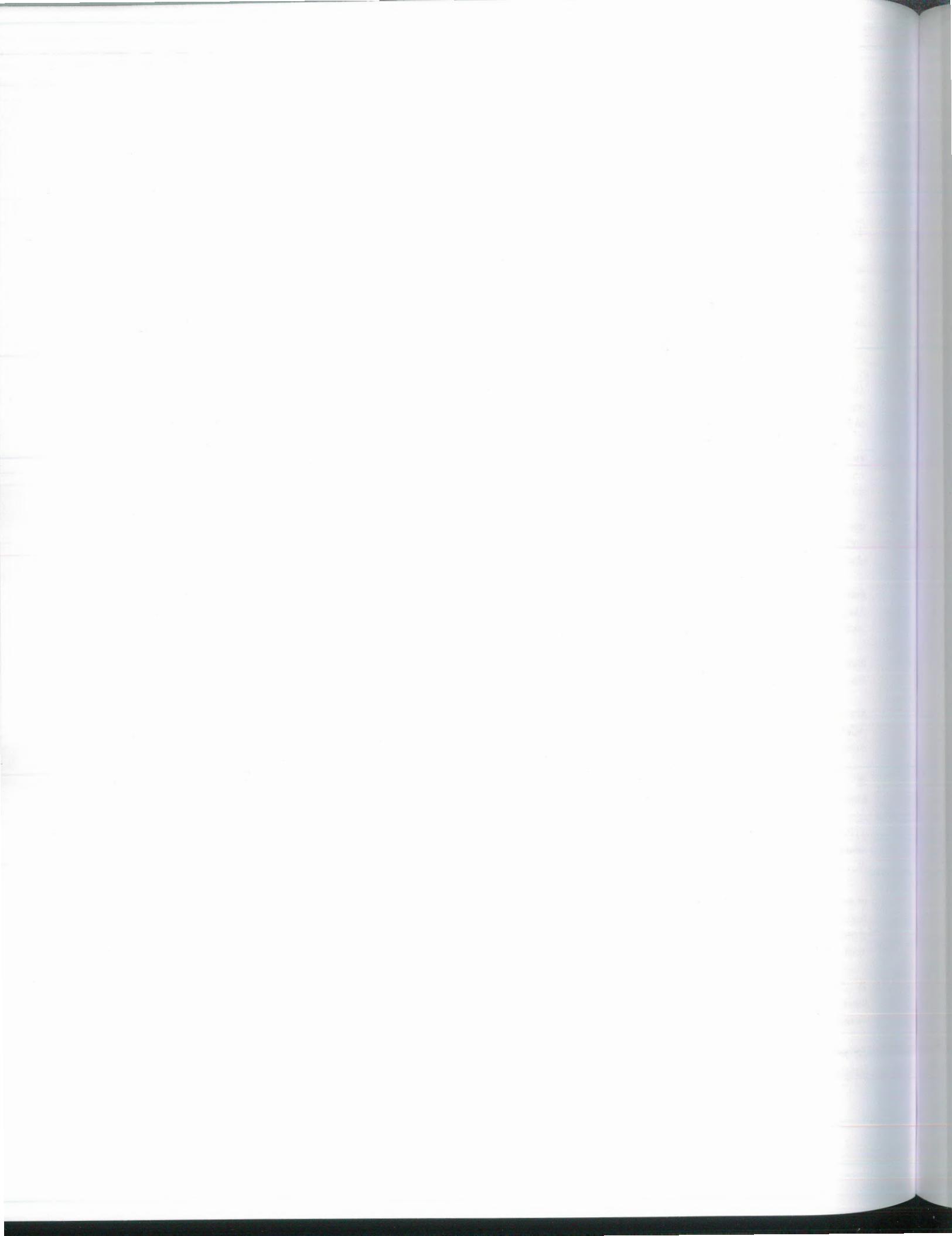


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Chapter 10

Hormones and reproductive cycles in crocodilians

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ABBREVIATIONS

ABT	assisted breeding technologies
AMH	anti-Müllerian hormone
AND	androstenedione
AR	androgen receptor
AVT	arginine vasotocin
CAM	chorioallantoic membrane
CORT	corticosterone
<i>CYP17A1</i>	17a-hydroxylase/17,20b-lyase
<i>CYP19A1</i>	aromatase
DEGs	differentially expressed genes
DMRT3	doublesex and mab-3-related transcription factor 3
E ₂	estradiol-17β
ESR1	estrogen receptor alpha
ESR2	estrogen receptor beta
FOXL2	forkhead box L2
FSH	follicle-stimulating hormone
FSH _b	FSH subunit b
FSH-R	follicle-stimulating hormone receptor
GnRH	gonadotropin-releasing hormone
GR	glucocorticoid receptor
GSI	gonadosomatic index
GTH	gonadotropin
HPG axis	hypothalamic-pituitary-gonadal axis
IGF-I	insulin-like growth factor 1
IGF-II	insulin-like growth factor 2
IP ₃	Inositol triphosphate
Kiss1	kisspeptin 1
Kiss1r	kisspeptin receptor
LH	luteinizing hormone
miRNA	micro-RNA
ncRNA	noncoding RNA
P ₄	progesterone
PCNA	proliferating cell nuclear antigen
PG	prostaglandin
PR	progesterone receptor
RNA-seq	RNA sequencing
<i>SF-1/NR5A1</i>	steroidogenic factor-1
SOX9	SRY-box transcription factor 9
SVL	snout-vent length
T	testosterone

TSP	thermosensitive period
3β-HSD	3β-hydroxysteroid dehydrogenase

1 INTRODUCTION

The order Crocodylia shares a position with birds and dinosaurs on the archosaurian branch of the diapsid amniotes (Laurin & Gauthier, 2012). Evolutionarily, crocodilians share many reproductive traits homologous to those found in birds and presumably dinosaurs and retain some ancestral characteristics common to lepidosaurian and chelonian reptiles. For instance, crocodilians and birds share similar regional specialization of the oviducts for the secretion of albumen, the formation of noncalcified eggshell membranes, and the shelling of eggs, whereas oviparous squamates and chelonians exhibit a temporal rather than spatial separation of the secretory and shelling processes (Palmer & Guillette, 1992). On the other hand, crocodilians, like other reptiles, ovulate and process an entire clutch simultaneously, whereas birds ovulate and process individual eggs sequentially.

Many crocodilian species must reach a total length of nearly two meters or more before they are capable of successful reproduction. It can take more than a decade to reach sexual maturity in the wild; thus, key events related to sexual differentiation and maturation span many years. During embryogenesis and neonatal development, egg incubation temperature and hormones influence the formation of primary and secondary reproductive traits. Subtle sexually dimorphic cloacal/genital morphologies observed at hatching increasingly diverge during posthatching years. As juvenile crocodilians mature, they transition into an extended pubescent period during which they experience attenuated annual hormonal cycles that amplify seasonally until they parallel those of adults. Upon reaching sexual maturity, most crocodilian species exhibit highly seasonal and synchronous reproduction. Reproductive activity within

a species typically corresponds to seasonal changes in temperature or rainfall, depending on whether that species inhabits a temperate, subtropical, or tropical environment. Whereas overall copulatory mechanics of male phallic eversion and female cloacal intromission are conserved among taxa, divergent, species-specific phallic glans morphologies suggest dissimilar insemination strategies among major Crocodylia clades (Moore, Francis, et al., 2020; Moore et al., 2022). Energetic investment in reproduction for female crocodilians is relatively high, with clutches of 12–50 or more megalecithal eggs, weighing 45–160g each (Thorbjarnarson, 1996). Many species also exhibit nest-guarding behavior throughout egg incubation and provide varying degrees of parental care stimulated by hatchling vocalization (Chabert et al., 2015; Hunt & Watanabe, 1982; Vergne et al., 2011). Although the maximal life span of crocodilians is not known, long-term recapture data suggest that female American alligators (*Alligator mississippiensis*) in South Carolina, the United States, attain sexual maturity at approximately 16 years, exhibit determinate growth which ceases around 31 years, have a reproductive lifespan of up to 46 years, and may live for 70 years or more (Wilkinson et al., 2016).

Crocodylians are a taxonomic group of aesthetic and commercial interest in many countries. Applied conservation measures, which include captive propagation of endangered crocodilians and ecotourism, as well as financial interests in farming or ranching of crocodilians, have stimulated many of the advances in our understanding of crocodilian reproductive physiology. With only three recognized families (Alligatoridae, Crocodylidae, and Gavialidae) and 27 species (The Reptile Database, 2023), crocodilians are less diverse than squamates and turtles, and their basic reproductive anatomy and physiology appear highly conserved. It should be noted, however, that the available primary literature examining crocodilian reproduction is biased toward *A. mississippiensis*. For each topic covered within this chapter, every attempt will be made to point out characteristics generally considered to be representative of all crocodilians. Similarly, special attention will be paid to interspecific variation and how it relates to phylogenetic or environmental influences.

2 DEVELOPMENT

Early embryonic development of the reproductive system in crocodilians is quite similar to birds with a key exception being the absence of heteromorphic sex chromosomes. All crocodilians studied to date exhibit temperature-dependent sex determination. Data that include incubation temperatures and resulting sex ratios in 11 species suggest that all crocodilians follow the same female-male-female pattern with increasing incubation temperature (Lang &

Andrews, 1994). Considering the latitudinal range and environmental variation that exists across species, it is interesting to note that the viable range (approximately 28–34°C) and male-producing range (approximately 31–33°C) are relatively consistent among species from all three families. Key events related to sexual differentiation and maturation in crocodilians are summarized in Table 1. In addition to sex determination, development and differentiation of secondary sex traits and hormone-dependent tissues begins *in ovo*. Upon hatching, crocodilians exhibit markedly male or female characteristics; however, neonates continue to undergo significant morphological differentiation of the gonads and reproductive tract over the ensuing months, much of which is under hormonal control. Juvenile crocodilians exhibit an extended period analogous to puberty, a developmental stage not well studied in nonmammalian vertebrates. During these years they exhibit increasingly pronounced cyclic patterns of hormone production as they grow larger and show signs of seasonal responsiveness in the gonads and reproductive tract.

2.1 Sexual differentiation

2.1.1 Gonadal differentiation

The length of egg incubation time in crocodilians averages about 70 days and varies from 2 to 4 months depending on the species. The rate of embryonic development is proportional to incubation temperature, and incubation time within a species can vary by 3 weeks under constant incubation temperatures that span the viable range (Deeming & Ferguson, 1989; Ferguson, 1985; Lang & Andrews, 1994; Piña et al., 2003). As a result of interspecific variation in size and the effect of temperature on the rate of development, embryo size and age are not reliable bases for comparison of development across species or under different incubation conditions. To accommodate this variability, a standardized series of 28 embryonic stages of development in *A. mississippiensis* was presented by Ferguson (1985). Each stage is based upon several diagnostic morphological features originally present at that point in development, rather than on morphometrics. The series was cross-referenced against the freshwater crocodile (*Crocodylus johnstoni*) and the saltwater crocodile (*Crocodylus porosus*) development (Ferguson, 1985), and more recently against the broad-snouted caiman (*Caiman latirostris*) (Iungman et al., 2008) (Table 1). Although some morphological discordance exists between consecutive stages among species, the series is generally accepted as applicable to other crocodilians and will be used to define embryonic events discussed in this chapter.

Several detailed accounts of the morphological differentiation of the bipotential gonad in *A. mississippiensis* have been published (Forbes, 1940; Joss, 1989; Smith & Joss, 1993, 1994a).

TABLE 1 Key events related to sexual differentiation and maturation in crocodilians.

Embryonic stage ^a /size ^b	Description	References
12	Genital ridge composed of germinal epithelium and medullary sex cords	Forbes (1940), Smith and Joss (1993)
21–23	Thermosensitive period of sex determination. Significant decrease in yolk steroid concentrations, and sexual differentiation of gonad.	Lang and Andrews (1994), Conley et al. (1997)
24	Morphologically and steroidogenically distinct testis and ovary.	Smith and Joss (1994b), Smith et al. (1995), Gabriel et al. (2001), Milnes, Roberts, et al. (2002)
25–28 (hatching)	Males: Sertoli cells and germ cells enclosed in seminiferous tubules. Müllerian duct begins to regress. Females: Cortical proliferation and development of lacunae in the ovary. Oogonial nests and primary oocytes present at hatching.	Austin (1989), Smith and Joss (1993), Smith and Joss (1994a), Moore et al. (2008)
≈40 cm snout-vent length (SVL)	Males and females begin to show an attenuated seasonal cycle with regard to testosterone and estradiol-17 β , respectively.	Coutinho et al. (2000), Rooney et al. (2004)
≈90 cm SVL	Sexual maturity.	Thorbjarnarson (1996)

^aEmbryonic stages according to Ferguson (1985) and lungman et al. (2008). ^bSizes are approximate for most species and not necessarily applicable to the genus *Paleosuchus* (dwarf caiman) or *Osteolaemus* (dwarf crocodile).

