initial mesenchyme (Fig. 1). Temperature plays the most important role in the differentiation of the bipotential gonad into a testis (see Chapter 1, this volume). At the male-producing temperature, sex cords envelop the germ cells and differentiate into typical testis cords; otherwise, they degenerate (Fig. 2; Yao & Capel, 2005). Many conserved genes are involved in sex differentiation and gonadal development in reptiles (Fig. 3). Among these, steroidogenic factor 1 (*Sf1*) and Wilms tumor 1 (*Wt1*) genes are important for the initial formation of the bipotential gonad. Double sex and mab-3-related transcription factor 1 (*Dmrt1*) is an important testis-determining gene as its expression increases during the temperature-sensitive period (TSP) and remains high during testicular development. The action of *Dmrt1* in testes development is downstream to temperature and upstream to another important gene, anti-Müllerian hormone (*Amh*). The expression of *Amh* increases at the end of the TSP, indicating its role at later stages of testicular differentiation probably in inducing the regression of Müllerian ducts (Yao & Capel, 2005). The potential action of Amh on sexual differentiation was evaluated also in the Chinese tortoise *Pelodiscus sinensis*. In this species, the authors demonstrated that *Amh* RNA was initially expressed in male embryonic gonads at stage 15, prior to gonad sexual differentiation, showing a male-specific expression pattern throughout embryogenesis. In detail, *Amh* is rapidly upregulated during sexual reversal from female to male induced by the aromatase inhibitor letrozole, as well as loss of *Amh* function by RNA interference, resulting in complete feminization of the male genetic gonads (ZZ), suppression of the testicular marker Sox9 and upregulation of the ovarian regulator Cyp19a1, characterized by the formation of a testicular structure, ectopic activation of Sox9 and a significant decline of Cyp19a1. These data provided the authors with strong evidence that Amh is necessary and sufficient to lead testicular development in a reptilian species (Zhou et al., 2019). Downstream molecular events in testis differentiation need to be

☆ Sunil Kumar, Brototi Roy, and Umesh Rai are the authors of the original version of this chapter.

Hormones and Reproduction of Vertebrates, Volume 3. https://doi.org/10.1016/B978-0-443-16022-6.00015-9
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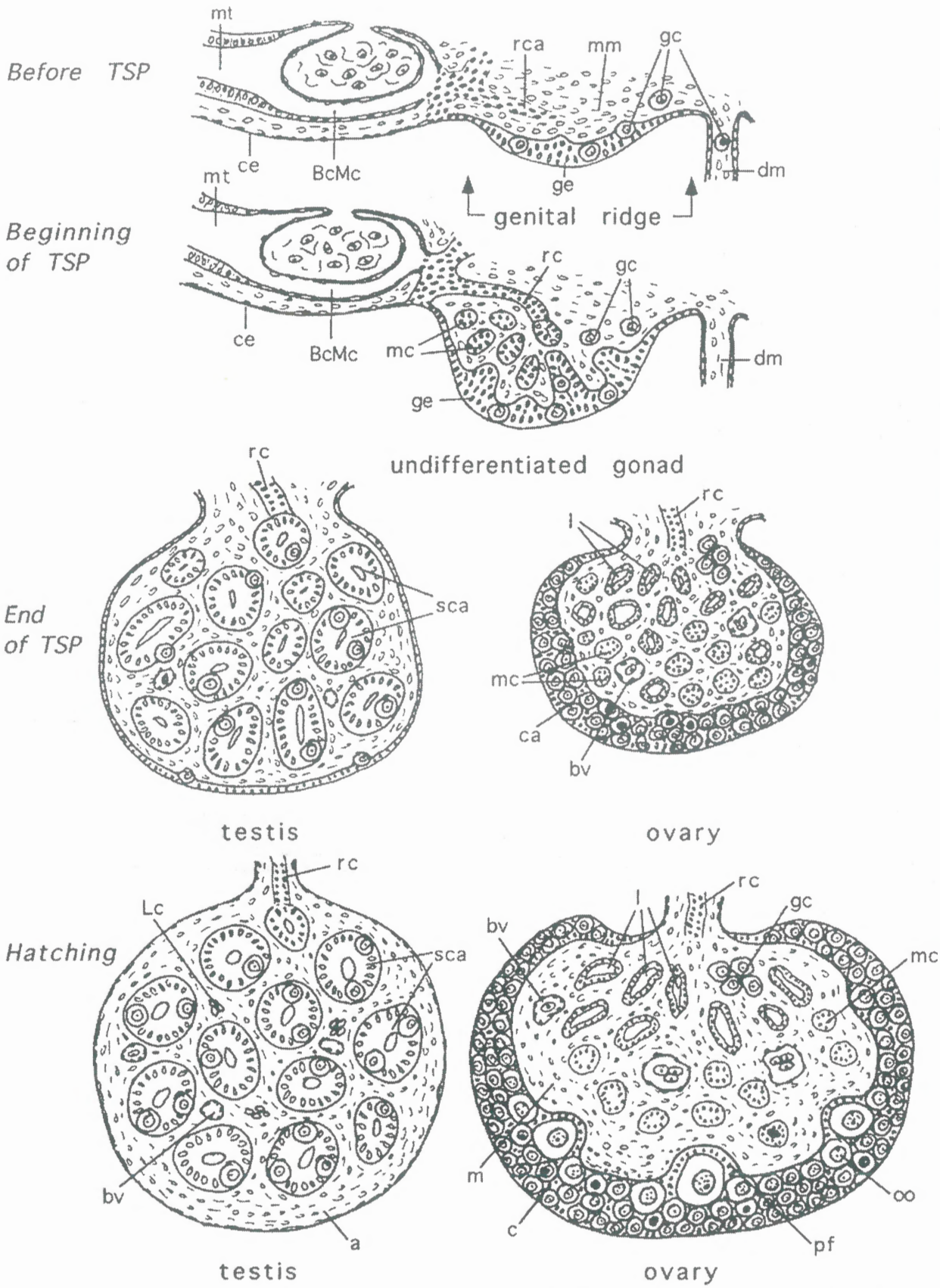

FIG. 1 Development and differentiation of gonads in reptiles before, during, and after the thermosensitive period (TSP). a, albuginea; BcMc, Bowman's capsule of Malpighian corpuscle; bv, blood vessel; c, cortex; ca, cortex anlage; ce, coelomic epithelium; dm, dorsal mesentery; gc, germ cells; ge, germinal epithelium; l, lacunae; Lc, Leydig cells; m, medulla; mc, medullary cords; mm, mesonephric mesenchyme; mt, mesonephric tube; oo, oocyte; pf, primordial follicle; rc, rete cord; rca, rete cord anlage; sca, seminiferous cord anlagen. *(Courtesy: Pieau, C., Dorizzi, M., & Richard-Mercier, N. (1999). Temperature-dependent sex determination and gonadal differentiation in reptiles.* Cellular and Molecular Life Sciences *55, 887–900.)*

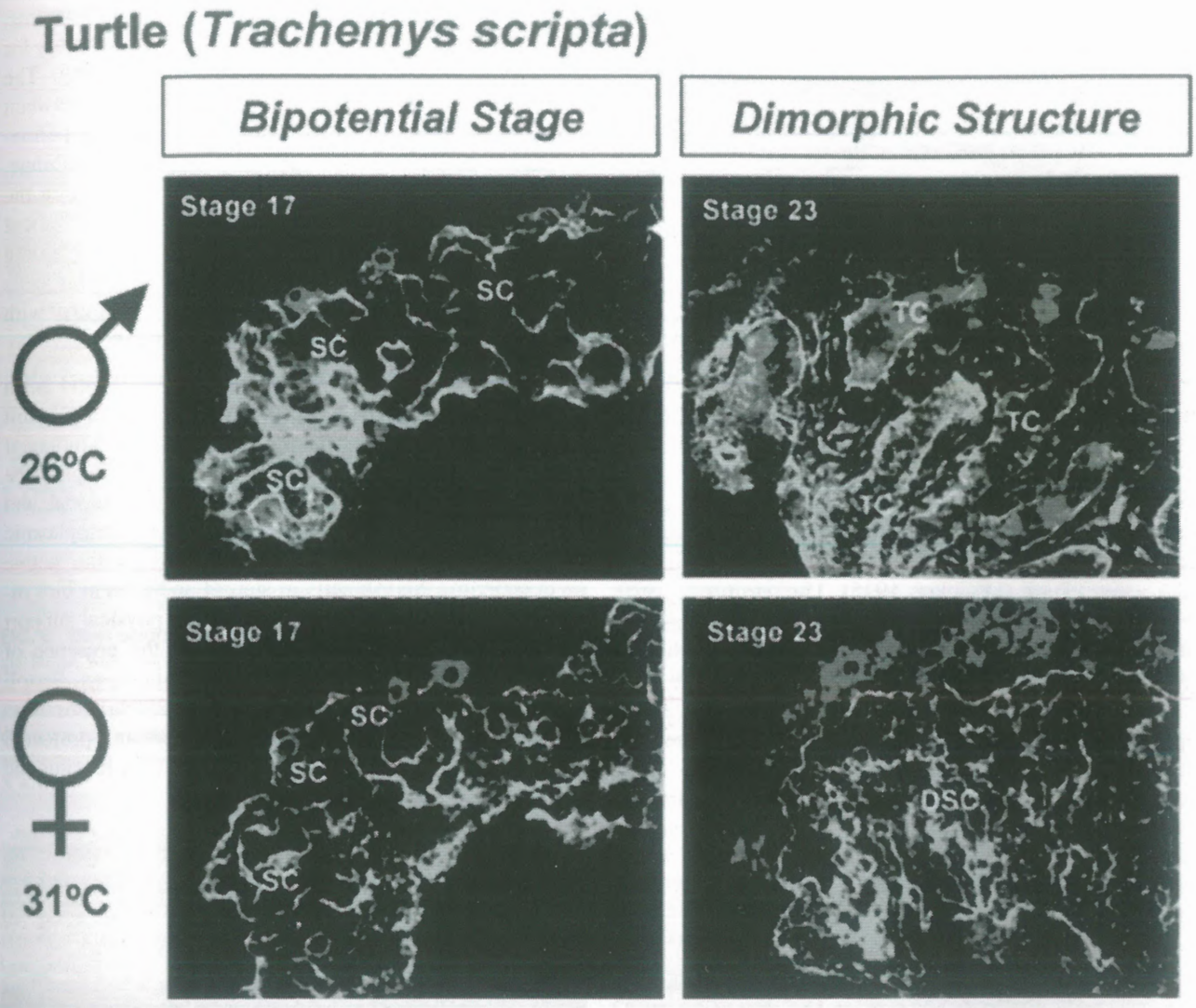

FIG. 2 Temperature-dependent cellular reorganization in the bipotential gonad of a turtle *Trachemys scripta*. Primordial germ cells (PGCs, *red*) are associated initially with the coelomic epithelium. At the testis-determining temperature (26°C), PGCs are enclosed within the testis cord by stage 23. At the ovary-producing temperature (31°C), sex cords degenerate (DSC) and PGCs remain in the periphery. The primordial germ cells are highlighted by an anti-VASA antibody (*red*) and sex cords (SC) and testis cords (TC) are outlined by an anti-laminin antibody (*green*). *(Modified from Yao, H.-C.H., & Capel, B. (2005). Temperature, genes, and sex: A comparative view of sex determination in* Trachemys scripta *and* Mus musculus. Journal of Biochemistry *138, 5–12.)*

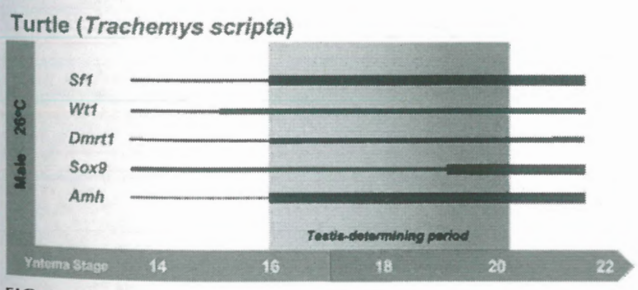

FIG. 3 Temporal expression of genes that play critical roles in sex determination and differentiation in the male red-eared slider turtle (*Trachemys scripta*). The *blue shaded* area represents the temperature-sensitive period. Anti-Müllerian hormone, *Amh*; *Drosophila Doublesex* and *C. elegans Mab-3* related transcription factor 1, *Dmrt1*; Sry-like HMG-box protein 9, *Sox9*, steroidogenic factor 1, *Sf1*; Wilm's tumor gene 1, *Wt1*. *(Modified from Yao, H.-C.H., & Capel, B. (2005). Temperature, genes, and sex: A comparative view of sex determination in* Trachemys scripta *and* Mus musculus. Journal of Biochemistry *138, 5–12.)*

explored in many reptiles to develop conceptual knowledge of the molecular regulation of sex differentiation.

There are two important functions of the testis: the production of male gametes (spermatogenesis) and the synthesis of sex steroids (steroidogenesis). These functions are regulated by the complex interaction of extra- and intratesticular factors. However, purification and characterization of intratesticular factors regulating testicular functions in a paracrine manner in reptiles are yet to be done. In addition to endocrine and paracrine factors, environmental cues such as photoperiod, temperature, and rainfall are implicated in control of reproductive activity by influencing the release of hypothalamic hormones, particularly gonadotropin-releasing hormone (GnRH). This in turn stimulates secretion of gonadotropins (GTHs) and consequently regulates testicular functions.

2 TESTICULAR STRUCTURE

The reptilian testis differs from testes of anamniotes that are characterized by a cystic form of spermatogenesis (Pudney, 1995). However, like other vertebrates, the two important testicular functions occur in two distinct compartments of the testis, i.e., the tubular and interstitial compartments.

2.1 Tubular compartment of the testis

The tubular compartment is the site of spermatogenesis. It consists of seminiferous tubules. Each tubule is lined with basement membrane and cellular peritubular (fibroblast/myoid-like) cells. These cells make their first phyletic appearance in the reptilian testis (Unsicker, 1975) since they are absent in anamniotes. Depending on the reproductive state, peritubular cells change from fibroblast-like in the unreproductive phase to myoid-like in the spermatogenically active phase (Unsicker, 1975). The peritubular cells are arranged in multilayers during the quiescent phase in the Indian wall lizard, *Hemidactylus flaviviridis*, whereas during the breeding phase they are arranged in a single layer (Fig. 4; Khan & Rai, 2004a). The peritubular myoid cells in reptiles, like their mammalian counterparts, share several ultrastructural characteristics with smooth muscle cells. They contain 50-70 Å thick intracytoplasmic filaments, plasmalemmal and intracellular dense attachment sites for thin filaments, and smooth membrane inpocketing. The presence of desmosomes and tight junctions between adjacent myoid cells suggests the existence of a permeability barrier even at the level of the basement membrane. The myoid-like appearance of peritubular cells during the spermatogenically active phase suggests their involvement in the contraction of seminiferous tubules, thereby aiding sperm transport. The transformation of peritubular cells from fibroblast to myoid-like is closely associated with the increase of steroidogenic activity of Leydig cells.

In addition to peritubular cells, the Sertoli cells and germ cells constitute the tubular compartment of the testis. Sertoli cells are irregular in shape and extend from base to lumen of the tubule. Their irregular-shaped nucleus occupies the basal position. The presence of numerous spherical and elongated mitochondria along with smooth endoplasmic reticulum (SER) and Golgi bodies suggests the active involvement of Sertoli cells in steroid and protein biosynthesis (Fig. 5). Also, Sertoli cells provide physical support to developing germ cells as evident by the presence of various adhering junctional complexes between Sertoli and germ cells (Fig. 6; Khan & Rai, 2004a). Another important function of the Sertoli cell is to create a protected microenvironment for developing germ cells by forming the

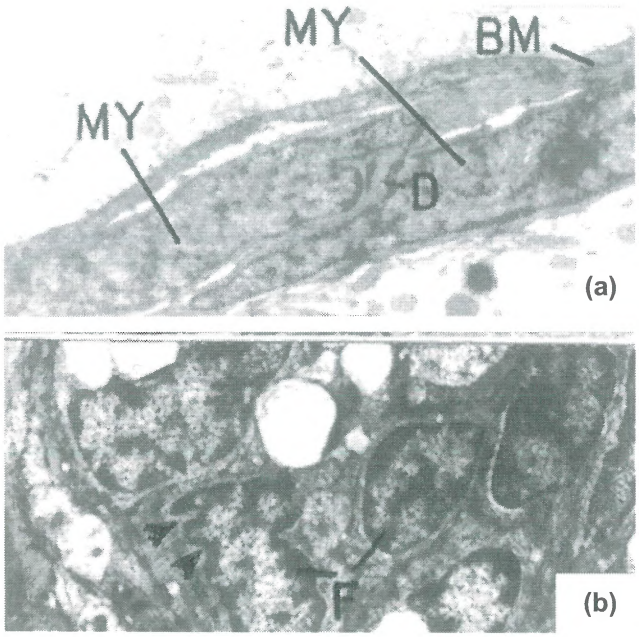

FIG. 4 Electron micrograph of basal lamina of seminiferous tubules during (A) breeding (X 7, 950) and (B) regressed phase (X 6, 150) in the Indian wall lizard, *Hemidactylus flaviviridis*. The peritubular cells change from a myoid-like, single layer in the breeding phase to a fibroblast-like multilayer during the regressed phase. MY, myoid cells; BM, basement membrane; F, fibroblast-like peritubular cells; D, desmosomes-like junctions; arrowheads, nuclear indentations.

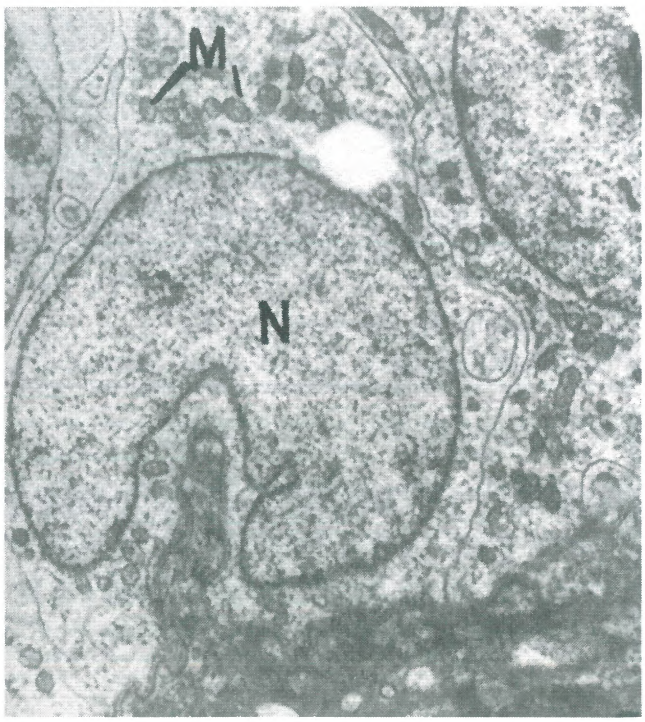

FIG. 5 Electron micrograph of a regressed testis showing the basal portion of Sertoli cells with a horseshoe-shaped nucleus (N) and basement membrane invaginations. Numerous spherical mitochondria (M) can be seen. X 7, 950.

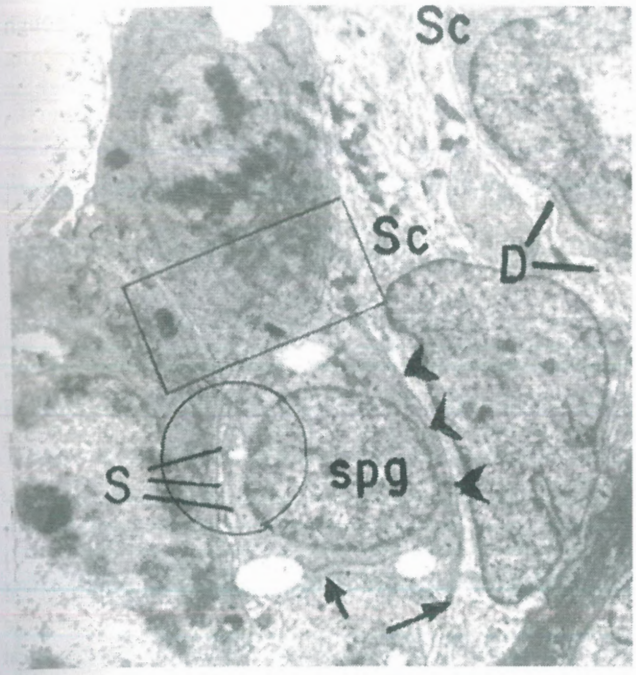

FIG. 6 Basal portion of seminiferous epithelium of a regressed testis in the Indian wall lizard *Hemidactylus flaviviridis* showing different types of junctional interactions between the testicular cells: desmosome-like junctions between Sertoli cells (D), Sertoli cell-germ cell (arrow), and germ cell-germ cell (rectangle); septate-like junctions between germ cells (S), and Sertoli cell-germ cell (arrowhead); spg, spermatogonia; Sc, Sertoli cell. X 4, 650.

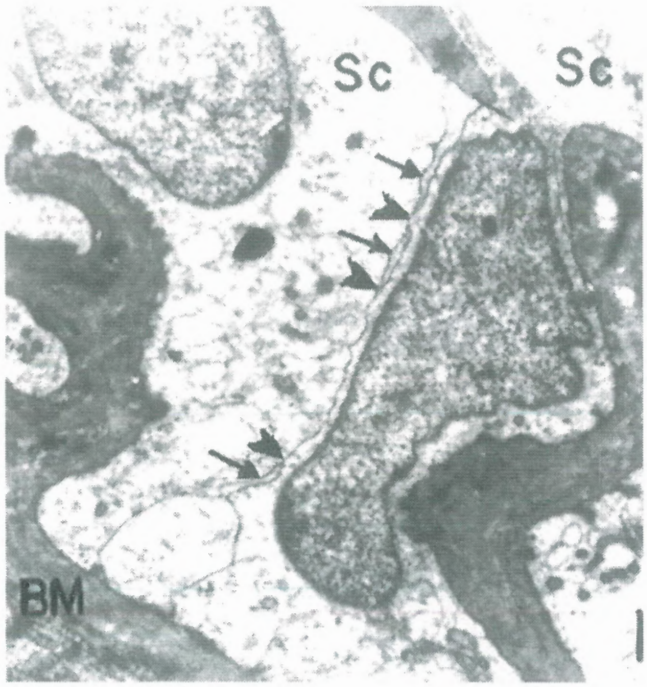

FIG. 7 Electron micrograph of blood-testis barrier between Sertoli cells of the Indian wall lizard. Occluding junctions including tight junctions (arrow heads) and gap junctions (arrows) between Sertoli cells (Sc), BM, and basement membrane. X 6, 150.

blood-testis barrier. This is formed by occluding junctional complexes between adjacent Sertoli cells (Fig. 7). The blood-testis barrier demarcates the seminiferous tubule into two distinct compartments (basal and adluminal). The basal compartment faces the interstitial space, houses the spermatogonia, and has direct access to the serum components. The adluminal compartment includes primary and secondary spermatocytes, and spermatids. Germ cells of the adluminal subcompartment derive their nutrients from the Sertoli cells. Lactate, a Sertoli cell metabolite, is utilized by germ cells as an energy substrate (Nirmal & Rai, 1999). In addition to their nursing function, a role for Sertoli cells in spermiation is possible, based on the presence of residual bodies within the cytoplasm (Fig. 8). The residual bodies and phagocytosed degenerated germ cells are eventually disposed of by the Sertoli cells (Dubois et al., 1988; Khan & Rai, 2004a).

2.2 Interstitial compartment

The interstitial compartment is the extra-tubular space between the seminiferous tubules. This compartment consists of blood and lymphatic vessels, nerve fibers, a large population of steroid-secreting Leydig cells, and immune cells (macrophages and a few mast cells). Leydig cells, the primary site for steroid biosynthesis, are characterized by the presence of elongated mitochondria with tubular cristae, smooth endoplasmic reticulum (SER), lipid droplets, and 3β-hydroxysteroid dehydrogenase (3βHSD) enzyme activity. Since reptiles are seasonal breeders, marked variations in Leydig cell ultrastructural features are observed during different phases of the reproductive cycle (Khan & Rai, 2004a). The cytoplasm of Leydig cells during the regressed phase contains numerous large lipid droplets, irregular-shaped nuclei, sparsely distributed SER, and tubular mitochondria. Very weak steroidogenic activity of Leydig cells is evidenced by weak 3β-HSD activity. During recrudescence, Leydig cells resume their steroidogenic activity with a marked decrease in size and number of lipid droplets. Leydig cells in the spermatogenically active phase are characterized by abundant SER and a large number of tubular mitochondria (Fig. 9; Dubois et al., 1988; Khan & Rai, 2004b).

Regarding other testicular interstitial cells, studies are limited and confined to a few species. Macrophages have been isolated from the testes of Indian wall lizards and identified by their morphological and functional characteristics (Figs. 10 and 11; Khan & Rai, 2007). They constitute the second-largest population of the testicular interstitium. Corresponding to Leydig cells, the number of testicular macrophages varies in different phases of the reproductive cycle. With the onset of recrudescence, macrophages increase in

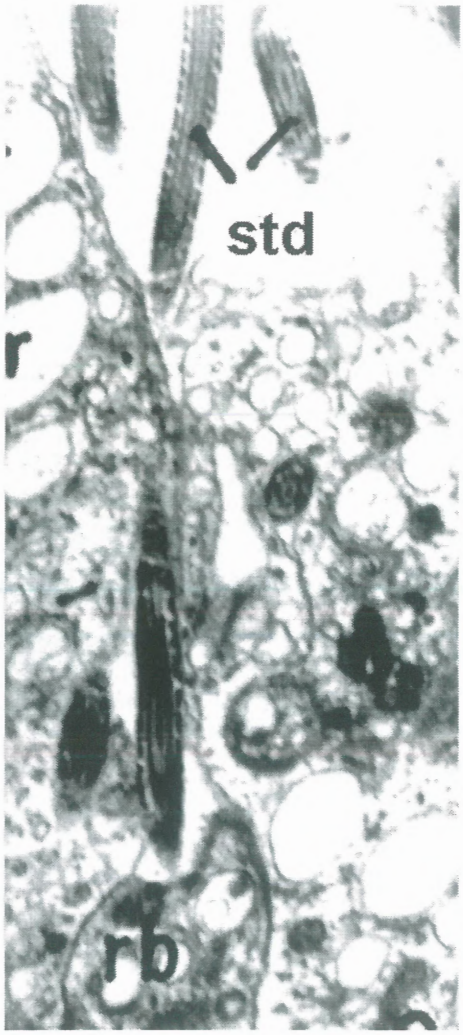

FIG. 8 Degenerated residual body (rb) deep within Sertoli cell cytoplasm during spermatogenically active phase in *Hemidactylus flaviviridis*. Elongated spermatids (std) embedded in apical portion of the Sertoli cell cytoplasm can be seen. Some empty recesses (r) after spermiation are also visible X 4, 350 (Khan & Rai, 2004a).

number and reach a maximum in the breeding phase. Interestingly, the ratio of macrophages to Leydig cells remains constant throughout the reproductive cycle, implying a close relationship between these two cell populations. The presence of mast cells has been demonstrated in the testis of *Podarcis sicula sicula* using electron microscopy. The number varies during the year with peaks in spring and winter (Minucci et al., 1995). Unlike macrophages, mast cells have an inverse relationship with Leydig cell number. Administration of a Leydig-cell specific cytotoxin, ethane dimethane sulfonate, increases mast cell number. The influence of interstitial cells on the spermatogenesis process has also been described in the soft-shell turtle, *P. sinensis*. In detail, interstitial cells such as the telocytes (TCs) and the peritubular cells (Pc) (with smooth muscle-like characteristics) in contact with surrounding structures in the testis have been shown. TCs connect with each other through homocellular and heterocellular junctions with Pcs, Leydig cells, and blood vessels. The authors hypothesized that the interactions of interstitial cells with their surroundings in the *P. sinensis* testes might offer new insights into the understanding of testis function (Yang et al., 2015).

3 TESTICULAR FUNCTIONS

3.1 Spermatogenesis

Spermatogenesis is a complex biochemical and morphogenetic process, regulated by both systemic and local factors (Rosati, Agnese, et al., 2020; Rosati et al., 2022), occurring in the testis and consisting of the differentiation of spermatogonia into spermatozoa. In anamniotes, temporal spermatogenesis occurs within a cyst in which a cohort of germ cells progress through the phases of spermatogenesis. All the germ cells present in a cyst are in the same stage of spermatogenesis. When spermatogenesis is completed, the cysts rupture and sperm are released as a single spermiation event. Although reptiles resemble other amniotes in possession of seminiferous tubules lined with seminiferous epithelium, spermatogenesis in temperate species is generally aligned with anamniotes. Instead of spatial germ cell development as in mammals and birds, a strategy of temporal germ cell development has been described in some temperate species representing different orders of reptiles.

In the slider turtle, *Trachemys scripta*, the initiation of spermatogenesis is characterized by the proliferation of spermatogonia near the basement membrane of seminiferous epithelium in June and early July. Three types of spermatogonia are seen: resting, A, and B. The resting spermatogonia located toward the base of tubules are the smallest in size and do not undergo mitosis. It is the type A spermatogonia that undergoes mitosis to form type B spermatogonia. With the initiation of meiosis, primary spermatocytes are formed from type B spermatogonia. They give rise to spermatids after the completion of the second meiotic division. These haploid germ cells are transformed into sperm by the process of spermiogenesis, which is completed by late October to early November. Based on acrosomal development, nuclear condensation, cytoplasmic elimination, and flagellum formation, spermiogenesis in the slider turtle is divided into eight recognizable steps (Gribbins et al., 2003).