Nothing is known concerning germ cell migration in crocodilians, but the primordial gonad is first seen as a thickening of the coelomic epithelium along the ventromedial surface of the mesonephric kidney. By stage 12, the genital ridge is composed of an overlying germinal epithelium that becomes increasingly distinct from the medullary sex cords as development progresses. The embryonic gonad remains morphologically indistinguishable at male- and female-producing temperatures through stage 20, at which time it consists of a distinct cortex and medulla. Temperature shift experiments between strongly biased male- and female-producing temperatures indicate that the thermosensitive period (TSP) of sex determination in *A. mississippiensis* occurs between stages 21 and 24 (Lang & Andrews, 1994). This period corresponds to the appearance of morphological differences in the embryonic gonad; however, recent studies suggest that temperature influences gonadal differentiation prior to the canonical TSP and commitment to testicular differentiation is not finalized until the very end of the TSP. Eggs incubated at female-biased temperatures prior to the TSP—as early as stage 15—are more likely to produce females when subsequent development occurs at an intermediate temperature (McCoy et al., 2015). Moreover, susceptibility to estrogen-induced ovarian development at male-producing temperature remains until stage 25 (Kohno et al., 2020). Between stages 21 and 23, enlarged pre-Sertoli cells begin to appear in the medulla of the putative testis, while the putative ovary shows signs of medullary cord fragmentation and proliferation of

germ cells in the cortex (Smith & Joss, 1993). By stage 24 at male-producing temperatures, seminiferous cords are widespread, and the germinal epithelium is greatly reduced, whereas oogonial nests and prefollicular cells are forming in the cortex of the embryonic ovary.

Throughout the TSP, de novo steroidogenic activity, measured by enzyme histochemistry of 3 β -hydroxysteroid dehydrogenase (3 β -HSD) activity in *C. porosus*, is present in adrenal tissue but nonexistent in the adjacent embryonic gonad (Smith & Joss, 1994b). Aromatase (CYP19A1) enzyme activity, measured by the tritiated water assay in *C. porosus* and *A. mississippiensis*, is present in the gonad-adrenal-mesonephros complex throughout the TSP, but does not increase in a sex-specific manner until the end of the TSP, when signs of morphological differentiation in the gonad are already present (Milnes, Roberts, et al., 2002; Smith et al., 1995; Smith & Joss, 1994b). Extragonadal aromatase activity follows a similar pattern, increasing in the brain throughout the TSP, but failing to exhibit sexual dimorphism until stage 24 (Milnes, Roberts, et al., 2002). It is not known if adrenal enzyme activity provides the substrate for aromatase activity in the embryonic gonad or brain, but the undifferentiated gonad is capable of binding estradiol-17 β (E₂) throughout the TSP (Smith & Joss, 1994c). Although no difference in the uptake of ³H-E₂ was observed between male- and female-producing temperatures, the occurrence of E₂-binding in the undifferentiated gonad provides a possible

mechanism for the temperature-overriding effects of exogenous estrogens on gonadal sex determination.

Maternally derived yolk steroids provide additional potential sources of steroids and steroidogenic substrates. Progesterone (P_4), androstenedione (AND), testosterone (T), and E_2 are found in ng/g concentrations in the yolk prior to the TSP (Conley et al., 1997; Hamlin et al., 2010). The concentration of these steroids declines dramatically throughout the period of sex determination, with the most significant decrease in AND, T, and E_2 occurring between stages 21 and 23 (Conley et al., 1997). The decline in yolk AND concentration is inversely proportional to incubation temperatures; however, this is not necessarily linked directly to sex determination as a large percentage of females are produced at both the lower and upper range of viable incubation temperatures. Temporal patterns of embryonic aromatase activity and declines in yolk steroids during egg incubation do not appear to direct gonadal sex determination. On the other hand, the presence of measurable 3β -HSD in the adrenal gland, the dramatic decline in yolk steroid concentrations throughout the TSP, the uptake of E_2 in the medulla of the embryonic gonad at male- and female-producing temperatures, and the presence of sexually dimorphic aromatase activity following the TSP suggest that steroids have a role in directing embryonic development and sexual differentiation in crocodilians. The mechanism for preferential uptake and metabolism of yolk steroids during the TSP is not known, but the chorio-allantoic membrane (CAM) is a potential site of embryonic steroid synthesis and metabolism. The CAM of *A. mississippiensis* expresses mRNA for steroidogenic factor-1 (*NR5A1*) and steroidogenic enzymes 3β -HSD, 17 α -hydroxylase/17,20 β -lyase (*CYP17A1*), and *CYP19A1*. The CAM is also potentially responsive to changes in steroid hormone concentrations, as it expresses progesterone receptor (*PR*), glucocorticoid receptor (*GR*), androgen receptor (*AR*), and estrogen receptor alpha (*ESR1*) and beta (*ESR2*) (Cruze et al., 2012).

Significant differentiation of gonadal cell types and structures continues from late embryogenesis through neonatal and early juvenile development. From stage 25 to hatching, the testes are distinguished from previous stages by increasing basal nuclei and apical endoplasmic reticulum in Sertoli cells enclosed in seminiferous tubules (Smith & Joss, 1994a). At hatching, clearly identifiable germ cells are present within the seminiferous cords, and the germinal epithelium consists of only a squamous cell monolayer (Smith & Joss, 1993). Over the ensuing months of neonatal development, a compact tunica albuginea encapsulates the testis, and the germinal epithelium continues to regress so that only remnant patches remain (Forbes, 1940). By 6 months posthatching, well-organized seminiferous tubules, Sertoli cells, and

Leydig cells are present in testes, and germ cells are undergoing mitotic divisions (Guillette et al., 1994). Although it is probably several years under natural environmental conditions until complete spermatogenesis occurs, Forbes (1940) noted the presence of a narrow seminiferous tubule lumen and occasional spermatocytes at 18 months after hatching.

Over the final stages of development in female embryos, cortical proliferation continues as lacunae, or fluid-filled spaces, develop within the fragmented cords of the medulla (Smith & Joss, 1993). Oogonia and primary oocytes are scattered throughout the germinal epithelium at hatching, and prefollicular cells, adjacent to spherical germ cells containing meiotic chromosomes, are present by day seven in *A. mississippiensis* (Moore et al., 2008). A dramatic increase in the thickness of the lacunar system separating the germinal epithelium from the mesonephros develops over the next few months while folliculogenesis proceeds. By 3 months posthatching, some oocytes have advanced to the diplotene stage of meiosis, marked by the appearance of lampbrush chromosomes and a single layer of cuboidal follicular cells surrounded by a fibrous theca (Moore et al., 2008). Detailed ontogenetic studies of oocyte and follicular maturation in older juvenile and subadult crocodilians have yet to be published, but presumably, previtellogenic follicles persist in immature crocodilians as a potential source of estrogens at the onset of puberty. This is supported by the finding that ovine follicle-stimulating hormone (FSH) injections into 5-month-old, female *A. mississippiensis* result in elevated plasma E_2 and increased steroidogenic mRNA expression (Moore et al., 2012).

2.1.2 Urogenital tract differentiation

Following the initial stages of gonadal differentiation, sex steroids and other hormones begin directing the development and differentiation of secondary sex characteristics in crocodilians. The Müllerian duct, precursor to the oviduct, is present in all early embryos, but begins to regress approximately 2 weeks prior to hatching (stage 24/25) at male-producing temperatures in *A. mississippiensis*. The regression of the Müllerian duct following the grafting of testicular explants into newly hatched and ovariectomized females implicates anti-Müllerian hormone (AMH, also termed Müllerian-inhibiting substance, MIS) secreted from testes as the cause (Austin, 1989). Ovariectomized females lacking testis grafts but supplemented with T implants do not exhibit Müllerian duct regression, supporting the role of AMH as opposed to T as the causal agent for regression (Austin, 1990). Cloning of the AMH gene from *A. mississippiensis* and the observation that AMH mRNA expression substantially increases from stages 23 to 25 in putative males and the lack of expression in females

confirms AMH as the cause of Müllerian duct regression (Urushitani et al., 2011; Western et al., 1999). Finally, ovariectomized female hatchlings receiving testis grafts and supplemented with E₂ do not exhibit Müllerian duct regression, thus indicating that E₂ antagonizes the effects of AMH (Austin, 1990).

Additional experimental data from *A. mississippiensis* suggest that the presence of the Müllerian duct is independent of estrogen signaling, but growth and regional differentiation may be dependent upon signaling through ESR1. *In ovo* exposure to an aromatase inhibitor does not disrupt the formation of the Müllerian duct (Lance & Bogart, 1992), whereas *in ovo* exposure to E₂ results in hypertrophy (Crain et al., 1999). Doheny et al. (2016) demonstrated that an ESR1 agonist promotes hypertrophy and precocial regionalization of the duct, but an ESR2 agonist does not. Four days of ovine FSH injections in a 5-month-old female *A. mississippiensis* resulted in increased circulating E₂ and elevated expression of proliferating cell nuclear antigen (PCNA) in the oviduct (Moore et al., 2012). Galoppo et al. (2016) thoroughly characterized postnatal and juvenile development of the oviduct in *C. latirostris* up to 31 months of age. As the oviduct enlarges and folds along its anterior-posterior axis, the simple columnar epithelium lining the lumen transitions to a complex pseudostratified and ciliated epithelial layer interrupted by glandular invaginations. Additionally, the muscularis become more clearly delineated from the mucosa as indicated by immunopositive staining for smooth muscle α -actin and desmin. Immunopositive nuclear staining for ESR1 is strongest in the developing glands of late postnatal and juvenile caiman. Staining for PR gradually increases in intensity in the luminal and glandular epithelium from the neonate to the juvenile stage.

Less is known about hormonal influences on the development of male secondary sex characteristics compared to those of female crocodilians. The Wolffian (mesonephric) duct develops parallel to the dorsal margin of the mesonephros and becomes continuous with medullary sex cords that extend into the mesonephros as rete cords (Forbes, 1940). It has a similar appearance in both sexes throughout embryonic and early posthatching growth, functioning as the excretory duct of the active mesonephros and emptying into the cloaca (Ferguson & Joanen, 1983; Forbes, 1940). At 1-year posthatching, the Wolffian duct is only slightly larger in males *A. mississippiensis* compared to females (Ferguson & Joanen, 1983), but is unresponsive to exogenous androgens from early posthatching through 2 years of age (Forbes, 1938; Ramaswani & Jacob, 1965). The onset of regional differentiation of the Wolffian duct and hormonal responsiveness has yet to be studied in detail.

Sexual differentiation of external genitalia is a gradual process spanning embryonic to juvenile stages with greater current understanding of male phallic form and function, as compared to the understudied female clitoris. In male differentiation, the embryonic genital tubercle elongates into a proximal dense fibrous corpus fibrosum and elaborates into a distal inflatable glans of corpus spongiosum terminating in the sperm-carrying sulcus spermaticus. These structures facilitate female cloacal intromission and insemination upon phallic eversion and copulation (Kelly, 2013). Although the generalized male phallic form and copulatory function is conserved across taxa, the distal glans shape is clade specific, with *Alligatoridae* presenting a more rounded glans and filiform glans tip (Moore, Fitri, et al., 2020; Moore, Francis, et al., 2020; Moore et al., 2016) and *Crocodylidae* presenting a notched glans with a blunt deflected glans tip (Fitri et al., 2018; Johnston et al., 2014; Moore, Groenewald, et al., 2020). The clitoris of *C. latirostris* is much smaller overall and has less sulcus-adjacent smooth musculature, greater innervation, and a less developed glans corpus spongiosum in comparison to male phalli (Tavalieri et al., 2019).

Growth of the cliterophallus during embryonic development is somewhat temperature- and sex-dependent. The length and width of the cliterophallus of neonatal *A. mississippiensis* increase with egg incubation temperature in both sexes, and there is some overlap in size of this homologous structure in males and females incubated at temperatures that produce both sexes (Allsteadt & Lang, 1995). Males and females can usually be distinguished by examination of externalized genitalia within months of hatching in *C. porosus* and *C. johnstoni* (Webb et al., 1984), Nile crocodile (*Crocodylus niloticus*) (Hutton, 1987), mugger crocodile (*Crocodylus palustris*) (Lang et al., 1989), Siamese and Morelet's crocodiles (*Crocodylus siamensis* and *Crocodylus moreletii*, respectively) (Lang & Andrews, 1994), *C. latirostris* (Ciocan et al., 2020), and common caiman (*Caiman crocodilus*) (Lang & Andrews, 1994). A noted exception is gharial (*Gavialis gangeticus*), in which males cannot be distinguished from females based on cliterophallus morphology until 1 m or more in total length is reached (Lal & Basu, 1982). Increasing dimensions of the male phallus relative to that of females as body size increases suggests that the male phallus responds to ontogenetic increases in androgens (Allsteadt & Lang, 1995). This is consistent with findings that hatchling male crocodilians respond to exogenous T with pronounced growth of the phallus, whereas the structural homolog in females does not respond (Forbes, 1938; Ramaswani & Jacob, 1965). To that end, histochemical study of hatchling and juvenile *C. latirostris* cliterophalli showed greater immunoreactive anti-AR and ESR1 protein staining in male phalli tissues compared to the clitoris, with

greater staining of smooth muscle, sulcus epithelium, and ductus deferens (Tavalieri et al., 2019). Furthermore, a positive relationship exists between plasma androgen concentration and phallus size in juvenile *A. mississippiensis*. More variation in this relationship is explained by plasma dihydrotestosterone than plasma T, suggesting that 5 α -reductase activity is particularly important to phallus development in crocodilians as it is in mammals (Guillette et al., 1999; Pickford et al., 2000).

2.2 Juvenile growth and peripubertal seasonality

Puberty refers to the period during which the immature hypothalamic-pituitary-gonadal (HPG) axis of a juvenile transitions into that of a sexually mature adult capable of producing offspring. It is a complex process that involves the resetting of endocrine homeostasis and concomitant changes in reproductive anatomy, physiology, and behavior.

As crocodilians mature, gonadal activity and magnitude of response along the HPG axis to hormonal stimulation increases. For example, plasma T and E₂ concentrations increase in juvenile males and females, respectively, with increasing body size (Crain et al., 1998; Milnes, Woodward, et al., 2002). Although pituitary activity in juvenile crocodilians has not been studied directly, experimental evidence suggests that responsiveness to gonadotropin (GTH) stimulation is correlated with size. Injections of ovine pituitary extract cause hypertrophy of the testes, ovaries, and the female reproductive tract in immature *A. mississippiensis*, and the degree of this response increases with the size of the animal (Forbes, 1934, 1937). In juvenile males, plasma T concentrations increased following injections of ovine follicle-stimulating hormone (FSH). Larger males produced more T, and although still immature, the largest alligators in the study exhibited a more pronounced seasonal variation in response to FSH stimulation than smaller males (Edwards et al., 2004).

Crocodilians mature at a rate somewhat analogous to humans and the transition from juvenile to sexual maturity occurs over several years. The synchronized and seasonal nature of crocodilian reproduction has enabled researchers to characterize the onset of seasonal cyclicity and maturation by sampling across size classes throughout the year. Seasonal variation in plasma sex steroids has been characterized in wild-caught juvenile *A. mississippiensis* (Fig. 1), and a size threshold of approximately 38 cm snout-vent length (SVL) was observed (Rooney et al., 2004). Animals below this size show little discernable pattern with regard to seasonal variation in plasma E₂ or T concentrations, whereas males and females above 38 cm SVL begin to show pronounced seasonal variation in T and E₂, respectively, suggesting the onset of puberty. Similarly, male yacare caiman (*Caiman yacare*) show signs of spermatogenesis

at ~40 cm SVL, and subadults (70–90 cm SVL) exhibit a seasonal rise in plasma T that coincides with peak spermato-genic activity (Coutinho et al., 2000). At 40 cm SVL, wild *A. mississippiensis* and *C. yacare* are approximately 3–5 years in age (Coutinho et al., 2000; Milnes, Woodward, et al., 2002), and are less than half the size at which both species are generally considered fully mature (~90 cm). In *C. moreletii*, an inflection point in body size, phallic development, and skull morphology is indicative of a male pubertal transition. At 65 cm SVL, an increase in body condition index is associated with an increase in male phallic glans size and the development of prominent maxillary and mandibular caniform teeth (Moore et al., 2019). At this size, the rate of increase in body mass surpasses the longitudinal growth rate, and the relation between head size and body size decreases (Padilla et al., 2020). These findings suggest that crocodilian puberty influences a broad range of sexually dimorphic and morphometric characteristics in addition to reproductive function.

3 REPRODUCTIVE CYCLES

3.1 Seasonality

All species of crocodilians examined show a pattern of annual reproductive activity. No species are known to produce more than one clutch per year in the wild, but multiple clutches deposited by individuals in a single season have been documented in *C. palustris* in captivity, a species that ranges from equatorial to temperate central Asia (Whitaker & Whitaker, 1984). These observations indicate that the frequency of reproduction in the wild is likely dictated by environmental factors rather than inherent physiological limitations. The first published descriptions of reproductive seasonality in crocodilians came from naturalists' accounts of *A. mississippiensis* in the late 19th and early 20th centuries. Clarke (1891) noted that male alligators in Florida, the United States, were most frequently on the move and bellowing in May and June, and newly laid eggs in nests were found only in early to mid-June. McIlhenny (1935) offered the first detailed accounts of mating, nest building, and egg laying from his observations of alligators maintained in seminatural enclosures on Avery Island, LA, the United States. He remarked on the increased interest and bellowing carried on by males in late spring and early summer toward females in adjacent enclosures. He also noted that all egg deposition took place between May 20 and June 25, and egg incubation lasted approximately 9 weeks. American alligators and Chinese alligators (*Alligator sinensis*) are among the most temperate crocodilian species and both species exhibit highly synchronous and seasonal reproductive activity (Guillette et al., 1997; Lance, 1989; Thorbjarnarson et al., 2001). The window of reproductive activity in temperate species

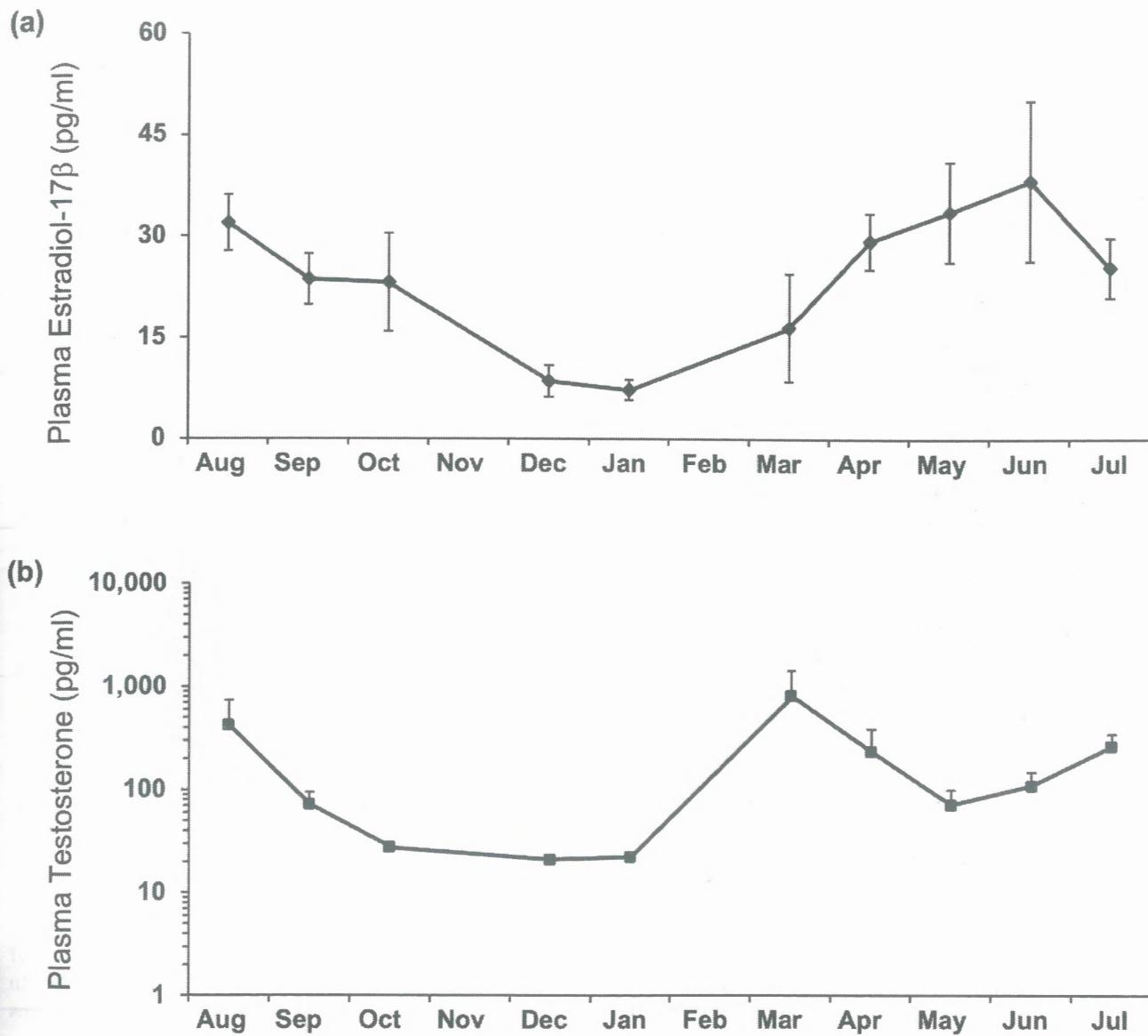


FIG. 1 Seasonal variation in plasma steroid concentrations of juvenile *Alligator mississippiensis* ≥ 38 cm snout-to-vent length. Monthly variation is observed in (A) plasma estradiol-17 β in juvenile females, and (B) plasma testosterone in juvenile males. Error bars represent 1 standard error. (Reproduced with permission from Rooney, A. A., Crain, A. D., Woodward, A. R., & Guillette Jr., L. J. (2004). Seasonal variation in plasma sex steroid concentrations in juvenile American alligators. General and Comparative Endocrinology, 135(1), 25–34.)

is limited by the onset of cooler temperatures in the late fall that bring about a period of metabolic dormancy that lasts through the winter months. Most reproductive activity in temperate species corresponds to periods of warm ambient temperatures (Fig. 2). Warmer temperatures and increasing photoperiod in the spring trigger HPG activity and gametogenesis, which culminates with copulation and oviposition in late spring and early summer.

Tropical species also exhibit patterns of annual seasonality, but the timing corresponds to local rainfall patterns rather than temperature or photoperiod (Fig. 2). In these species,

ambient temperatures are permissive of year-round metabolic activity, and periods of reproductive activity tend to be more protracted compared to their temperate counterparts. Many tropical environments have distinct wet and dry seasons that affect population density and food availability. As water begins to recede during the dry season, food resources, such as fish, initially become more concentrated. Eventually, however, the crocodilian population density also increases to a point where food and suitable habitat become limiting resources. Studies of captive crocodilians have shown that plasma corticosterone (CORT)

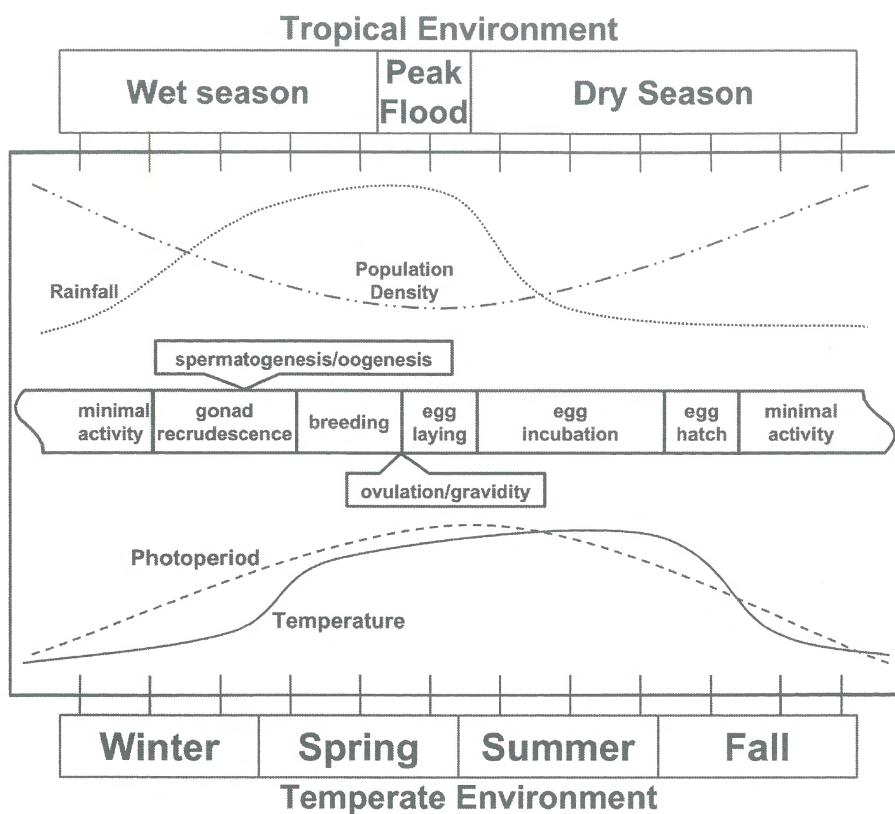


FIG. 2 Relationship between environmental factors and reproductive cycles in crocodilians from tropical and temperate environments. In tropical environments, the lack of rainfall during the dry season leads to a reduction in wetlands area covered by water, and a subsequent increase in crocodilian population density. Reproductive activity is suppressed during periods of high population density, resumes with the onset of seasonal rains, and culminates with egg laying near the time of peak water levels. In temperate environments, reproductive activity is suppressed during periods of cooler temperatures and shortened day length. The resumption of reproductive activity corresponds to increasing temperature and photoperiod in early spring.

increases with population density, even when food is not a limiting factor, and females with the greatest plasma CORT concentrations had the poorest nesting success (Elsey et al., 1990). Interspecific competition for food and high population density leading to elevated plasma glucocorticoids might inhibit HPG activity. This hypothesis has yet to be specifically tested in wild crocodilians but is supported by observational data. Tropical and subtropical species that experience conspicuous annual wet and dry seasons, such as *C. crocodilus* and *C. moreletii*, exhibit gonadal recrudescence and mating late in the dry season or early in the wet season as water levels begin to rise and animals are dispersing from areas of high density (Platt et al., 2008; Thorbjarnarson, 1994). Nest building and egg deposition occur as water levels peak, possibly an adaptation to avoid flooding and limit accessibility by terrestrial nest predators.

Although many aspects of crocodilian reproduction appear to be conserved among species, little is known about the intraspecific variability in species with extensive ranges that inhabit tropical and semitropical regions (e.g., *C. niloticus*) or are well adapted to freshwater and saltwater environments (e.g., *C. porosus*). Abiotic factors such as

photoperiod, temperature, rainfall patterns, and salinity are variable within these species' ranges and may be affected by climate change. Deliberate investigations of the geographic variation in reproductive seasonality in wide-ranging species may shed light on how crocodilians will respond to a changing environment.