In the turtle, *Graptemys pseudogeographica kohnii*, a postnuptial spermatogenic cycle has been described that is characterized by an important autumn spermatogenesis event. In detail, most germ cell populations in the May specimens are represented by resting spermatogonia, type A spermatogonia, type B spermatogonia, preleptotene spermatocytes, and numerous nuclei of Sertoli cells near the

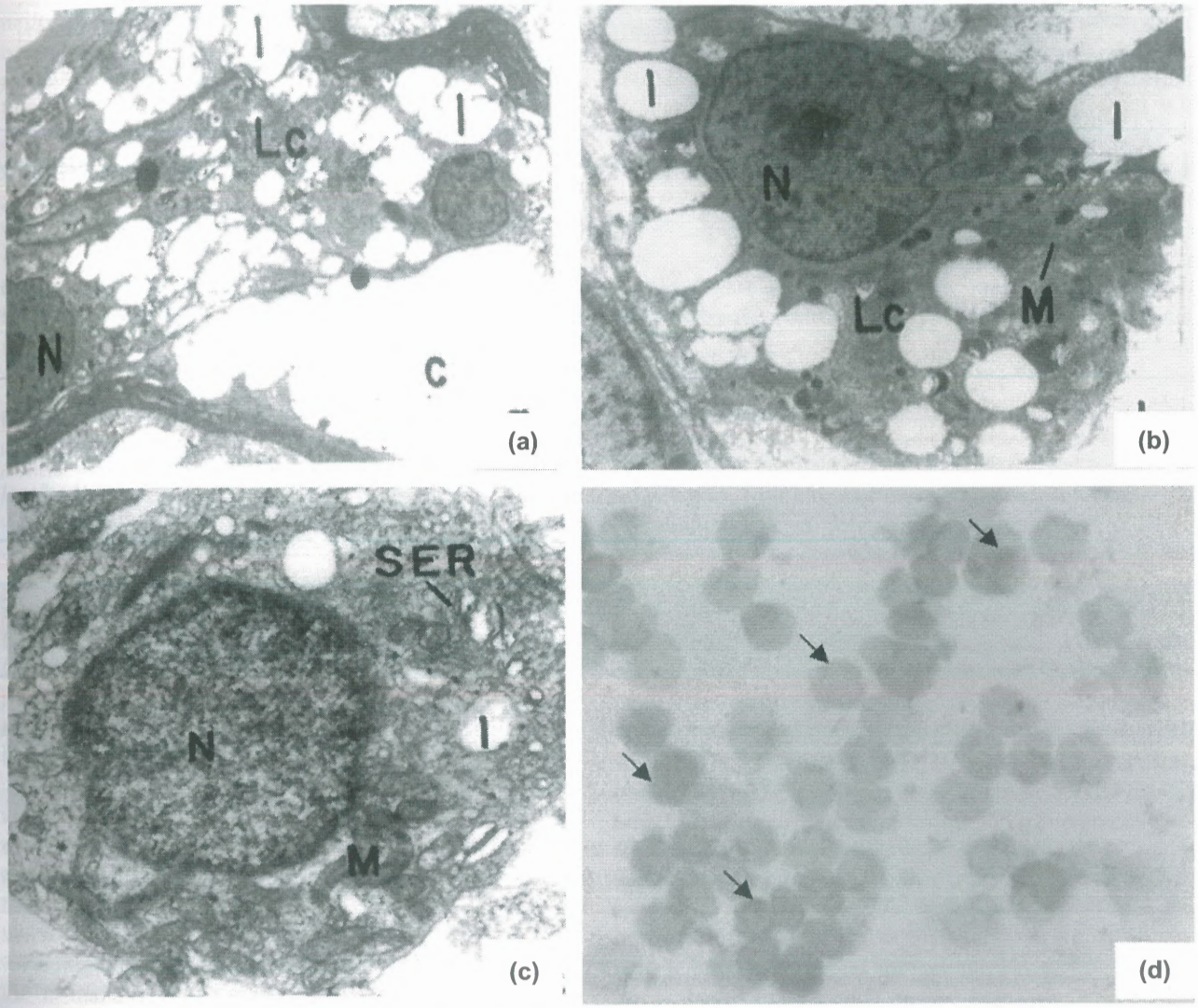

FIG. 9 Ultrastructural features of Leydig cells (Lc) during different phases of the reproductive cycle of the Indian wall lizard. (A) In regressed testis, abundant lipid droplets (l) are seen in the Leydig cell lying adjacent to a blood capillary (c) X 3450. (B) In the recrudescent phase, a few elongated mitochondria (M) with a decreased number of lipid droplets are observed X 10,000. (C) The voluminous cytoplasm of Leydig cells during the spermatogenically active phase includes an increased number of mitochondria with tubular cristae and an increased amount of smooth endoplasmic reticulum (SER). Lipids droplets (l) are rarely seen. The chromatin is evenly distributed in the nucleus (N) X 12,600. (D) Leydig cells showing Δ^5-3β-hydroxysteroid dehydrogenase enzyme activity after incubation with ovine follicle-stimulating hormone (oFSH) for 24 h (800×) (Khan & Rai, 2004a).

basement membrane. During June, meiotic processes begin with increasing numbers of spermatocytes resulting in an increase in the height of the seminiferous epithelium and the diameter of the seminiferous tubules. In September, the main germ cell population completed the meiosis process whereby the seminiferous epithelium was dominated by abundant round and first elongated spermatids. October seminiferous epithelia are characterized by a decrease in height as mature spermatozoa fill the luminal space, ready to be released and stored in the epididymis until the following spring mating season. After the spermiation event, the testes enter a period of quiescence that lasts until the next spermatogenic cycle, which begins the following spring (Lancaster et al., 2015).

Changes in the seminiferous epithelium during different times of the reproductive cycle have also been described in *P. sinensis*. Specifically in this turtle, the phase of maximum spermatogenic activity is recorded during summer and autumn, while the quiescent phase corresponds to the rest of the year. Germ cells progress through the spermatogenic stages according to a temporal rather than spatial pattern, while apoptotic germ cells mainly appear during the spermatogenic blocked stage, they are rarely present during active spermatogenesis. It is inferred that apoptosis may be one reason for the loss of germ cells during the resting phase of spermatogenesis. During the period of spermatogenesis, Sertoli cells become very tight and are in contact with several round/elongated spermatids. Many residual

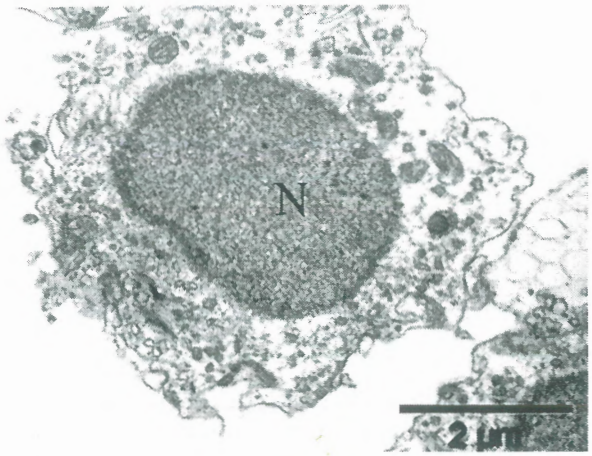

FIG. 10 Electron micrograph of lizard testicular macrophage with ruffled border, indented nucleus (N) and presence of numerous phagolysosomes.

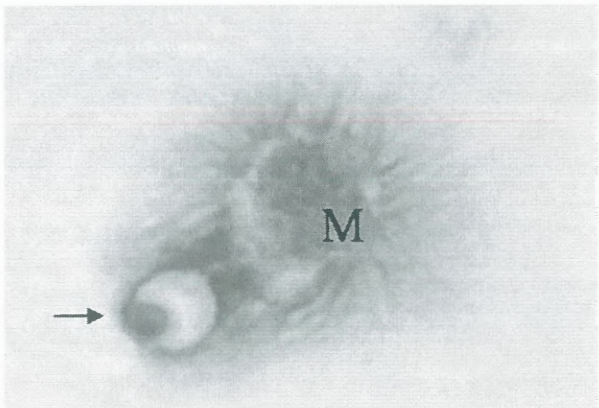

FIG. 11 The testicular macrophage (M) of the Indian wall lizard showing phagocytosis of a heat-killed yeast cell (arrow). Numerous phagocytic processes can be seen; X: 480.

spermatozoa can be internalized and degraded within Sertoli cells by endocytosis during the the spermatogenic blocked stage, which precedes the next reproductive cycle in *P. sinensis*. In the late spermatogenic phase, the round-shaped mitochondria of the spermatids elongate and swell, later forming a crescent shape and developing into "onion-shaped" mitochondria. As spermiogenesis progresses, the endoplasmic reticulum of the spermatids is transferred to a specialized structure called the "chrysanthemum flower center," which may be a source of autophagosomal membranes (Chen et al., 2020).

The male European wall lizard *Podarcis muralis* differs from *T. scripta* in having a spermatogenically active seminiferous epithelium throughout the year with a very short quiescent phase. Spermatogenesis slows down during the quiescent phase instead of ceasing completely. Only two types of spermatogonia, namely A and B, are present within the seminiferous epithelium, and spermiogenesis is divided into seven recognizable steps (Gribbins & Gist, 2003). In contrast, in the Italian wall lizard *Podarcis siculus* (until recently known as *P. sicula*), the spermatogenic cycle has been divided into six periods, two of which are completely refractory, coinciding with February and July. In general, there is a seminiferous epithelium that is spermatogenically active for almost the entire year, so spermatogenic activity can be divided into three phases: proliferative, meiotic, and differentiative. Germ cells develop through the stages of spermatogenesis within the seminiferous tubules, where spermatogonia can be distinguished in the basal compartment and spermatozoa in the apical part; in particular, in the lizard's seminiferous epithelium, two types of spermatogonia can be identified, namely A and B. Spermatogonia A have an ovoid shape and are present in the basal epithelium throughout the year; these cells divide mitotically to give rise to spermatogonia B, which are like spermatogonia A but are round in shape and smaller in size. After dividing repeatedly, the B spermatogonia enter meiosis I. Inside the seminiferous tubules, from the basal portion to the lumen, are the other stages of differentiation, spermatocytes I and II, the round and elongated spermatids, and finally the spermatozoa, which are very abundant during the reproductive phase that takes place in May. The only other cells present within the seminiferous epithelium are the Sertoli cells, which are characterized by a triangular nucleus and are present within the tubules at any time of the year (Rosati, Agnese, et al., 2020). In addition, in the testis of *H. flaviviridis*, genes associated with the progression of spermatogenesis during the active phase and conserved in the mouse were highlighted for the first time through molecular studies (Gautam et al., 2013). Important genes such as *Hk1*, *Nme5*, *Akap4*, *Arih1*, *Rassf7*, and *Tubb4b* were found to be closely associated with active spermatogenesis in both mice and lizards. Factors that interfere with the expression of one gene can potentially abrogate the process of spermatogenesis and lead to infertility (Gautam et al., 2013; Sarkar et al., 2016). As in the European wall lizard, a prenuptial pattern of sperm development (described later) occurs in the American alligator, *Alligator mississippiensis*, though spermatogenesis is confined to a short period from February to June (Gribbins et al., 2006). Since both these species have an actively dividing population of spermatogonia A and B throughout the year; so a favorable temperature as well as GTHs and testosterone (T) administration during the quiescent phase can initiate the spermatogenesis. Thus, alligators and European wall lizards lack a refractory period. On the other hand, a long refractory period is observed in slider turtles in which testes contain only resting spermatogonia during the quiescent phase and no mitotically active A and B spermatogonia. The resting spermatogonia fail

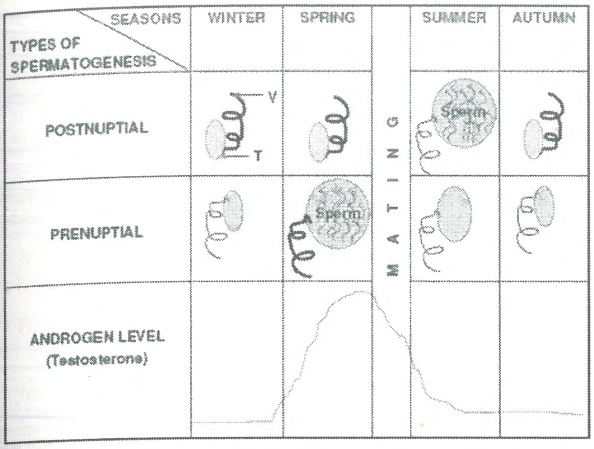

FIG. 12 Schematic representation of two major patterns of spermatogenesis and its correlation with the androgen profile. T, testes; V, vas deferens. *(Modified from Licht, P. (1984). Reptiles. In G.E. Lemming (Ed.),* Marshall's physiology of reproduction: Reproductive cycles of vertebrates *(4th ed.), Vol. 1 (pp. 206–282). Edinburgh: Churchill Livingstone.)*

to respond to temperature or hormonal manipulations (Gribbins et al., 2003).

Based on seasonal timing of spermatogenesis and mating, two major patterns for testicular cycles are recognized in reptiles: prenuptial and postnuptial (Fig. 12; Saint, 1963). In the prenuptial species, spermatogenesis takes place before or during the mating season, and a single annual peak of androgen is generally seen corresponding to the culmination of spermatogenesis. On the other hand, timing of spermatogenesis does not coincide with mating in postnuptial species. In these species, spermatogenesis takes place after the mating season, and sperm are stored in the male or female ducts for long periods to be used in the next mating season. Peak plasma androgen levels and sexual behaviors occur when the seminiferous tubules are regressed. In general, lizards exhibit a prenuptial type of spermatogenesis, while chelonians are characteristically postnuptial. Snakes are less consistent and may fall into either category (Licht, 1984).

3.2 Steroidogenesis

The synthesis of androgens begins with the mobilization of cholesterol from cholesterol depots present in the form of conspicuous lipid droplets within the cytoplasm. Cholesterol is transported to the inner-mitochondrial membrane where it is converted to pregnenolone by the C_{27} cytochrome P_{450} side-chain cleavage enzyme complex. Thereafter, pregnenolone is carried to the microsomal compartment where the membrane-bound enzyme 17 α-hydroxylase and $C_{17,20}$ lyase converts it into dehydroepiandrosterone (DHEA). 3β-HSD along with $\Delta^{5\text{-}4}$-isomerase converts DHEA into androstenedione (AND), the immediate precursor of Testosterone (T). The synthesis of T is regulated by 17β-hydroxysteroid dehydrogenase (Fig. 13).

Based on 3β-HSD activity and ultrastructural features (Callard et al., 1976, 1978), both Leydig and Sertoli cells are sites of steroid biosynthesis in reptiles. Although the importance of Sertoli cell-secreted steroids is not very clear, they may contribute meaningfully to maintaining high intratubular androgen concentrations required for spermatogenesis, or in maintaining accessory sex organs during the mating season when the testes are regressed in postnuptial species. On the other hand, Leydig cell-secreted androgens enter the peripheral circulation and control the development of accessory sex organs and sexual behavior. In most reptiles, T is the principal androgen and shows pronounced seasonal variation (see Table 1). However, estrogen synthesis by somatic and germ cells has recently been shown in reptilian testes, with a role of these hormones in both cell differentiation and in determining the quiescent phase of spermatogenesis, depending on levels (Rosati, Agnese, et al., 2016). In detail, in the turtle, *T. scripta*, aromatase, the rate-limiting enzyme regulating estrogen biosynthesis, was immunocytochemically demonstrated in the somatic cells, Leydig cells, and Sertoli cells, and detected within germ cells, particularly spermatids and spermatozoa. Aromatase immunoreactivity shows an inverse relationship with the testicular cycle. It is strong during the quiescent phase, but very weak when germ cells are in the meiotic phase (Gist et al., 2007). Aromatase immunolocalization is also reported in the testes of the Chinese rat snake *Zaocys dhumnades*. The presence of the enzyme has been shown in spermatids and spermatozoa within the testis and in the seminiferous spherules of the vas deferens, which in this species represent alternative sites for spermiogenesis (Liang et al., 2011). In the wall lizard *P. siculus* during the reproductive cycle, steroidogenic enzymes (StAR, 3β-HSD, 17β-HSD, P450 aromatase, and 5α-Red) are present in epididymal and testicular (both germ cells and somatic cells) cells (Rosati, Agnese, et al., 2016; Rosati, Prisco, et al., 2020; Rosati, Santillo, et al., 2017). The levels of steroidogenic enzymes are the key to the local action of sex hormones on spermatogenesis (Rosati, Agnese, et al., 2016; Rosati, Andreuccetti, & Prisco, 2017; Rosati, Prisco, et al., 2020). In contrast, no such relationship is reported in crocodiles and alligators, probably due to very low levels of estrogens that are detectable only by using a highly sensitive assay system. The plasma estrogen levels in male alligators range from 0.23 to 3.14 pg/mL. Based on much higher mRNA expression of aromatase in brain than in testes, this extragonadal site is suggested to contribute majorly to estrogen biosynthesis (Lance et al., 2003). However, Milnes et al. (2002) reported plasma E_2 levels of 10–60 pg/mL in juvenile alligators from Florida wetlands. The discrepancies in estrogen levels in the different studies are attributed in part

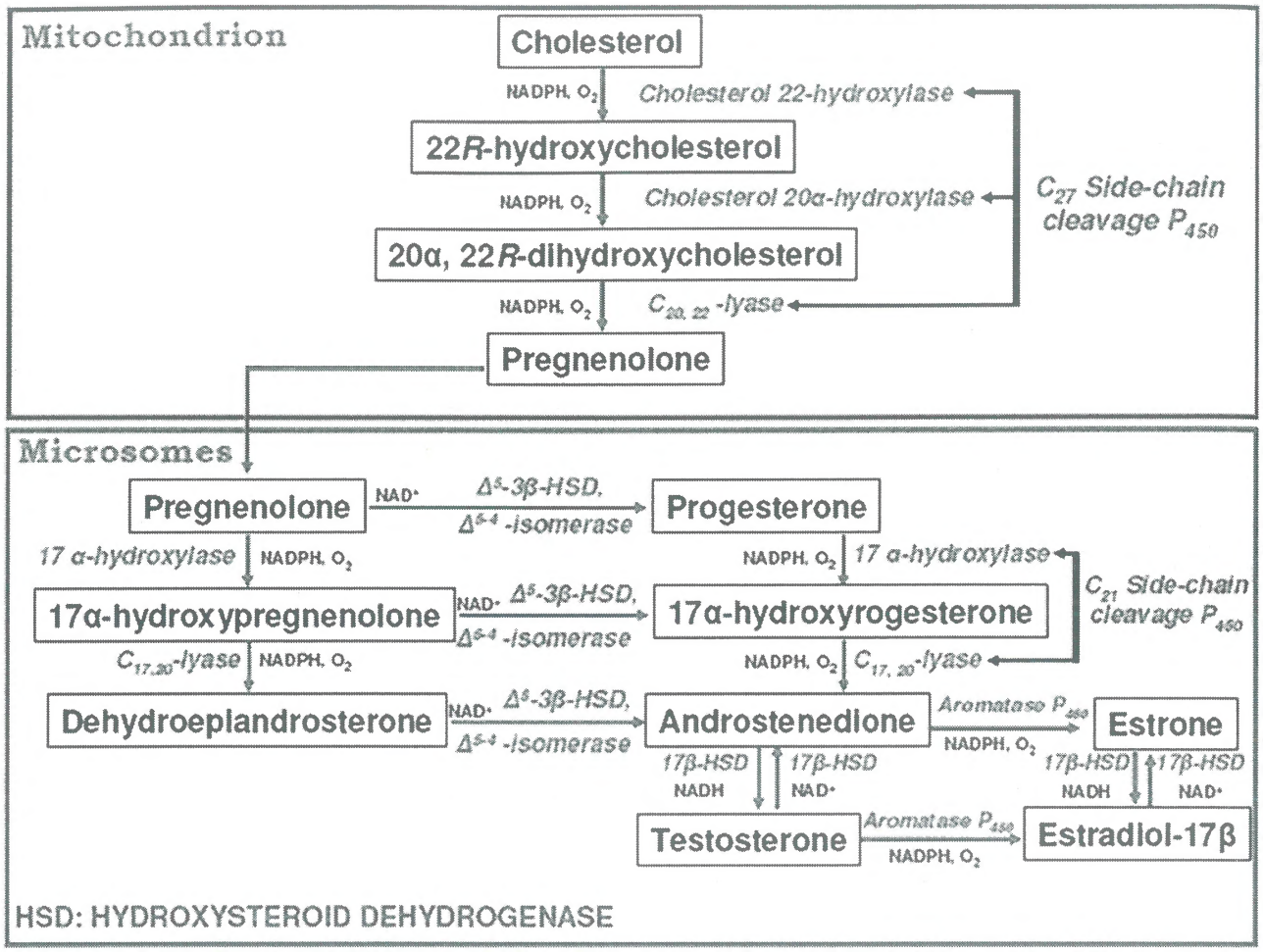

FIG. 13 The biosynthetic pathway of sex steroids. The conversion of cholesterol to androgen is carried out by three primary enzyme systems, namely C_{27} side-chain cleavage P_{450}, C_{21} side-chain cleavage P_{450} and Δ^5-3β-HSD = and $\Delta^{5\text{-}4}$-isomerase. The C_{27} side-chain cleavage P_{450} enzymes are in the mitochondria, whereas the other two enzyme systems are in the smooth endoplasmic reticulum.

to differences in the sensitivity of the assay system used, and to the presence of Endocrine-disrupting chemicals (EDCs) in the Florida wetlands.

4 REGULATION OF TESTICULAR FUNCTIONS

4.1 Environmental factors

Studies on the role of environmental factors in control of testicular functions are few and scattered. Temperature acts as the main cue for testicular recrudescence in temperate species. For example, in *Anolis carolinensis*, the responsiveness of testes to mammalian/chelonian GTHs is reported to be maximal at 30°C compared to 20°C (Licht, 1975). Similarly, androgen production is temperature-dependent in the lizard *Tiliqua rugosa* (Bourne et al., 1986) and the turtle *Pseudemys scripta* (Licht et al., 1989). However, temperature is ineffective in influencing the in vitro effect of GnRH on luteinizing hormone (LH) secretion from pituitary cells in *T. scripta* (Licht et al., 1989). On the other hand, in tropical species, rainfall/moisture regulates gonadal recrudescence and breeding because temperature remains relatively constant throughout the year (Crews, 1984; Licht, 1984; Lofts, 1987; Whitter & Crews, 1987). The largest testicular size in four tropical gecko species from Brazil is observed during the rainy season (Vitt, 1986). It is of interest to note that the Indian wall lizard *H. flaviviridis* inhabiting the temperate zone of India (North India) shows distinct phases of the testicular cycle from regressed to spermatogenically active, whereas testes in *Hemidactylus brooki* that inhabit the tropical region of India (South India) are reported spermatogenically active throughout the year (Shanbhag et al., 2000). In some reptiles, photoperiod is an important regulator of testicular functions. An increase in photoperiod results in testicular

TABLE 1 Peak plasma androgen level in certain prenuptial and postnuptial reptilian species.

Species	Peak(s)			Correlation
	Number of peaks	Large peak	Small peak	
Postnuptial type				
Chrysemys picta (Callard et al., 1976; Licht et al., 1985)	Double	10–16 ng/mL	~5 ng/mL	Neither peak coincides with spermatogenesis
Chelydra serpentina (Mahmoud et al., 1985)	Double	50 ng/mL	20 ng/mL	Neither peak coincides with spermatogenesis
Chelonia mydas (Licht et al., 1985)	Double	27.39 ng/mL	3 ng/mL	Exceptionally large peak of androgens coincides with spermatogenesis
Prenuptial type				
Geochelone nigra (Schramm et al., 1999)	Single	28.94 ± 6.38 ng/mL		With spermatogenesis
Podarcis s. sicula (Andò et al., 1990	Single	174.8 ng/mL		With spermatogenesis
Calotes versicolor (Radder et al., 2001)	Single	~13 ng/mL		With spermatogenesis
Sternotherus odoratus (McPherson et al., 1982; Mendonca & Licht, 1986)	Single	90–125 ng/mL		With spermatogenesis

Generally, two androgen peaks are observed in postnuptial species, whereas a single peak parallel to spermatogenesis is seen in prenuptial species.

recrudescence and active spermatogenesis in the red-eared turtle, *Trachemys scripta elegans* (Burger, 1937), the desert night lizard, *Xantusia vigilis* (Bartholomew Jr., 1950, 1953), and *A. carolinensis* (Underwood & Hall, 1982). Further, the pineal hormone, melatonin (MEL), is implicated in mediating photoperiod effects on testes since pinealectomy in *A. carolinensis* induced testicular development during the fall when testes normally are regressed. This effect is reversed after MEL replacement therapy (Underwood, 1995). The antigonadal role of MEL is also reported in the lizard *Calotes versicolor* (Radder et al., 2001). Administration of MEL to intact animals resulted in the inhibition of gonadal activity, whereas an increase in gonadal weight and accessory sex organs occurs in pinealectomized lizards (Haldar-Misra & Thapliyal, 1981a). In *P. siculus*, temperature and photoperiod are considered the most important environmental factors, which control gonadal activity (Alberts et al., 1992; Andò et al., 1990, 1992; Angelini et al., 1976; Angelini & Botte, 1992). These animals have a cyclic gonadal activity in which the reproductive phase occurs during spring and a refractoriness begins in summer and becomes less intense as the weeks pass and only disappears in late autumn when the temperature drops (Angelini et al., 1976). In winter, even if refractoriness is over, spermatogenesis remains blocked because environmental temperatures remain too low (Angelini et al., 1976). Spring recovery depends above all on temperature, so it only begins and continues when the temperature reaches 20–23°C.

Higher temperature levels are necessary for the endocrine activity of the testis and the development of secondary sexual characters (Angelini et al., 1976). Temperatures below 20°C allow partial recovery; on the other hand, with temperatures around 8°C, the gonad is unable to respond to external stress. However, the photoperiod only affects the reactivation of spermatogenesis if the appropriate temperatures are reached. Furthermore, it has been suggested that the increase in temperature during late summer (August) induces the refractoriness, assisted by the decrease in photoperiod after the summer solstice (Angelini et al., 1976); although, alterations in temperature and photoperiod (spring-summer) do not prevent refractoriness, but anticipate or delay it, whereby refractoriness appears to be a genetically programmed reproductive strategy mechanism (Angelini et al., 1980; Angelini & Ghiara, 1984).

4.2 Extragonadal factors

4.2.1 Hypothalamic hormones

GnRH was first identified by its stimulatory effect on synthesis and release of pituitary GTHs that in turn regulate reproductive functions (Burgus et al., 1972; Matsuo et al., 1971). The lack of GnRH expression or mutation in the gene encoding GnRH receptors produces sterility in all vertebrates including reptiles (Licht et al., 1987; Somozo et al., 2002; Tsai & Licht, 1993a). To date, 16 GnRH variants have

been isolated and characterized from different vertebrates and protochordate nervous tissue (Adams et al., 2002; Kah et al., 2007; Kavanaugh et al., 2008). Although varying forms of GnRH are encoded by different genes, they are grouped into one family since all are decapeptides with conserved amino acids at 1, 4, 9, and 10 positions. Among the different structural variants, chicken GnRH-II (cGnRH-II) is universally present in all vertebrates without any sequence alterations. In fact, cGnRH-II appears to have remained unchanged during the evolution of jawed vertebrates.

In reptiles, many forms of GnRH have been demonstrated using various techniques. For example, two forms of GnRH, cGnRH-I and cGnRH-II, are present in *A. mississippiensis* (Lovejoy et al., 1991), crocodiles, *Crocodylus niloticus* (Millar & King, 1994), slider turtles, *T. scripta* (Sherwood & Whittier, 1988; Tsai & Licht, 1993b). In the lacertilians, *Cordylus nigra*, and *P. siculus*, cGnRH II and salmon GnRH (sGnRH) are present (Powell et al., 1985, 1986). Recently, in mature females, the gene and amino acid sequence of the GnRH 1 form was identified. GnRH1 cDNA consists of 282 bp of open reading frame, highly conserved with GnRH1 orthologs among crocodiles (*A. mississippiensis,* 93.5%; *Crocodylus porosus*, 94.6%; and *Gavialis gangeticus* 93.5%), which encodes a 93 amino acid protein that has several conserved regions with greater proximity to avian species than to other reptile species. This hormone has also been immunolocalized and is present not only in the brain, diencephalon, and medulla, but also in other peripheral tissues such as the ovary. This evidence suggested that in this animal model GnRH1 regulates many functions including vitellogenesis (Nie et al., 2021). Many unidentified GnRH-like forms are also reported in *P. siculus* (Powell et al., 1985, 1986), striped skink, *Mabuya capensis,* and a tortoise, *Chersine angulata* (King & Millar, 1979, 1980). However, the physiological significance of varying forms of GnRH is still obscure. The synthetic cGnRH-II stimulates pituitary GTH secretion. But its anatomical location raises doubts about its availability to reach the pituitary gonadotropes through portal vessels. On the other hand, cGnRH-I, whose axons terminate on the hypophysial portal vessels that perfuse the pituitary, does not always stimulate LH secretion in vivo (Licht & Porter, 1985; Tsai & Licht, 1993b). Nonetheless, cGnRH-I is shown as a potent GTH releaser in vitro (Licht et al., 1987; Licht & Porter, 1985).

With increasing knowledge of multiple GnRH variants in the brain, a search for distinct GnRH-receptors (GnRH-Rs) in target tissues began. The first complete study demonstrating the presence of distinct GnRH-receptor subtypes was performed in goldfish (Illing et al., 1999). Phylogenetic analysis classifies the vertebrate GnRH-Rs into four groups: I/III, 2/no mammalian I, 3/II, and 4/mammalian I (Ikemoto & Park, 2005). In reptiles, the first GnRH-R was cloned and characterized by the leopard gecko *Eublepharis macularius* (Ikemoto et al., 2004). Molecular phylogenetic analysis revealed that the cloned GnRH-R belongs to type-2/no mammalian I. As its expression levels were quite low in the pituitary gland of reproductively active leopard geckos, the possibility of some other type of GnRH-R responsible for GTH secretion in reptiles was suggested. In 2007, three GnRH-Rs that respond to GnRH ligand, cGnRH I, and cGnRH II were demonstrated in the lizard *E. macularius*. It is noteworthy that two of the three GnRH-R subtypes (GnRH-R 2 and GnRH-R 3) could not be detected in the anterior pituitary, rather these are present in the posterior or intermediate lobe (Ikemoto & Park, 2007). Further studies are required to explain the structure, expression pattern, and functions of GnRH-Rs in reptiles.

Studies on the role of GnRH in regulation of testicular functions are limited, and exhibit inconsistencies. Mammalian GnRH (mGnRH) or its agonist does not affect plasma LH and gonadal steroids in male turtles *Chelonia mydas*, *Sternotherus odoratus*, and *Lepidochelys olivacia* (Licht et al., 1982; Licht et al., 1984; McPherson et al., 1982; Mendonca & Licht, 1986). Similarly, cGnRH-I is ineffective in *S. odoratus*. On the contrary, mGnRH stimulates plasma T secretion in male alligators as well as the injection of a GnRH agonist, chicken-II LH-RH, stimulates plasma T secretion in males in a viviparous lizard, *Tiliqua nigrolutea* (Lance et al., 1985; Edwards & Jones, 2017). The inconsistency in results might be due to GnRH variants, dose, or frequency of treatment as diverse effects of GnRH based on these factors are reported in Italian lizard *P. s. sicula*. In this species, a single dose of mGnRH, cGnRH-II, or sGnRH increases plasma T levels, though mGnRH and cGnRH-II are more potent than sGnRH. In contrast to single injection, prolonged GnRH treatment inhibits testicular and epididymal activity, possibly due to desensitization of pituitary receptors (Ciarcia et al., 1989). The concept of GnRH-induced desensitization of pituitary receptors was reinforced by in vitro observations in female *T. scripta* in which prolonged cGnRH-I or cGNRH-II treatment attenuated LH release from pituitary cells (Tsai & Licht, 1993b).

In addition to GnRH, a novel hypothalamic dodecapeptide, called gonadotropin-inhibitory hormone (GnIH), was identified in the avian pituitary and may play an important role in the regulation of GTH secretion (Tsutsui et al., 2000). The circadian rhythmicity of GnIH, regulated by light and seasons, can influence reproductive activity, mating behavior, aggressive behavior, and feeding behavior in animals (Teo et al., 2021). GnIH is localized in the neurons of the avian paraventricular nucleus (PVN), ventral paleostriatum, septal area, preoptic area (POA), other areas of the hypothalamus as well as in the optic tectum, median

eminence, and the dorsal motor nucleus of the vagus in the medulla oblongata. GnIH fibers are observed near GnRH neurons in the avian POA. Moreover, GnIH significantly inhibits GTH release and consequently testicular development and functions in birds (Tsutsui & Osugi, 2009). Although homolog peptides of GnIH sharing a common C-terminal LPXRFamide motif have been identified in the hypothalamus of no avian vertebrates including reptiles, their role in testicular function has not been explored in reptiles (Osugi et al., 2014).