3.2 Hypothalamic-pituitary regulation

Like other vertebrates, reproductive activity in crocodilians is presumed to be regulated by the HPG axis. Both FSH and luteinizing hormone (LH) have been isolated from *A. mississippiensis* pituitary extracts (Licht et al., 1976), with minced alligator testes exhibiting greater *in vitro* sensitivity to LH at low concentrations but higher maximum T secretion when stimulated by FSH (Tsui & Licht, 1977). To date, there are no studies examining seasonal fluctuations in circulating GTHs, but a series of studies on female *A. sinensis* have begun to shed light on HPG regulation of reproductive activity as ambient temperature increases following hibernation. For instance, gonadotropin-releasing hormone (GnRH) peptide concentration in the hypothalamus increases gradually following hibernation, with a sharp increase

corresponding to maximum gonadosomatic index (GSI) and the presence of late-stage vitellogenic follicles (Nie et al., 2021). Similar patterns of *GnRH1*, *GnRH2*, kisspeptin 1 (*Kiss1*), and kisspeptin receptor (*Kiss1r*) mRNA expression in the hypothalamus and pituitary (Zhang, Nie, et al., 2020) and follicle-stimulating hormone receptor (*FSH-R*) mRNA expression in the ovary (Zhang et al., 2015a) are observed. Interestingly, *Kiss1* and FSH subunit β (*FSHβ*) mRNA are also highly and seasonally expressed in ovarian tissue, suggesting a potential paracrine role for these signaling molecules (Zhang, Nie, et al., 2020; Zhang et al., 2015b).

3.3 Male reproductive cycles

A relatively consistent pattern of plasma T, testis mass, and/or spermatogenesis corresponding to male reproductive seasonality has been documented in *A. mississippiensis* (Hamlin et al., 2011; Lance, 1989), *C. crocodilus* (Thorbjarnarson, 1994), *C. jacare* (Coutinho et al., 2000), and *C. niloticus* (Kofron, 1990). Gonadal recrudescence in male crocodilians follows a period of postnuptial regression and quiescence. A subtle but sustained increase in plasma T over the postnuptial nadir corresponds to the earliest signs of spermatogonia proliferation (Fig. 3). In *A. mississippiensis*, the initial onset of steroidogenic and spermatogenic activity precedes and lasts through the winter (Hamlin et al., 2011; Lance, 1989). The proliferation of spermatogonia and the appearance of primary spermatocytes is evident in *A. mississippiensis* in late winter, and early spermatids are

present about 1 month prior to the formation of spermatozoa (Gribbins et al., 2006). A more substantial increase in plasma T coincides with increasing testicular mass and spermatogenic activity beginning approximately 4 months prior to maximum breeding and nesting activity (Hamlin et al., 2011; Lance, 1989).

In *A. mississippiensis*, mature spermatozoa are abundant in seminiferous tubules for about 1 month following the peak of plasma T, which decreases abruptly at the end of the breeding season, after which the testes regress and enter a period of relative quiescence (Lance, 1989). Similarly, live spermatozoa in the penile groove and elevated plasma T concentrations were observed in *C. niloticus* in the winter months preceding peak breeding activity in July (Kofron, 1990). In tropical species, such as *C. crocodilus* and *C. jacare*, gonadal recrudescence is initiated in the dry season and peak testis mass and spermatogenesis occurs in the wet season shortly before courtship and nesting (Coutinho et al., 2000; Thorbjarnarson, 1994).

3.4 Female reproductive cycles

3.4.1 Vitellogenesis and oogenesis

Similar to male crocodilians, annual hormonal and ovarian cycles in females (Fig. 4) have only been described in a few species, but these data generally support the postulate that reproductive endocrinology is conserved among crocodilians. The most notable difference among species is the

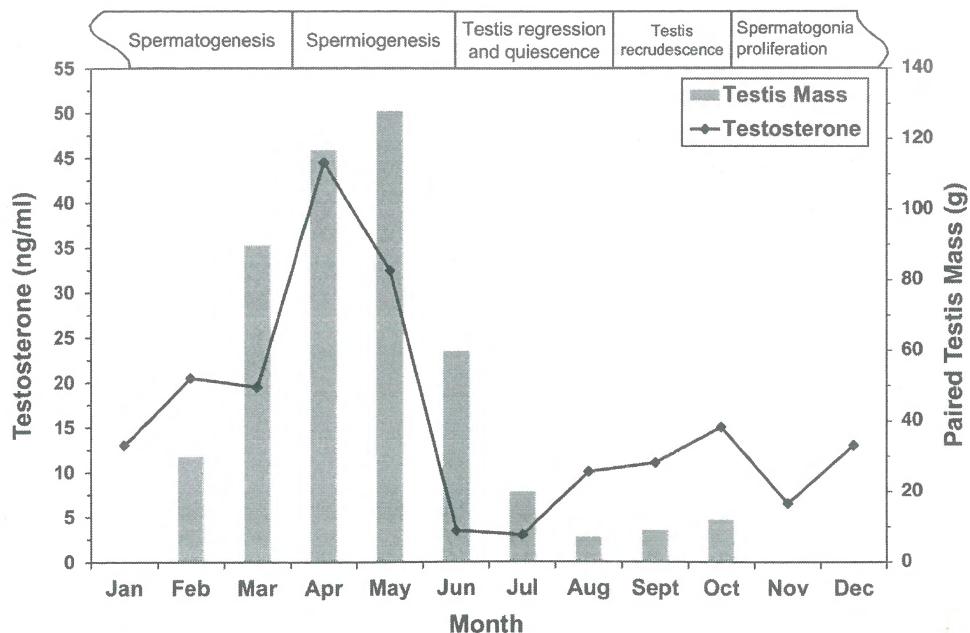


FIG. 3 Plasma testosterone concentration and paired testis mass in relation to the annual reproductive cycle of adult male *Alligator mississippiensis*. (Data taken from Hamlin, H. J., Lowers, R. H., & Guillette Jr., L. J. (2011). Seasonal androgen cycles in adult male American alligators (*Alligator mississippiensis*) from a barrier island. *Biology of Reproduction*, 85(6), 1108–1113 and Lance, V. A. (1989). Reproductive cycle of the American alligator. *American Zoologist*, 29(3), 999–1018.)

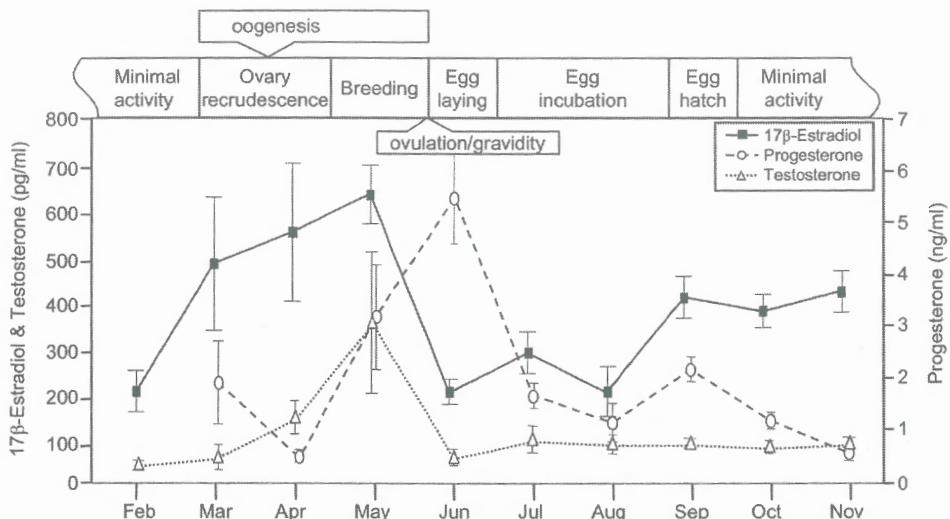


FIG. 4 Mean (± 1 standard error) plasma steroid concentrations in relation to the annual reproductive cycle of adult female *Alligator mississippiensis*. (Based on Guillette Jr., L. J., Woodward, A. R., Crain, D. A., Masson, G. R., Palmer, B. D., Cox, M. C., You-Xiang, Q., & Orlando, E. F. (1997). The reproductive cycle of the female American alligator (*Alligator mississippiensis*). General and Comparative Endocrinology, 108(1), 87–101.)

variation in the onset, length, and continuity of ovarian activity that corresponds to differences in temperature, photoperiod, and/or rainfall patterns. In *A. mississippiensis*, a postnuptial period of moderately elevated plasma E_2 and early follicular recruitment occurs in the fall, approximately 8 months prior to ovulation (Guillette et al., 1997; Hamlin et al., 2014). The enlarged follicles from this first wave of oogenesis may serve as a ready source of E_2 after a period of ovarian quiescence in the winter, rather than contributing mature oocytes. This hypothesis is supported by the simultaneous presence of large atretic follicles and smaller vitellogenic follicles in reproductively active *A. mississippiensis* examined in early spring (Guillette et al., 1997), and the presence of atretic follicles in nesting *C. latirostris* (Portelinha et al., 2015). In *C. niloticus* in Zimbabwe, plasma E_2 and calcium, both indicative of vitellogenesis, begin to rise as temperatures cool in April and May. Unlike *A. mississippiensis*, these animals continue to feed throughout the coolest months, and plasma E_2 and calcium concentrations continue to increase in reproductively active females until ovulation in early August (Kofron, 1990). In tropical species such as *C. crocodilus* and *C. yacare*, vitellogenic follicles and/or elevated plasma E_2 concentrations are observed in the months following the dry season and immediately preceding ovulation (Coutinho et al., 2000; Thorbjarnarson, 1994).

Despite the diversity in the environments that different species inhabit, reproductively active female crocodilians typically exhibit the most substantial increase in circulating E_2 in the months prior to nesting (Guillette et al., 1997; Hamlin et al., 2014; Kofron, 1990; Lance, 1989). Elevated E_2 stimulates vitellogenin production, which accompanies an approximately 10-fold increase in the size of preovulatory

follicles (Lance et al., 2009; Portelinha et al., 2015). As the follicles increase in size, the thecal layer composed of fibroblasts begins to form and the zona pellucida develops and thickens into two layers consisting of the striated zona radiata and an outer hyaline layer (Calderon et al., 2004; Uribe & Guillette, 2000). As vitellogenesis advances, small yolk platelets begin to appear inside vacuoles and differences develop in the animal and vegetal poles based on peripheral placement of the nucleus and the morphology and size of the yolk platelets. The granulosa cells gradually transform from a single squamous to cuboidal to columnar layer throughout vitellogenesis (Calderon et al., 2004; Uribe & Guillette, 2000), and show intense staining for 3 β -HSD activity indicating active steroidogenesis (Lance, 1989). The theca continues to thicken and by late vitellogenesis consists of large blood vessels, fibroblasts, collagen fibers, flattened lacunae, and smooth muscle cells. Prior to ovulation, the oocyte reaches its greatest diameter, which ranges from 35 to 45 mm. The preovulatory increase in plasma E_2 also corresponds to the rapid and dramatic growth of the oviduct that occurs during the 4 months prior to ovulation. The length of the oviduct in a nonreproductive female alligator averages 45 cm, whereas reproductive tracts of vitellogenic females reach 1.5–2.5 m in length by the time of ovulation (Guillette et al., 1997).

3.4.2 Copulation, ovulation, gravidity, and oviposition

During late vitellogenesis, an increase in plasma T in females corresponds to the onset of courtship and copulatory behavior (Guillette et al., 1997; Lance, 1989). It is unclear what role T has in female crocodilians, but in songbirds it has been linked to territorial aggression toward

intruding same-sex individuals and breeding pairs (Gill et al., 2007). Copulation in crocodilians can precede ovulation by a month or more, and females may breed with multiple males during this time. Multiple paternity within clutches was first reported by Davis et al. (2001) and appears widespread among crocodilian species (Isberg, 2021; Muniz et al., 2011). Because a relatively small number of dominant males may be disproportionately represented in a large number of nests (Barragán-Contreras et al., 2021; Lance et al., 2009; Rossi Lafferriere et al., 2016; Zajdel et al., 2019), the promiscuous nature of crocodilian mating systems does not necessarily result in increased heterozygosity.

Crocodilian copulation often takes place in water with males approaching females laterally from the rear and assuming a superior position. Prior to intromission, the male phallus is protruded from intracloacal storage to its external copulatory position by coordinated cloacal muscle contractions that rotate the shaft and glans cranially via a suspensory connective tissue pivot point (Kelly, 2013). With applied muscular force, intromission is facilitated by the rigid corpus fibrosus element of the phallus shaft that, in turn, places the distal male glans in the proctodeum abutting the female reproductive tract openings in the distal ventral folds of the urodeum, the second internal chamber of the cloaca. Increased blood flow into distal corpus spongiosum elements expands the glans into a species-specific shape that creates a copulatory fit with female cloacal elements (Moore et al., 2021, 2022). These complex interactions advantageously exclude external water from the site of

semen transfer, produce compression pressure on the highly innervated female clitoris, and align the glans tip and the terminus of the sulcus spermaticus with the vaginal openings of each oviduct. The mechanical insemination differences between the filiform glans tip of Alligatoridae and the blunt terminus seen in Crocodylidae are yet to be studied between these clades, along with corresponding morphological and/or functional differences in female cloacal structure and vaginal openings. Although many of the species-specific details of insemination mechanisms still require further functional clarification, the sequence of the copulatory process and related functions of male phallic and female cloacal structures allowing insemination are conserved among crocodilian taxa.

At the time of ovulation, a sharp decrease in plasma E₂ and a rise in plasma P₄ are observed in female crocodilians, and elevated plasma P₄ concentration is maintained throughout the period of gravidity (Guillette et al., 1997; Lance, 1989; Lance et al., 2009; Portelinha et al., 2015). In *A. mississippiensis*, granulosa cells associated with the newly formed corpora lutea show intense 3 β -HSD staining (Lance, 1989), and plasma P₄ concentrations are positively correlated with corpora luteal volume (Fig. 5) (Guillette et al., 1995). Elevated P₄ is also closely associated with insulin-like growth factor 1 (IGF-I) and oviductal secretions during the 3 weeks of gravidity (Fig. 6). Plasma concentrations of IGF-I are greatest at this time and both IGF-I and insulin-like growth factor 2 (IGF-II) have been identified in the albumen of *A. mississippiensis* (Guillette et al., 1996; Guillette & Williams, 1991). Elevated IGF-I mRNA

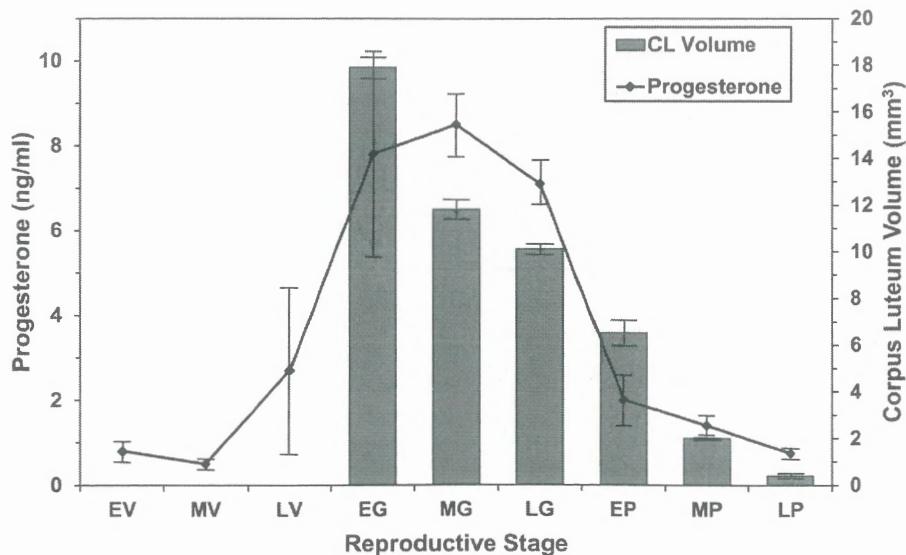


FIG. 5 Mean (± 1 standard error) plasma progesterone and corpora luteum volume during early (E), mid (M), and late (L) vitellogenesis (V), gravidity (G), and postoviposition (P) in female *Alligator mississippiensis*. (Data taken from Guillette Jr., L. J., Woodward, A. R., You-Xiang, Q., Cox, M. C., Matter, J. M., & Gross, T. S. (1995). Formation and regression of the corpus luteum of the American alligator (*Alligator mississippiensis*). Journal of Morphology, 224(1), 97–110 and Guillette Jr., L. J., Cox, M. C., & Crain, D. A. (1996). Plasma insulin-like growth factor-I concentration during the reproductive cycle of the American alligator (*Alligator mississippiensis*). General and Comparative Endocrinology, 104(1), 116–122.)

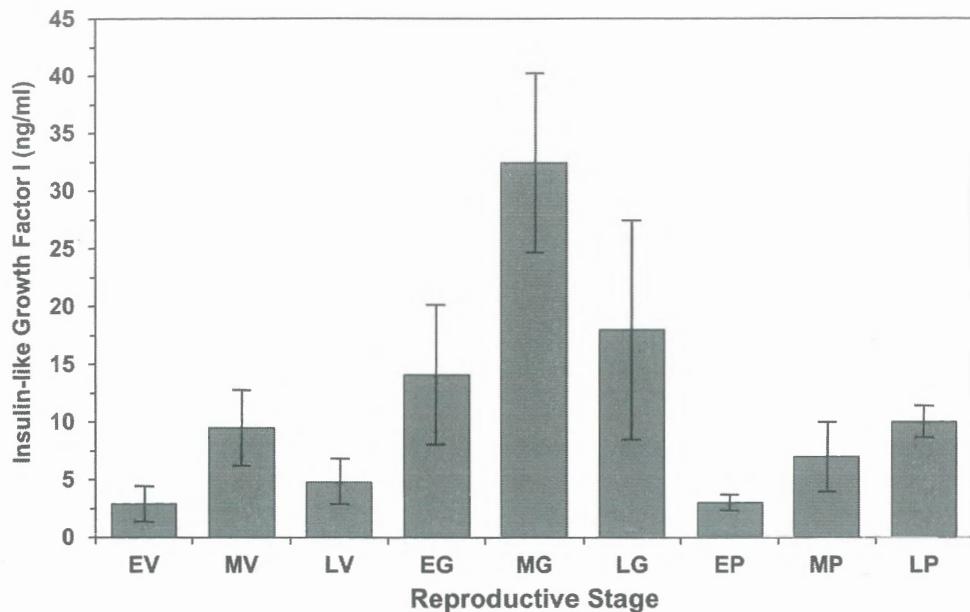


FIG. 6 Mean (± 1 standard error) plasma insulin-like growth factor I (IGF-I) during early (E), mid (M), and late (L) vitellogenesis (V), gravidity (G), and postoviposition (P) in female *Alligator mississippiensis*. (Reproduced with permission from Guillette Jr., L. J., Cox, M. C., & Crain, D. A. (1996). Plasma insulin-like growth factor-I concentration during the reproductive cycle of the American alligator (*Alligator mississippiensis*). General and Comparative Endocrinology, 104(1), 116–122.)

expression has also been reported in the oviducts of *A. sinensis* in the active season in comparison to hibernation (Zhu et al., 2017). Immunolocalization of IGF-I to tubal glands of the oviduct that secrete albumen indicates that this hormone is synthesized locally for incorporation into the egg and suggests a role for maternal growth factors in embryonic development (Cox & Guillette, 1993).

The bifurcated oviduct of crocodilians consists of the infundibulum, tube, utero-tubular junction or isthmus, anterior and posterior uterus, and vagina (Bagwill et al., 2009; Gist et al., 2008; Palmer & Guillette, 1992). The anterior infundibulum is funnel shaped, opens to the coelom, and has thin transparent walls. The posterior infundibulum is tubular and consists of muscular walls with mucosal foldings and secretory cells. The occurrence of branched acinar glands distinguishes the tube from the posterior infundibulum. Immunocytochemical data and de novo synthesis in tissue explants suggest that both the posterior infundibulum and tube function in albumen formation (Buhi et al., 1999; Palmer & Guillette, 1991). Thin, translucent walls and a lack of mucosal glands distinguish the utero-tubular junction. Both functionally and ultrastructurally, the anterior uterus of the alligator resembles the avian isthmus in which eggshell membrane formation takes place. Endometrial glands in the anterior uterus secrete proteinaceous fibers similar in structure and diameter to those of the eggshell membrane (Palmer & Guillette, 1992). The posterior uterus is histologically similar to the avian shell

gland, which secretes the calcareous eggshell. Eggs are retained in this portion of the crocodilian uterus for most of the gravidity, where eggshell calcification takes place simultaneously on the entire clutch.

It is unclear exactly where fertilization takes place or when in temporal relation to ovulation, but during and shortly after the mating season, sperm are found in the lumen of the vagina near the opening of deep mucosal folds, and aggregates of sperm are found within tubular glands located near the tubal-isthmus and uterovaginal junctions (Bagwill et al., 2009; Gist et al., 2008). Across taxa, crocodilian sperm share many conserved morphological features; however, features of the sperm midpiece differ between Alligatoridae and Crocodylidae (Gribbins et al., 2011). This divergence aligns with comparable variation in penile glans structure between these clades, as discussed in Section 2.1.2. Direct evidence of female-induced sperm capacitation in crocodilians is lacking, but exposure of ejaculated *C. porosus* spermatozoa to media formulated to achieve capacitation in mammals increases cyclic-AMP-dependent motility and results in differential phosphorylation of proteins in capacitated versus noncapacitated spermatozoa (Nixon et al., 2016, 2019).

Oviposition in crocodilians is marked by a sharp decline in plasma P₄ concentration and corpora luteal volume. The rate of luteolysis slows after the first month of postoviposition in *A. mississippiensis*, and persistent remnants of the corpora lutea in the fall can be used as an indicator that ovulation

occurred the previous spring (Guillette et al., 1995). Hormonal control of oviposition is poorly understood, but limited experimental data suggest it is similar to other vertebrates. For instance, injection of oxytocin has been used to treat dystocia and induce oviposition in *C. porosus* (Carmel, 1991). Arginine vasotocin (AVT), the homolog of mammalian oxytocin, stimulates inositol triphosphate (IP₃) production in *A. mississippiensis* uterine endometrium in vitro. Stimulation of IP₃, a secondary messenger that stimulates prostaglandin (PG) production in mammals, is greatest in uterine endometrium from late gravid females (Mirando & Guillette, 1991). In birds, PGs inhibit P₄ production in corpora lutea, stimulate uterine contractions, and relax the uterovaginal sphincter near the time of oviposition (Hertelendy et al., 1984). Circulating PG concentrations have not been reported in any crocodilian, but PGF_{2α} and PGE₂ are secreted by the uterus, and secretions are greatest in postoviposition animals (Dubois & Guillette, 1992). Oddly, AVT inhibits PG secretion in uterine explants, indicating that more work is required to fully understand this aspect of crocodilian reproduction.

4 CURRENT AND FUTURE RESEARCH DIRECTIONS

Of the 23 crocodilian species recognized by the International Union for Conservation of Nature and Natural Resources (IUCN), 7 are listed as critically endangered and 4 are listed as vulnerable (IUCN, 2023). In addition to threats from habitat loss and modification, crocodilians are at risk from exposure to and bioaccumulation of persistent pollutants that make their way into aquatic systems (see Chapter 13 in this volume). A better understanding of crocodilian reproductive biology will render conservation-oriented breeding programs more effective and improve our ability to assess detrimental effects of ecosystem disturbances on reproduction.

Refinements in assisted breeding technologies (ABT) are establishing innovative farming practices and have the potential to augment endangered species breeding efforts (Johnston et al., 2021). Semen collection and preservation techniques have been characterized and refined in *C. porosus* (Fitri et al., 2018; Johnston et al., 2014). In addition to providing detailed semen characteristics, these efforts provide the material required to improve artificial insemination efforts. The most recent successes in ABT have been related to male gamete collection and cryopreservation. The next challenge is developing techniques for minimally invasive monitoring of the female reproductive cycle and increasing our understanding of cloacal functional anatomy and postinsemination gamete dynamics to successfully produce offspring in assisted breeding settings.

At the forefront of recent advances in crocodilian reproductive biology is the application of advanced molecular biology techniques and the ever-growing database of crocodilian genome and transcriptome sequences. The genomes of *A. mississippiensis*, *A. sinensis*, *C. porosus*, and *G. gangeticus* have been sequenced and published (Green et al., 2014; Wan et al., 2013). This information has shed light on the evolutionary relationships between the major clades within Crocodylia and the relationship of crocodilians to other amniotes, thus providing important context for comparisons among taxa (St John et al., 2012). Additionally, whole genome sequences provide greater context for the analyses of transcriptomes. Rice et al. (2017) used a contiguous assembly of the *A. mississippiensis* genome to predict regions enriched for genes with female-biased expression based on proximity to ESR1 or zinc finger protein CTCF-binding sites. Female-biased genes are more likely to be found in proximity to the predicted estrogen-responsive regions based on RNA sequencing (RNA-seq) of gonads from embryos incubated at female-producing temperatures. RNA-seq analyses of embryonic gonads from *A. mississippiensis* and *A. sinensis* eggs incubated at male- and female-producing temperatures revealed hundreds of differentially expressed genes (DEGs) associated with sexual differentiation (Lin et al., 2018; Yatsu et al., 2016). These analyses not only reconfirmed the differential expression of numerous genes long associated with sexual differentiation (e.g., *AMH*, *SOX9*, *FOXL2*, *DMRT3*), but also identified genes not previously linked to sexual development and implicate micro-RNA (miRNA) and noncoding RNA (ncRNA) as potential mediators of sexual differentiation (see Chapter 1 in this volume). Similar approaches are revealing transcriptomic variation associated with seasonal changes in activity. Zhang, Cai, et al. (2020) characterized DEGs, miRNA, and ncRNA in the hypothalamus, liver, and skeletal muscle in a comparison of hibernating versus active season *A. sinensis*. Numerous DEGs associated with biological rhythms in mammals were identified in the hypothalamus, providing insight into the transduction of environmental signals into changes in activity. Future comparisons of crocodilian transcriptomes from reproductive and endocrine tissues are likely to reveal novel regulatory mechanisms and effectors of physiological and anatomical changes associated with annual reproductive cycles.