4.2.2 Pituitary hormones

The hypothalamus produces GnRH (gonadotropin-releasing hormone) that stimulates the adenohypophysis to release gonadotropins, FSH (follicle-stimulating hormone), and LH, which in turn induce the activity of gonads (Li & Arimura, 2003). Pituitary gonadotropins act by binding to specific membrane receptors present on target cells in which they stimulate the secretion of steroid sex hormones (androgens and estrogens) and/or protein factors (inhibin and activins) (Li & Arimura, 2003). These molecules are released into the blood and act as feedback regulators on the hypothalamic–pituitary axis. In reptiles as well as in other vertebrates, gonadal activity depends on the pituitary FSH-like and LH-like gonadotropins (Licht, 1984). In Chelonia and crocodilia, two distinct gonadotropins, such as mammalian FSH and LH have been isolated and characterized (Licht et al., 1976; Licht & Papkoff, 1985; Mahmoud et al., 1985; Papkoff et al., 1976). It is further confirmed by cloning of partial cDNA sequence for FSH β-subunit and full-length cDNA sequence for LH β-subunit from the pituitary of Reeve's turtle *Geoclemys reevesii* (Aizawa & Ishii, 2003). These two GTHs, follicle-stimulating hormone (FSH) and LH, are no covalently linked heterodimeric glycoproteins consisting of α- and β-subunits encoded by distinct genes. The α-subunit of both GTHs is identical and interchangeable, while β-subunit is hormone-specific as well as species-specific and confers biological action through signal transduction. Both subunits are synthesized as precursor proteins that are posttranslationally glycosylated, assembled, and secreted into the circulation.

In testes, FSH normally regulates Sertoli cell functions, and Leydig cell androgen synthesis is controlled by LH. However, the precise role of FSH and LH in the regulation of testicular functions in chelonia and crocodilia is still not clear. Mammalian FSH (mFSH) is reported to be more potent in regulating steroidogenesis in turtles and alligators as compared to mLH (Callard & Ryan, 1977; Lance & Vliet, 1987; Tsui & Licht, 1977). But altogether different results are reported with homologous GTHs in alligators. The in vitro incubation of interstitial cells with alligator LH, not FSH, results in an increase in androgen production (Tsui & Licht, 1977).

The confusion regarding "two gonadotropins-two testicular functions" is further exacerbated in the squamates. A single pituitary GTH was purified from five species of snakes belonging to three families (Licht et al., 1979). The binding affinity for snake pituitary GTH was observed only in the testes of squamates, and not in other vertebrates. When tested for biological activity in reptiles, squamate GTH provoked FSH-like response in squamates, but was LH-like in no squamates. Based on these observations, Licht and his group (Licht et al., 1985) suggested that the snake pituitary GTH is like neither FSH nor LH but shares biological action of both FSH and LH depending on the animal model in which it is tested. Other studies using mammalian GTHs report that a single FSH-like molecule regulates both the testicular functions in squamates (Eyeson, 1971; Licht, 1972; Licht & Hartree, 1971; Licht & Midgley, 1977; Licht & Papkoff, 1971; Licht & Pearson, 1969; Rai & Haider, 1986; Reddy & Prasad, 1970a, 1970b). A high-affinity binding site for only mFSH is present in both the tubular and interstitial compartmelichtnts of the testes of the whiptail lizard, *Cnemidophorus tigris* (Licht & Midgley, 1977). In vitro experiments in the Indian wall lizard provide direct evidence of oFSH action in control of Sertoli as well as Leydig cell activities (Khan & Rai, 2005). However, instead of only an FSH-like GTH, the single GTH present in Japanese the grass lizard *Takydromus tachydromoides* seems to be LH-like as a cDNA encoding only for the LH β-subunit has been cloned in this species (Aizawa & Ishii, 2003). The concept of the existence of a single GTH, however, is contradicted in some of the squamates. In the male lizard *P. sicula campestris*, two GTHs are immunocytochemically demonstrated in the pars distalis using hFSHβ and hLHβ antibodies (Desantis et al., 1998). In another study, Southern blot analysis shows that the *P. siculus* genome contains a nucleotide sequence encoding β-subunits of both FSH and LH. However, it is not clear whether these nucleotide sequences are translated into two functional GTH β-subunits or a single β-subunit that is similar to mammalian FSHβ as well as LHβ (Borrelli et al., 1997). The "two GTHs testicular functions" are validated in the lizard *Agama agama*. In hypophysectomized *A. agama*, mFSH maintains spermatogenesis, whereas mLH regulates interstitial (Leydig) cell function (Eyeson, 1971). Nevertheless, to date only a cDNA for FSH receptor has been cloned from the gonads of squamates (Bluhm et al., 2004; Borrelli et al., 2001), strengthening the "one-GTH-two function" hypothesis. Finally, although there are studies that contradict each other about the presence of one or two gonadotropins, the hypothesis that gametogenesis in some reptiles may be regulated by two gonadotropins has recently been supported by a study conducted in mature male *A. carolinensis*, in which administration of bovine LH and FSH resulted in increased testicular size and blood levels of testosterone (Kang et al., 2020).

4.2.3 Other hormones

The importance of the thyroid gland in the regulation of testicular activities had been recognized long ago in reptiles based on seasonal variation of plasma thyroid hormones (Bermudez et al., 2005; Bicho et al., 2013; Kar & Chandola-Saklani, 1985; Naulleau et al., 1987). An inverse correlation between plasma thyroid hormones and androgens is reported in the Chinese cobra, *Naja naja*, and six-lined racerunner, *Cnemidophorus sexlineatus* (Bona-Gallo et al., 1980; Sellers et al., 1982). A further correlation between thyroxine (T_4) levels and sex hormones has been recorded in the desert tortoise, *Gopherus agassizii*. Animals taken under observation for 2 years showed adult reproductively active animals to have T4 peaks in July concomitantly with significantly higher testosterone levels. The increase in T4 in desert tortoises is therefore associated with periods of increased feeding and reproductive activity, supporting a role for thyroid hormones in these energy-demanding activities (Kohel et al., 2001). Experimental studies show a direct role for thyroid hormones in spermatogenesis. Either hypothyroidism induced by thyroidectomy in the variegated lizard *Coleonyx variegatus* (Plowman & Lynn, 1973) and the garden lizard *C. versicolor* (Haldar-Misra & Thapliyal, 1981b) or hyperthyroidism caused by administration of thyroid hormones to intact *Calotes* (Haldar-Misra & Thapliyal, 1981b) leads to impairment of testicular functions, indicating that abnormalities in thyroid functions adversely affects spermatogenesis. The direct involvement of thyroid hormone was evidenced by the demonstration of its α receptor in the testis of *P. siculus* (Cardone et al., 2000). Thyroid hormones were reported to increase the expression also of *AR* mRNA levels and thus, enhance the androgen responsiveness of testicular cells during spermatogenesis (Cardone et al., 2000). The correlation between thyroid function and spermatogenesis was confirmed by a toxicological investigation conducted in the lizard *Podarcis bocagei*. The administration of a specific thyroid endocrine disruptor caused not only thyroid damage but also alterations in male gametogenesis. Indeed, the seminiferous tubules of treated animals showed significantly larger diameters, a significantly higher number of spermatogenic layers, and exhibited upregulation of receptors for thyroid hormones compared to lizards from reference areas. Data on testicular sensitivity to thyroid hormones are lacking for chelonians and crocodilians.

Another hormone that appears to have a potential effect on spermatogenesis is leptin; a positive effect of this hormone on spermatogenesis has been demonstrated in the lizard. In *P. siculus* during the onset of the refractory phase, leptin treatment caused a slowing of testicular inactivation with effects on testicular morphology, spermatogenesis, peroxisome proliferator-activated receptor (PPAR)a, 17β-hydroxysteroid dehydrogenase, 17β-estradiol, and testosterone in both testis and blood (Putti et al., 2009).

Lizards treated with leptin injections showed seminiferous tubules with germ cells at all stages and larger lumens than controls. Furthermore, in all groups, the epithelium of the epididymis showed better development compared to controls, with mitosis and binucleated cells. In both control and treated animals, spermatozoa and spermatids were immunoreactive to the leptin receptor and PPARa. In treated animals, intercellular interstitial cells and peritubular fibrocytes were also immunoreactive to the leptin receptor, while PPARa immunoreactivity translocated from the cytoplasm to the nucleus. 17β-HSD immunoreactivity was present in spermatids and interstitial cells of control lizards and in secondary spermatocytes and spermatozoa of treated lizards.

These observations indicate that leptin causes a delay in the summer regression of testosterone in *P. siculus*, playing a regulatory role in reproduction in this species, as already hypothesized for mammals (Putti et al., 2009).

Glucocorticoids are also known to influence testicular functions in reptiles (see also Chapter 7, this volume). Stress induces an increase in plasma corticosterone (CORT) with a parallel decrease in sex steroid levels (Greenberg & Wingfield, 1987; Guillette et al., 1995; Lance & Elsey, 1986). Administration of CORT decreases plasma androgen levels in the lizards *Uta stansburiana* (DeNordo & Licht, 1993), *Anolis sagrei* (Tokarz, 1987) and *P. s. sicula* (Manzo et al., 1994). In addition to steroidogenesis, exogenous CORT adversely affects spermatogenesis in male brown anoles, *A. sagrei* (Tokarz, 1987) and *Mabuya carinata* (Yajurvedi & Nijagal, 2000). It has been postulated that CORT affects testicular functions by acting directly on the gonads in *M. carinata*, as CORT interferes with spermatogenesis even in the presence of GTHs (Yajurvedi & Nijagal, 2000).

4.3 Intratesticular factors

Sertoli cells, Leydig cells, and testicular immune cells in mammals are reported to secrete steroidal and nonsteroidal factors that regulate testicular functions in a paracrine manner. Studies in reptiles are scarce and concern only a few species.

4.3.1 Androgens

Earlier studies in reptiles present a confusing picture pertaining to the role of androgens in the regulation of spermatogenesis; in particular, the function of these hormones is highly dependent on the relationship between systemic and intratesticular levels of testosterone and the stage of the reproductive cycle studied. For example, androgen

administration in juvenile terrapins stimulates spermatogenesis (Lofts & Chiu, 1968), whereas this treatment is ineffective in alligators (Forbes, 1939) as well as in the lizard *Sceloporus occidentalis* (Gorbman, 1939). However, in *A. mississippiensis* a recent study showed that *AR* mRNA responds to the administration of all naturally occurring androgens and that their effects are inhibited by co-treatment with antiandrogens, such as flutamide, *p,p*-dichlorodiphenyldichloroethylene, and vinclozolin. Furthermore, it is interesting to note that a spliced form of AR was also found in the alligator cDNA, which lacks seven amino acids within the ligand-binding domain and shows no response to androgens. According to the authors, the spliced form of AR could have a dominant-negative function against androgen-induced AmAR (Miyagawa et al., 2015). An inhibitory effect of androgens is observed in some species such as *Eumeces fasciatus* (Reynolds, 1943), *Uromastix hardwickii* (Ramaswami & Jacob, 1963), and *A. agama* (Eyeson, 1971). This androgen-induced inhibition of spermatogenesis is attributed to the decrease of basal GTH level by negative feedback (Eyeson, 1970). However, in these studies, the reproductive state of animals was not considered. In the Korean soft-shelled turtle (*Pelodiscus maackii*) testis, it has been shown that the expression of Claudin 11, an important protein involved in the formation of the blood-testicular barrier, is under the control of testosterone (Park et al., 2015). In the Indian wall lizard, depending on its reproductive state, differential effects of androgens on spermatogenesis are demonstrated. During the breeding phase, administration of AR antagonists, such as cyproterone acetate (CA) and flutamide, inhibits spermatogenesis (Haider & Rai, 1986, Haider & Rai, 1987). This provides a clue as to why a direct correlation exists between Leydig cell steroidogenic activity and spermatogenesis in prenuptial species. In the spermatogenic blocked stage, androgen administration leads to a decrease of spermatogonial cells in the Indian wall lizard (Rai & Haider, 1986). In vitro experiments in wall lizards have resolved these discrepancies. Sertoli cells were collected from regressed testes and subjected to androgen treatment in the presence or absence of FSH. Dihydrotestosterone (DHT) alone inhibited lactate production from inactive Sertoli cells while enhancing metabolic activity of the Sertoli cells pretreated with FSH or simultaneously incubated with FSH and DHT. This suggests that the observed discrepancies of androgens acting on spermatogenesis are dependent on the inactivated/GTH-activated state of the Sertoli cells (Khan & Rai, 2004b). In *P. siculus*, androgens upregulate AR expression; therefore, studies conducted in primary cultures of Podarcis testicular cells revealed that testosterone administration increases AR expression in a dose-dependent trend (Cardone et al., 1998). Testosterone (T) autoregulates the expression of its own receptor mRNA whereas this effect was reversed by flutamide and cyclohexiimide (Cardone et al., 1998). However, the precise location of AR within a testis has not been demonstrated; hence, it is difficult to explain precisely the autocrine/paracrine role of androgens on testicular functions at this time.

4.3.2 Estrogens

In the testis of reptiles, de novo biosynthesis of estrogens is reported. In the turtle *T. scripta*, aromatase P450 (enzyme of estrogen biosynthesis) has been immunolocalized and its distribution changes during the different phases of the reproductive cycle; during the no reproductive period, the protein is present in somatic cells, Leydig and Sertoli cells, while during the reproductive season, it is also detected within germ cells, particularly spermatids and spermatozoa (Gist et al., 2007). In lizard *P. siculus*, it is known that high levels of estrogens are responsible for blocking spermatogenesis, while high levels of testosterone are responsible for resuming this process (Angelini et al., 1987; Ciarcia et al., 1986; Rosati, Agnese, et al., 2016, 2020). Also in this species, aromatase P450 was detected in testicular somatic cells and germ cells during the annual cycle, especially in spermatids and spermatozoa, except in the autumn recovery, when the enzyme was only detected in Leydig cells (Rosati, Agnese, et al., 2016, 2020; Rosati et al., 2021). Through molecular biology and biochemical investigations, it has also been shown that the mRNA and protein reach their highest levels in summer and in winter stasis, when the highest levels of estrogens, responsible for the block of spermatogenesis typical of this species, are also present (Rosati, Agnese, et al., 2016). In contrast, in the autumn recovery, P450 aromatase levels decreased, along with 17β-estradiol levels, while testosterone concentration increased, leading to the resumption of spermatogenesis (Rosati, Agnese, et al., 2016, 2020; Rosati et al., 2021). The presence of aromatase has also been reported within germ cells of the *Z. dhumnades*. The enzyme has been immunolocalized in spermatozoa within the testis, as well as in the seminiferous spherules of the vas deferens, which represent alternative sites for spermiogenesis in this species (Liang et al., 2011). The involvement of estrogens in control of testicular functions is implicated by the presence of estrogen receptors (ER) in the testis. ERα is localized in Leydig cells, while ERβ is found in spermatogonia, Sertoli cells, and epithelial cells of excurrent ducts in the *T. scripta* (Gist et al., 2007). The presence of ERβ in germ cells as well as *ERs* mRNA (Verderame & Scudiero, 2018) are reported also in *P. s. sicula* (Chieffi & Varriale, 2004). In this lizard, estrogens are implicated in postreproductive refractoriness and induce regression of testes and epididymis during the reproductively active phase (Angelini et al., 1986). This is corroborated by induction of the precocious resumption of spermatogenesis and sperm release in the autumnal early recrudescent phase using the aromatase inhibitor, fadrazole (Cardone et al., 2002). Furthermore, estrogens decrease *AR*

mRNA expression in androgen-primed testicular cells in the European wall lizard (Cardone et al., 1998), and are hypothesized to cause testicular regression by either inhibiting the hypothalamus-pituitary-gonad (HPG) axis (Angelini et al., 1986), and/or by directly downregulating *AR* gene expression. In Indian wall lizards, estrogens decrease the metabolic activity of Sertoli cells (Khan & Rai, 2004b). It appears that estrogens might inhibit spermatogenesis and could influence the nursing function of the Sertoli cells since these cells are aromatase positive even during the period of full spermatogenesis.

In parallel to lacertilians, estrogens may maintain testes in the quiescent state in chelonians. An intense immunoreactivity for aromatase along with higher expression of ERα and β is reported in the quiescent testes of the turtle, *T. scripta* (Gist et al., 2007). However, a key role for estrogens in early phases of spermatogenesis is suggested in *P. s. sicula* based on the observations that estrogens induce proliferation of type A spermatogonia through activation of ERK1/2 and serine/threonine kinase Akt 1 (present in spermatogonia (SPG), spermatocytes (SPC), and spermatids (SPT)), and that pretreatment with an antiestrogen (ICI 182-780) inhibits spermatogonia mitotic divisions (Chieffi et al., 2002; Russo et al., 2005). Finally, in *P. siculus* the regulation of spermatogenesis mediated by estrogen receptor activation can be altered by the presence in the environment of xenobiotics that have estrogen-like activity. In particular, it has been shown how alkylphenols as well as the herbicide glyphosate, a substance recently classified as an EDC, alter the normal course of spermatogenesis in the lizard. The authors described in specimens taken during the period of maximum spermatogenic activity and treated with these substances a reduction in the number of spermatozoa, where the seminiferous epithelium acquires a configuration similar to a refractory period. All these damages have been associated with alterations in estrogen receptor levels and distribution (Chianese et al., 2023; Di Lorenzo et al., 2021; Rosati et al., 2023; Verderame et al., 2022). The role of estrogens in spermatogenesis has been questioned in alligators because estrogen levels as well as aromatase expression are very low in males (Lance et al., 2003).

4.3.3 Other paracrine factors

In addition to systemic control, spermatogenesis is also regulated by cell–cell interaction as well as by a wide range of local endogenous no steroidal factors that act by paracrine mechanisms. About this type of control, in reptiles, the literature is scarce and limited mainly to studies of the action of several factors on the whole testis and individual isolated cells in lizard and turtle species.

4.3.3.1 Testicular factors

Among the various factors produced in the testis that directly control reptile spermatogenesis, we can include the neuropeptides PACAP (Pituitary adenylate cyclase-activating polypeptide) and VIP (Vasoactive intestinal peptide) (Agnese et al., 2010, 2014, b; Rosati et al., 2014, 2015; Rosati, Andreuccetti, & Prisco, 2017; Rosati, Prisco, et al., 2016). These are bioactive neuropeptides, members of the growth hormone-releasing hormone (GHRH)/VIP/glucagon family, much studied in the control of male gametogenesis in *P. siculus* (Arimura, 1998; Rosati, Agnese, et al., 2020; Sherwood et al., 2000; Vaudry et al., 2009). Indeed, using different experimental approaches such as RT-qPCR, in situ hybridization, and immunohistochemical investigations, it has been shown that these molecules are widely expressed in the lizard testis throughout the reproductive cycle (Agnese et al., 2010, 2014, b; Rosati et al., 2014, 2015; Rosati, Prisco, et al., 2016). qPCR experiments revealed a cyclic pattern of *PACAP* and *VIP* mRNA levels during the different phases of the reproductive cycle, with peaks during the phases of maximum testicular activity. Immunohistochemistry investigations demonstrated extensive localization of PACAP and VIP at both somatic and germ cell levels, particularly at the level of spermatids and spermatozoa (Rosati et al., 2014, 2015; Rosati, Andreuccetti, & Prisco, 2017; Rosati, Prisco, et al., 2016) In *P. siculus*, organ cultures have shown that in vitro treatment of the testicles with PACAP and VIP at different concentrations stimulates the secretion of steroid hormones such as testosterone and 17β estradiol. The authors have therefore suggested a direct action of neuropeptides with the steroidogenic enzymes since all these molecules are co-localized in the same cells (Rosati et al., 2015; Rosati, Prisco, et al., 2016). An effect on sex hormone secretion in the testis of *P. siculus* seems also to be determined by D-aspartic acid (D-Asp), an endogenous amino acid present in the neuroendocrine tissues of invertebrates and vertebrates (D'Aniello, 2007), where a substantial amount of D-Asp was recorded in the testis and showed cyclic changes of activity during the annual reproductive cycle in parallel with sex hormone levels. Administration of exogenous D-Asp to lizards has revealed that the testis is specifically able to take up and accumulate this amino acid causing an increase in testosterone levels both in the testis and in the plasma, with a consequent significant increase in the mitotic activity of the testis (Di Fiore et al., 2014, 2016, 2019; Raucci et al., 2005). Further studies have also shown that an endogenous opioid peptide, β-endorphin (β-EP), can influence hormone secretion in male testis of *P. siculus*. In addition to the presence of β-EP in the testis at the level of interstitial cells, the administration of exogenous β-endorphin to the lizard results in a reduction of testosterone release through inhibition of pituitary gonadotropin production (Ciarcia et al., 1994, 1996). More recently, it has been hypothesized in the *P. siculus* testis that spermatogenesis is also under the control of an endogenous factor known as Vasa. Through

biochemical and molecular investigations, the expression of the Vasa gene has been demonstrated at three significant moments of the reproductive cycle: reproductive period, summer stasis, and autumn recovery (Milani & Maurizii, 2015). By immunohistochemical analysis the authors also demonstrated the presence of the Vasa protein in germ cells, from spermatogonia to spermatids, but not in spermatozoa. No Vasa signal has been reported in somatic cells (Milani & Maurizii, 2015). Recently, VASA *mRNA* was also isolated in the testis of the Chinese soft-shell turtle (*P. sinensis*). By in situ hybridization investigations, the distribution of *VASA* mRNA in testicular germ cells was demonstrated. *VASA* mRNA was not detected in resting spermatogonia, but it was shown to be present in proliferating spermatogonia, and becomes abundant in spermatocytes and detectable in spermatozoa, suggesting its possible involvement in the control of meiotic and differentiation processes (Li et al., 2017) as demonstrated in the Asian yellow pond turtle, *Mauremys mutica* (Liu et al., 2021). Finally, in a recent study in *H. flaviviridis*, an involvement of a novel factor in the control of spermatogenesis, described for the first time in reptiles, Nesfatin-1, was demonstrated. Nesphatin-1 is a pleiotropic hormone involved in various physiological functions, including reproduction. The authors showed that expression of Nefastin-1 in the lizard testis varies significantly depending on the reproductive phase, being highest in the active phase and lowest in the regression phase. In vitro treatment with Nesfatin-1 resulted in a dose- and time-dependent stimulatory effect on the expression of proliferation and differentiation markers such as SCF, C-kit, and PCNA, suggesting a spermatogenic role of Nesfatin-1 in the lizard testis. Nesfatin-1 also induced significantly higher expression of StAR and a concomitant increase in testosterone production by the lizard testis. All these data taken together suggested that Nesfatin-1 is an intratesticular factor that plays a role in the activation of spermatogenesis (Dotania et al., 2023).

4.3.3.2 Cellular factors

4.3.3.2.1 Sertoli cell-secreted factors Sertoli cell-conditioned medium (SCCM) is shown to regulate the activities of Leydig cells collected from regressed testes. The spent culture medium collected from oFSH-preactivated or unactivated Sertoli cells increases Leydig cell steroidogenesis, although oFSH-preactivated SCCM is more potent than unactivated SCCM. Interestingly, preactivation of Leydig cells with oFSH increases their responsiveness to SCCM. Perhaps the Sertoli cells secrete a steroidogenic factor that regulates basal as well as FSH-induced T production by Leydig cells. In addition to this hypothesized steroidogenic factor, FSH-activated Sertoli cells secrete an unknown mitogenic factor that induces proliferation of Leydig cells but only when they are in an activated state in response to GTH (Khan & Rai, 2005).

4.3.3.2.2 Testicular macrophage-secreted factor Macrophages lie near the Leydig cells in the testicular interstitium. As discussed in Section 2.2, there is a direct relationship between the Leydig cell number and size of the testicular macrophage population. The ratio of macrophages to Leydig cells (i.e., 1:3–4) remains constant throughout the reproductive cycle, though their total numbers increase from the regressed to the spermatogenically active phase. This reflects a close communication between these two cell populations. In vitro experiments substantiate the role played by macrophages in the regulation of Leydig cell activities. The conditioned-medium of testicular macrophages (TMCM) enhances T production by Leydig cells collected from regressed testes of lizards. The steroidogenic effect of TMCM is more pronounced when testicular macrophages are preactivated with FSH. This suggests that macrophages secrete a steroidogenic factor that regulates basal as well as FSH-induced T production by Leydig cells. Further, FSH has a stimulatory effect on the secretion of this presumed steroidogenic factor from testicular macrophages. Also, testicular macrophages in response to FSH are reported to secrete a mitogenic factor that induces proliferation of Leydig cells only when the latter are activated with the GTH (e.g., oFSH). Khan and Rai (2008a) examined the role of testicular macrophages in the regulation of Leydig cell activities under inflammatory conditions. The testicular macrophages were treated with a bacterial lipopolysaccharide (LPS) and the spent medium was collected. LPS-treated TMCM inhibits Leydig cell steroidogenesis as well as proliferation. Thus, it is concluded that testicular macrophages differentially regulate Leydig cell functions under normal physiological conditions and during inflammation. In a recent study conducted in *H. flaviviridis*, Leydig cells were shown to use 25-hydroxycholesterol (25-HC) derived from testicular macrophages for steroidogenesis. Specifically, Leydig cells (LCs) collected from testes when incubated with 25-HC obtained from a fraction of the conditioned medium from testicular macrophages produced significantly more testosterone. In addition, to examine the gonadotropic regulation of steroid biosynthesis using 25-HC as a substrate, ovine FSH was used in the following experimental combinations: 0 h FSH + 12 h 25-HC, 0 h 25-HC + 12 h FSH. A marked increase in testosterone production was observed in FSH-treated cells compared to controls, suggesting to the authors that gonadotropin regulates the uptake of 25-HC as a substrate for testosterone biosynthesis (Singh et al., 2021). This latter effect, by a feedback mechanism when present at high levels, inhibits the synthesis of 25-HC (Singh et al., 2017).

4.3.3.2.3 Histamine The mast-cell secreted factor, histamine, is ineffective in influencing basal T production by Leydig cells. However, histamine affects FSH-induced

Leydig cell steroidogenesis in a biphasic manner, depending on its concentration. In detail, histamine at low concentrations increases T production while, at high concentrations determines a reduction of the hormone levels (Khan & Rai, 2007). The biphasic effects of histamine apparently are mediated by two histaminergic receptors. The inhibition of steroidogenesis at the high concentration of histamine is mediated by H1 receptors, while the stimulation at low concentration works through H2 receptors. However, histamine does not influence Leydig cell proliferation and therefore it appears that the increased T production in response to histamine is due to altered steroidogenic enzyme levels.

4.3.3.2.4 Leydig cell-secreted factor Leydig cell-secreted paracrine factors appear to regulate the nursing function of Sertoli cells. FSH-activated Leydig cell-conditioned medium (LCCM) that contains FSH as well as FSH-induced Leydig cell-secreted factor increases lactate production by Sertoli cells. On the other hand, FSH-preactivated LCCM that contains FSH-induced Leydig cell-secreted factor but not FSH inhibits metabolic activity in Sertoli cells (Khan & Rai, unpublished data). In a separate study, DHT is reported to exert diverse effects on the metabolic activity of Sertoli cells depending on their state of activation, i.e., inhibitory on uactivated and stimulatory on FSH-activated cells (Khan & Rai, 2004b). Perhaps the Leydig cell-secreted factor is an androgen that differentially affects lactate production by Sertoli cells.

5 REGULATION OF TESTICULAR IMMUNE FUNCTIONS

The immune cells in the interstitial compartment ensure an infection-free microenvironment for the developing germ cells. Although the presence of lymphocytes in testicular interstitium has not been reported in reptiles, macrophages lying near Leydig cells immunologically regulate the testis microenvironment by phagocytosis and release of cytotoxic substances (Fig. 14). The functions of testicular macrophages are regulated by pituitary GTH as well as by paracrine factors secreted from neighboring cells. A study in the Indian wall lizard demonstrates the stimulatory role of oFSH in the regulation of macrophage-based immune responses (Khan & Rai, 2008a). Leydig cell-secreted factor also affects the phagocytic and respiratory burst activities of testicular macrophages (Khan & Rai, 2008b). Androgens can differentially regulate phagocytosis. In the presence of FSH, DHT enhances phagocytosis, but it downregulates phagocytosis in the absence of FSH. In contrast, DHT suppresses the respiratory burst activity of uactivated or even FSH-activated testicular macrophages.

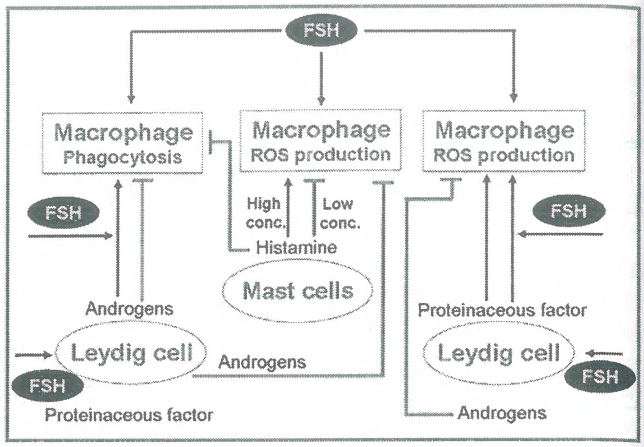

FIG. 14 Schematic representation of the regulation of testicular immune responses in the Indian wall lizard *Hemidactylus flaviviridis*.

Intriguingly, FSH-activated/FSH-preactivated LCCM increases superoxide production, and hence implicates the involvement of an FSH-induced Leydig-cell secreted proteinaceous factor. This has been corroborated using heat-inactivated FSH-activated LCCM that dramatically decreased superoxide production. Since FSH and DHT have distinct signaling pathways, it would be an interesting area of research to explore the crosstalk between these hormones in control of testicular functions.

The mast cell-secreted product histamine is also important in the regulation of testicular immune responses. Histamine differentially regulates phagocytic and respiratory burst activities depending on concentrations. Both phagocytosis and superoxide production are inhibited by high concentrations of histamine. On the other hand, superoxide production increases at a low concentration of histamine, and phagocytosis remains unaltered (Fig. 14).

6 CONCLUSION

Reptiles are the first phylogenetic group in which spermatogenesis occurs in seminiferous tubules lined by peritubular cells. Based on the timing of spermatogenesis, Leydig cell steroidogenic activity, and mating, spermatogenesis is categorized into two major types: prenuptial and postnuptial. Testicular functions are regulated by a sophisticated network of interactions between endocrine and paracrine factors synthesized in the testis. Several variants of GnRH occur in the brains of reptiles, cGnRH-II being the most widespread although GnRH1 is probably the most important for releasing GTHs. The presence and role of another potential hypothalamic hormone, GnIH, need to be determined for reptiles. GnRH stimulates the GTH release from the anterior pituitary. Two distinct GTHs regulating different testicular functions are established in Chelonia and Crocodilia. However, in squamate reptiles, even after four

decades of research, it is ironic that our knowledge about GTHs is rudimentary. The concept of a single GTH resembling mammalian FSH that regulates all testicular functions is supported in lizards and snakes. Two separate GTHs have not been cloned successfully or characterized for any squamate species. Sex steroids, androgens as well as estrogens, play important roles in the regulation of testicular functions. Androgens differentially regulate spermatogenesis, depending on the reproductive state of the testis. Stimulation of spermatogenesis by androgens is seen during the breeding phase, but no effect is observed during the unbreeding phase. Surprisingly, no efforts have been made to investigate the exact location of ARs in testicular cells. Hence, it is not clear whether androgens regulate spermatogenesis by acting directly on the germ cells or indirectly through Sertoli cells/peritubular myoid cells, or both. In recent years, ERα as well as ERβ have been localized in the reptilian testis. Also, aromatase enzyme has been immunocytochemically demonstrated in germ and somatic cells. Estrogens are implicated in the testicular regression by inhibiting the HPG axis and/or by downregulating AR expression, but also in the differentiative process as spermiohistogenesis. The inhibitory effect of estrogens on the nursing function of Sertoli cells might be instrumental in decreasing spermatogenesis.