5 RESEARCH GAPS

Significant advances in our understanding of crocodilian reproductive endocrinology have been made since the first edition of this chapter was published, but some of the same gaps in our knowledge still exist. For instance, egg incubation temperature is now known to affect posthatching growth rates (Piña et al., 2007), but how? Bock et al.

(2022) showed that incubation temperature affects genome-wide DNA methylation in hatchling blood cells independent of gonadal sex and differential methylation is tissue specific. Does incubation temperature lead to nongenomic effects that affect the expression of thyroid hormones, growth factors, hormone receptors, synthetic or degrading enzymes, etc.? Ontogenetic changes related to posthatching sexual differentiation, puberty, and the onset of reproductive maturation and senescence are also only partially understood. The characterization of pituitary GTH secretion in relation to variation in life history stages and the annual reproductive cycle of adults is needed. As a taxonomic group of long-lived species, a better understanding of changes in peripheral hormone receptor expression related to responsiveness to hormone stimulation would make crocodilians an interesting model for reproductive aging. Finally, what environmental stimuli have the greatest impact on the frequency and success of reproduction, particularly for tropical species and species with broad latitudinal ranges? As environments are altered by pollution and global climate change, the ability to predict the impact of those changes on reproduction and evolution in crocodilians will enhance future conservation efforts.

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Chapter 11

Hormones and reproductive cycles in lizards

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ABBREVIATIONS

AND	androstenedione
CORT	corticosterone
DHT	dihydrotestosterone
E ₂	17 β -estradiol
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
GTH	gonadotropin
HPG	hypothalamic-pituitary-gonadal
LH	luteinizing hormone
P ₄	progesterone
T	testosterone
TSD	temperature-dependent sex determination

1 INTRODUCTION

1.1 Why study lizards?

It is common for researchers to extol the virtues of their model organism, and those of us who work with lizards are no exception. But, why study lizards? One valid argument is that understanding the world around us without need for an immediate application or justification of that knowledge is worthwhile. However, there additionally are advantages of working with lizards in particular, and benefits that come from studying their reproductive cycles, and they fall into one of three general categories: practical considerations; evolutionary considerations; and environmental and conservation considerations.

1.1.1 Practical considerations

There are numerous practical considerations that make lizards good study organisms. Many species are relatively easy to observe repeatedly in the field because of their conspicuous reproductive morphology and behavior, comparatively restricted space use and fidelity, and terrestrial lifestyle. Additionally, many species are relatively small and pose little to no threat to the investigator (e.g., in terms of bites or other injury-causing behaviors, use of venoms or

toxins, etc.), thus making them amenable to capture and handle for measurement and manipulation. One practical trade-off to relatively small body size, however, is that it often is not feasible to conduct repeated-measure designs on hormone profiles in the blood given the volume of blood drawn vs. total blood volume and presumed effects on subsequent hormone profiles, although sufficiently large lizards, sufficiently small samples, time between samples, or other, empirically based justifications may allow for it (e.g., Husak et al., 2009; Rhen et al., 2000; Smith & John-Alder, 1999; Wack et al., 2008). Many species also can be maintained in the laboratory in naturalistic enclosures that promote the full range of reproductive behaviors seen in the field, permitting integrative studies using both field and laboratory approaches. Finally, due to comparatively high population densities for many lizard species, laboratory colonies can be maintained and supplemented with easily obtained wild stock as necessary to prevent unwanted genetic separation over successive generations in the laboratory. Overall, these practical considerations give us the ability to examine endogenous hormone profiles, manipulate hormone levels, and examine hormone—reproductive cycle relationships while maintaining both high internal and external validity of study design and ecological relevance (Miller, 1994).

1.1.2 Evolutionary considerations

Reptiles are a diverse and ancient assemblage of species, nearly global in distribution, that yield insight into present-day adaptations and historical traits that made terrestrial living possible over 300 million years ago. The extant amniotes—taxa possessing extraembryonic membranes that form early in development and allow the transition from an aquatic to a terrestrial environment—are a monophyletic clade comprised of reptiles (including birds, which are nested within Reptilia) and mammals that arose from a common, reptile-like ancestor 200–300 million years ago (Pough et al., 2004).

Given high species diversity and widespread geographic distribution, it is not surprising that lizards show variety in their reproductive biology and associated life histories as the following considerations illustrate. First, although most lizard species are oviparous, approximately 20% of species are viviparous (Blackburn, 1982, 1985). Viviparity has evolved independently in approximately half of all lizard families (reviewed in Pianka & Vitt, 2003), multiple times within the same genus (e.g., *Sceloporus*; Benabib et al., 1997; Méndez-De La Cruz et al., 1998), and a few species even show oviparity or viviparity depending on location within the species range (Whittington, 2021). This currently is an exciting area of study; our understanding of the physiology, genetics, and evolution of viviparity is expanding rapidly (e.g., Blackburn, 2015; Foster et al., 2020).

Second, typical clutch sizes can vary tremendously across oviparous lizard species. Some species exhibit one-egg (e.g., *Anolis*; Smith et al., 1973) or two-egg clutches (e.g., many geckos including *Eublepharis*; LaDage et al., 2008) produced repeatedly over the course of a breeding season, whereas other species produce clutches averaging approximately 20–30 eggs produced once per breeding season (e.g., *Phrynosoma*; Ballinger, 1974; Endriss et al., 2007). Within species, female body size can play a significant role in determining clutch size. For example, Nile monitor lizards (*Varanus niloticus*) average 21 eggs per clutch overall, but small females average less than 15 eggs per clutch whereas large females average over 50 (de Buffrénil & Rimblot-Baly, 1999).

Third, there is considerable diversity in how reproductive effort is allocated over the lifespan. Males and females of some lizard species take several years to become reproductively mature but then reproduce across multiple years over a comparatively long lifespan (20+years in some cases), whereas in other species maturation occurs within the year of birth or hatching and reproduction is limited to one or a few breeding events (Pianka & Vitt, 2003; Shine & Charnov, 1992; Tinkle et al., 1970). In general, high probability of annual adult survival is related to slow reproductive maturation and low annual fecundity, whereas low probability of annual adult survival is related to fast maturation and high annual fecundity. In an extreme case that appears to represent the shortest lifespan for a tetrapod, the chameleon *Furcifer labordi* lives only 4–5 months outside of the egg (in contrast to 8–9 months as an embryo) during which time growth to sexual maturity, mating, and oviposition by females must occur before senescence and death take place in the entire adult population (Hudel & Kappeler, 2022; Karsten et al., 2008).

Fourth, sex determination is genetically controlled in most species of lizards, similar to the large majority of vertebrate

species overall. However, temperature-dependent sex determination (TSD), in which the incubation temperature experienced by the egg is the trigger for sexual differentiation into male (typically intermediate temperatures) or female (typically low and high temperatures), occurs in many lizard species, distributed across at least five lizard families—Iguanidae, Agamidae, Eublepharidae, Gekkonidae, and Lacertidae (Viets et al., 1994; see also Chapter 1, this volume).

Fifth and even more fundamental than variation in sex-determining systems, lizard species differ in whether they reproduce sexually or asexually. Although most lizards reproduce sexually, there are approximately 30 all-female species that reproduce asexually by parthenogenesis (Pianka & Vitt, 2003). Parthenogenetic species occur in at least seven lizard families (Agamidae, Chameleonidae, Gekkonidae, Gymnophthalmidae, Lacertidae, Teiidae, and Xantusiidae) and have arisen as the result of sexual reproduction between two parental species (Godwin & Crews, 2002; Pianka & Vitt, 2003).

Lizards are a rich taxonomic group for integrative and comparative studies in part due to the diversity of life-history and reproductive traits outlined earlier. Adding to their appeal is the fact that both shared and novel hormonal targets and mechanisms have been documented in the process of investigating the endocrine regulation of these traits. Many of the hormones, e.g., the sex steroids testosterone (T) and 17 β -estradiol (E₂), are identical in structure and importance across amniotes, and they modulate activity of homologous central and peripheral targets via structurally similar or identical receptors; this conservation of endocrine mechanisms is amply supported in Section 2.3. However, work on lizards frequently has revealed novel relationships, as well, between hormones, their targets and mechanisms, and association (or lack thereof) with specific behavioral and physiological events. For example, the study of lizards in the laboratory and field has helped to reveal that large taxonomic differences exist in hormone concentrations found in the plasma and associated responsiveness to these hormones, that hormones and the behaviors or processes they regulate do not have to be temporally associated, and that hormones previously thought to be primarily important in one sex often play an equally important role in the opposite sex, e.g., the role of progesterone (P₄) not only in female but also in male sexual behavior (reviewed in Crews & Moore, 2005; Godwin & Crews, 2002). Indeed, such phenomena first documented in lizards often lead to the identification of previously unrecognized but fundamental regulatory pathways and endocrine relationships in the other amniotes (Godwin & Crews, 2002).

1.1.3 Environmental and conservation considerations

Amphibians as well as reptiles are integral components of many ecosystems and our study of these groups contributes much to our understanding of organismal biology overall. Unfortunately, there is good evidence that many amphibian and reptile populations, including lizards, are indeed declining, a striking fact considering how little is known about the status of the majority of species to begin with (Pianka & Vitt, 2003). Furthermore, the decline in reptile populations appears to be at least on par with that of the more widely reported amphibian declines (Gibbons et al., 2000; Whitfield et al., 2007). Numerous factors are involved: habitat alteration and loss (e.g., due to deforestation, conversion to agricultural land, development), human population growth, release of pollutants and agrichemicals into the environment, negative impacts of nonnative species, disease and parasitism, and overcollecting all have been implicated depending on the taxon in question (reviewed in Gibbons et al., 2000; Pianka & Vitt, 2003). Additionally, studies continue to support the idea that global climate change negatively impacts amphibian and reptile populations, again including lizards, across a variety of geographic locations and habitats (Huey et al., 2009; Kearney et al., 2009; Pounds et al., 2006; Sinervo et al., 2010; Whitfield et al., 2007; but see Araújo et al., 2006; Cosendey et al., 2023; Rohr et al., 2008).

Efforts to monitor and protect amphibian and reptile populations are important simply because preserving biodiversity is a worthy goal. However, as a strictly practical consideration, continued study and monitoring of these organisms may allow us to use them as indicators of environmental stability and health in the face of anthropogenic disturbance leading to environmental change. In this light, the study of reproductive cycles and their endocrine regulation will become increasingly important as many amphibians and reptiles are sensitive to environmental cues, such as temperature, for determining the onset of breeding or even the resulting sex ratios of offspring in the case of species with TSD. Indeed, effects of warming on the onset of breeding and offspring sex ratios already have been demonstrated (e.g., Kusano & Inoue, 2008; Mitchell et al., 2008; Schwanz & Janzen, 2008; Wapstra et al., 2009). Additionally, amphibians and reptiles are susceptible to pollutants such as endocrine disruptors that interfere with hormonal and developmental processes, thus rendering breeding a particularly affected stage of the life cycle (e.g., Hayes et al., 2006; Orlando & Guillette Jr., 2007; Willingham & Crews, 2000; reviewed in Marlatt et al., 2022). Knowledge of “baseline” breeding patterns and their hormonal regulation will allow us to identify when disturbance is present as well as take advantage of potentially unique opportunities for uncovering novel hormone-reproduction pathways.

1.2 Overview for remainder of chapter

The remainder of this chapter reviews lizard reproductive cycles and their endocrine regulation. First, the seasonality of reproduction, which synchronizes breeding events with environmental conditions most amenable to reproductive success, will be discussed (Section 2.1). This will be followed by a brief review of the general patterns of reproductive cycles that have been revealed through studies of hormones, physiology, and behavior (Section 2.2). The role of hypothalamic, pituitary, and gonadal hormones in regulating reproductive cycles will then be considered in detail, examining available data across a majority of extant lizard families (Table 1, Section 2.3). Throughout this review, the emphasis will be on information that has become available in the time since several excellent reviews of lizard (or more generally reptilian) reproductive physiology were published (primarily Crews, 1979, 1980; Duvall et al., 1982; Licht, 1984; Moore & Lindzey, 1992; Whittier & Crews, 1987; Whittier & Tokarz, 1992). More specifically, the current chapter will add to the review of Lovorn (2011), weighted especially in the areas of less well-represented taxa where possible.

TABLE 1 Classification, description, and approximate number of genera and species of lizard^a.