Studies on cell–cell interaction in the testis are very limited and confined to a few species, such as the wall lizard Indian *H. flaviviridis* and Italian *P. siculus* (Fig. 15). Hence, it is difficult to generalize about the paracrine regulation of testicular functions in reptiles. Nonetheless, factors secreted from Sertoli cells, testicular macrophages, and mast cells appear to regulate Leydig cell functions. Steroidal as well as no steroidal factors from Leydig cells are implicated in control of the nursing function of Sertoli cells. Additionally, Leydig cell-secreted factors contribute to maintaining immune responses in the testis. Unfortunately, not all factors have yet been identified or characterized; only in the case of neuropeptides (PACAP and VIP) has their cooperation in controlling hormone secretion been demonstrated. Thus, to reach a logical conclusion on phylogenetic relationships with respect to GTH specificity and testicular functions, cloning and expression of pituitary GTHs and their biological activities in homologous systems as well

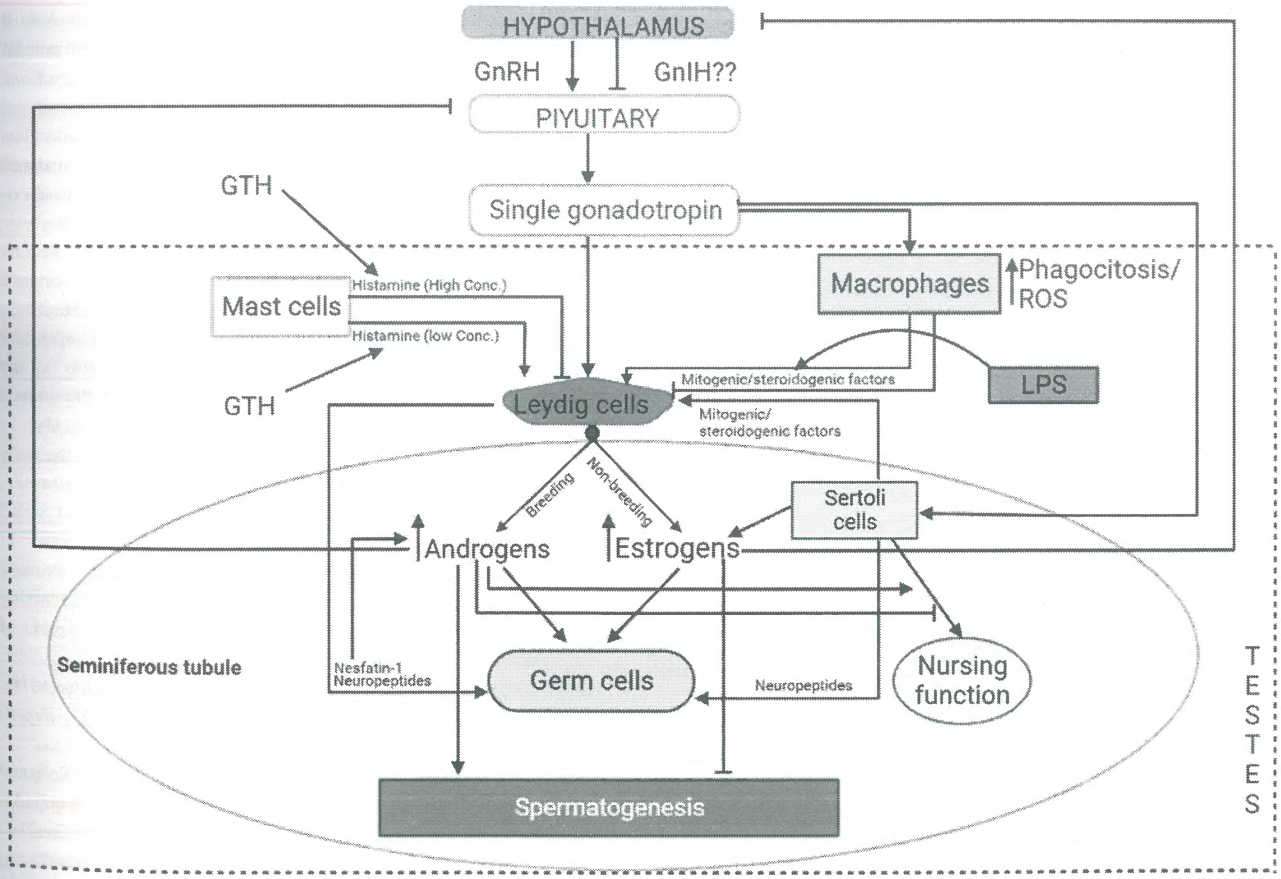

FIG. 15 Proposed model for endocrine and paracrine regulation of testicular function in squamates. Lines without a tip indicate inhibition/negative feedback, *while arrows* with a tip represent stimulation of a particular response. See the text for abbreviations (unless abbreviations are not in the text; in that case, they need to be defined in the legend).

as isolation and characterization of testicular paracrine factors and their functions need to be investigated in a large number of species across the reptilian orders.

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Chapter 4

Hormonal regulation of ovarian function in reptiles

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ABBREVIATIONS

20β-HP	20β-hydroxypregn-4-ene-3-one
3β-HSD	3β-hydroxysteroid dehydrogenase
AR	androgen receptor
ART	assisted reproductive technique
CA	corpus atretica
CBG	corticotrophin-binding globulin
cGnRH-II	chicken II gonadotropin-releasing hormone
CORT	corticosterone
DHEA	dehydroepiandrosterone
DHT	5β-dihydrotestosterone
E_1	estrone
E_2	17β-estradiol
EDC	endocrine-disrupting chemical
ER	estrogen receptor
ERE	estrogen response element
ESD	environmental sex determination
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
GH	growth hormone
GnIH	gonadotropin release inhibiting hormone
GnRH	gonadotropin-releasing hormone
GTH	gonadotropin
HPG	hypothalamic-pituitary-gonadal
IGF-1	insulin-like growth factor 1
IgG	immunoglobulin
LH	luteinizing hormone
P_4	progesterone
PG	prostaglandin
PR	progesterone receptor
PRE	progesterone response element
S-BP	steroid-binding protein
SHBP	sex-hormone binding protein
T	testosterone
TSD	temperature-dependent sex determination
VLDL	very-low-density lipoprotein
Vtg	vitellogenin

1 INTRODUCTION

The reptilian ovary has two primary functions: the production of gametes and the secretion of hormones with regulatory effects on the female reproductive system. As in all vertebrates, endocrine control of ovarian development and physiology primarily involves the hypothalamic-pituitary-gonadal axis (HPG axis). However, our knowledge of HPG axis functioning in reptiles remains sketchy compared with current understanding of the mammalian system.

1.1 Ovarian structure

In reptiles, the ovaries are paired, hollow, oval (or, in snakes, elongate) structures attached to the dorsal body wall by a mesovarium (Jones, 1978). Each ovary is covered by a thin epithelium and contains follicles and corpora lutea embedded in a stroma of connective tissue. Small regions of the dorsal surface of the ovary represent the germinal beds, where multiplication of oogonia and formation of primordial follicles occur (Guraya, 1989). In reptiles, unlike birds and mammals, the ovarian stroma is sparse, and there is little interstitial tissue.

1.2 Ovarian development

Ovarian differentiation begins at about Stage 24–30 (sensu Dufaure & Hubert, 1961) of embryonic development, corresponding roughly to the time of oviposition in oviparous squamates. However ovarian development is not always complete at the time of hatching or birth: in alligators, for example, ovarian development continues during the first few months of life (Moore et al., 2008).

Hormones and Reproduction of Vertebrates, Volume 3. https://doi.org/10.1016/B978-0-443-16022-6.00001-9
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Studies of gonadal differentiation in reptiles have largely focused on oviparous species with temperature-dependent sex determination (TSD) due to interest in the underlying mechanisms of TSD (see Chapter 1 in this volume). Detailed stage-by-stage descriptions of the differentiation and development of the ovary are available for the oviparous lizards *Sceloporus undulatus* (Austin, 1988) and *Calotes versicolor* (Doddamani, 1994) and the viviparous lizards *Niveoscincus ocellatus* (Neaves et al., 2006) and *Lacerta vivipara* (Dufaure, 1966, in Merchant-Larios, 1978), and a major review of the embryonic development of the genital system in both female and male reptiles is available (Raynaud & Pieau, 1985). The following description of ovarian development in squamates is derived from these sources.

Six stages of gonadal development can be identified in embryonic lizards (Table 1). At embryonic stage 24–30 (Dufaure & Hubert, 1961), the gonad first develops as a thickening of undifferentiated mesenchymal tissue underlying the coelomic epithelium, extending down the ventromedial surface of each mesonephros. This forms the genital ridge, which grows and bulges into the coelom. At this early stage, the gonad is indifferent, or bipotential, and contains both medullary and cortical elements. The bipotential stage in lizards lasts for a much shorter period than in turtles and crocodilians (see below). Primordial germ cells migrate into the developing gonad, and multiply by mitosis; rete cords form as single rows of cells extending from the mesonephros into the medulla of the gonad. Differentiation of the ovary begins as the primordial germ cells congregate in the cortex and begin to differentiate into oogonia at stages 31–32. By stages 33–35, gonadal sex can be recognized; the ovary contains a prominent cortex with one or two layers of oogonia, the tunica albuginea begins to develop, and medullary oogonia becomes scarce. At this stage, oocytes begin to appear in the rete and cortex, particularly in the anteriodorsal region of the gonads: these regions may later form the germinal beds (Austin, 1988). During the final stages of embryonic development (stages 36–40), the ovarian cortex becomes well developed, containing 6–10 layers of oogonia and oocytes, the medulla degrades and becomes filled with stromal tissue, and the rete cords are absent or degenerating. Folliculogenesis begins after hatching or birth. The ovaries of neonatal lizards contain clearly defined germinal beds, and some primordial follicles, consisting of an oocyte surrounded by a single layer of monomorphic granulosa cells, are apparent about 10 days after birth. In *C. versicolor*, follicles with a polymorphic granulosa including pyriform cells (see Section 3.2) are present in 2-month-old hatchlings (Doddamani, 1994).

TABLE 1 Stages of ovarian development in lizards.

Stage	Embryonic stage	Gonadal component	Description
1	24–30	Genital ridge	Genital ridge first appears on either side of the dorsal mesentery; some primordial germ cells present
2	28–32	Undifferentiated, bipotential, gonad	Ridge develops several layers of cells, forms ovoid, and bulges into coelom
3	31–32	Undifferentiated gonad	Rete present; outer cortex and inner medulla develop; germ cells in both cortex and medulla; primordial germ cells begin to differentiate into oogonia
4	33–35	Ovary	Cortex prominent and 2–3 cells thick; Oogonia mainly in cortex and rete; oocytes in meiotic prophase appear
5	36–38	Ovary	Cortex 3–5 cells thick; vascularity of ovary increases
6	37–40	Ovary	Cortex is 6–10 cells thick; rete degenerating or absent; cortex filled with oogonia and oocytes
6+	Neonate	Ovary	Germinal beds apparent; primordial follicles appear sometime after birth

Embryo stages are sensu Dufaure and Hubert (1961).

Based on Austin, H. B. (1988). Differentiation and development of the reproductive system in the iguanid lizard, *Sceloporus undulatus*. *General and Comparative Endocrinology*, *72*, 351–363, Doddamani, L. S. (1994). Histoenzymological studies on embryonic and posthatching development of the ovary in the tropical oviparous lizard, *Calotes versicolor*. *Journal of Morphology*, *222*, 1–10, Neaves, L., Wapstra, E., Birch, D., Girling, J. E., & Joss, J. M. P. (2006). Embryonic gonadal and sexual organ development in a small viviparous skink, *Niveoscincus ocellatus*. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, *305*, 74–82.

The development of the ovary follows a similar pattern in other reptilian taxa. The main morphological stages are similar in turtles and alligators: in many, but not all, species, primordial follicles are present in the ovary by the time of hatching (Pieau et al., 1999). In alligators, three broad stages can be recognized: genital ridge formation, the period of bipotentiality, and, finally, differentiation of the gonad. Differentiation takes place during the last third or quarter of embryonic life, as the gonads enter a rapid development phase (Joss, 1989). Ovarian development is incomplete at hatching although the gonad is clearly differentiated. Moore et al. (2008) provide a thorough description of ovarian development in neonatal American alligators, *Alligator mississippiensis* (to 3 months posthatching), and review comparative literature from birds. The ovary develops rapidly during the first few months posthatching: cortical production of oocytes continues, folliculogenesis is initiated and the medullary region undergoes considerable restructuring into the complex of vascularized lacunae which is a distinctive feature of crocodile, turtle, and bird ovaries. These lacunae may allow space for follicular development or provide nourishment to developing oocytes: further research is required (Smaga et al., 2022).

1.2.1 Hormonal control of ovarian development

Differentiation of the bipotential embryonic gonad into an ovary, rather than a testis, is dependent upon the endocrine milieu during the organizational period. Estrogen is the proximate stimulus critical to ovarian development (Smaga et al., 2022) which is mediated via the estrogen receptor (ER) (Crews, 1996). There are, however, two ultimate mechanisms that may determine whether estrogen is produced by the developing embryo. In many reptiles (but almost exclusively squamates), sex is determined by genetics, that is, genes carried on heteromorphic sex chromosomes trigger a genetic cascade that results in the bipotential gonad developing into either an ovary or a testis. In contrast, many reptilian species exhibit environmental sex determination (ESD). The form of ESD characteristic of reptiles is TSD: the sex of an individual embryo is determined by the environmental temperature experienced during a critical window of development (Bowden et al., 2000).

In species with TSD, temperature is transduced into an endocrine signal. That signal directs the sex-determination process and establishes the sex of the individual embryo by influencing endogenous steroid production during embryogenesis. Females result from the activation of an "ovary-determining cascade and simultaneous inhibition of a testis-determining cascade" (Crews, 1994) during the thermosensitive period. Estrogen levels remain low in testes, but rapidly increase and stimulate development of the cortex as the ovaries differentiate (Pieau et al., 1999). The embryonic stage at which sex is labile varies between taxa, and higher temperatures may produce predominantly either males or females, depending on the species. In crocodilian embryos, for example, incubation temperatures of 30°C produce females, and the steroidogenic enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and aromatase, which catalyzes the conversion of aromatizable androgens to estrogens, are active in the developing gonads of embryos incubated at this temperature. A marked increase in aromatase activity occurs coincident with germ cell proliferation and development of the ovarian cortex (Smith & Joss, 1994). Experimentally, TSD may be overridden by application of exogenous hormones, hormone agonists, or aromatase inhibitors such as fadrozole, which blocks the conversion of androgens to estrogens, implicating temperature-dependent regulation of aromatase activity as a primary mechanism of TSD. (For further discussion, see Chapter 1 in this volume.)

1.3 Major ovarian hormones

Reproductive activity in female reptiles is associated with dramatic increases in circulating concentrations of the primary sex steroids, 17β-estradiol (E_2), testosterone (T), and progesterone (P_4) (e.g., Edwards & Jones, 2001; Taylor et al., 2004). The ovary is the endocrine gland primarily responsible for secretion of the sex steroids in female reptiles, although P_4 may be synthesized by other tissues such as the adrenal gland (Highfill & Mead, 1975), or the uterus and placenta in viviparous reptiles (Girling & Jones, 2003; Painter et al., 2002). These hormones belong, respectively, to the C_{18} (estrane), C_{19} (androstane), and C_{21} (pregnane) series of steroids.

The two classes of steroids most closely associated with female reproduction are the estrogens and the progestins. 17β-Estradiol is generally assumed to be the primary gonadal estrogen in reptiles, as it is in many other vertebrates (Kime, 1987; Norris, 2007), and plasma concentrations of this steroid are routinely assayed in studies of female reproduction. However, this assumption may not always hold true. The very low plasma concentrations, even during vitellogenesis, of E_2 in the lizard *Pogona barbata* (Amey & Whittier, 2000) led the authors to speculate that a different estrogen may be more important physiologically in *P. barbata*, with E_2 simply being a short-lived metabolite of that hormone. A possible alternative to E_2 has also been postulated for the blotched blue-tongued lizard, *Tiliqua nigrolutea* (Edwards, Jones, & Davies, 2002) in which an unidentified, highly polar estrogen (rather than E_2) is the end product of steroid biosynthesis from pregnenolone. Similarly, the red-sided garter snake *Thamnophis sirtalis parietalis* produces a 6-substituted E_2 that represents 30%–60% of the immunoreactive estrogens in the plasma

of this species (Whittier & Hess, 1992). This species may also produce an alternative progestin (Whittier et al., 1987). However, P_4 is generally assumed to be the primary progestin in reptiles, as in other vertebrates (Callard et al., 1992).

The ovary also produces a third class of steroids: the androgens. Androgens such as testosterone fulfill an important function as precursors of estrogens, but the presence of tissue androgen receptors (AR) and significant circulating levels is good evidence that androgens have a wider biological significance in female as well as male vertebrates (Staub & De Beer, 1997). Testosterone is certainly synthesized by the reptilian ovary (Edwards et al., 2003; Gobbetti et al., 1993b), and plasma concentrations of testosterone vary significantly through the female reproductive cycle (e.g., Callard et al., 1978; Edwards & Jones, 2001), providing circumstantial evidence for a physiological role for this hormone.

Some nonsteroidal hormones are also produced by the reptilian ovary. Both ovarian follicles and corpora lutea have the capacity to synthesize prostaglandins (Gobbetti et al., 1993a; Guillette et al., 1988, 1991), which may have roles in promoting vitellogenesis and in ovulation, as well as influencing the expression of reproductive behaviors (Whittier & Crews, 1986; Whittier & Tokarz, 1992). Gonadotropin-releasing hormone-1 (GnRH-1) and bradykinin, plus their respective receptors, have been identified in ovaries of several nonmammalian vertebrates including the lizard *C. versicolor* (Singh et al., 2007). Gonadal GnRH appears to have autocrine or paracrine actions within the ovary and may play an important role in regulating ovarian development and activity (reviewed by McGuire & Bentley, 2010).

1.4 Hypophysial regulation of ovarian function

In vertebrates, ovarian secretion of steroids is regulated by the HPG axis. Gonadotropin (GTH) release is regulated by hypothalamic GnRH. In females, the production of gonadal P_4 and estrogens is stimulated by GTHs. These steroids may exert feedback control on GTH secretion at the level of the hypothalamus (Callard, Chan, & Potts, 1972). In reptiles, as in other vertebrates, multiple molecular forms of GnRH are present in the brain of a single individual (Montaner et al., 2000). Implantation of osmotic pumps containing chicken II (cGnRH-II) analog or mammalian GnRH-I induced a fivefold increase in plasma estrogens and elicited receptive behaviors in female iguanas (Phillips et al., 1987): this chicken hormone was about twice as potent as the mammalian form. In an investigation of the role of GnRH in the activation and deactivation of the HPG axis in an irregularly breeding lizard, reproductively quiescent females of *T. nigrolutea* responded to a single intraperitoneal injection of chicken-II GnRH with increased plasma T, but not estrogen, concentrations (Edwards & Jones, 2017). A gonadotropin release-inhibiting hormone (GRIH) has been identified in reptile brains (Tsutsui et al., 2018). The structure of this neuropeptide hormone and its primary role as an inhibitor of gonadotropin-releasing hormone (GnRH) and pituitary gonadotropin release is considered to be well-conserved across vertebrates (Teo et al., 2021) but functional studies in reptiles are lacking.

Sexual maturation in reptiles is dependent upon maturation of the HPG axis: at puberty (defined as the attainment of fertility), the ovaries become receptive to gonadotropic stimulation (Masson & Guillette, 1985). In mammals, increased secretion of GnRH at puberty activates the pituitary-gonadal axis (Ebling, 2005). Administration of synthetic mammalian GnRH-I to adult *Chrysemys picta* 3 weeks before ovulation stimulates both follicular development and steroidogenesis (Al-Kindi et al., 2001), presumably via stimulation of GTHs. However, we know very little of the neuroendocrine initiation of puberty in reptiles. A study of testicular responses to injections of ovine follicle-stimulating hormone (FSH) suggested that puberty may be extended over several years in large reptiles such as alligators (Edwards et al., 2004). In female reptiles, puberty is often associated with the attainment of a threshold body size or mass. For example, in the green iguana (*Iguana iguana*), males attain puberty at 15–17 months of age and 170–200 mm snout-vent length. Females exhibit a marked increase in plasma concentrations of E_2 at 15–17 months of age but do not mature for at least another year: they do not ovulate unless they reach the critical snout-vent length of 250 mm (Pratt et al., 1994). The estrogen surge in juveniles at 15–17 months occurs concurrently with reproductive activity of mature females and may serve to synchronize the juvenile iguana females before their first reproductive season.

In eutherian and marsupial mammals, the separate roles of the two pituitary GTHs in regulating ovarian function are well established. FSH regulates oogonial proliferation, oocyte growth, and differentiation, while luteinizing hormone (LH) stimulates ovulation and steroidogenesis. However, the picture in reptiles is less clear, and for many taxa it is still uncertain whether one or two GTHs are present. Fractionation studies showed that turtles and alligators have two distinct GTHs that appear to be homologous to the mammalian LH and FSH (Licht et al., 1976; Papkoff et al., 1976), but that snakes have only a single GTH (Licht et al., 1979). Reviewing (then) current knowledge of pituitary GTHs in reptiles, Licht (1980) concluded that most tetrapod vertebrates do have two distinct GTHs, but that squamate reptiles deviate from this pattern in having a single pituitary GTH that lacks FSH/LH specificity. More recent work has shown that the lizard genome contains

nucleotide sequences homologous to those that code for the β subunits of both mammalian FSH and LH (Borrelli et al., 1997). However, Bluhm et al. (2004) could not find evidence for an LH receptor in the snake *Bothrops jararaca* although they were able to clone a cDNA for the FSH receptor (FSHR), and FSHR was expressed in ovary and testis. That study provided further evidence that squamate reptiles have diverged from the fundamental pattern in tetrapod vertebrates in possessing only a single GTH that combines FSH- and LH-like activity (Licht, 1983). A recent exhaustive review of the evolution of vertebrate neuroendocrine hormones (Dufour et al., 2020), including patterns of gene duplication and gene loss, incorporated tantalizingly little new information from reptilian models.

2 OVARIAN STEROIDS

Estrogens, androgens, and progestins are synthesized and secreted by the ovary, and carried in the bloodstream to their target tissues where they exert their biological effects via specific receptors. The dominant end product of steroid synthesis varies among stages of the reproductive cycle. Broadly speaking, the follicular phase is characterized by the production of estrogen by the developing follicles, while P_4 is produced by the corpora lutea during the postovulatory phase. Steroids secreted by the ovary may be later modified by other tissues through further metabolism, either into biologically active metabolites or into conjugated forms to facilitate excretion.

2.1 Steroid synthesis

Steroids are synthesized from cholesterol, which may be taken up from the bloodstream or synthesized de novo within the endocrine cell. Cholesterol is converted to the C_{21} steroid pregnenolone, the key intermediate in steroidogenesis, by side-chain hydrolysis. All other steroid hormones are synthesized from pregnenolone via a series of enzyme-catalyzed steps, including conversion of pregnenolone to P_4 by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) (Norris, 2007). Gore-Langton and Armstrong (1994) and Kime (1987) provide detailed descriptions of the various pathways of steroid biosynthesis. There are two alternative pathways through which androgens may be synthesized (Fig. 1). The 17α-hydroxylase:C-17,20-lyase enzyme complex can utilize either P_4 or pregnenolone as its substrate to form androstenedione or dehydroepiandrosterone (DHEA) via the intermediates 17α-hydroxy-progesterone or 17α-hydroxy-pregnenolone respectively. These pathways are known as the Δ^4 (4-ene-3-oxo) and Δ^5 (5-ene-3β-hydroxy) pathways. The preferred pathway may vary between taxa, or between sexes within taxa, or with reproductive stage.

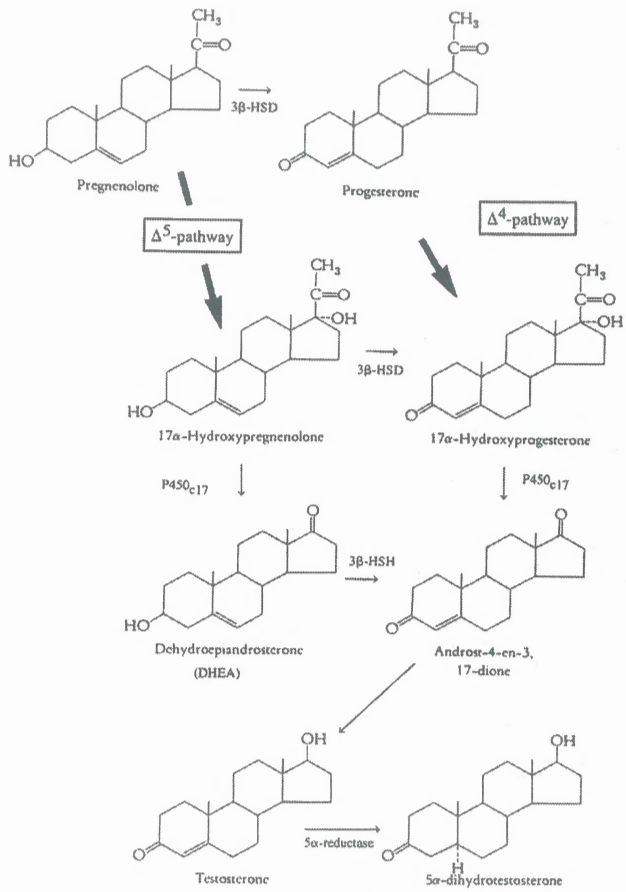

FIG. 1 The Δ^4- and Δ^5-pathways of androgen steroidogenesis from pregnenolone. The Δ^4-pathway is preferred in reptilian ovaries, although both pathways may operate. Estrogens are synthesized from androgens such as testosterone and androstenedione by aromatization. *(From Norris, D. O. (2007).* Vertebrate endocrinology *(p. 83). San Diego, CA and London: Academic Press, with permission.)*

Steroidogenic pathways in reptilian ovaries were investigated in a series of early studies (c.1965–72) in which chromatographic techniques were used to identify pathway intermediates synthesized by tissues incubated in vitro with unlabeled or radioactively-labeled steroid precursors (Yaron, 1972). Alternatively, histochemical localization and semiquantification of key steroidogenic enzymes, particularly 3β-HSD, can be used to infer steroidogenic activity in particular tissues (Norris, 2007). The advent of sensitive assays for plasma hormones in the early 1970s led to a new research emphasis on documenting cycles of plasma steroids (Chieffi & Pierantoni, 1987), and more recent studies of steroidogenic pathways in reptiles are sparse. Indeed, Lincoln et al. (2023) explicitly noted that investigation of a wider range of steroids, DHEA in particular, would help to elucidate how steroidogenic pathways are regulated in red-sided garter snakes (*T. s. parietalis*). It is clear, however, that the pathways of steroid biosynthesis in reptilian ovaries

are basically similar to those in mammals, reflecting their evolutionary conservation among higher vertebrates.

Progesterone occupies a position early in the steroidogenic pathway and is therefore a key precursor for other steroids as well as being secreted as an active hormone during the luteal phase. Corpora lutea of the viviparous lizard *Chalcides chalcides*, for example, produce high levels of P_4 during early gestation, and this production is reduced by addition of trilostane, a potent inhibitor of 3β-HSD activity, to the culture medium (Guarino et al., 1998). In-vitro incubations of ovarian tissue from *L. vivipara* with ^{3}H-pregnenolone demonstrated that P_4 is the primary steroid produced during gestation, with 85% of metabolized pregnenolone appearing as P_4 in incubations of ovaries (with corpora lutea) from late-gestating females. In contrast, P_4 is rapidly metabolized into 5α- or 5β-reduced steroids at other stages of the reproductive cycle (Xavier, 1982). Hypertrophied (vitellogenic) ovaries of *T. nigrolutea* also rapidly convert P_4 into downstream metabolites (Edwards et al., 2003).

The little information available for female reptiles suggests that the Δ^4 pathway of androgen biosynthesis is more active in the ovary although the Δ^5 pathway may make some contribution to steroid synthesis. Pregnenolone, 17α-hydroxy-pregnenolone, DHEA, P_4, androstenedione, E_2, and estrone (E_1) were all detected in extracts of ovaries of the oviparous lizard *Lacerta sicula* (Lupo Di Prisco et al., 1968), indicating that both major pathways of steroid biosynthesis were operating. Incubation of snake *Naja naja* (Lance & Lofts, 1977), turtle, and lizard ovaries (Chan & Callard, 1974) with radioactively labeled pregnenolone precursor indicated that androgens were preferentially synthesized via the Δ^4 pathway, but both pathways appear to operate in ovaries (and testes) of *Tiliqua rugosa* (Bourne, 1981). Pathway preference may vary within a species, with sex, season, and/or reproductive condition. A detailed study of steroid biosynthetic pathways in the lizard *T. nigrolutea* demonstrated activity of the Δ^4 pathway in both female and male gonads. Intermediates of the Δ^5 pathway were detected only in males, and only when regressed rather than hypertrophied (active) testes were incubated (Edwards et al., 2003).

The final steps of the steroidogenic pathway are those through which certain androgens are aromatized to estrogens (Fig. 2). As already noted, the end product of estrogen biosynthesis in reptiles is generally assumed to be E_2, but alternative estrogens, including E_1 (Chan & Callard, 1974; Lupo Di Prisco et al., 1968), may also be synthesized. In mammals, there is considerable evidence to support the "two cell-type, two gonadotropin" theory of estrogen biosynthesis. In brief, androgens are produced by cells of the theca interna under LH stimulation and are then transported across the follicular basement membrane to the granulosa, where they are aromatized into estrogens under the influence of FSH (Gore-Langton & Armstrong, 1994). To date, we have no experimental evidence that a similar system operates in reptiles.

Finally, steroid synthesis is a dynamic process, and shifts in pathway preference or the activity of specific enzymes may represent proximate mechanisms for regulating ovarian steroid production. At least some of the enzymes of the steroidogenic pathways appear to be temperature-sensitive in reptiles (Bourne et al., 1986; Girling & Jones, 2006), suggesting a mechanism whereby environmental temperature may cue seasonal shifts in activity of the HPG axis.

2.2 Tissue receptors

Gonadal steroids exert their physiological effects by binding to specific, high affinity, tissue receptors. Steroid receptors were traditionally considered to be nuclear transcription factors: after ligand binding, the dimerized steroid-receptor complexes are translocated to the nucleus where they interact with the genome to alter patterns of mRNA expression (Ho et al., 1985). These nuclear receptors have six functionally distinct domains (identified as A–F). The E domain is responsible for ligand binding, while the C domain binds to the DNA via a zinc finger. More recently, however, a different type of steroid receptor has been identified: these are membrane-linked, extra-nuclear steroid receptors that act via G-protein coupling, causing posttranslational modifications or phosphorylation of existing proteins. These receptors initiate rapid signaling actions of steroid hormones (see reviews by Hammes & Levin, 2007; Kohno et al., 2008). Research into this type of receptor has focused on mammals, although membrane-bound P_4 receptors have been identified in amphibian oocytes (Revelli et al., 1998).

There are separate classes of receptor for each major group of sex steroids (estrogens, androgens, and progestogens) (Callard & Callard, 1987). Reptilian steroid hormone receptors have a high degree of sequence homology with those of other vertebrates (Young et al., 1994). Differential expression of sex steroid receptors in different tissues or at different times of the reproductive cycle provides a mechanism for regulating sensitivity to their respective hormones. Three steroid hormone receptors have been identified in males of the leopard gecko, *Eublepharis muscularis* and were quantified in different tissues using mRNA expression of each receptor. Receptor expression appeared to vary with environmental conditions (low temperature and short photoperiod upregulate androgen receptor), and both AR and ER were expressed in a wide variety of tissues (Endo & Park, 2003).

Two major forms of the ER, ERα and ERβ, with differential ligand activation and different tissue distributions, are ubiquitous among vertebrates, although a third type, ERγ, is present in fishes (Lange et al., 2003). During the evolutionary sequence from fish to mammals, the affinity of

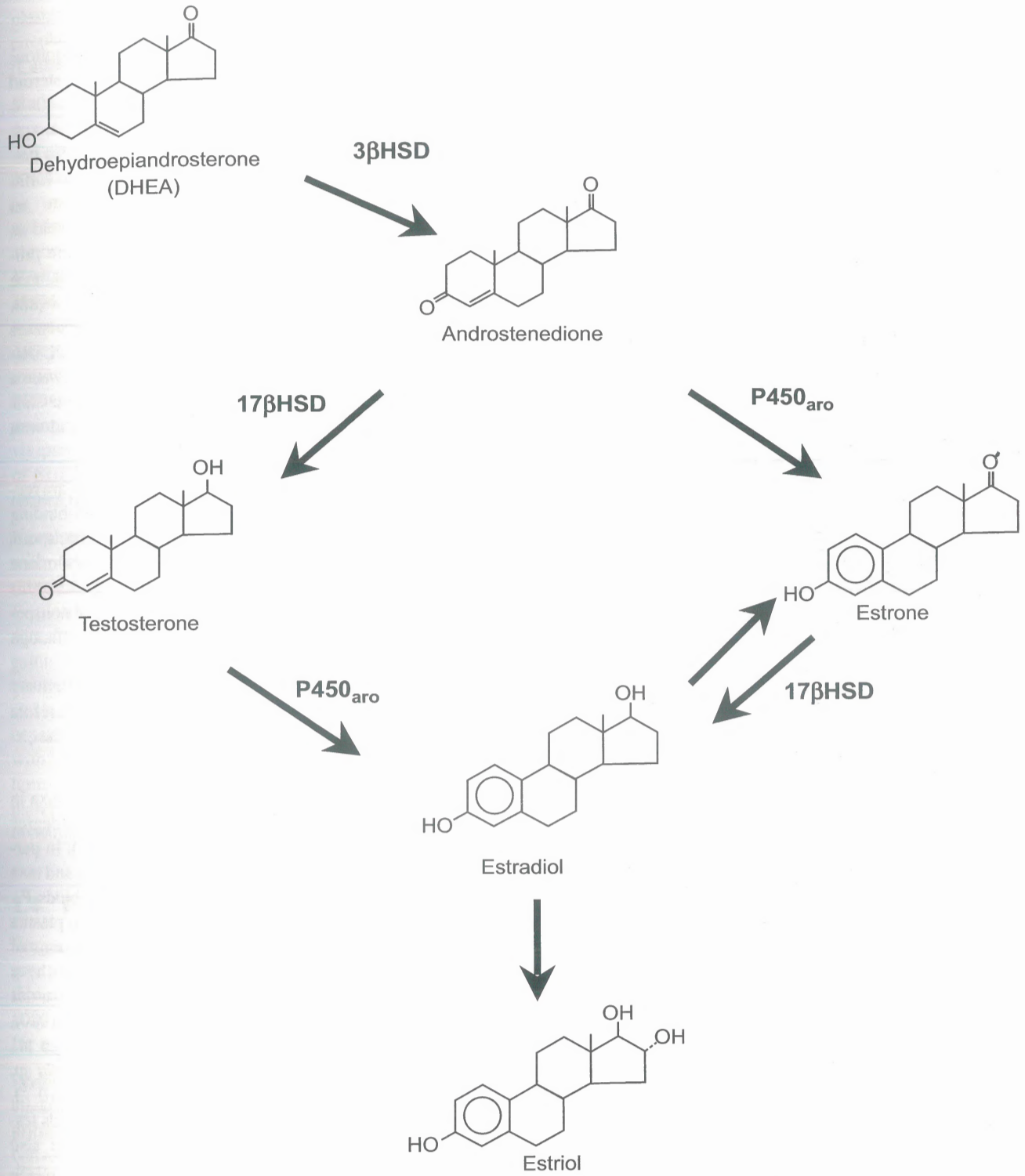

FIG. 2 Synthesis of estrogens from androgens. *(From Norris, D. O. (2007).* Vertebrate endocrinology *(p. 84). San Diego, CA and London: Academic Press, with permission.)*

the ER for its hormone has increased about 1000-fold, which is mirrored by a corresponding decrease in endogenous plasma concentrations of E_2. Sequence homologies demonstrate that the estradiol-binding pocket has been conserved (Lange et al., 2003). The ERα has been partially or fully sequenced in a range of reptilian species, including full sequences for a caiman (*Caiman crocodilus*) and a lizard (*Cnemidophorus uniparens*) (Sumida et al., 2001). Both ERα and ERβ have been cloned and sequenced for the American alligator (Katsu et al., 2006).

Progesterone receptors (PRs) have been identified in tissues of a range of reptilian taxa including the viviparous snake *Nerodia* (Kleis-San Francisco & Callard, 1986), the lizard *Podarcis sicula* (Paolucci & Di Cristo, 2002), house gecko *Hemidactylus flaviviridis* (Al-Amri et al., 2012), turtle *C. picta* (Giannoukos & Callard, 1995), and American alligator (Vonier et al., 1997). In the lizards *Mabuya* sp. (Duarte-Méndez et al., 2018) and *Uromastyx acanthinura* (Sadjia et al., 2007), PR is expressed in tissues that produce P, suggesting an autocrine action for that hormone. PR concentrations in the oviduct and liver of *P. sicula* vary differentially across the reproductive cycle: in particular, PR is detectable by binding assays and western blotting in the liver during periods when vitellogenin is not being synthesized, and in the oviduct except during the passage of eggs (Paolucci & Di Cristo, 2002). Two isoforms of PR, PRA, and PRB, have been identified in both oviduct and liver of *C. picta* (Giannoukos & Callard, 1995, 1996) and in ovary of *Podarcis siculus* (Motta et al., 2020). Differential expression of these isoforms during the ovarian cycle may provide a mechanism through which P_4 inhibits vitellogenesis (see Section 3.3.2). Additionally, expression of PRB in turtle ovary suggests a role for P_4 in the regulation of oocyte maturation in reptiles (Custodia-Lora & Callard, 2002).

The physiological roles of androgens in female vertebrates are poorly understood (Staub & De Beer, 1997). Perhaps for this reason, ARs in female reptiles have received little attention. AR have, however, been identified in some female reptiles. Patterns of expression of AR-mRNA in brain and oviduct of the female leopard gecko *Eublepharis macularius* vary across the reproductive cycle, and high levels of AR in the brain appear to be correlated with elevated circulating concentrations of androgen. These results suggest physiological roles for androgens in regulation of the female reproductive tract as well as female receptivity (Rhen et al., 2003), in addition to their function as aromatizable precursors to estrogens.

In recent years, there has been a great deal of research into endocrine-disrupting chemicals (EDCs). Many such environmental contaminants exert their effects via their capacity to bind to endogenous steroid receptors. Some endocrine-disrupting chemicals act as receptor agonists by binding to the receptors to induce conformational changes similar to those resulting from binding of the natural ligand, inverse agonists if inappropriate changes occur, or receptor antagonists if they occupy the receptor without leading to conformational change and a physiological response (Orchnik & Propper, 2006; Rooney & Guillette, 2000). Interactions of EDCs with receptors for reproductive steroids may have profound consequences for reptilian reproductive success and sexual development. For further discussion of endocrine disruption in reptiles, see Chapter 13 in this volume.

2.3 Steroid-binding proteins in plasma

Nonreceptor steroid-binding proteins (S-BPs) are ubiquitous among vertebrates. They are distinguished from steroid receptors by their higher binding capacity ($B_{max} = 10^{-6}$–10^{-9}) but lower affinity ($K_d = 10^{-7}$–10^{-9} M) (Callard & Callard, 1987), and by their more limited specificity for particular steroids. Plasma binding proteins are thought to fulfill several important functions. Binding proteins are an important means of maintaining a reservoir of steroid in the circulation. Steroids bound to binding proteins are protected from excretion, thus reducing the metabolic clearance rate of the hormones (Ho et al., 1987; Siiteri et al., 1982). Binding proteins also regulate access of steroids to receptors in target cells because only free steroid can enter the cell. Differences in affinity drive the dynamic equilibrium between a hormone, its binding protein, and its receptor, and thus influence the proportion of steroid that is free to bind with tissue receptors (Callard & Callard, 1987).

As a generality, two major classes of binding proteins are recognized in vertebrates: the first is a corticotropin-binding globulin (CBG, or transcortin) that primarily transports adrenal (C_{21}) steroids, and the second is a sex-hormone binding protein (SHBP) that binds estrogens and androgens (C_{18} and C_{19} steroids). In addition, steroids may bind nonspecifically to other plasma proteins, notably albumin. Although albumin has a lower affinity for steroids than do the binding proteins, it has high capacity because it is present in relatively high concentrations in the blood: albumin may therefore make an important contribution to steroid transport in the circulation (Callard & Callard, 1987).

There are phylogenetic differences among vertebrate taxa in the type and concentration of S-BP present, and in their degree of specificity (reviewed by Callard & Callard, 1987). In particular, there is considerable variation among species and taxa as to whether either of the major binding proteins binds P_4. High levels of a transcortin-type protein are present in plasma of gestating *L. vivipara*, in which plasma concentrations of P_4 are about 100× basal levels (Xavier, 1982). SHBPs have been identified in crocodilians (Ho et al., 1987), turtles (Ikonomopoulou et al., 2006; Salhanick & Callard, 1980), and squamates (Martin & Xavier, 1981; Paolucci et al., 1992; Riley et al., 1988). The viviparous snake *Nerodia* sp. appears to have a single medium-high affinity (10^{-7}–10^{-8}), limited capacity (0.09–0.7 M), binding protein that binds testosterone, E_2 and 5α-dihydrotestosterone (DHT), but also binds corticosterone and P_4 (Riley et al., 1988). In contrast, the lizard *L. vivipara* has two distinct plasma S-BP: a steroid hormone binding globulin (SHBG), with high affinity but limited capacity for E_2, and a CBG with high affinity (10^{-8} M) for P_4. Testosterone showed a similar affinity for both the CBG and SHBG molecules: this high concentration of testosterone-binding proteins may reflect the relatively high

plasma concentrations of T, which are five times higher than circulating E_2 concentrations in females of this species (Callard et al., 1978).

Studies of plasma sex steroids in female reptiles invariably report total (rather than free) concentrations of hormones and can, therefore, give a false picture of hormone activity. Annual variations in plasma concentrations of SHBPs may be very important in regulating the availability of the ovarian steroids to their target tissues but have received almost no attention in the literature. In the viviparous *Nerodia* sp., significantly lower concentrations of SHBG are present during mid-late pregnancy although there is little variation at other times of the cycle. During mid-late pregnancy, plasma P_4 is at a peak, but plasma estrogen concentrations are low: the authors suggested that the decrease in SHBG may facilitate the supply of P_4 for "uterine maintenance" (Riley et al., 1988). Comparative studies of viviparous and oviparous species could elucidate the role of SHBPs in maintaining appropriate availability of P_4 to tissues during gestation.

It is also important to appreciate that binding affinity varies with temperature. For example, Ikonomopoulou et al. (2006) found that E_2 binds with high affinity to a SHBG in plasma of nesting *Chelonia mydas*, but that binding was abolished at 36°C, so at high core body temperatures most of the E_2 would become biologically available to the tissues and for excretion. In contrast, T binding was thermostable, with the binding protein showing similar affinity and capacity at 4°C, 23°C, and 36°C. Shifts in binding affinity with body temperature, which would alter the availability of hormones to tissues, may play a role in regulating seasonal patterns of endocrine activity in reptiles, and warrant further investigation.

2.4 Peripheral metabolism of steroids

Enzymes that catalyze the metabolism of a single functional group on a steroid molecule can either activate or deactivate that steroid. Selective expression of such enzymes, particularly in peripheral tissues, provides an important mechanism for regulating steroid hormone action (Baker, 2004).

Peripheral metabolism of steroids may involve either derivatization or conjugation. Even though it is considered a primary steroid, T secreted by the gonad may be regularly converted by peripheral tissues into more biologically active derivatives with greater affinity for the tissue receptors: such derivatives are, in effect, a more active form of the hormone. Testosterone may be derivatized by reduction to DHT via the 5α-reductase enzyme, or aromatized to E_2: for example, T is readily converted to DHT in epididymis, cloaca, and skin of the male lizard *T. nigrolutea* (Edwards et al., 2005). Conjugation to either sulfate or glucuronide moieties deactivates steroids and converts them into water-soluble forms that do not bind to plasma binding proteins, rendering them readily excretable (Kime, 1987; Norris, 2007). Either metabolites or conjugates may play a physiological role as pheromones. Chemical communication is highly developed in terrestrial reptiles, and steroid metabolism, particularly by cloaca or skin, may be the means through which pheromones are produced (see Chapter 7 in this volume for further discussion). The most important site of steroid conjugation is the liver although other tissues, including the gonads themselves, can also metabolize steroids. Injection of radioactive P_4 in vivo into females of the lizard *T. rugosa* showed that P_4 is rapidly metabolized to free derivatives and conjugated steroids, The major free derivative was 20β-hydroxypregn-4-ene-3-one (20β-HP), which is the major metabolite of P_4 in other vertebrates. High levels of radioactivity associated with conjugate fractions from liver and kidney suggested that both organs are routes of steroid excretion (Bourne, 1981).

Only one study has directly examined steroid metabolism by peripheral tissues of a reptilian species. In female *T. nigrolutea*, steroid conjugates are produced by skin, kidney, cloaca, skeletal muscle, adrenal, oviduct, and ovary, although liver is most active (Edwards et al., 2005). In this in vitro system, liver conjugated about 75% of the initial substrate, with approximately 10% being converted to highly polar free derivatives. The proportion of E_2 metabolized by each tissue in vitro varied with reproductive stage: tissues from gestating females produced the greatest proportion of conjugated steroids, suggesting that this could be a mechanism for protecting tissues from high levels of E_2 during gestation. In mammals, steroid sulfates may be reconverted to active steroids via the sulfotransferase/sulfatase pathway, particularly in the fetoplacental unit where the system serves to protect developing embryos from high levels of maternal hormones (Kime, 1987). No-one has yet explored this possibility in viviparous female reptiles. However, in oviparous reptiles the sulfotransferase/sulfatase pathway serves to buffer developing embryos from maternal yolk steroids (Paitz & Bowden, 2008).

3 FOLLICULAR DEVELOPMENT

Among vertebrates, an ovarian follicle comprises the oocyte, with its acellular zona pellucida, plus associated surrounding layers of granulosa and thecal cells separated by a membrana propria (Norris, 2007). In reptiles, in contrast to birds and mammals, oogenesis and folliculogenesis continue through the female's reproductive lifetime (Jones et al., 1982). In most reptiles, follicle growth and vitellogenesis (yolk production) occur seasonally so that ovarian morphology and hormone production vary greatly over

the annual cycle, although ovarian activity is continuous in some tropical species (Licht, 1984).

3.1 Recruitment

Oogonial proliferation and differentiation begin in the embryonic ovary and continue in adult female reptiles. Mitotic divisions of oogonia in the germinal beds produce groups of naked oocytes that then enter meiotic prophase and become surrounded by a single layer of granulosa cells, forming primordial follicles. Such recruitment often occurs seasonally, as in the oviparous lizard *Ctenosaura pectinata* (Uribe et al., 1996) and the viviparous lizard *Sceloporus torquatus torquatus* (Uribe et al., 1995).

3.2 Follicular development

In reptiles, new follicles are produced from oogonia located in germinal beds on the dorsal surface of the ovary: these germinal beds are remnants of the embryonic ovary (Miller, 1963). In lizards, each ovary generally contains two germinal beds, although some species of lizard possess only one germinal bed per ovary, whereas others have several (Guraya, 1989; Jones et al., 1982). In snakes, the germinal epithelium is distributed as scattered irregular patches over the outer border of the ovarian stroma (Lance & Lofts, 1978). Oogonia in the germinal beds proliferate via mitotic activity and then enter meiotic prophase to form oocytes. Such activity often occurs seasonally, particularly in temperate reptilian species (Guraya, 1989).

Most reptilian oocytes are telolecithal, and so follicular development is typically associated with extensive vitellogenesis. Some features of follicular development vary among squamate, chelonian, and crocodilian reptiles. In squamates, the follicular epithelium, or granulosa, initially consists of small cuboidal cells, but differentiates during the previtellogenic phase to become multilayered and polymorphic. At this stage, the epithelium is characterized by the presence of unique flask-shaped pyriform cells, in addition to small and intermediate cells (Uribe et al., 1996) (Fig. 3A and B). These pyriform cells differentiate from small somatic follicular cells early in follicular development via the intermediate cell stage to become "nurse cells," in direct contact with the developing oocyte (Maurizii et al., 2004). Differentiation of the small cells into pyriform cells appears to be linked to the progressive appearance of glycoproteins with terminal α-*N*-acetylgalactosamine residues on the cell surface, which may be involved in fusion between the oocyte and the follicle cell membranes as well as maintenance of the differentiated pyriform cells. The pyriform cells are connected to the oocyte via intercellular bridges containing a cytoskeleton of α-tubulin and cytokeratin microtubules (Maurizii et al., 2004). It has been suggested that both intermediate and pyriform cells synthesize and then transfer RNA, ribosomes, vesicles, and other cellular materials to the oocyte across these bridges (Motta et al., 1995); however, at least in the lizard *Gerrhonotus coeruleus*, only relatively small molecules such as ribosomal RNA are transferred (Klosterman, 1987). Immunolabeling of ERα, ERβ, and PR in pyriform cells of the lizard *Sceloporus torquatus* suggests that these cells have some role in mediating autocrine and paracrine actions of E_2 within the developing follicles (Cruz-Cano et al., 2023; Sadjia et al., 2007). After the end of the previtellogenic phase, and as active sequestration of yolk into the ooplasm begins, the intermediate and pyriform cells regress, so that the granulosa is again composed of a homogenous single layer of cells (Uribe et al., 1996) (Fig. 3C).

Crocodilians, being archosaurs, share some key features of folliculogenesis with birds: these features include the morphology of yolk sphere development. Uribe and Guillette (2000) provided a detailed description of oogenesis and folliculogenesis in the American alligator, *A. mississippiensis*, and a study of *C. crocodilus* (Calderón et al., 2004) showed that these processes are similar in both species. In crocodilians, in contrast to squamate reptiles, the oocytes are surrounded by a granulosa consisting of a single layer of follicular cells that are squamous during early oogenesis, becoming cuboidal during mid to late stages of oogenesis: a well-defined theca forms during previtellogenesis. As vitellogenesis is initiated, small yolk granules appear in the ooplasm. Later, large, acidophilic yolk platelets accumulate in vacuoles containing numerous yolk spheres of different sizes. Platelets located toward the center of the oocyte contain larger yolk spheres than those toward the periphery. At all stages of follicular development, the ovary exhibits a well-developed system of large lacuna in both cortex and medulla, which may provide expansion space for the large developing follicles. In addition, bundles of smooth muscle present around the lacuna and the theca externa of the follicles appear to support the growing follicles; similar structures are observed in bird ovaries (Schroedder & Talbot, 1985).

3.2.1 Roles of hormones in recruitment and folliculogenesis

The hormonal regulation of follicle recruitment and, therefore, oocyte number, appears to involve both GTHs and ovarian factors. In the adult female reptile, follicular recruitment is typically initiated via exogenous environmental cues, presumably acting via neuroendocrine stimulation of the HPG axis. Temperature and/or photoperiod are the environmental factors most likely to be involved. Follicular growth and development in female *P. sicula* held at different temperatures suggest that ovarian sensitivity is

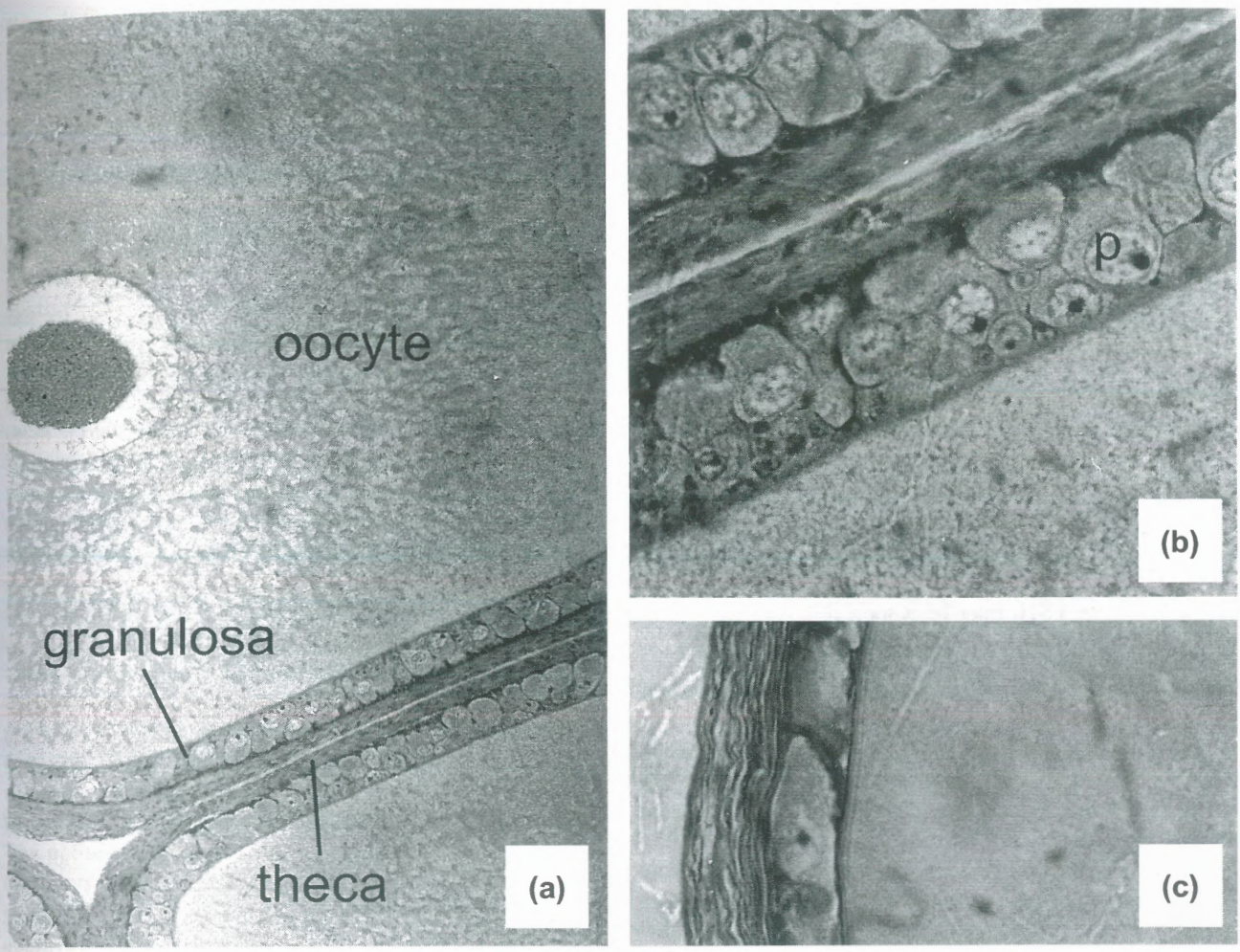

FIG. 3 Micrographs of the follicular wall in the lizard *Tiliqua nigrolutea*. (A) shows the follicular walls of two adjacent previtellogenic oocytes, with the inner granulosa and outer thecal layers clearly differentiated. The two follicular walls are shown at high power in (B). Note the large pyriform cells (p) that are characteristic of the granulosa layer in early follicles of squamates, but do not appear in follicles from other reptilian taxa. (C) shows a high-power view of the follicular wall of a vitellogenic follicle of *T. nigrolutea*. Note that the granulosa is now composed of a single layer of monomorphic cells. *(Photographs courtesy of Dr. Ashley Edwards.)*

cued by temperature in that species. Similarly, in garter snakes (*T. s. parietalis*), ovarian recrudescence appears to require exposure to low temperatures during hibernation: holding females at warmer hibernation temperatures induced ovarian regression and loss of FSH receptors (Bona-Gallo & Licht, 1983).

In seasonally breeding reptiles, there may be a refractory period during which the ovary is unresponsive to environmental cues. Females of *Anolis carolinensis* become unresponsive to long photoperiods and warm temperatures after the breeding season, and it has been suggested that this ovarian refractory period may be due to the presence of large corpora atretica (CA) (Crews & Licht, 1974). In *P. sicula*, the number of prefollicular oocytes and the sensitivity of the ovary to exogenous heterologous FSH (assessed by the responsiveness of adenylate cyclase), vary with season. There are two peaks of maximal sensitivity to FSH that correspond to peaks of ovarian activity: June, during the reproductive period, and December, when oogonial recruitment resumes despite apparently unfavorable environmental conditions (Angelini et al., 1985).

Other environmental factors such as stress or social conditions, including sexual composition of the group and male reproductive status, may also influence ovarian recrudescence. In *A. carolinensis*, for example, the presence of intact males appears to be necessary for GTH secretion (Crews et al., 1974; DeNardo & Autumn, 2001). It is well known that stress inhibits reproductive function in reptiles, as in other vertebrates (see Chapter 7 in this volume). In at least some species, β-endorphin, which is released in response to stress, may have a mediating role in determining the timing of ovarian recrudescence: β-endorphin inhibits both

seasonal and FSH-induced recrudescence in *Mabuya carinata* (Ganesh & Yajurvedi, 2003). Alternatively, postreproductive refractoriness and seasonal regression of the ovaries may be attributed to reduced sensitivity to gonadotropins (Angelini et al., 1985). In other species, mating appears to initiate a neuroendocrine reflex that activates female reproduction. Mating is a facultative and, in some circumstances, obligatory stimulus for vitellogenesis in red-sided garter snakes (Mendonca & Crews, 1990) and blood pythons (DeNardo & Autumn, 2001). Presumably, this system ensures that females commit to mobilizing resources for vitellogenesis only if males are present to ensure fertilization.

Autocrine and paracrine factors may also influence folliculogenesis in reptiles. In *P. sicula*, oocyte numbers decreased in excised germinal beds cultured in vitro for 48 h unless FSH or follicles were also present (Sica et al., 2001). The cultured follicles released a recruiting factor that appears to be a low molecular weight (c. 21 kDa) diffusible protein. The authors hypothesized that this factor, which is not dependent upon FSH, may be responsible for recruitment of oocytes from the pool of oogonia.

During folliculogenesis, follicles not only differentiate and grow, but also acquire the capacity to respond to gonadotropins. In mammals, granulosa cells of committed preantral follicles must express FSH receptors to progress to the gonadotropin-dependent stage; otherwise, they would become atretic. Growth factors, particularly activin and inhibin, and cytokines interact to promote and sustain folliculogenesis through paracrine or autocrine regulation of gonadotropin receptors. In mammals, inhibin has local stimulatory effects on LH-induced androgen synthesis by thecal cells, although it probably also acts as a peripheral hormone, regulating the activity of the HPG axis via negative feedback effects on the secretion of pituitary FSH (Findlay, 1993). Immunocytochemical analyses have revealed the presence of an inhibin-like protein in the cytoplasm of pyriform cells in previtellogenic follicles of *P. sicula*, which may reflect a role for inhibin in the maintenance of a follicular hierarchy (Limatola et al., 2002). However, inhibin has been little studied in reptiles.

3.2.2 Determinants of clutch size

In reptiles, clutch size, or the number of follicles ovulating in any given cycle, varies with species. Some gekkonid lizards lay only one egg per clutch, although average clutch size in lizards is usually 2–10 eggs; snakes lay clutches of 6–15 eggs, and crocodilians 13–55 eggs, with some sea turtles laying 50–100 eggs at a time (Thompson & Speake, 2004). Radder et al. (2008) recently reviewed literature on germinal beds in lizards and showed that, among lizards, clutch size is linked to ovarian morphology. Species that produce large clutches have multiple germinal beds in each ovary, whereas species ovulating small clutches have only one germinal bed per ovary. Most species exhibit polyautochronic ovulation, in that several ova are ovulated simultaneously from both ovaries. Gekkonid lizards, on the other hand, ovulate two ova simultaneously, one from each ovary (i.e., monoautochronic ovulation) (Boyd, 1940). *A. carolinensis* exhibits monoallochronic ovulation in that only one ovum ovulates at a time, with left and right ovaries alternating every 2 weeks (Jones et al., 1973).

At the functional level, clutch size is determined by the number of follicles that become vitellogenic, and the proportion of these follicles that become atretic before ovulation. Etches and Petitte (1990) provided a review of follicular recruitment and the establishment of ovarian hierarchies in reptiles and birds, but the underlying hormonal mechanisms are still poorly understood. A suite of early papers (e.g., Jones et al., 1973, 1976) investigated endocrine influences on clutch size in *A. carolinensis*, reflecting the particular opportunities for experimental manipulation afforded by a species exhibiting monoallochronic ovulation. However, due to the highly derived pattern of the anole ovarian cycle, those results are unlikely to be representative of reptiles in general. More typically, squamate reptiles ovulate larger, single clutches of variable size. In such species, the number of vitellogenic follicles may be increased by administration of exogenous FSH at appropriate times, although those follicles may not ovulate. Treatment with FSH at doses of 1, 10, or 50 μg stimulated follicular growth, but not ovulation, in the lizard *Leiolopisma laterale* (Jones et al., 1973), while in the skink *Niveoscincus metallicus*, additional follicles were recruited only if exogenous FSH was administered during early vitellogenesis (Jones & Swain, 2000).

3.2.3 Follicular atresia

In vertebrate ovaries, a large proportion of follicles undergo atresia to form CA before completing their development (Guraya, 1989). Through its influence on the number of eggs produced, atresia may be the primary mechanism through which clutch size is regulated (Cummins, 1995; Méndez-de la Cruz et al., 1993). Atresia may occur at any stage of follicular development. Among lizards, atresia is common during the previtellogenic stages (Fox & Guillette, 1987; Uribe et al., 1995), although in six species of Chilean *Liolaemus* lizards, follicular atresia rates were highest in gravid females (Leyton & Valencia, 1992). Exogenous FSH induced follicular development in female *Uta stansburiana*; however, atresia of large follicles was observed after cessation of FSH treatment, suggesting that GTH stimulation is required to maintain follicular maturation (Ferguson, 1966). High levels of atresia observed during gravidity are positively correlated with elevated plasma P_4 concentrations during this reproductive phase

(Fox & Guillette, 1987) and may reflect inhibition of GTH secretion by P_4, resulting in inhibition of follicular development.