Family	Description	No. genera (species)
Agamidae	Agamas, common lizards, frill-necked lizards, bearded dragons, flying dragons, jacky dragons	58 (582)
Alopoglossidae	Largescalce lizards, shade lizards	1 (32)
Amphisbaenidae	Worm lizards	12 (183)
Anguidae	Glass lizards, alligator lizards	10 (87)
Anolidae ^b	Anoles	1 (440)
Bipedidae	Two-legged worm lizards	1 (3)
Blanidae	Worm lizards	1 (7)
Cadeidae	Worm lizards	1 (2)
Carphodactylidae	Geckos	7 (34)
Chamaeleonidae	Chameleons	12 (228)
Cordylidae	Spinytail lizards	10 (69)
Corytophanidae ^b	Basilisks, casque-headed lizards	3 (11)

Continued

TABLE 1 Classification, description, and approximate number of genera and species of lizard—cont'd

Family	Description	No. genera (species)
Crotaphytidae ^b	Collared lizards, leopard lizards	2 (12)
Dibamidae	Blind lizards	2 (25)
Diplodactylidae	Geckos	26 (192)
Diploglossidae	Galliwasp, glass lizards	12 (56)
Eublepharidae	Geckos	6 (47)
Gekkonidae	Geckos	60 (1576)
Gerrhosauridae	Plated lizards	6 (38)
Gymnophthalmidae	Spectacled lizards	53 (288)
Helodermatidae	Gila monster, beaded lizard	1 (5)
Hoplocercidae ^b	Wood lizards, clubtails	2 (20)
Iguanidae	Iguanas	9 (44)
Lacertidae	Wall lizards	38 (373)
Lanthanotidae	Borneean earless monitor lizard	1 (1)
Leiocephalidae ^b	Curly-tailed lizards	1 (29)
Leiosauridae ^b	Anole-like lizards	5 (35)
Liolaemidae ^b	Ground lizards	3 (342)
Opluridae ^b	Madagascar ground lizards	2 (8)
Phrynosomatidae ^b	Earless lizards, tree lizards, side-blotched lizards, spiny lizards, and horned lizards	9 (172)
Phyllodactylidae	Geckos	10 (164)
Polychrotidae ^b	Anoles	1 (8)
Pygopodidae	Geckos (legless lizards)	7 (47)
Rhineuridae	Worm lizards	1 (1)
Scincidae	Skinks	156 (1741)
Shinisauridae	Crocodile lizard	1 (1)
Sphaerodactylidae	Geckos	12 (231)
Teiidae	Whiptails and tegus	18 (173)
Tragonophidae	Shorthead worm lizards	4 (6)
Tropiduridae ^b	Neotropical ground lizards	8 (146)
Varanidae	Monitor lizards	1 (86)

TABLE 1 Classification, description, and approximate number of genera and species of lizard—cont'd

Family	Description	No. genera (species)
Xenosauridae	Knob-scaled lizards	1 (14)
Xantusiidae	Night lizards	3 (38)
Total		552 (7597)

^aUetz et al. (2023).^bFamilies that were previously considered subfamilies of Iguanidae.

2 REPRODUCTIVE CYCLES

2.1 Seasonality of reproduction •

Classic life-history theory describes variation in growth, reproduction, survival, and aging with the goal of understanding how energy is invested to maximize reproductive success (Stearns, 1992, 2000). Critically, energy is limiting, such that energy used for reproduction cannot be used for growth or maintenance and vice versa; thus trade-offs can exist between growth, maintenance, and reproduction in that energy allocation that benefits one represents energy loss to the detriment of the others (Stearns, 1989), although not all tissues need be maintained at a constant cost year-round. For example, hibernation can greatly reduce energetic demands both overall and targeted toward specific organ systems (e.g., Naya et al., 2008; Zena et al., 2020). Finally, reproduction is costly; the nature of the costs can vary widely across species and may include increased mortality due to injury or predation as well as direct metabolic costs of reproduction (Crews, 1998; Shine, 1980; Whittier & Crews, 1987). Typically, males expend energy on reproduction via higher activity levels during times of breeding to secure reproductive access to females (e.g., territory maintenance, direct aggressive interactions with other males). For example, experiments using a field population of mountain spiny lizards (*Sceloporus jarrovii*) have demonstrated that T implants given to breeding males lead to increased aggressive activity as well as mortality (Marler & Moore, 1988, 1989). Further work demonstrated that this increased mortality was most likely due to associated high energy demands rather than predation, as food supplementation caused T-implanted males to survive as well as control males (Marler & Moore, 1991). In contrast to males, energy expenditure by females primarily is in the form of allocation to eggs or developing young in the case of viviparous species. For example, whereas male green anoles (*Anolis carolinensis*) expend approximately 48% of their total metabolizable energy on reproduction, females expend

approximately 66% on reproduction, including 36% specifically on egg production (Orrell et al., 2004). Other studies examining energy allocation by females to their eggs have found that it ranges from approximately 10% to 25% of the energy budget per clutch (reviewed in Whittier & Crews, 1987). Although data are limited for lizards overall, it is found that females in general allocate more energy to reproduction than do males (reviewed in Orrell et al., 2004).

As discussed earlier, reproduction is costly and utilizes resources that could be allocated to other necessary functions such as growth and maintenance. Therefore, it is not surprising to find that reproduction typically occurs when the environment is most amenable to offspring survival and when parents are able to take on the physiological demands of reproduction at the least cost to themselves. In many cases, this leads to seasonal reproduction, i.e., a restricted time of year when individuals are breeding. Seasonal reproduction is widespread for lizard species in temperate zones (Fitch, 1970; Licht, 1984; Pianka & Vitt, 2003), in which climate produces well-defined seasons and lizards typically mate in the spring and offspring hatch or are born in the summer. This coordinates reproduction with the time of year providing the necessary sunlight, heat, moisture, and/or availability of food resources necessary for offspring production and survival (Duvall et al., 1982; Rubenstein & Wikelski, 2003; Whittier & Crews, 1987). Although most temperate zone lizards reproduce in the spring, nearly all lizards of the genus *Sceloporus* that are viviparous (~40% of the genus) breed in the fall and give birth in the late spring or early summer, this timing presumably associated with increased survival and reproductive opportunities for offspring (reviewed in Guillette Jr. & Méndez-De La Cruz, 1993; Méndez-De La Cruz et al., 1998). Even within-species variation in the seasonality of reproduction can occur, as is the case for the brown anole, *Anolis sagrei*, in which Florida populations show a strong seasonality in reproduction consistent with temperate zone climate (Lee et al., 1989), but tropical Caribbean (Licht & Gorman, 1970; Sexton & Brown, 1977) and Hawaiian (Goldberg et al., 2002) populations show a less pronounced seasonality in which reproductively active individuals can be found throughout the year.

The fact that most lizard species in temperate zones have restricted time periods of reproduction does not mean that lizards living in tropical environments—in which moisture, day length, and temperature may be amenable to reproduction year-round—reproduce continuously. In fact, the diversity of reproductive patterns seen in tropical lizards nicely demonstrates that generalizations from temperate zone species do not necessarily apply to lizards in other habitats. For example, among species of tropical lizards in Australia (genera *Carlia*, *Cryptoblepharus*, *Ctenotus*, *Morosia*, *Sphenomorphus*, *Diporiphora*, and *Gemmatophora*),

some breed during the wet season, some during the dry season, and other species breed across both seasons (James & Shine, 1985). Embryonic moisture tolerance and phylogenetic constraints appear to be more likely factors than embryonic thermal tolerance or resource availability contributing to these broad differences in breeding (James & Shine, 1985).

Additional factors may complicate the determination of the seasonal nature of breeding, or lack thereof, and the environmental correlates for a given species. First, males and females may have evolved to respond to different environmental features to cue reproductive activity. For example, in the Mexican gecko *Phyllodactylus lanei*, the male gonadal cycle cues to photoperiod, but the female gonadal cycle cues to mean monthly temperature, producing overlapping but not identical durations of reproductive activity for males and females (Ramírez-Sandoval et al., 2006).

Second, although some populations of lizards may be described as breeding continuously, this description does not necessarily apply to the individual nor does it necessarily apply equally across the sexes. This is because no individual within the population is breeding year-round, although at any time within the population breeding individuals can be found. Such is the case for many tropical lizard species, e.g., the anoles *Anolis acutus* (Ruibal et al., 1972), *Anolis limifrons* (Sexton et al., 1971), and *Anolis opalinus* (Jenssen & Nunez, 1994), as well as the gecko *Cyrtodactylus malyanus* and the flying lizard *Draco melanopogon* (Inger & Greenberg, 1966), and the parthenogenetic, oviparous whiptail lizard *Cnemidophorus nativo* (Menezes et al., 2004). In these examples, the proportions of breeders are not equal across all 12 months or between the sexes (when two sexes exist). In general, slightly more breeders are present during the wet than during the dry season, and males are more likely to be gametogenic during the dry season than females, largely due to moisture requirements for eggs (e.g., Jenssen & Nunez, 1994). Reproductive patterns in these species can be described as cyclic for the individual but asynchronous for the population (Crews, 1998).

Third, some species may breed seasonally, but not necessarily annually, as is the case when breeding occurs at a particular time each year for a population but individuals—typically females—with the population do not breed each year, a phenomenon known as multiennial reproduction. This pattern may result when individuals skip a breeding season, or because it takes longer than a year to complete egg production or gestation. Multiennial reproduction occurs in a variety of lizards, including the viviparous New Zealand gecko, *Hoplodactylus maculatus* (Cree & Guillette Jr., 1995), the oviparous Argentinian gecko, *Homonota darwini* (Ibargüengoytía & Casalins, 2007), viviparous ground

lizards, *Phymaturus patagonicus* (Ibargüengoytía, 2004), *Phymaturus antofagastensis* (Boretto & Ibargüengoytía, 2006), *Phymaturus punae* (Boretto et al., 2007), *Liolaemus pictus* (Ibargüengoytía & Cussac, 1996), and the viviparous blue-tongued skink, *Tiliqua nigrolutea* (Edwards et al., 2002). The species in the examples mentioned earlier occur on different continents but share the constraint of living in relatively cool environments that result in short activity periods and a suite of life-history traits adapted to survival and successful reproduction in such a climate (e.g., Adolph & Porter, 1996). In fact, populations of the geographically widespread *Hoplodactylus maculatus* that live in warmer regions produce offspring annually rather than biennially (Cree & Guillette Jr., 1995).

Fourth, although there are many examples of phenotypic flexibility, it is clear that the phylogenetic history of lizard species can be a critical influence, even constraint, on reproductive pattern, such that species sharing a close evolutionary history but living in very different environments may have more similar reproductive patterns than species sharing a common environment but which are more evolutionarily distant. Whether breeding occurs in the wet season, dry season, or both differs based on phylogeny rather than converges based on habitat in Australian and Brazilian tropical skinks, agamids, and teiids (reviewed in Pianka & Vitt, 2003). Thus, environmental cues frequently are critical to successful reproduction, but environmental considerations alone will not explain the full range of reproductive patterns seen in lizards. Lizards from one taxon may reproduce very differently than lizards from a different taxon, even within a common environment.

2.2 Types of reproductive cycle

Reproductive cycles, regardless of the extent to which they are seasonal, can be described in a variety of ways. Work by Fitch and colleagues extensively cataloged reproductive cycles in lizards and snakes based on mode and degree of seasonality of reproduction, size at reproductive maturity, fecundity, and other life-history-based assessments (e.g., Fitch, 1970). Licht, Saint Girons, and their respective colleagues were among the first to systematically characterize reproductive cycles based on relationships among mating behavior, sex steroid production, and gametogenesis (summarized in Licht, 1984; see also Saint Girons, 1963). Although it was acknowledged that not all species fit neatly into such categorization, two general types of reproductive cycles were recognized—prenuptial and postnuptial cycles (Type I and Type II reproductive cycles, respectively, as reviewed in Lance, 1998a, 1998b). Prenuptial reproductive cycles are those in which gonadal recrudescence, sex steroid production, and gametogenesis occur in advance of mating whereas in postnuptial cycles they occur following mating.

The potential relationships among mating behavior, sex steroids, and gamete production were further generalized by Crews (1984). Associated reproductive patterns were defined as those for which maximal sex steroid secretion and gametogenesis immediately preceded or coincided with mating behavior, roughly equivalent to the prenuptial cycles discussed earlier. In contrast, dissociated reproductive patterns were defined as those for which mating behavior is uncoupled from maximal sex steroid secretion and gamete production, similar to postnuptial cycles discussed earlier. Around the time of publication of Crews (1984), the large majority of work in reproductive endocrinology had been conducted on mammalian species, principally laboratory rodents, that have an associated reproductive pattern. This resulted in what was perhaps a too narrowly restricted view of the relationship between hormones and reproduction. The paper by Crews (1984) has been highly influential and has stimulated investigation of reproductive patterns in reptiles, amphibians, and other “nontraditional” vertebrate model organisms.

With respect to the framework proposed by Crews (1984), most lizard species studied to date exhibit an associated reproductive pattern, as the available data support a positive relationship between peak gametogenesis, sex steroid production, and mating behavior for both males and females. This is true for virtually all the widely studied species (Table 2). But, there are exceptions. Several species of viviparous sceloporines, e.g., *Sceloporus formosus*, *Sceloporus malachiticus*, and some populations of *Sceloporus grammicus* and *Sceloporus mucronatus*, exhibit dramatic sex differences in the onset of gametogenesis, in which testicular recrudescence occurs in the spring but ovarian recrudescence does not begin until the fall and the timing of mating determines which sex is associated/dissociated (Guillette Jr. & Méndez-De La Cruz, 1993; Méndez-De La Cruz et al., 1998). Timing of mating is highly variable among species and even among populations of the same species. In a high elevation population of *Sceloporus grammicus* in Parque Nacional de Zoquipan in central México, breeding occurs in the early fall, and thus is dissociated from testicular recrudescence in males but associated with the initiation of ovarian recrudescence in females (Guillette Jr. & Casas-Andreu, 1980, 1981; Zúñiga-Vega et al., 2008). This contrasts with *Sceloporus grammicus* from Teotihuacán, México, in which testicular recrudescence and breeding occur in the summer and fall, at the onset of female ovarian recrudescence (Jiménez-Cruz et al., 2005). In *Sceloporus mucronatus* from Valle de la Cantimplora, México, peak testicular recrudescence and mating occur during the summer, prior to ovarian recrudescence which does not occur until several months later (Ortega-León et al., 2009); this is distinct from many fall-breeding populations elsewhere (Villagrán-Santa Cruz et al., 1994).