There has been some debate about whether atretic follicles are steroidogenic. Guraya (1989) described the stages of atresia in previtellogenic follicles in squamates, and reviewed evidence for the presence of several steroid dehydrogenases in early CA in lizard ovaries, suggesting "the possibility of transient luteinization or endocrine activity." Histochemical visualization of steroidogenic enzymes suggested that granulosa cells of CA in *C. versicolor* do have some steroidogenic capacity (Gouder et al., 1979) although this could simply reflect relict activity persisting before degradation. Guraya (1989) concluded that degenerating, yolky follicles have no endocrine function, but are simply vitellogenic follicles in the process of resorption by follicle cells or phagocytes. It has been postulated that atretic follicles may represent an alternative source of P during luteolysis in gestating lizards (Duarte-Méndez et al., 2018). In contrast, Duarte-Méndez et al. (2018) detected no immunohistochemical localization of 3B-HSD in atretic follicles of gestating *Mabuya* sp. and concluded that the CA were unlikely to represent an alternative source of P_4. Interestingly, the presence of CA suppresses ovarian sensitivity to GTHs or to environmental stimulation in *A. carolinensis*, and surgical removal of the CA significantly increases the response of the ovary to exogenous GTHs (Crews & Licht, 1974). The authors suggested that the mechanism could involve secretion of a local factor that inhibits vitellogenesis or steroidogenesis. Conversely, the relatively large size of the CA could lead to its appropriating the majority of circulating GTH, depriving other follicles of stimulatory hormone.

3.3 Vitellogenesis

In reptiles, follicular growth and maturation typically involves the deposition of large amounts of yolk. The bulk of that yolk is derived from the precursor molecule vitellogenin, a large molecular weight lipophosphoglycoprotein that is synthesized in the liver (Ho, 1991). Vitellogenesis is the process of hepatic synthesis of vitellogenin and its transport to the oocytes. This requires the mobilization of significant maternal reserves and is therefore dependent upon a threshold level of body condition (Bonnet et al., 1994). This requirement precludes annual reproduction in females of some species such as the viviparous vipers (*Vipera* spp.) (Bonnet et al., 1994) and the blue-tongued lizard, *T. nigrolutea* (Edwards, Jones, & Wapstra, 2002). Activation of the HPG axis to initiate vitellogenesis in appropriate seasons could involve leptin, a hormone produced primarily by adipocytes, which acts as a metabolic signal to the reproductive system in mammals. Leptin has been little studied in reptiles, but plasma leptin concentrations varied seasonally in *S. undulatus* (Spanovich et al., 2006) and increased during the previtellogenic period in *P. sicula* (Paolucci et al., 2001).

Vitellogenin itself has a very long evolutionary history. It is a yolk precursor in a wide range of egg-laying animals including insects, nematodes and vertebrates (Brandt et al., 2005). Vitellogenin provides the developing embryo with amino acids, lipids, phosphate and multivalent metal ions, but its lipid content of 12%–15% is not sufficient to fuel the needs of amniote embryos, which must reach an advanced stage of development at hatching. In oviparous amniotes, very-low-density lipoprotein (VLDL), also synthesized in the maternal liver, provides an additional source of yolk lipid, while a range of other proteins are also incorporated into the yolk: these include serum albumin, vitamin-binding proteins such as cholecalciferol, and immunoglobulins (IgGs) (Thompson & Speake, 2004).

The embryos of oviparous reptiles depend solely upon yolk for their nourishment during development. However, among viviparous reptiles, there is a spectrum of different degrees of dependence upon lecithotrophy (yolk nutrition) versus placentotrophy for embryonic nourishment. Most viviparous squamates do ovulate large yolky eggs, but, at the other end of the spectrum, viviparous species of *Chalcides* and *Mabuya* ovulate microlecithal eggs: for example, *Mabuya heathi* (Vitt & Blackburn, 1983), *Mabuya bistriata* (Vitt & Blackburn, 1991) and *Mabuya brachypoda* (Hernandez-Franyutti et al., 2005) ovulate eggs only about 1 mm in diameter. In such species, oogenesis and oocyte maturation occur as in other reptiles, but vitellogenesis is suppressed, resulting in relatively small eggs (Hernandez-Franyutti et al., 2005). Callard and Ho (1987) provide a pertinent review of vitellogenesis in the context of the evolution of viviparity in vertebrates. It has been suggested that P_4 plays a major role in the suppression of vitellogenesis (Callard et al., 1990a), but endocrine studies are still needed to elucidate the underlying mechanism(s).

3.3.1 *Mechanisms of vitellogenesis*

Vitellogenesis in vertebrates, and particularly in reptiles, has been the subject of several substantial comparative reviews (Callard & Ho, 1987; Callard et al., 1990a; Ho, 1991; Ho et al., 1982; Polzonetti-Magni et al., 2004). Among vertebrates, vitellogenin is synthesized not in the ovary but in the liver and is transported to the developing oocytes in the bloodstream. Vitellogenesis is accompanied by hypertrophy of the liver, signaling hepatic production of vitellogenin (Gavaud, 1986). Changes in hepatic protein synthesis in response to estrogens are linked to the transfer of yolk precursors to the developing oocytes (Ho, 1991). Calcium, which is transported to the oviduct in the bloodstream, binds to the vitellogenin molecule. Plasma calcium

concentrations have therefore been used as an indicator of vitellogenesis in reptiles (Rostal et al., 1998).

Vitellogenin is sequestered by the oocytes via receptor-mediated endocytosis and then stored in membrane-bound compartments (Polzonetti-Magni et al., 2004). Electron microscopic studies in *P. sicula* demonstrate that exogenous yolk precursors, visualized as electron-dense particles, are taken up by the oocyte by micropinocytosis and accumulate in the yolk globules. Although the chemical composition of these particles was not confirmed, they are presumably vitellogenin (Limatola & Filosa, 1989). In addition, a vitellogenin-binding protein has been identified in oocytes of *P. sicula*. In untreated animals, this protein is present in the plasma membranes of all classes of oocyte during the reproductive period, and treatment of winter animals with exogenous FSH induced vitellogenin-binding protein in all oocytes (Romano & Limatola, 2000). In the oocytes, vitellogenin is enzymatically cleaved into phosvitins and lipovitellin, which form the major yolk proteins that are destined to be utilized as an energy source by the developing embryos (Polzonetti-Magni et al., 2004).

3.3.2 Hormones and vitellogenesis

The proximate stimulus for the induction of vitellogenesis is the presence of circulating estrogens, generally produced by the ovary from the maturing follicles. Being estrogen-dependent, the production of vitellogenin is normally restricted to females. However, male vertebrates also carry the vitellogenin gene, so exposure to natural or xenobiotic estrogens can trigger its expression in the liver of males as well as females (Ho, 1991). Indeed, vitellogenin synthesis by males is a useful indicator of endocrine disruption by ecoestrogens (Kime et al., 1999).

Ovarian development and vitellogenesis are, therefore, typically associated with elevated plasma concentrations of E_2 in squamates, turtles, and crocodilians, and upregulation of hepatic ER may signal "readiness" for vitellogenesis (Custodia-Lora et al., 2004). For example, high plasma E_2 concentrations in vitellogenic females of the snake *Vipera aspis* are associated with mobilization of maternal reserves to fuel vitellogenesis; this is evidenced by high plasma levels of calcium, phosphorus, phospholipids, cholesterol, triglycerides, and proteins. Insertion of silastic implants containing E_2 into non-reproductive females (that typically exhibit low plasma E_2) resulted in mobilization of reserves as seen in vitellogenic females (Bonnet et al., 1994). Similarly, exogenous E_2 induced hypercalcemia, hyperproteinemia, and liver hypertrophy in ovariectomized lizards (*L. vivipara*), although several other steroids, including P_4 and T, had no such effect (Gavaud, 1986). Vitellogenin appears in the plasma six to eight hours after administration of exogenous estradiol to either sex of *C. picta* (Ho et al., 1981), and plasma E_2 concentrations rise significantly prior to nesting in loggerhead turtles (*Caretta caretta*) at a time when the ovaries contain hundreds of vitellogenic follicles but are minimal by the time of the final nesting for that season (Wibbels et al., 1992).

There do seem to be some exceptions to the general pattern of elevated estrogens during vitellogenesis. Circulating estrogen concentrations appear to be low during the presumed period of vitellogenesis in the green turtle *C. mydas* (Licht, 1979), and the lizard *P. barbata* exhibits very low plasma concentrations of E_2, with no elevation during vitellogenesis (Amey & Whittier, 2000) although the authors speculate that this could be because a different estrogen is more important in this species. In the cold-adapted tuatara, *Sphenodon punctatus*, vitellogenesis is slow and extraordinarily protracted, taking place over a period of 3 years in a reproductive cycle of 4 years. Plasma steroids still follow patterns characteristic of other reptiles: plasma E_2, testosterone, and P_4 are low during early vitellogenesis, rising only during the preovulatory period (Cree et al., 1992), and plasma P_4 and testosterone provide reliable indicators of whether a female will ovulate in any given year (Cree et al., 1991). In tuatara, plasma vitellogenin does not appear to be correlated with plasma concentrations of E_2 (nor of T or P_4) (Brown et al., 1994), despite the observation that exogenous E_2 does stimulate vitellogenesis.

However, estrogens are not the only hormones associated with vitellogenesis. Vitellogenesis is under multihormonal control (Callard et al., 1990a; Ho et al., 1982; Polzonetti-Magni et al., 2004), and the vitellogenic action of estrogens is modulated by other ovarian steroids, and by pituitary hormones. For example, in the House gecko *H. flaviviridis*, both plasma T and E_2 concentrations rise significantly during vitellogenesis (Al-Amri et al., 2012). The most detailed studies of the endocrine interactions associated with vitellogenesis have focused on the freshwater turtle, *C. picta* (Duggan & Callard, 2003), utilizing the specific radioimmunoassay for plasma vitellogenin developed for that species. Progesterone is generally considered to inhibit estrogen-induced vitellogenesis, probably by acting directly on the liver (Callard, Bayen, & McConnell, 1972; Callard et al., 1990b). Both P_4 and T inhibit estradiol-induced vitellogenesis in *C. picta* (Ho et al., 1981), and rising levels of these hormones during the periovulatory phase may summate to terminate vitellogenesis at the end of the cycle (Ho, 1991). Callard et al. (1990a) used evidence from chickens to suggest that the inhibitory action of P_4 on vitellogenesis could involve interaction of the PR with a progesterone response element (PRE) close to the estrogen response element (ERE) of the vitellogenin gene. Rarely, in some species such as the lizard *C. versicolor*, the key reproductive events of vitellogenesis and gravidity may, on occasion, overlap. In gravid females of *C. versicolor* with

vitellogenic follicles, E_2 concentrations rise, while plasma P_4 remains low (Radder et al., 2001), suggesting that elevated P_4 is not required for egg retention during late gravidity. Low P_4 at this stage may potentiate the growth of a new crop of follicles.

Progesterone may also have previtellogenic effects. Positive and negative effects of P_4 could be mediated via differential expression in the liver of the two isoforms of the PR. While PRA, which has been suggested to act as a general transcription inhibitor (Duggan & Callard, 2003), is expressed throughout the annual cycle of *C. picta*, PRB, a gene-specific positive regulator, is expressed only during follicular growth, in autumn and in spring (Giannoukos & Callard, 1995) and appears to be under estrogenic control. This variation, underpinned by changes in the ratio of circulating P_4 to estrogens (P:E ratio), suggests a mechanism whereby P_4 could modulate the estrogenic induction of vitellogenesis (Custodia-Lora et al., 2004).

Pituitary hormones may also be involved in the regulation of vitellogenesis in reptiles (Ho, 1991), although studies remain scarce. At the follicular level, uptake of yolk precursor into developing follicles of the lizard *P. sicula* was stimulated by exogenous FSH (Limatola & Filosa, 1989). At the hepatic level, hypophysectomy abolished the seasonal increase in hepatic ER concentrations observed in *C. picta* (Ho et al., 1989). Although the specific pituitary hormone(s) involved in the expression of hepatic ER was not determined, the authors suggested that it is likely to be a growth hormone (GH)-related factor. In their review of vitellogenesis and viviparity, Callard and Ho (1987) suggested that GH has synergistic effects on gonadotropin-stimulated vitellogenesis, while prolactin inhibits the synthesis and/or uptake of vitellogenin into follicles. However, in view of the paucity of evidence, further work, with a larger variety of species, is necessary to confirm and elucidate the putative roles of these pituitary hormones in the regulation of reptilian vitellogenesis. In addition, there is need for detailed studies of the endocrine control of vitellogenesis in reptiles other than turtles, in both viviparous and oviparous species.

The possibility that ovarian GnRH and bradykinin play regulatory roles in follicular development and vitellogenesis should also be explored. Although GnRH was originally identified as a hypothalamic neurohormone, multiple types of GnRH are now known to be expressed in a wide range of tissues, including ovary, and gonadal GnRH has been hypothesized to play an autocrine/paracrine role in ovarian development and function (Leung et al., 2003; McGuire & Bentley, 2010). Three GnRH subtypes and their receptors were detected in ovary of the leopard gecko, *E. macularius* (Ikemoto & Park, 2007), and seasonal differences in receptor expression suggest some role for ovarian GnRH in the regulation of seasonality. Bradykinin stimulates GnRH release from the hypothalamus but is also produced in the ovary, where it may be involved locally in follicular development and ovulation (Kihara et al., 2000). In *C. versicolor*, the greatest intensity of both GnRH and bradykinin immunoreactivity was in granulosa cells of previtellogenic or early vitellogenic follicles, suggesting some link with the regulation of follicular development (Singh et al., 2007, 2008).

3.3.3 Yolk steroids

In reptiles, yolk itself contains steroid hormones that are deposited into the yolk during vitellogenesis. Yolk steroids may play an important role in sex determination, particularly in oviparous reptiles, and influence offspring fitness in both oviparous and viviparous reptiles. Concentrations of individual steroids vary between layers of yolk within each egg (Bowden et al., 2001; Conley et al., 1997; Elf et al., 2002), and change during embryonic development. In alligators, concentrations of E_2, androstenedione, and T decline markedly during egg incubation, with the decline most striking at stages 21–23, when gonadal differentiation is occurring (Conley et al., 1997). The authors suggested that androstenedione and T sourced from the yolk may be metabolized to estrogens by the gonads of the developing embryos, and thereby play a key role in sex determination.

Steroid concentrations in yolk also vary among eggs, both within and among clutches. Steroid concentrations at the time of ovulation may passively reflect concurrent circulating maternal hormone levels: yolk and maternal plasma steroid concentrations tend to co-vary. For example, both plasma and yolk T concentrations were artificially elevated in female turtles with T implants during follicular development (Janzen et al., 2002). Seasonal variation in yolk steroids has been correlated with seasonal shifts in sex ratio in clutches of *C. picta*, a turtle with TSD (Bowden et al., 2000). Conversely, it has been hypothesized that differential allocation of yolk steroids to individual eggs within a clutch is an important mechanism through which mothers can influence offspring development and sex determination (Bowden et al., 2000; Lovern & Wade, 2003).

Yolk steroids are not, however, derived entirely from maternal sources. During embryonic development, as the embryonic endocrine organs begin to produce hormones, additional steroids may diffuse across into the yolk (Lovern & Wade, 2003). The CORT content of *Urosaurus ornatus* eggs, which is non-detectable at oviposition, was significantly increased by day 25 of incubation, strongly suggesting that its source is the fetal adrenals (Weiss et al., 2007), while in the viviparous *Sceloporus jarrovi*, yolk P_4 and CORT concentrations more closely mirrored fetal, rather than maternal, plasma concentrations of those

steroids (Painter et al., 2002). These and similar observations support the notion of a dynamic interaction between maternal and embryonic endocrine systems in both oviparous and viviparous reptiles.

Hormones other than steroids may also be deposited into yolk during vitellogenesis. Plasma concentrations of insulin-like growth factor 1 (IGF-1) are elevated during gravidity in American alligators, providing some circumstantial evidence that this hormone, which is a growth-promoting substance, may be sequestered into yolk (Guillette et al., 1996). The potential contributions of such endocrine factors to supporting embryonic development remain to be elucidated, but this could be an additional mechanism through which maternal effects on offspring characteristics may be exerted.

4 OVULATION

Ovulation, or release of the oocyte from the ovary, is the culmination of vitellogenesis and follicular maturation. Plasma concentrations of estrogens rise significantly during late vitellogenesis as the follicles mature. Following ovulation, plasma estrogen concentrations decline rapidly to prereproductive levels as the postovulatory follicle is transformed into a corpus luteum.

4.1 Mechanisms of ovulation in reptiles

Ovulation involves enzymatic degradation and rupture of the multilayered follicular wall and the escape of the mature oocyte. The point of follicular rupture is visible externally as a pale region known as the stigma. In reptiles, the stigma is avascular (Uribe & Guillette, 2000). In nonmammalian vertebrates, the stigma ruptures due to ischemia resulting in necrosis, rather than via an inflammatory response as occurs in mammals (Jones, 1987). Histological examination of follicles in FSH-treated *A. carolinensis* demonstrated a reduction in follicular blood flow immediately before rupture. There were dramatic changes in the stigma, including marked accumulation of extracellular fluid and dissociation of collagen bundles to form a "lacework of fibrils," but no evidence of inflammatory cells in the interstitial spaces (Jones et al., 1988). The mechanism of oocyte escape from the follicle has not been studied in reptiles, but the process is believed to involve contractile fibers. Follicles of *A carolinensis* undergo spontaneous rhythmic contractions (Jones et al., 1984) that may be related to this process.

4.2 Hormonal control of ovulation

From an endocrine perspective, ovulation marks the transition from the estrogen-dominated follicular phase to the P_4-dominated luteal phase. In female vertebrates, changing patterns of plasma steroids through the ovarian cycle influence GTH secretion via feedback effects at the level of the pituitary gonadotropes or the hypothalamic GnRH neurons. Peak plasma concentrations of estrogens precede ovulation, and the ovulatory surge is stimulated via a positive feedback action of estrogen at the level of the hypothalamus (Niswender et al., 2000).

In reptiles, most studies of plasma steroids have examined long-term patterns of variation over the reproductive cycle, and relationships between hormonal changes and physiological effects are usually inferred rather than established. There is, therefore, little direct evidence for feedback control of GTH secretion by steroids in reptiles. Some early studies examined the effects of hypothalamic implants of P_4 or estrogens in female *Sceloporus cyanogenys*. Estrogenic implants into the hypothalamus (but not other parts of the brain, nor in subcutaneous locations) inhibited ovulation and depressed ovarian steroidogenesis (Callard, Bayen, & McConnell, 1972), providing evidence for negative feedback inhibition of GTH release, while P_4 implants inhibited ovarian growth (vitellogenesis) but not ovulation. In general, P_4 also has a negative feedback effect on GTH secretion, so that folliculogenesis is inhibited during gravidity. In contrast, it has been suggested that P_4, not estrogens, may be responsible for positive feedback regulation of the ovulatory LH surge in turtles (Etches & Petitte, 1990). Ovulation is also correlated with elevated plasma testosterone concentrations in species including the Galapagos tortoise, *Geochelone nigra* (Rostal et al., 1998), the blue-tongued lizard (Edwards & Jones, 2001), and the American alligator (Lance, 1989), although a causal relationship has not yet been established.

In most vertebrates, a marked surge of plasma LH and/or FSH stimulates follicular rupture and initiates subsequent luteinization of the granulosa cells (Jones, 1987). In reptiles, the precise roles of the GTHs in ovulation remain unclear. The paucity of experimental studies and descriptions of changes in plasma GTHs around the time of ovulation reflects a lack of specific assays for these hormones in reptiles other than turtles. In sea turtles, plasma FSH concentrations are low except for a transient peak associated with the onset of oviposition, whereas plasma LH exhibits a marked "ovulatory" surge associated with nesting (Owens & Morris, 1985). The temporal dissociation between these two hormonal peaks suggests that in turtles FSH may prime the ovary to respond to LH. However, time series samples from individual loggerhead turtles (*C. caretta*) identified concurrent surges of LH and FSH in the periovulatory period (Wibbels et al., 1992), perhaps reflecting a nonspecific action of GnRH on GTH release by the pituitary. Peak LH levels preceded, or were coincident with, peak P_4 concentrations in plasma, providing evidence that LH stimulates ovarian production of P_4 by the postovulatory follicle.

In vertebrates, gonadotropin-induced ovulation is mediated by prostaglandins (Jones, 1987), and inhibitors of prostaglandin (PG) synthesis act at the ovarian level to inhibit ovulation. Thus, in *A. carolinensis*, indomethacin suppresses ovarian PGE secretion in vitro, inhibits preovulatory histological changes in the follicular wall, and significantly delays ovulation in response to exogenous FSH (Jones et al., 1990). Both $PGF_{2\alpha}$ and PGE_2 are produced by follicles of *P. sicula*. Preovulatory follicles produced the highest levels of $PGF_{2\alpha}$ while PGE_2 production was highest in early vitellogenic follicles. The effects of $PGF_{2\alpha}$ and PGE_2 on sex steroid production in vitro by follicles at different vitellogenic stages suggest that $PGF_{2\alpha}$ could be involved in ovulation, while PGE_2 has a stimulatory effect on E_2 release by the follicles, suggesting that PGE_2 is involved in maintaining vitellogenesis (Gobbetti et al., 1993b). More detailed information is still required regarding the roles of steroid hormones and PGs in the induction of ovulation in reptiles.

4.3 Oocyte maturation

The final stages of oocyte maturation involve completion of the first meiotic division; until this is completed, the oocyte is not fertilizable. To date, there have been no studies of the endocrine control of oocyte maturation in reptiles: our knowledge of this process in nonmammalian vertebrates is restricted to fishes and amphibians. It has been suggested that P_4 may act as a maturation-inducing substance (Nagahama, 1987), but this hypothesis is based solely upon patterns of plasma P_4 concentrations during the reproductive cycle of various reptiles.

5 THE POSTOVULATORY OVARY: THE CORPUS LUTEUM

All reptiles, whether oviparous or viviparous, develop true secretory corpora lutea from the postovulatory follicles. These corpora lutea are transitory endocrine structures, formed primarily from the granulosa cells, which hypertrophy after ovulation to form a luteal cell mass surrounded by thecal layers (Guraya, 1989). The luteal cells are steroidogenic and secrete P_4. Several major reviews have focused on corpora lutea in reptiles or, more generally, in nonmammalian vertebrates (Callard et al., 1992; Guraya, 1989; Jones & Baxter, 1991; Saidapur, 1982; Xavier, 1987). Jones and Baxter (1991) identify five types of corpora lutea among vertebrates, distinguished by their length of lifespan and type of endocrine regulation. They consider that reptilian corpora lutea fall into Type II, having an extended life span and being controlled by pituitary luteotropin(s). They suggest that the life of these corpora lutea is extended because the luteinizing hormone becomes a luteotropin, or another pituitary hormone such as prolactin takes over this function, but these ideas remain to be tested experimentally. The significance of the corpus luteum and its primary steroid product, P_4, in the evolution of viviparity is discussed further in Chapter 9 of this volume.

5.1 Morphological changes in the reptilian corpus luteum

The lifespan of a typical corpus luteum may be divided into three stages: luteogenesis, luteal maturity, and luteal regression, or luteolysis (Fox & Guillette, 1987). The associated morphological changes have been described in great detail in several reviews (e.g., Fox & Guillette, 1987; Saidapur, 1982; Xavier, 1987): a synopsis of those descriptions is given here.

After ovulation, the collapsed follicle appears flaccid and sac-like, with a large opening in one side through which the oocyte has escaped. The postovulatory follicle then shrinks in size as its walls become thickened and folded to produce a multilayered granulosa. The granulosa cells now become "luteinized": they hypertrophy, acquire secretory granules and large lipid droplets in their cytoplasm, and are characterized by a large central nucleus with one to three nucleoli. The ultrastructure of the luteinized cells, with abundant smooth endoplasmic reticulum, is characteristic of steroid-secreting cells. The granulosa involutes and hypertrophies to fill the central cavity of the collapsed follicle, forming the luteal cell mass, while the follicular lumen and the opening through which the oocyte emerged eventually disappear in most species, except crocodilians and tuatara (Guillette & Cree, 1997). The thecal cells also proliferate to form two well-defined layers, the theca externa and the theca interna, around the inner luteal cell mass. The thinner theca externa is a loose tissue containing collagen, fibroblasts, a few hypertrophied round cells and macrophages, while the theca interna is more cellular. At maturity, the corpus luteum is a compact, solid structure with three distinct zones: the outermost theca externa, the theca interna, and the luteal cell mass (Guraya, 1989) (Fig. 4).

Reptilian corpora lutea differ from those of mammals in that the cells filling the luteal cavity in reptiles are almost exclusively of granulosa origin. However, there are some differences among species in the cellular composition of the corpus luteum. Xavier (1987) distinguished three different morphological types of corpora lutea among reptiles in contrast to the physiological types defined by Jones and Baxter (1991). These morphological types are differentiated by the degree to which the cells of the theca interna invade the luteal cell mass. In some species, the granulosa cells alone form the luteal cell mass (type 1). In type 2 corpora lutea, fibroblasts from the theca interna invade to form superficial septae, but no capillaries penetrate among the luteal cells. In other species, the corpus

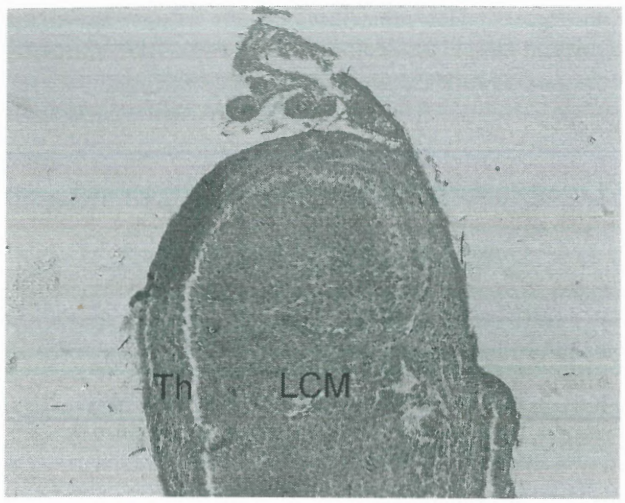

FIG. 4 Micrograph of a corpus luteum of the viviparous lizard *Niveoscincus metallicus* during mid-gestation, when the embryos were at Stage 29 (sensu Dufaure & Hubert, 1961). Note the well-defined inner luteal cell mass (LCM), and the outer thecal layers (Th).

luteum becomes richly vascularized, and the theca interna invades the luteal cell mass, forming fibrous dividing septae (type 3).

The functional significance of these morphological differences is unknown. However, the corpora lutea remain active and secretory for species-specific periods of time. In oviparous reptiles, the corpora lutea persist during gravidity (egg retention), and regress near the time of oviposition. In tuataras, which have an extraordinarily extended reproductive cycle, corpora lutea persist during the 8 months of egg retention (Guillette & Cree, 1997). Corpora lutea of viviparous reptiles persist for variable periods, but generally remain active until the end of gestation (Guraya, 1989; Xavier, 1987). The nuclear diameter of the luteal cells decreases gradually during gestation (Bennett & Jones, 2002), presumably reflecting changes in endocrine activity by these cells.

Eventually, the corpora lutea degenerate as luteolysis begins. Nuclei of the luteal cells become pycnotic, and vacuoles and sudanophilic lipid droplets appear in their cytoplasm; the theca becomes less vascular, fibrous connective tissue invades, and the thecal layers merge. Luteal cells eventually disintegrate and are replaced by connective tissue. The remaining scar is comparable to the corpus albicans of the mammalian ovary (Guraya, 1989).

5.2 Steroid production by the corpus luteum

Corpora lutea are generally considered to be the major source of circulating P_4 secreted by the reptilian ovary. This has been confirmed by in vitro tissue incubations and by histochemical studies. In vitro studies demonstrate that corpora lutea of oviparous turtles (Klicka & Mahmoud, 1972) and viviparous lizards (Girling & Jones, 2003; Xavier, 1982) can convert pregnenolone to P_4, and the rate of P_4 secretion by non-luteal ovarian tissues is insignificant compared with its rate of production by corpora lutea (Bennett & Jones, 2002; Xavier, 1987). The steroidogenic enzyme 3β-HSD, which converts pregnenolone to P_4, is detectable in luteinized granulosa cells by histochemistry. The intensity of 3β-HSD activity in the corpus luteum has been positively correlated with plasma P_4 concentrations in both oviparous and viviparous species (Martínez-Torres et al., 2003; Shanbhag et al., 2001), although in some viviparous species the placentae may take over the primary role in P_4 production during later gestation (Guarino et al., 1998).

Xavier (1987) distinguished two basic patterns of P_4 secretion by reptilian corpora lutea. The first is a preovulatory or ovulatory surge of plasma P_4, characteristic of turtles, in which the corpora lutea are poorly steroidogenic. The second pattern, characteristic of squamates, is a peak of plasma P_4 after ovulation, or at mid-late pregnancy in viviparous species. However, at variance with this pattern, the viviparous skink *Niveoscincus microlepidotus* (Girling et al., 2002) and viviparous snake *Natrix sipedon pictiventris* (Chan et al., 1973) exhibit preovulatory peaks of plasma P_4 concentrations, which may reflect P_4 synthesis by the preovulatory follicles. Luteolysis (Fergusson & Bradshaw, 1991; Fox & Guillette, 1987), luteectomy (Guillette & Fox, 1985; Highfill & Mead, 1975), or ovariectomy (Fergusson & Bradshaw, 1991) is associated with a marked drop in plasma P_4 concentrations as the luteal cells lose their capacity to secrete P_4 or the source of that P_4 is removed.

In some species at least, the corpus luteum may produce androgens and estrogens as well as P_4 (Xavier, 1987). Tissue concentrations of T and E_2 were higher in corpora lutea than in follicular tissue of gravid *Uromastix hardwicki*, although the concentration of P_4 in the corpora lutea was 100 times that of E_2 (Arslan et al., 1978) and corpora lutea of the lizard *Podarcis sicula sicula* released E_2 as well as P_4 when incubated in vitro (Ciarcia et al., 1993; Gobbetti et al., 1993a). The functional significance of these androgenic and estrogenic steroids is unclear, and it is possible that they simply reflect metabolic catabolism of P_4.

5.3 Relaxin

The mammalian corpus luteum secretes some nonsteroidal hormones including relaxin, a polypeptide hormone that plays important roles in mammalian pregnancy. Among these, relaxin supports oxytocin-stimulated uterine contractions, and widens the pelvic symphysis to facilitate parturition. Jones and Baxter (1991) considered that the "fundamental effects of relaxin… should all be present in

reptiles," but we still have surprisingly little knowledge of relaxin in reptiles, although relaxin-like molecules have been identified in several viviparous sharks (see review by Jones & Baxter, 1991). The possibility of relaxin production by reptilian corpora lutea has not yet been reported, although Koob and Callard (1982) speculated that in viviparous reptiles the corpora lutea may begin secreting relaxin as P_4 secretion drops toward the end of gestation.

5.4 Hormones and luteolysis

In mammals, $PGF_{2\alpha}$ elicits luteolysis through a series of actions, including reduction of blood flow to the corpus luteum, downregulation of receptors for luteotropic hormones, and inhibition of P_4 synthesis (Niswender et al., 2000). Although the proximate mechanisms of luteolysis of reptilian corpora lutea have not been positively identified, there is good evidence that PGs mediate this process. A single injection of $PGF_{2\alpha}$ induces luteolysis (evidenced by loss of ultrastructural features associated with steroid synthesis and a drop in plasma P_4) in *A. carolinensis* (Guillette et al., 1984) and *Chelydra serpentina* (Mahmoud et al., 1988), and reduces P_4 (but not androgen or E_2) secretion in vitro by corpora lutea of the lizard *P. s. sicula* (Gobbetti et al., 1993a). Whether the initiation of luteolysis is also associated with the withdrawal of a pituitary luteotropin remains to be tested in reptiles.

6 FUTURE DIRECTIONS: APPLICATION OF REPRODUCTIVE ENDOCRINOLOGY TO CONSERVATION

This review has highlighted a number of areas in which our knowledge of hormones and the reptilian ovary is still scanty compared with our understanding of mammalian systems, although it must be acknowledged that even for mammals we have extensive data for relatively few species. In particular, research into reptilian endocrine systems has been hampered by a lack of homologous preparations of peptide hormones, both for the development of specific assays and for experimental applications.

However, there is one area in which the ability to apply our fundamental knowledge of reptilian reproduction would be of great benefit. Species are at risk due to habitat loss, climate change, environmental pollution, introduced species, diseases, or unsustainable use and overcollecting. Knowledge of reptilian reproductive endocrinology may become vital to species conservation, especially if ex-situ captive breeding programs become necessary, but, to date, such avenues have not been widely exploited.

For example, plasma hormone concentrations can be used as indicators of reproductive condition in species of conservation concern when use of noninvasive techniques such as ultrasound is impractical. Gravid female tuatara can be identified by the criteria of plasma testosterone and P_4 concentrations of $\geq$ 2.0 ng/mL and $\geq$5.8 ng/mL plasma, respectively (Cree et al., 1991). Similarly, measurements of plasma sex steroids can be used to monitor reproductive cycles in Gálapagos tortoises in captivity, and to investigate the proximate cues that regulate seasonality in that species (Rostal et al., 1998). Noninvasive monitoring of sex steroids in feces or urine is widely utilized in mammalian conservation and captive breeding programs. Such techniques may be more problematic in reptiles, and need careful validation (Atkins et al., 2002) but fecal steroid monitoring has been applied in a limited number of reptilian species in conjunction with techniques such as ovarian ultrasonography (e.g., Bertocchi et al., 2018).

Knowledge of ovarian endocrinology may also be applied to stimulating reproduction by captive reptiles. One group has experimented with GnRH as a means of inducing female receptivity and ovulation in iguanas, with some limited success (Phillips et al., 1987). Exogenous FSH stimulates ovarian growth and receptivity in captive painted dragons, but the presence of males is required for fertilization (Uller & Olsson, 2005), demonstrating that an understanding of how environmental conditions influence ovarian physiology is essential if captive breeding is to succeed. In particular, the stress of captivity may severely disrupt the reptilian reproductive cycle via inhibitory effects on plasma concentrations of ovarian steroids (Guillette et al., 1995). Captive anoline lizards, for example, frequently exhibit cessation of follicular growth and the appearance of atretic follicles, apparently as a consequence of decreased hepatic expression of vitellogenin. In captive *Anolis pulchellus*, vitellogenin (Vtg) synthesis drops, and a marked downregulation of Vtg mRNA levels is observed, but these effects can be alleviated or reversed by treatment with E_2 (Morales & Sanchez, 1996). In contrast, captivity in outdoor, seminatural enclosures had little effect on plasma concentrations of sex steroids in the lizard *Sceloporus virgatus* during the reproductive period (Weiss et al., 2002), suggesting that such conditions would be appropriate for long-term breeding programs.

The diversity of reproductive strategies among reptiles means it is not always possible to make generalizations about all aspects of their reproductive endocrinology. However, as the need to preserve global biodiversity becomes more critical, the development and application of assisted reproductive techniques (ARTs) based on such understandings will become increasingly important tools in the conservation of reptilian species (Perry et al., 2021). A deeper understanding of the fundamental endocrinology of the HPG axis and how that axis is influenced by environmental cues may underpin the successful management of endangered species, both in situ and ex situ.

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Chapter 5

Hormones and the sex ducts and sex accessory structures of reptiles

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ABBREVIATIONS

AVT	arginine vasotocin
E_2	estradiol
ER	estrogen receptor
LESP	lizard epididymal secretory proteins
P_4	progesterone
PG	prostaglandin
PR	progesterone receptors
RER	rough endoplasmic reticulum
T	testosterone

1 INTRODUCTION

As a class, the Reptilia made their appearance in the Paleozoic, flourished during the Mesozoic, and became mostly extinct by the Cenozoic. There remain today only four major reptilian groups, the Testudines, Crocodilia, the Sphenodontia, and the Squamata. Most of the living reptiles are lizards and snakes, members of the Squamata. From a reproductive perspective, the Reptilia possess three features, none of which is unique to them but which together serve to distinguish the group. First, all living and most of the extinct reptiles possess an amniotic egg in which the embryo develops in a fluid-filled sac, the amnion. This is thought by some to have enabled reptiles to reproduce successfully in terrestrial, even arid environments. The second is internal fertilization, in which sperm are introduced directly into the female reproductive tract instead of being shed to the exterior. Internal fertilization has several implications. One is that it requires a copulatory organ to introduce sperm to the female. Another is that the male and female reproductive cycles must be synchronized so that sperm are present in the female tract when eggs are ripe. Alternatively, in cases in which the male and female cycles are dissociated (Crews, 1984), provisions must be made to preserve sperm, either in the male or female, until the eggs are ripe for fertilization. The third feature is that, once fertilized, eggs remain within the female reproductive tract for short (oviparous) or long (viviparous) periods of time prior to oviposition or parturition. This review concentrates on the sperm ducts of the male and the oviduct of the female, and their anatomy and endocrinology. The issue of viviparity is considered in Chapter 9, this volume; this review will focus on oviparous reptiles. None of these features described above are unique to reptiles, but the reptiles are the first vertebrate group to employ all of these features.

2 OVIPARITY

The oviparous mode of reproduction is characteristic of some fish and amphibians, most reptiles, and all birds. In this mode, ovulated eggs receive investments, including a shell in some species, from the oviduct wall, and the eggs are shed (oviposited) from the female prior to the completion of embryonic development. This mode has several features, one of which is the size of the egg. Since eggs are oviposited with the embryo at a relatively early stage of development, all materials essential for development must be incorporated into the egg prior to oviposition. Reptilian eggs are megalecithal, having an extremely large quantity of yolk, which occupies the largest part of the ovulated egg. Precursors to yolk are synthesized by the liver prior to ovulation (vitellogenesis) and incorporated into the developing follicle, a process controlled by ovarian hormones. Vitellogenesis is considered in Chapter 4, this volume.

Our knowledge of vertebrate reproduction as a whole is scanty. What has emerged, however, is that reproduction is quite species-specific, and many examples of mechanisms existing in some species not being applicable to others can be found in the literature (Van Tienhoven, 1983). In reptiles, as in most vertebrate taxa, our knowledge of reproductive mechanisms is based on a few intensely studied species. Despite the acknowledged pitfalls of such an approach, what is known for these few species is typically extended to the group as a whole.

Hormones and Reproduction of Vertebrates, Volume 3. https://doi.org/10.1016/B978-0-443-16022-6.00005-6
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3 REPRODUCTIVE CYCLES AND SEX HORMONES

Reproduction in most reptiles is cyclic (Callard & Kleis, 1987) and is associated with favorable times of the year, with temperature, photoperiod, and moisture serving as zeitgebers. As is the case with other vertebrates, environmental influences act via the hypothalamus to coordinate the release of gonadotropins from the pituitary gland to regulate the cyclicity. The gonadotropins follicle-stimulating hormone (FSH) and possibly luteinizing hormone (LH) in turn act on the gonads to regulate the secretion of gonadal sex hormones as well as the growth, maturation, and release of the gametes. It is the gonadal sex hormones that regulate either wholly or in part the function of the sex ducts, the oviduct of the female and the sperm duct of the male, and the secondary sex organs.

Two different types of male reproductive cycle have been described for reptiles. The first, the most common, is the associated (Crews, 1984) or prenuptial (Licht, 1984) cycle (see also Chapter 13, this volume). In this type, spermatogenesis and male sex hormone levels are temporally synchronized to ovulation in the female. Crocodilians, the tuatara, and most lizards and snakes possess this type of cycle. In the dissociated, or postnuptial, cycle, male sex hormone secretion and spermatogenesis are not linked to ovulation in the female, and the two events are separated in time. Some turtles and snakes possess this type of cycle. Species with dissociated cycles typically have a prolonged period of sperm storage, either in the epididymis of the male or in the oviduct of the female.

In both sexes, the primary sex ducts as well as the accessory sex organs are secondary sex organs, whose size and functional integrity are dependent on the secretion of sex steroids (androgens, estrogens). Over the past several decades, the identities of the gonadal sex steroids have been determined for a large number of vertebrates (see Kime, 1987). In general, testosterone (T), androstenedione, and dihydrotestosterone (DHT) predominate among the various androgen secretions of the male, and estradiol (E_2) and estriol predominate among the estrogenic secretions of females. All reptiles, including males and females, secrete progestins as well, with progesterone (P_4) being the one most commonly reported.

4 THE MALE

The embryology of the reptilian male reproductive system was reviewed by Fox (1977). Only recently has the detailed anatomy of the connections between the testis, efferent ductules, and ductus epididymis been clarified, and from these there appear to be only minor differences among the representatives of the various reptilian orders. Male reptiles do not possess accessory glands, as is common for mammals. The only exception is the sexual segment of the kidney, found in squamate reptiles. The gross anatomy of the reptilian male reproductive system (Fig. 1) resembles that of other amniote vertebrates.

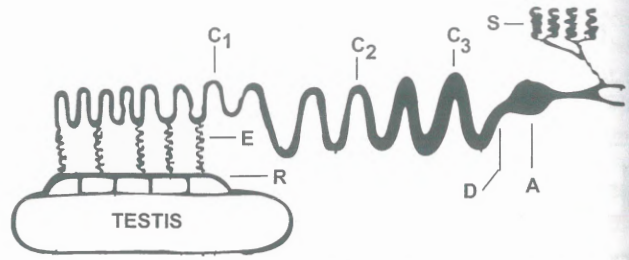


FIG. 1 Generalized diagram of the reptilian male reproductive system. A, ampulla; C_1, caput epididymis; C_2, corpus epididymis; C_3, cauda epididymis; D, ductus deferens; E, efferent ductules; R, rete testis; S, renal sex segment. Hemipenis not shown. *(Modified from Jones, 1998. Reproduced by permission, Society for Reproduction and Fertility.)*

4.1 Efferent ductules

The seminiferous tubules of the testis empty into a number of tubules emerging from each testis. The number of these tubules draining a testis is variable, estimated to be 6–8 in the lizard *Sitana ponticeriana* (Akbarsha et al., 2007), 20–30 in the turtle *Chrysemys picta* (Holmes & Gist, 2004), and 33 in the snake *Tropidonotus natrix* (Volsoe, 1944). They are lined by a simple nonciliated squamous to cuboidal epithelium and are surrounded by connective tissue. These tubules resemble the rete testis tubules of the mammal histologically (Robaire & Hermo, 1988), but in older reptilian literature (see Volsoe, 1944) were termed 'ductuli efferentes.' Despite the fact that only in the turtle do these tubules form a true rete (exterior to the testis), more recent authors have designated these tubules draining the testis as rete testis tubules (Holmes & Gist, 2004). In most reptiles, the rete testis tubules communicate with the efferent ductules and convey the seminal products to the epididymis (ductus epididymis). The junction between rete testis tubules and efferent ductules is usually abrupt, with the unciliated cuboidal epithelium of the rete testis changing to a columnar form with numerous cilia.

Efferent ductules are highly convoluted and run through the loose connective tissue before discharging their contents into the epididymis (ductus epididymis) proper. The junction with the epididymis usually occurs at the proximal portion of the epididymis, but can occur at any level; as in the mammal, blind endings are common. Older authors have identified the efferent ductules as ductuli epididymis. Subsequent histological examinations of these ducts reveal that both efferent ductules and ductuli epididymis have the same cell types. It seems prudent to adopt the more modern nomenclature if only to avoid confusion among comparable

structures in the vertebrate male duct (Akbarsha et al., 2007; Guerrero et al., 2004; Holmes & Gist, 2004). Variations do exist in the efferent ductules from different reptilian taxa, but the basic anatomy of these tubules is the same for all reptiles studied.

As they leave the rete testis, efferent ductules range in diameter from 18 to 60 μm and are surrounded by a thin layer of smooth muscle. They possess a simple cuboidal to columnar or pseudostratified epithelium consisting of ciliated and nonciliated cells. The nonciliated cells possess microvilli and outnumber the ciliated cells by 3–4 : 1 (Fig. 2). Cilia in the efferent ductules are unusually long (estimated to be 40 μm in the turtle). Other variations may exist along the length of the efferent ductules. For example, in the lizard *S. ponticeriana* and in *Sphenodon punctatus*, the tubules become less convoluted distally (Akbarsha et al., 2007; Gabe & Saint Girons, 1964). In the caiman (*Caiman crocodilus*) and turtle (*C. picta*), the epithelial cells, particularly the nonciliated cells, possess prominent secretory granules ranging in diameter from 0.5–5 μm (Guerrero et al., 2004; Holmes & Gist, 2004). In some species, different zones within the efferent ductules are recognized based on the distribution of granulated cells (Guerrero et al., 2004; Holmes & Gist, 2004; Volsoe, 1944). In addition, both large and small clathrin-lined vesicles have been observed in the efferent ductules of all reptiles examined.

In the mammalian testis, the efferent ductules are similar in appearance to those of reptiles and are specialized for secretion and absorption of both liquid and particulate matter (Hess, 2003; Robaire & Hermo, 1988). It is probable that the reptilian efferent ductules have similar functions. In addition, spermiophagy within the efferent ductules is reported in *S. ponticeriana* (Akbarsha et al., 2007) and β-N-acetylglucosaminidase activity, associated with sperm remodeling, is present in the tortoise *Testudo hermanni* (Kuchling et al., 1981).

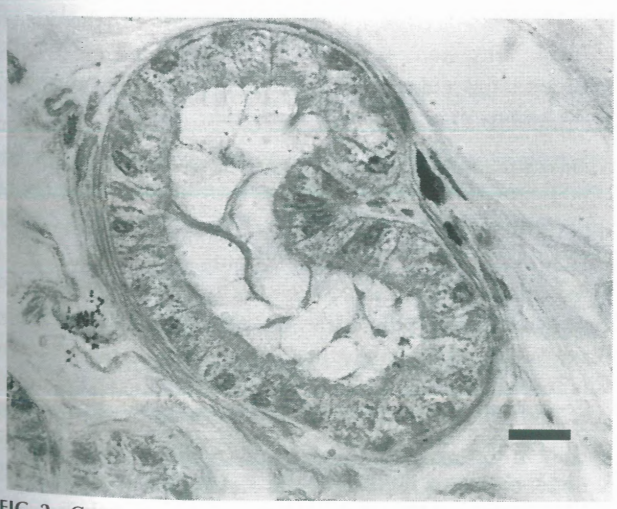


FIG. 2 Cross section of efferent ductule from the turtle *Chrysemys picta*. Bar = 50 μm.

4.2 Ductus epididymis

The efferent ductules discharge into a single duct, the ductus epididymis. The reptilian epididymis is similar in many respects to its mammalian counterpart. It lies laterally to the testis and consists of a single highly coiled tube embedded in loose connective tissue. It maintains connections with the testis via the connective tissue and the efferent ductules. Subdivisions resembling the mammalian initial segment, caput, corpus, and cauda epididymis are recognized in a number of reptiles (Akbarsha et al., 2006a; Akbarsha & Manimekalai, 1999; Averal et al., 1992; Haider & Rai, 1987; Robaire & Hermo, 1988) but not in others (Guerrero et al., 2004; Holmes & Gist, 2004). For the purposes of this review, the proximal portion of the ductus epididymis will be subdivided into an initial segment and, caudally, the caput epididymis; a middle corpus epididymis; and the distal region, the cauda epididymis (Fig. 3). Where these recognizable subdivisions exist, as in the lizard *S. ponticeriana* (Akbarsha et al., 2006b), the initial segment is a large, thin-walled chamber that receives many of the efferent ductules. The caput, corpus, and cauda epididymis generally consist of an epithelium that is highest in the caput and decreases gradually to be lowest in the

FIG. 3 Epididymis of the lizard *Sitana ponticeriana*. ED, efferent ductules; IS, initial segment of epididymis; CA, caput epididymis; CO, corpus epididymis. Cauda epididymis not shown. Bar = 0.17 mm. *(Reproduced with permission from Akbarsha et al., 2006a.)*

cauda. In addition, the diameter of the ductus epididymis tubule increases in size from caput to cauda.

The epididymis is the primary sperm storage organ for the male. Following spermatogenesis, the epididymis is engorged with sperm and can exceed the testis in size. During breeding, sperm not transferred to females become concentrated in the cauda epididymis. During the time of testicular rest, the epididymis of some species may be devoid of sperm, but in others some sperm remain in the epididymis throughout the year. The reptilian epididymis is lined by a pseudostratified epithelium and is surrounded by a layer of 5–7 smooth muscle cells. The organ is well-vascularized. The epithelium lining the epididymis varies greatly among species both with respect to the distribution of ciliated vs. nonciliated cells and with the presence and size of cytoplasmic secretory granules (Dufaure & Saint Girons, 1984).

The most abundant cell type in the reptilian epididymis, accounting for >80% of the epithelium, is variously identified as a principal (Akbarsha et al., 2006a), vesicular (Holmes & Gist, 2004), columnar (Guerrero et al., 2004), or secretory cell (Desantis et al., 2002). The shape of principal cells changes from columnar in the caput epididymis to cuboidal distally; they possess microvilli and appear to have tight junctions. Some may be ciliated. In all species examined, the apical cytoplasm of principal cells contain many small (<1 μm) vesicles, some with coated membranes. More basally, multivesicular bodies and large (>1 μm) vesicles are numerous. These properties are suggestive of an endocytotic function. In *C. picta*, spermiophagy by principal cells (Holmes & Gist, 2004) has been reported. In addition, principal cells of most species contain varying quantities of rough endoplasmic reticulum (RER) and apically located secretory vesicles; some have secretory blebs.

Most of the variation in epididymal histology that exists among reptiles lies in the presence and nature of secretory granules. Dufaure and Saint Girons (1984) examined the epididymides of 89 reproductively active squamates (lizards and snakes) and classified the secretory granules in the cytoplasm into five categories ranging from no granulation to cells containing several large (10–12 μm) secretory granules. Subsequent studies have documented extensive variation in other groups; for example, no secretory granules have been detected in the caiman (*C. crocodilus*) (Guerrero et al., 2004) or turtle (*C. picta)* epididymis (Holmes & Gist, 2004). Granules 4–8 μm in diameter are found in the lizard (*Lacerta vivipara*), and smaller granules are reported in the initial segment and caput regions of another turtle, *Lissemys punctata punctata* (Akbarsha & Manimekalai, 1999).

The second most abundant cell in the reptilian epididymal epithelium is a basal cell, so identified on the basis of its location. As in mammals, this cell is believed to replenish the principal cells. Three other cell types, similar to their mammalian counterparts and collectively accounting for about 10–20% of the epithelium, have been identified in the reptilian epididymis. The first is a narrow cell possessing an elongated nucleus and dense cytoplasm. It is found in the cauda epididymis of *C. picta* (Holmes & Gist, 2004) and in the initial segment of *S. ponticeriana* (Akbarsha et al., 2006b). The second is a cell containing numerous mitochondria and relatively few vesicles. In *S. ponticeriana* it is expanded apically and is restricted to the initial segment, whereas in *C. picta* it is columnar and found in the cauda epididymis. A third cell type, a clear cell, is found in the cauda epididymis of *S. ponticeriana*; it contains endocytotic vesicles, multivesicular bodies, and lysosomes (Akbarsha & Manimekalai, 1999). The functions of these latter three cell types are unknown.

4.3 Ductus deferens

Caudally, the ductus epididymis continues as the ductus deferens. It terminates in the copulatory organ, where a penile groove (sulcus spermaticus) receives the sperm and transfers them to the female. The ductus deferens is relatively undifferentiated in most reptiles, consisting of a simple epithelium, a thin lamina propria, and smooth muscle of varying thickness. With the exception of the renal sex segment of squamates, there are no accessory glands along the ductus deferens contributing to the seminal fluid. As a result, reptilian semen tends to be highly viscous, with few fluid components. There have been few detailed studies on the epithelial cells lining the ductus deferens, but most consider the pseudostratified epithelium to consist of cells similar to the principal and basal cells of the epididymis (Fox, 1977; Gist, unpublished observations; Sever, 2004; Volsoe, 1944). However, in a detailed examination of the lizard *Mabuya carinata*, Aranha and coworkers describe five different cell types in the epithelium lining the ductus deferens, with narrow cells, apical cells, and clear cells (intraepithelial leukocytes) in addition to principal and basal cells (Arahna et al., 2004; Aranha et al., 2006).

In a few squamate reptiles, the terminal portion of the ductus deferens is swollen and structurally differentiated to form an ampulla that is used for sperm storage. In the rattlesnake (*Crotalus durissus*), the entire ductus deferens functions in sperm storage (Almeida-Santos et al., 2004). The morphology of the ampulla varies among species. In the black swamp snake *Seminatrix pygaea*, the epithelium of the ampulla remains undifferentiated as in the ductus deferens, but is highly folded and truncated and sperm are stored in the lumen throughout the year (Sever, 2004). In the lizard *Calotes versicolor*, the ampulla is differentiated into storage (ductal) and secretory portions, the latter containing epithelial secretory cells that vary with reproductive condition

(Akbarsha & Meeran, 1995). Spermiophagy has been observed. Both transport and at least three types of secretory function are suggested by the ultrastructure of the epithelial cells in the secretory portion of the ampulla of the lizard *S. ponticeriana* (Akbarsha et al., 2005). In the ampulla of lizards, sperm are absent except during reproductive activity.

4.4 Reproductive cycles and hormonal dependence

In most reptilian species, the excurrent canal system varies greatly during the reproductive cycle. The variations are associated with specific phases of the reproductive cycle and are orchestrated by the secretion of androgenic hormones. Androgen dependence of the male reproductive tract has been demonstrated in a number of ways. In reptiles possessing an associated reproductive cycle, hypertrophy of the epididymis is associated with gonadal recrudescence, with peak size in most species being found at the time of highest T levels (e.g., Dufaure et al., 1986). Castration of reproductively active lizards results in an atrophy of the hypertrophied epididymis as the result of a diminished diameter of the ductus epididymis and a regression of its epithelium (Morel et al., 1986), while the same operation in non-reproductive lizards has little or no effect. Administration of FSH to sexually quiescent lizards results in hypertrophy of the efferent ductules and the ductus epididymis, resulting from increased epithelial height and, in the latter, tubule size (Haider & Rai, 1987). Follicle-stimulating hormone- or T-induced increases in epididymal size are blocked by the anti-androgens flutamide or cyproterone acetate (Rai & Haider, 1991, 1995). The hemipenes of lizards became enlarged in response to T (Ananthalakshmi et al., 1991). In sum, these data indicate that the male reproductive tract is sensitive to the male sex hormones and undergoes seasonal changes specifically in response to them.

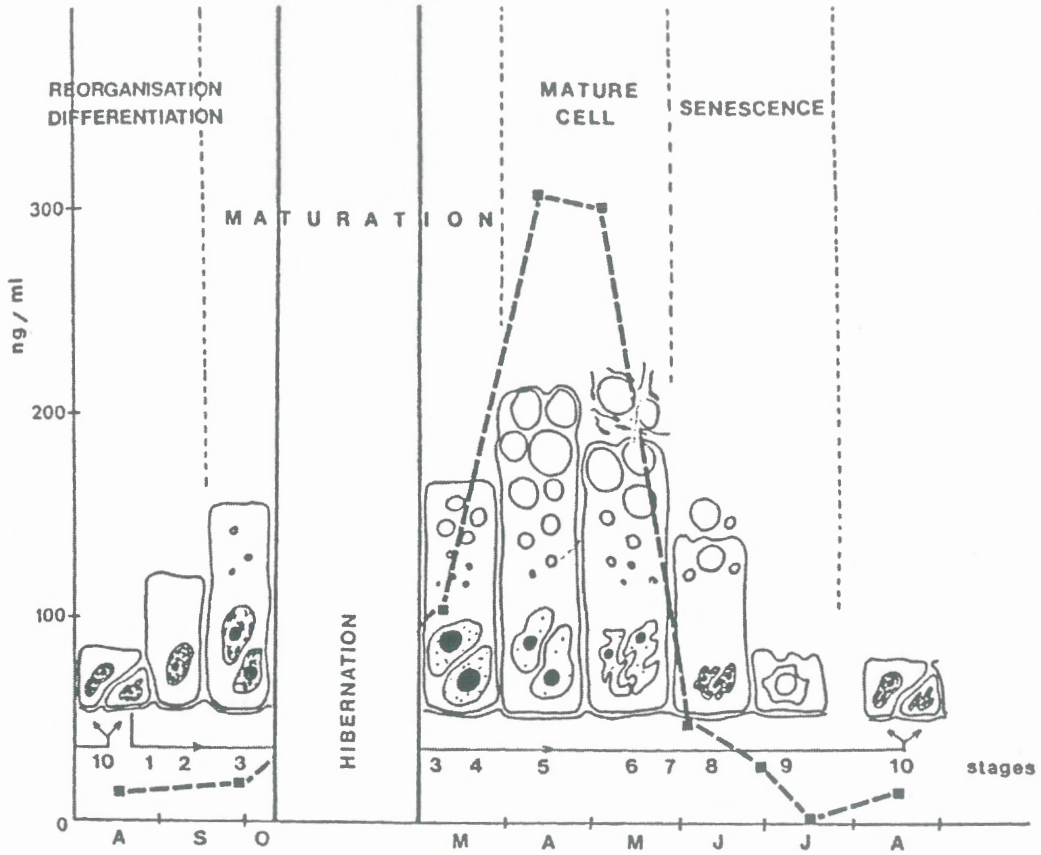
In reptiles possessing dissociated reproductive cycles, the hormonal dependence is not as strong. For example, in the turtles *Sternotherus odoratus* (McPherson et al., 1982; McPherson & Marion, 1981), *C. picta* (Callard et al., 1976; Gist et al., 2001), and *Chelydra serpentina* (Mahmoud & Cyrus, 1992; Mahmoud & Licht, 1997), plasma androgen levels do not closely follow testicular or epididymal sizes. The same is true for the garter snake, *Thamnophis sirtalis* (Krohmer et al., 1987). However, T administration to immature soft-shelled turtles (*L. p. punctata*) will stimulate development of the efferent ductules and ductus epididymis (De & Maiti, 1985). The asynchrony between hormone levels and epididymal stimulation may relate to the ability of the epididymis to store sperm from one reproductive season to the next.

4.5 Epididymis

The granulations in the principal cells of the lizard epididymis have been the subject of intense study. Through the efforts of Dufaure and his associates, the epididymis of the viviparous lizard (*L. vivipara*) has emerged as a classic example of steroid effects on secretory cells. The secretory granules of the principal cells in this species at the height of the breeding season are large (5–7 μm) and occupy the apical cytoplasm of the principal cells (Mesure et al., 1991). The granules first make their appearance as the testes are undergoing recrudescence; castration at that time prevents their appearance and replacement therapy with testosterone or other androgens results in their reappearance (Dufaure et al., 1986). They are found in all regions of the epididymis except for the cauda epididymis. The granules themselves are membrane-bound and consist of a central, insoluble protein (protein H) surrounded by a soluble protein (protein L) (Gigon-Depeiges & Dufaure, 1977). While little is known of protein H, the soluble form, protein L, has been shown to be a mixture of preproteins, all of approximately the same size (19 000 daltons) and immunological properties, but differing in their pI (Depeiges et al., 1988). Secreted L proteins, identified as lizard epididymal secretory proteins (LESP), include both glycosylated and phosphorylated forms (Morel et al., 1993; Ravet et al., 1991). All are known to be members of the lipocalin family of proteins, a family of low-molecular-weight proteins whose function broadly is to transport small lipophilic molecules. Included among the lipocalin proteins are retinol-binding protein and rat epididymal secretory protein I, an androgen-induced protein that has amino acid and mRNA similarities to LESP (Morel et al., 1993, 2000).

In *L. vivipara* at the time of maximal sexual activity, the contents of the secretory granules (containing both insoluble H and soluble LESPs) are discharged into the lumen of the epididymis, where they mix with sperm and bind to sperm heads (Depeiges & Dufaure, 1983). This androgen-induced secretion of proteins that bind to (and potentially influence) sperm closely resembles what occurs in mammals during epididymal sperm maturation (Robaire & Hermo, 1988). Whether this binding confers additional properties to the sperm remains undetermined, although sperm have been reported to increase their motility as they traverse from the caput to the corpus epididymis (Depeiges & Dacheux, 1985).

The details of the androgen stimulation of epididymal secretory granule formation and the synthesis of its proteins are known. The annual cycle of the epididymis of *L. vivipara* has been divided into 10 stages (Fig. 4) on the basis of histological and cytological characteristics of principal cells, and related to tissue and circulating testosterone



REORGANIZATION - DIFFERENTIATION

[^{3}H]thymidine incorporation in:

— basal cells → mitosis → basal cell / new epithelial cell generation

— epithelial cells → mitosis → increase in number of epithelial cells.

MATURATION

Important [^{3}H]thymidine incorporation.
Polynucleisation (2 or 3 nuclei/cell).
Hyperploidisation (→ 8c).
→ *DNA synthesis*

Increase of nucleolar volume and granular component.
→ *rRNA synthesis*

Concomitant increase of nuclear volume and chromatin decondensation.
→ *transcription activity*

MATURE CELL

No [^{3}H]thymidine incorporation.
→ *no DNA synthesis*

Decrease of nucleolar volume and granular component.
→ *diminution of rRNA synthesis*

Very lobate and voluminous nucleus, decondensed chromatin.
Polynucleation.
Polyploidy.
→ *great transcription activity*

SENESCENCE

No [^{3}H]thymidine incorporation.
Decrease in nucleus and nucleolus volume, in ploidy, and in number of nuclei/cell.
Chromatin condensation.
→ *no more nuclear and nucleolar activity*

Necrotic phenomena.
→ *cell death*

FIG. 4 Stages 1–10 of the annual cycle of principal cells from the epididymis of the lizard *Lacerta vivipara*. Changes in secretory granule, nuclear, and nucleolar morphology and activity are related to the level of plasma testosterone (dotted line). The time of year is given on the X axis from August (A) to July (J). *(Reprinted with permission from Faure et al., 1987.)*

levels (Dufaure et al., 1986; Faure et al., 1987). During the period of sexual inactivity characteristic of mid-summer (stage 1), androgen levels are low but the principal cells begin to divide and hypertrophy. Later on, in the fall, (stage 3) but prior to hibernation, androgen levels remain low but secretory granules make their appearance in the cytoplasm. What stimulates these initial cytological changes remains unknown. Following hibernation (March; stage 4), androgen levels are increased and at the same time increased nucleolar volume, ^{3}H-thymidine incorporation, DNA

content per cell, and number of nuclei are noted in the principal cells along with the formation of mature secretory granules. Maximal stimulation (stage 5) occurs in May, followed by release of the secretory granules; subsequently, androgen levels decline, the nuclei cease DNA synthesis, nuclei and nucleoli both become reduced in size, and the cells become necrotic (July; stage 9).

In the cytoplasm of principal cells, protein synthesis, evidenced by amino acid incorporation into secretory granule protein (Gigon-Depeiges & Dufaure, 1977; Ravet et al., 1987), is underway by stage 3, along with increased amounts of rough endoplasmic reticulum, Golgi formation, and, later, the condensation of vesicles. This is accompanied by accelerated mRNA synthesis, including mRNA specific for LESP (Courty, 1991; Courty et al., 1987). Castration or the administration of the anti-androgen cyproterone acetate obliterates, and testosterone restores, these changes (Morel et al., 1986). The mature granules, consisting of the insoluble protein H at the core and protein L at the periphery, are released from the apical surface of the principal cell at stage 6 (May) into the lumen of the epididymis. Here they bind to sperm heads (Depeiges & Dufaure, 1983; Dufaure et al., 1986).

A different mode of secretory granule formation is reported by Akbarsha et al. (2006a) for the fan-throated lizard, *S. ponticeriana*. Here, the soluble and insoluble proteins are synthesized in separate vesicles that coalesce within the principal cell to form the mature, secretory vesicle. The mature, secreted product in this species is the soluble form of the protein which, in turn, is replaced in vesicles by degradation of the insoluble protein.

Since the epididymis is an androgen-dependent organ that in mammals and certain lizards is involved in sperm maturation, any changes in sperm occurring during epididymal transport are presumed to be hormone-dependent. Whether this is true awaits experimental verification. The presence of hormone receptors has been reported for a number of species. Androgen receptor (AR) levels of 85 fmol/mg protein were reported in the epididymis of *L. vivipara* (Courty, 1991) at stage 6, the peak of both sexual activity and plasma T levels, but similar studies in other squamates are lacking. Estrogen receptor (ER) levels of 20 fmol/mg protein are present in epididymal cytosol from the turtle *C. picta* during the autumn (Dufaure et al., 1983) when sperm are undergoing maturation, and both ERα and ERβ are reported to be present in epithelial nuclei from the same species (Gist et al., 2007) following spermatogenesis. The function of estrogens in the reptilian epididymis remains unknown but, in mammals, estrogens, through ERα, promote fluid uptake from the efferent ductules and epididymis (Hess, 2003).

Sperm acquire motility as they move through the epididymis of *L. vivipara* (Depeiges & Dacheux, 1985) or *Hemidactylus flaviviridis* (Nirmal & Rai, 1997), but actual motility is highly variable. Motility values are reported to be in the 70% range (percent of total sperm) in the corpus epididymis of the lizards *H. flaviviridis* and *L. vivipara*, approximately 40% in whole epididymides of the turtles *T. scripta* (Garstka & Gross, 1990) and *S. odoratus* (Gist et al., 2000), and in the 1–10% range in sperm harvested from the epididymides of *C. picta* or *T. scripta* (Gist et al., 2000). Phosphodiesterase inhibitors such as caffeine or 3-isobutyl-1-methylxanthine increase motility whenever examined (Depeiges & Dacheux, 1985; Gist et al., 2000), suggesting a cAMP-dependent mechanism for sperm motility. Whether hormones have any direct effect on sperm motility or its acquisition is not known. Other hormone receptors present in the reptilian epididymis include natriuretic peptide and endothelin receptors in the turtle *Amyda japonica* (Kim et al., 2000). This may be relevant since sodium is reported to stimulate sperm motility in the lizard *H. flaviviridis* (Rai & Nirmal, 2003).

In summary, there is abundant evidence that the male sex hormones functionally control epididymal function, at least in lizards. They stimulate secretion of epididymal proteins by principal cells in those species containing prominent secretory granules and may do so in species containing less prominent secretory granules. The function of epididymal secretions, however, remains unknown. Although sperm acquire some motility as they move through the ductus epididymis, there is little evidence of a hormonal effect on the acquisition or maintenance of sperm motility, a property that in mammals is associated with fertility.

The other function of the epididymis is sperm storage. Following spermatogenesis and release from the testis, sperm are stored in the epididymis, vas deferens, or ampulla until mating. This interval can be short—as in reptiles possessing an associated reproductive cycle—or long (overwinter or longer)—as in reptiles with a dissociated cycle (Crews, 1984). In the garter snake *T. sirtalis*, spermatogenesis is completed in late summer (Krohmer et al., 1987). Although mating can occur in the autumn, most individuals mate upon emergence the following spring. Thus, sperm remain in the vas deferens of the male over the winter. In the freshwater turtles *C. picta* and *T. scripta*, spermatogenesis is completed in the fall; however, matings can occur at any time throughout the year. In these species, sperm may be found in the epididymis throughout the year (Gist et al., 2001). What maintains the viability/fertility of sperm held in the epididymis is not known. One factor may lie in the gametes themselves. Whereas vertebrate spermatozoa are typically short-lived (Harper, 1982), sperm harvested from turtle (*C. picta*, *T. scripta)* epididymides at various times of the year maintained equivalent motility parameters and sensitivity to stimuli for up to 30 days following collection (Gist et al., 2000). In the turtle *T. scripta*, epithelial cells

of the efferent ductules and ductus epididymis possess estrogen receptors (ERα, ERβ) (Gist et al., 2007). Estrogens, acting via ERα on the efferent ductules, are essential for fertility in mammals (Hess, 2003).

4.6 Renal sex segment

The renal sex segment is a structure found only in squamate reptiles. It consists of the terminal portions of the renal tubule and sometimes extends into the collecting ducts of the kidneys and ureters. Cells lining these portions of the tubule contain prominent secretory granules (Fig. 5). In lizards, comparable regions of the renal tubule in females have some granulation and can respond to androgen stimulation, but not to the same extent as males (Del Conte & Tamayo, 1973; Krohmer, 2004; Sever & Hopkins, 2005). Renal sex segment secretions are released into the ducts and ureter, where they mix with seminal products from the testes. Since the squamate male reproductive tract contains no other sex accessory glands contributing to seminal fluid, the secretion of the renal sex segment represents a major component of the semen that is transferred to the female at copulation. The renal sex segment varies in size and granulation during the reproductive cycle of the male, being greatest at the time of spermiation and less so during other phases of the cycle (see Fox, 1977). It is clearly androgen-dependent (Krohmer, 2004; Prasad & Reddy, 1972), as castration causes regression and androgen therapy results in hypertrophy.

The secretory granules of the renal sex segment cells have been studied histochemically and shown to contain carbohydrate, lipid, and protein components (Sever & Hopkins, 2005; Weil, 1984) that change in proportion during the annual cycle. Studies of changes in renal sex segment morphology and those comparing renal sex segment development to circulating androgen levels reveal that granules are synthesized under androgen stimulation at the level of the RER, are packaged, and undergo maturation in condensing vacuoles into electron-dense, mature granules (Fig. 5) that occupy progressively greater portions of the cytoplasm (Krohmer, 2004; Sever & Hopkins, 2005; Sever et al., 2002, 2008; Weil, 1984). Mature secretory granules are homogeneous in some species and are secreted from the cell in an apocrine manner. In other species, the homogeneous granule undergoes vesiculation prior to being released from the cell in a merocrine manner; many squamate reptiles possess both types of secretion. Renal sex segment secretions are released at mating, a time of declining androgen secretion. Following mating, granules of the renal sex segment cells in lizards disappear (Sanyal & Prasad, 1966; Sever & Hopkins, 2005), whereas those of snakes merely become reduced in number (Sever et al., 2002, 2008). Krohmer (2004) attributes the retention of granulation in renal sex segment cells of nonreproductive snakes to adrenal androgens.

Surprisingly, little is known regarding the function of renal sex segment secretions. It is clear that, in some snakes, renal sex segment secretions form a copulatory plug that effectively blocks the oviduct of the female from subsequent

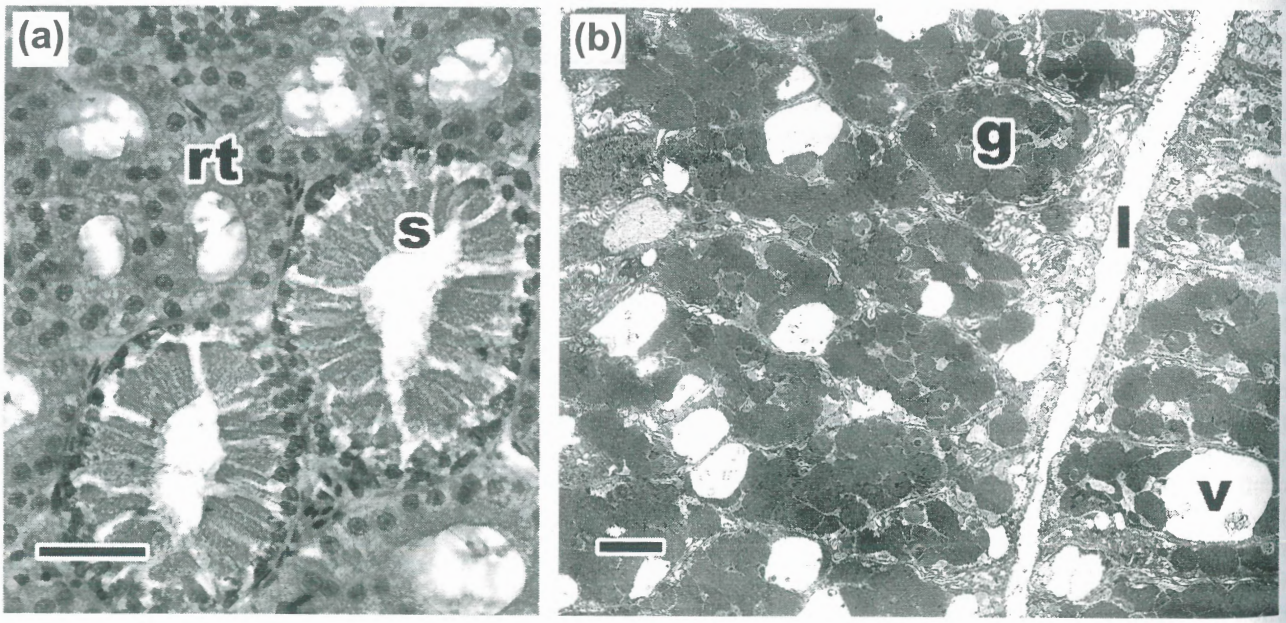


FIG. 5 Light (A) and electron (B) micrographs of the renal sex segment of the snake *Agkistrodon piscivorous*. g, sex segment secretory granule; l, lumen of sex segment tubule; rt, renal tubule; s, renal sex segment tubule; v, vacuole in renal sex segment cell. Bar = 50 μm (A) and 5 μm (B). *(Micrographs courtesy of David Sever.)*

inseminations. In the garter snake, *Thamnophis* sp., renal sex segment secretions are ejaculated as a bolus following the seminal products. These secretions harden to form a copulatory plug, preventing both subsequent matings and sperm loss (Devine, 1975). In the adder (*Vipera berus*), the renal sex segment secretions do not form a plug, but induce muscular contractions in the uterus so as to physically prevent sperm entrance (Nilson & Andrén, 1982). In addition to forming a physical barrier for inseminations, the copulatory plug serves a pheromonal function in that its presence inhibits male courtship behaviors (Devine, 1977; Nilson & Andrén, 1982; Ross & Crews, 1977). Even less is known regarding the function of renal sex segment secretions in lizards. Earlier suggestions that renal sex segment secretions maintained sperm within the oviduct are consistent with more recent observations of sperm storage within the squamate oviduct (Gist & Jones, 1987), but experimental data are absent. Cuellar et al. (1972) observed a higher motility of epididymal sperm from the lizard *Anolis carolinensis* incubated in the presence of kidney extracts, suggesting an activating effect. However, the study by Conner and Crews (1980) of sperm transfer in this same species made no mention of renal sex segment secretions.

4.7 Femoral glands

Femoral glands are epidermal structures present on the hindlimbs of certain, primarily iguanid, lizards. Found in both sexes, the femoral glands of males tend to be larger and vary to a greater extent with reproductive condition than those of females, despite the fact that femoral glands of females are sensitive to androgens (Fergusson et al., 1985). Earlier literature on femoral glands and their function were reviewed by Cole (1966). Femoral glands are exocrine, secreting in a holocrine manner a waxy mixture of lipids and protein to the exterior that may be smeared on vegetation or substrate (Alberts, Sharp, et al., 1992; Imparato et al., 2007). Femoral gland secretions have a pheromonal function. The tongue flick response (number of tongue flicks recorded in response to exposure to femoral gland secretions or extracts) has been used as a behavioral assay of femoral gland function. Exposure of lacertid lizards (*Podarcis hispanica*) to femoral gland secretions indicates that these secretions can serve to identify sex and reproductive condition since males were able to distinguish nongravid females from males or gravid females (Cooper & Pèrez-Mellado, 2002). Further, females of this species react to cholesta-5,7-diene-3-ol, a component of male femoral gland secretions, in a dose-dependent manner, suggesting a role in mate selection (Martin & Lopez, 2006). While reports suggest a complex role(s) for femoral gland secretions, it seems clear that sex recognition is among them.

Femoral glands are under androgen control. Castration causes atrophy of the femoral glands in the male lizard *Amphibolurus ornatus* and a cessation of secretion, and replacement therapy with either T or DHT reverses the degeneration (Fergusson et al., 1985). The size, secretory ability, and quantity of lipid in the secretion from femoral glands of the iguana (*Iguana iguana*) as well as the degree of social dominance are all correlated with circulating T levels (Alberts, Pratt, & Phillips, 1992).

4.8 Unresolved questions

Despite the well-documented action of T in stimulating epididymal protein synthesis in lizards, our knowledge of reptilian sperm maturation and capacitation is in its infancy. Based on the variability in epididymal anatomy and function, one may anticipate that different mechanisms of sperm maturation may exist among different reptilian groups as well as within a given group. Many reptilian species are becoming increasingly endangered, partly because of human predation, which targets larger, reproductively active individuals. To facilitate their recovery, it may be necessary to utilize assisted reproductive techniques such as sperm preservation and artificial insemination coupled with captive breeding programs. As they exist today, these techniques are based on a sound understanding of the endocrine and other influences on sperm maturation and/or fertility; therefore, their use in reptiles awaits further research in this area.

5 THE FEMALE

The reptilian oviduct receives eggs ovulated from the ovary and conveys them to the cloaca, where they are expelled to the environment. The oviducts are derived from the mesonephric ridge (Fox, 1977). In adults, they are typically paired and open rostrally to the coelom in the vicinity of the ovaries and caudally to the cloaca. In snakes, the oviducts fuse into a single uterus that in turn communicates with the cloaca. In other squamates, the left oviduct is reduced or absent.

The basic oviduct structure consists of a simple epithelium lining the lumen and an underlying lamina propria. Together, these two elements are referred to as the mucosa, and the compound glands lying within the lamina propria as mucosal glands. The lamina propria in turn is surrounded by an inner circular and outer longitudinal layer of smooth muscle. The oviduct is covered on the exterior by the serosa.

Ovulatory patterns in reptiles are diverse, ranging from monoallochronic, in which the ovaries alternate in ovulating one egg as in *A. carolinensis*, to polyautochronic, in which many eggs are ovulated from both ovaries simultaneously, as in sea turtles. Fertilization of ova is thought to occur at the level of the ovary or at the infundibulum of the oviduct, although some have suggested alternative locations within the oviduct. Following ovulation, additional materials such as egg white proteins may be added prior to shell formation.

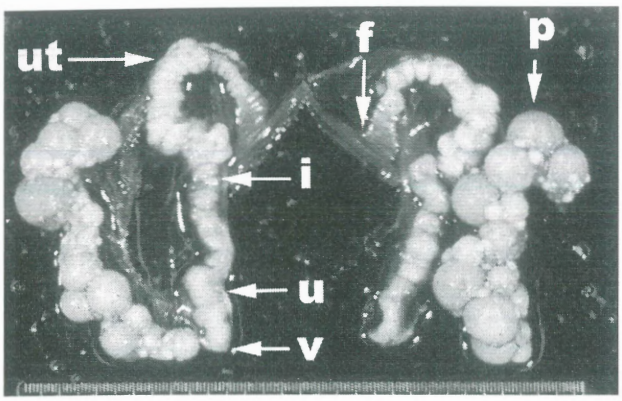


FIG. 6 Vitellogenic ovaries and oviducts from the turtle *Trachemys scripta*. f, infundibulum; i, isthmus; p, vitellogenic follicle; ut, uterine tube; u, uterus; v, vagina. Scale = 1 mm.

These additional materials, as well as eggshell membranes and mineral components, if any, are secreted by the wall of the oviduct.

Unlike the male sperm duct, in which a summary of recent information is lacking, the reptilian oviduct is the subject of two excellent reviews (Blackburn, 1998; Girling, 2002) covering recent findings and concentrating on squamates. Because of this, the present review will emphasize findings from non-squamate reptiles, the oviduct of which differs in some respects. Nomenclature of the various regions of the reptilian oviduct has led to confusion largely because of species differences (Fig. 6). Girling (2002) has addressed these problems and proposed terminology that will be used in this review.

5.1 Infundibulum

The infundibulum is found at the anterior end of the oviduct and receives the ovulated egg. The infundibulum is variable in length, ranging from 12% of the oviduct length in the snake to 20% in the turtle and 25% in the lizard (Blackburn, 1998; Gist & Jones, 1987; Perkins & Palmer, 1996). It is thin and highly convoluted, and opens via the ostium to the coelom in the vicinity of the ovary. Near the time of ovulation, the ostium moves to cover the ovarian surface and receive ovulated eggs (Alkindi et al., 2006; Cuellar, 1970). The infundibulum is devoid of glands in the lamina propria and because of this the wall of the infundibulum is thin. The simple epithelium consists of cuboidal to columnar ciliated and nonciliated cells, the proportion of which varies with species and reproductive condition. Epithelial cells at the ostium are mostly ciliated, and the proportion declines caudally (Alkindi et al., 2006; Girling et al., 1998; Motz & Callard, 1991). Nonciliated cells react with Periodic Acid Schiff reagent, can contain secretory blebs, and possess other ultrastructural features of secretory cells (Girling et al., 1998; Palmer et al., 1993); the secretion is most likely mucous, and commences at the time of ovulation. The infundibular epithelium may be thrown into folds; these folds are more numerous caudally and prior to ovulation. In some squamates, the cavities and secondary tubules in between folds can act as sperm receptacles (see Gist & Jones, 1987; Sever & Hamlett, 2002). The smooth muscle layer of the infundibulum is typically reduced.

5.2 Uterine tube

Caudal to the infundibulum is the uterine tube. This portion of the oviduct is absent in squamate reptiles, in which the infundibulum connects directly to the uterus. In crocodilians and turtles, the uterine tube contains glands that secrete egg white proteins and is homologous to the avian magnum. The uterine tube occupies approximately 40% of the length of the oviduct in both alligators and turtles (Gist & Jones, 1987; Palmer & Guillette, 1992). The simple epithelium of the uterine tube consists of ciliated and nonciliated columnar cells. The proportion of ciliated cells increases in preovulatory animals (Alkindi et al., 2006; Motz & Callard, 1991) and the nonciliated cells most likely secrete mucus. The oviduct wall in the uterine tube is thrown into a number of longitudinal folds that generally run the length of the uterine tube (Fig. 7). These are broken by shallow and deep furrows that occasionally contain ducts from tubuloalveolar glands in the lamina propria underlying the epithelium. These

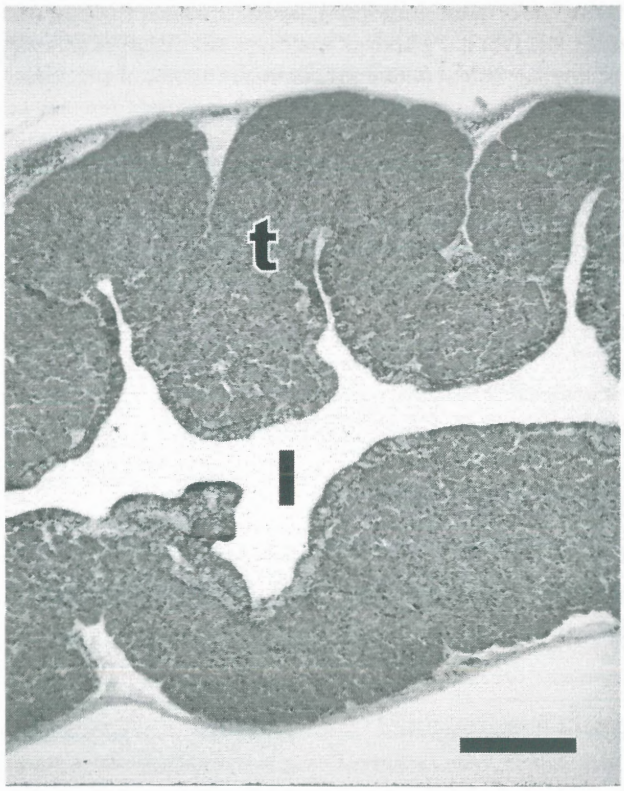


FIG. 7 Transverse section through the uterine tube of the turtle *Chrysemys picta*, showing longitudinal folds. l, oviduct lumen; t, tubuloalveolar glands in the lamina propria. Bar = 0.1 mm.

glands are extensive and can account for up to 80% of the volume of the oviduct wall in vitellogenic animals (Motz & Callard, 1991). The cells of the glands contain prominent secretory granules (Gist & Fischer, 1993; Palmer & Guillette, 1992) that resemble those in the avian magnum. The glands are believed to be the source of egg white proteins.

Mahmoud et al. (2004) identified 11 different proteins present in the epithelium and tubuloalveolar glands of the uterine tube of the turtle *C. serpentina* that were similar to those found in eggs. Most were present throughout the year. Based on their molecular weights, at least one of these proteins has been identified as an ovalbumin-like protein (Rose et al., 1990) and another as transferrin (Ciuraszkiewicz et al., 2007). Avidin is likely to be another since it is present in the 'tube' portion of the lizard oviduct (Botte et al., 1974). In turtles, the glands communicate with the oviduct lumen via ducts and through breaks in the epithelium, whereas in alligators there is a prominent duct system conveying tubuloalveolar secretions to the oviduct lumen (Fig. 8). At either end of the uterine tube, the glands become less numerous and occupy less of the lamina propria. At the caudal end, ducts connecting the tubuloalveolar glands to the oviduct lumen become both prominent and more numerous. These tubuloalveolar glands with prominent ducts function in sperm storage (Gist et al., 2008; Gist & Jones, 1989). Exterior to the glands are thin but prominent layers of smooth muscle.

As reptilian eggs are ovulated, they enter the infundibulum and begin their descent down the oviductal tube. Of the myriad of studies of reptilian reproductive events, only a few have mentioned observing eggs in the infundibulum or uterine tube (Palmer et al., 1993). Thus, the conclusion must be made that eggs traverse these regions of the oviduct rapidly, and spend most of their time in the uterine portion of the oviduct, where they receive eggshell investments. There is little doubt that the megalecithal egg descending though the uterine tube produces a distension of the wall. Such distension is thought to account for secretion of the egg white proteins from the glands (see Palmer & Guillette, 1991). However, in crocodilians, these same glands are connected to the lumen via well-formed ducts, suggesting a more complicated mechanism of secretion (Fig. 8). Questions that remain today regarding this region of the oviduct include whether species without a uterine tube secrete egg white proteins, how egg white proteins are secreted, and how egg white proteins are allocated among the many eggs comprising an egg clutch.

5.3 Isthmus

Located between the uterine tube and the uterus proper is an aglandular region known as the isthmus. Since squamate reptiles lack a uterine tube, some authors have questioned whether an isthmus exists in these taxa. This issue is addressed

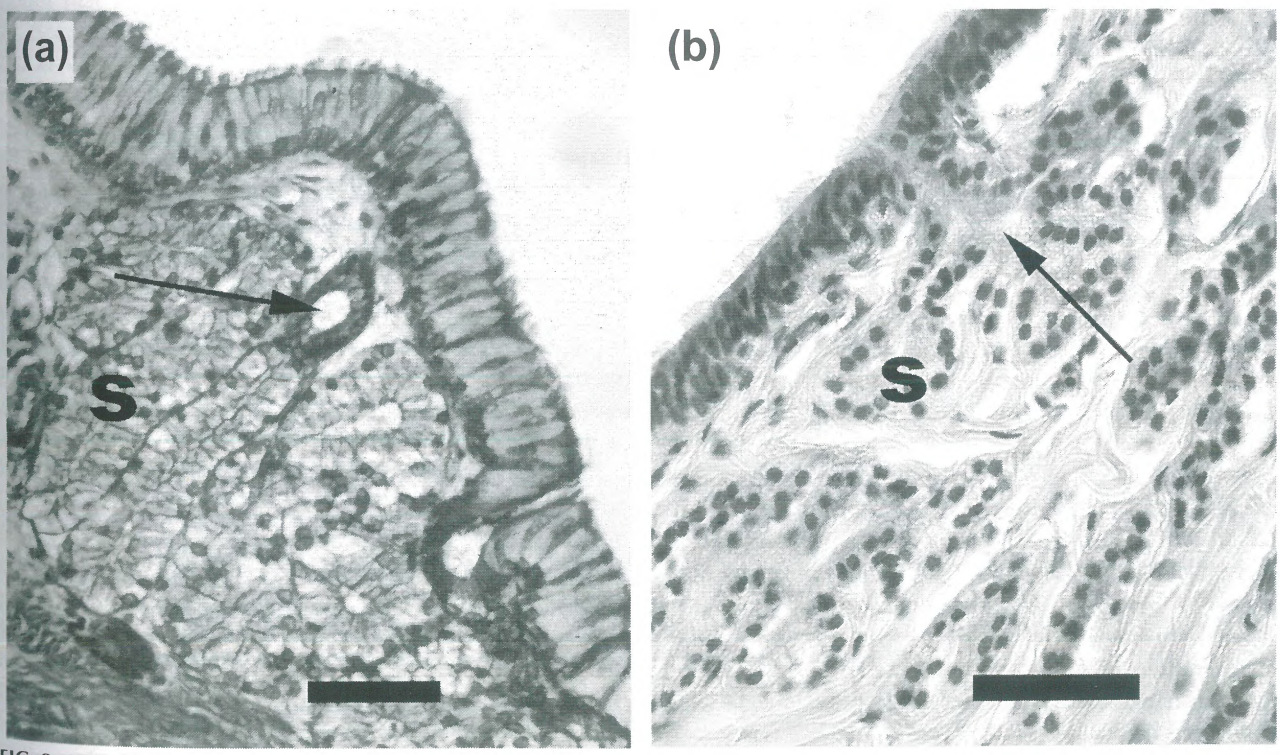


FIG. 8 Micrographs of the uterine tube of (A) the turtle *Chrysemys picta* and (B) *Alligator mississippiensis*, showing the organization of the tubuloalveolar secretory ducts. Arrows, secretory ducts; s, secretory cells. Bars = 10 μm.

by Blackburn (1998) and will not be dealt with here. The isthmus of turtles and crocodilians is typically short (less than 5% of total oviduct length). The simple epithelium is identical to that of the uterine tube, consisting of columnar ciliated and nonciliated cells. The lamina propria is devoid of glands, and the muscle layers are similar to those of the uterine tube.

5.4 Uterus

The uterus represents a major portion of the reptilian oviduct, and accounts for approximately 60% of its length. The uterine epithelium is similar to that in other regions of the oviduct, consisting of varying combinations of ciliated and secretory nonciliated columnar cells. The lamina propria is filled with glands that are variously described as tubuloalveolar to branched acinar (Girling, 2002). The mucosa of the uterus is thrown into randomly arranged mounds separated from each other by furrows. The glands deliver their secretions to the lumen via ducts. The thickness of the uterine epithelium, the number of glands in the lamina propria and the nature of their secretion, and the degree of vascularization are highly variable depending on the parity mode of the reptile (Blackburn, 1998). The wall of the uterus is thicker than the preceding portions of the oviduct because of the enlarged smooth muscle layers.

The uterus is the portion of the oviduct involved in eggshell formation. Reptilian eggshells have an inner fibrous protein and an outer calcareous component, each of which is highly variable among species and parity modes (Packard & DeMarco, 1991). The synthesis of these different components of the eggshell differs among crocodilian and non-crocodilian reptiles. The uterus of squamate and testudine reptiles is a single structure in which protein secretion and formation of the calcareous portion of the eggshell are separated in time, whereas in crocodilians the protein and calcareous components are produced in different regions of the uterus (Palmer et al., 1993).

Only a few studies have focused on the cellular origin of these eggshell components. There is little doubt that the glands of the lamina propria secrete the eggshell protein fibers. In the lizard *Sceloporus woodi*, Palmer et al. (1993) observed proteinaceous fibers emanating from the ducts of uterine glands 12 hours following ovulation. They speculated that these fibers covered the egg as the egg rotated in the uterus. Similar observations have been made in the turtle *C. serpentina* (Alkindi et al., 2006) and in the anterior portion of the alligator (*Alligator mississippiensis*) uterus (Palmer & Guillette, 1992). Even less is known of the cellular origins of the calcium that forms the shell. Guillette et al. (1989) found that postovulatory epithelial cells of the uterus in the lizard *Crotaphytus collaris* stained more intensely with Alizarin red S, a calcium stain, than other regions of the uterus. Immunofluorescent detection of Ca^{++} ATPase pumps indicates maximal activity in the apical and basolateral surfaces of uterine epithelial cells in lizards (*Lampropholis guichenoti*) containing shelled eggs (Thompson et al., 2007). More direct measurements of calcium concentrations by atomic absorption spectrophotometry in uterine epithelial tissue from the turtle *C. serpentina* (Alkindi et al., 2006) confirm high concentrations of Ca^{++} in uteri containing unshelled eggs and lower levels in uteri containing shelled eggs. Possible endocrine controls of protein deposition and calcification within the same uterus remain unstudied.

In contrast, the uterus of crocodilians is separated into anterior and posterior components and deposition of the two eggshell components is a function of egg location. The two regions of the uterus are similar except for the thicker layer of tubuloalveolar glands and myometrium in the posterior uterus (Palmer & Guillette, 1992). Fiber deposition is thought to occur in the anterior uterus while egg calcification is thought to occur in the posterior uterus. Palmer and Guillette (1992) observed calcification of the eggshell in the posterior uterus of *A. mississippiensis*.

The caudal terminations of the uterine glands in the oviduct of turtles and crocodilians are similar in morphology to the termination of the glands of the uterine tube noted above. The glands are less numerous, have prominent ducts open to the uterine lumen, and can serve as locations for sperm storage (Gist et al., 2008; Gist & Congdon, 1998).

5.5 Vagina

The terminal portion of the reptilian oviduct, the vagina, is short, devoid of glands in the lamina propria, and highly muscular. The paired vaginae (except in snakes) serve as a connector to the cloaca and open directly to it, acting like a sphincter. The simple epithelium of the vagina is composed of cuboidal to columnar cells, most of which are ciliated. The mucosa is thrown into high folds running longitudinally. In some squamates, the grooves between the folds can serve as sperm storage structures and in still others the grooves can become pinched off into tubules that serve the same function (Gist & Jones, 1987; Sever & Hamlett, 2002). The vagina is surrounded by thick inner circular and outer longitudinal layers of smooth muscle.

5.6 Reproductive cycles and hormonal dependence

The secretion of female sex hormones is linked to events of the ovarian cycle. Estradiol, T, the aromatizable precursor to E_2, and P_4 are the most commonly measured hormones; in most species, circulating levels of $P_4 > T > E_2$. The pattern of annual changes in these hormones is variable depending on the duration of ovarian events, the number of egg clutches,