TABLE 2 Examples of well-studied lizard species in which both males and females exhibit a positive association between gametogenesis, sex steroid production, and mating behavior.

Common name	Scientific name	Supporting reference(s)
Green anoles	<i>Anolis carolinensis</i>	Reviewed in Crews (1980) and Lovern et al. (2004)
Brown anoles	<i>Anolis sagrei</i>	Tokarz (1986, 1987, 1995), Lee et al. (1989), and Tokarz et al. (1998, 2002)
Little striped whiptails	<i>Aspidoscelis^a inornatus</i>	Lindzey and Crews (1986, 1988), Moore and Crews (1986), Sakata et al. (2003), and Crews (2005)
Garden lizards	<i>Calotes versicolor</i>	Reviewed in Shanbhag (2003)
Leopard geckos	<i>Eublepharis macularius</i>	Tousignant et al. (1995); Rhen et al. (1999, 2005, 2000)
Common lizards	<i>Lacerta vivipara</i>	Courty and Dufaure (1982), Uller and Olsson (2006), Heulin et al. (2008), and Vercken and Clobert (2008)
Wall lizards	<i>Podarcis sicula</i>	Andò et al. (1990, 1992), Carnevali et al. (1991), and Putti et al. (2009)
Mountain spiny lizards	<i>Sceloporus jarrovi</i>	Moore (1986, 1987) and Woodley and Moore (1999a, 1999b)
Eastern fence lizards	<i>Sceloporus undulatus</i>	Marion (1970), McKinney and Marion (1985), Klukowski and Nelson (1998), Smith and John-Alder (1999), and Cox et al. (2005)
Tree lizards	<i>Urosaurus ornatus</i>	Moore et al. (1998) and French and Moore (2008)

^aFormerly *Cnemidophorus*.

The examples mentioned earlier demonstrate that gonadal activity and mating behavior clearly are variable, but hormone analyses have not been performed in these species so endocrine relationships cannot be assessed at this point. More complete data are available for the viviparous Tasmanian skink *Niveoscincus ocellatus*. This species has a primary breeding period in the austral fall, associated with peak testis volume (and therefore spermatogenesis) and plasma T concentrations in males but dissociated from ovulation, fertilization, and pregnancy in females, which occur the following spring (Jones, Lopez, et al., 1997; Jones,

Wapstra, & Swain, 1997). Additionally, some animals mate a second time in the austral spring, associated with ovulation in females but when testis volume and T concentrations are basal in males (Jones, Lopez, et al., 1997; Jones, Wapstra, & Swain, 1997). A similar reproductive pattern is seen in another viviparous skink found in Tasmania, *Niveoscincus metallicus* (Jones & Swain, 1996; Swain & Jones, 1994), and likely *Niveoscincus coventryi*, *Pseudemoia entrecasteauxii*, and *Hemiergis decresiensis* as well (Murphy et al., 2006; hormones were not measured in this study). Thus, males and females in these skink species possess asynchronous gonadal cycles that are neither strictly associated nor dissociated.

Species that possess an associated reproductive pattern nevertheless exhibit flexibility in hormone-behavior relationships. For example, in the fall-breeding mountain spiny lizard, *Sceloporus jarrovi*, castration (and consequent removal of gonadal sex steroids like T) does not completely abolish sexual and territorial behavior (Moore, 1987), nor does exogenous T administered during the summer nonbreeding season increase territorial and reproductive behavior to levels typical of the fall breeding season (Moore & Marler, 1987). In green anoles, *Anolis carolinensis*, headbobbing-display behavior is critical to communication during courtship and, not surprisingly, display rates are highest during the April-July breeding season, associated with peak T (Jenssen et al., 1995, 2001). We also know that experimental manipulation of T directly affects display behavior; castration diminishes it and replacement T, or T given to gonadally intact juveniles, increases display behavior (Adkins & Schlesinger, 1979; Lovern et al., 2001; Mason & Adkins, 1976; Winkler & Wade, 1998). Yet, display behavior still is expressed during the nonbreeding season, when reproduction is absent and T concentrations are basal (Jenssen et al., 1996, 1995, 2001).

Given our current understanding of relationships among breeding, gonadal state, and sex steroids, what can we say of the utility of using the associated-dissociated framework for understanding reproductive cycles and their physiological correlates? Numerous authors have noted that while the majority of amphibian and reptile species studied to date fit within this framework, many clearly do not; some have viewed this situation as one in which the associated-dissociated model for describing mating patterns has general utility but numerous exceptions (e.g., Woolley et al., 2004), whereas others view the situation as one in which the model has been useful for stimulating interest in mating patterns but generally has led to confusion and obfuscation of potentially revealing mechanisms because they do not fit within the framework (e.g., Benner & Woodley, 2007; Schuett et al., 1997, 2006). Over the last several decades it has become clear that reproductive

physiology and behavior can be functionally, or at least temporally, uncoupled, that males and females of the same species may express different reproductive patterns, and that studying a diversity of species with different reproductive patterns can lead to a better understanding of the evolutionary determinants of hormone-behavior relationships.

2.3 Endocrine regulation and the hypothalamic-pituitary-gonadal (HPG) axis

The hypothalamic-pituitary-gonadal (HPG) axis is the main regulatory pathway for reproduction in male and female vertebrates, including reptiles, and it is extensively reviewed elsewhere, including Chapters 3 and 4 of this volume. Briefly, gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the release of gonadotropins (GTHs) from the anterior pituitary (adenohypophysis). In mammals, these GTHs are follicle-stimulating hormone (FSH) that primarily influences gamete development and luteinizing hormone (LH) that primarily influences sex steroid production and gamete release (Norris, 2007).

At the level of the gonads, different cell populations are responsible for different aspects of reproductive function. Like the mammalian testis, in the reptilian testis Sertoli cells of the seminiferous tubules play a direct role in spermatogenesis whereas Leydig cells outside of the seminiferous tubules are responsible for androgen production. However, unlike what is seen in mammals, in at least some reptiles it is likely that both Leydig and Sertoli cells are capable of significant androgen production and that one or the other may be the primary source of androgen depending on the time of year (e.g., Gist, 1998; Loft, 1987; Mesner et al., 1993). This may help to explain how T production in some species is maximal and associated with reproductive behavior at a time of year when testes are regressed and not producing sperm. The Sertoli cells may produce sufficient androgens that, along with upregulation of androgen receptor and production of androgen-binding proteins, maintain spermatogenesis when the testes are spermatogenetically active, whereas during testicular regression it may be the Leydig cells that are producing androgens that result in a rise in plasma androgen concentrations associated with reproductive behavior (Benner & Woodley, 2007; Gist, 1998).

In the mammalian ovary, granulosa cells of the follicular wall are responsible for egg maturation whereas thecal cells, more exterior in the follicular wall, are responsible for the initiation of sex steroid production (Norris, 2007). Thecal cells primarily synthesize androgens, most of which subsequently are converted to estrogens by the granulosa cells; granulosa cells also form the bulk of the corpus luteum following ovulation and produce P₄ (Norris, 2007). The

reptilian ovary functions very similarly to that described earlier for mammals, although squamate reptiles possess multiple granulosa cell types, one of which is unique to the group—pyriform cells, which form cytoplasmic connections to the egg in previtellogenesis follicles that degenerate once follicular development begins (Chieffi & Pierantoni, 1987; Lance, 1998a, 1998b; Norris, 2007).

In the discussion that follows, I review the taxonomic breadth of studies across a majority of lizard families that have yielded insight into the relationship between hormones and reproductive cycles in lizards. My focus is on the role that gonadal steroids play in initiating and/or maintaining reproductive condition—primarily T, E₂, and P₄. The androgen dihydrotestosterone (DHT), which results from the biochemical reduction of T, also is considered when possible. In vertebrates, plasma DHT profiles generally mirror those of T but at lower concentrations (Meisel & Sachs, 1994; Norris, 2007). Details about the role of the central nervous system in the regulation of reproduction in reptiles are considered elsewhere (Beck et al., 2008; Godwin & Crews, 2002; Wade, 2005; Chapter 2, this volume). In taxa lacking studies on hormones and reproductive cycles, I briefly review what (if any) information is available on reproductive cycles more generally, including related information on species traits that could be of interest for future studies. The order of presentation generally follows Table 1.

2.3.1 Agamidae

Agamids are a diverse group of lizards classified into 58 genera with over 500 species. Like Chamaeleonidae, a closely related family, most agamid lizards are oviparous but a few are viviparous (Pianka & Vitt, 2003). Among agamids, there has been considerable work on hormones and reproduction in the tropical or oriental garden lizard, *Calotes versicolor*. This lizard is widespread throughout India and southern Asia and is an oviparous, multiclutching, seasonal breeder (Shanbhag, 2003; Shanbhag et al., 2000). In males, plasma T is highest during the breeding season, correlated with testis mass and reproductive behavior (Radder et al., 2001). In females, plasma E₂ and P₄ are higher during the breeding season than during the nonbreeding season, and within the breeding season E₂ and P₄ were inversely related (Radder et al., 2001). Plasma E₂ peaked before ovulation and plasma P₄ peaked after ovulation. Interestingly, prior to oviposition when vitellogenesis is initiated for a subsequent clutch of eggs, plasma E₂ again is elevated and plasma P₄ concentrations decline, demonstrating that P₄ is not necessary for oviductal egg retention under normal conditions (Radder et al., 2001). However, under unfavorable environmental conditions, a secondary rise in plasma P₄ of adrenal rather than luteal origin appears to promote a cessation of vitellogenesis and extended oviductal egg retention (Shanbhag et al., 2001).

Hormone relationships with reproduction also have been studied in the Australian bearded dragons, *Pogona barbata*, and jacky dragons, *Amphibolurus muricatus* (Ameys & Whittier, 2000; Jones, 2017; Watt et al., 2003), as well as in mountain lizards, *Japalura mitsukurii formosensis* (Chen et al., 1987). In males of each species, plasma T is positively associated with reproduction. In male bearded dragons, plasma P₄ also was measured and was consistently detectable but did not vary with season. Surprisingly, in female bearded dragons, plasma E₂ may be unimportant in the reproductive cycle, as it was low or non-detectable in females across all reproductive states (nonreproductive, vitellogenic, gravid) and all months of the year. Plasma P₄ is higher during breeding than nonbreeding, with peak levels during vitellogenesis that slowly decline following ovulation. This may indicate an earlier peak in plasma P₄ than is typical for other lizards, in which plasma P₄ is highest after ovulation; such a pattern could be important particularly if E₂ is not necessary to stimulate vitellogenesis (Ameys & Whittier, 2000).

2.3.2 Alopoglossidae and Gymnophthalmidae

Also known as microteiids, these two families are comprised of over 300 species of spectacled lizards, largescale lizards, and shade lizards, and are found mostly in the tropical latitudes. They often live together in community assemblages of up to 10 species (Pianka & Vitt, 2003). As for Teiidae, some microteiids are parthenogenetic, including *Gymnophthalmus underwoodi* (Hardy et al., 1989). Terrestrial as well as fossorial niches are exploited, and activity patterns may be diurnal or nocturnal depending on the species. Numerous studies have used lizards in these families to study evolutionary ecology and phylogeny (e.g., Bars-Closel et al., 2018), but information on hormones and reproductive cycles is not available.

2.3.3 Amphisbaenidae, Bipedidae, Blanidae, Cadeidae, Rhineuridae, and Tropidophoridae

These six families of lizards are commonly known as worm lizards and total just over 200 species. Worm lizards are fossorial and in association with this environment they possess reduced vision, elongated bodies, and all but Bipedidae have lost their limbs (Bipedidae retains front limbs) (Pianka & Vitt, 2003). Most species studied thus far are oviparous (Pianka & Vitt, 2003). No endocrine studies have been conducted to date, although some work on reproductive ecology and behavior has been done (e.g., in *Anoplagonius kingii* (Vega, 2001) and *Monopeltis anchetae* and *Zygaspis quadrifrons* (Webb et al., 2000)). In the Iberian worm lizard, *Blanus cinereus*, it has been shown that chemical communication allows individuals to discriminate between males and females and that precloacal secretions of both sexes contain

numerous lipophilic compounds including steroids (Cooper Jr. et al., 1994; López & Martín, 2005). Chemosensory communication, given the fossorial lifestyle, is likely widespread among worm lizards (Martín et al., 2020). Overall, these lizard families remain understudied and data deficient (Colli et al., 2016).

2.3.4 Anguidae, Diploglossidae, and Xenosauridae

These families are comprised of 23 genera and 157 species of lizards generally known as glass lizards, legless lizards, alligator lizards, galliwasp, and knob-scaled lizards. Some species are oviparous and some viviparous; some are terrestrial and some fossorial (Pianka & Vitt, 2003). Species of several genera including *Anguis*, *Ophisaurus*, and *Diploglossus* are known to be comparatively late-maturing and long-lived for lizards (over 40 years for one captive male in the genus *Anguis*); this is probably the case for many more (Pianka & Vitt, 2003). Few studies exist on the reproductive biology of these taxa, in part because of the secretive or cryptic nature of many of the species. Reproduction is seasonal in the species studied thus far (e.g., alligator lizard, *Gerrhonotus coeruleus principis* (Vitt, 1973) and the glass snake, *Ophiodes fragilis* (Pizzatto, 2005)); in the California legless lizard, *Aniella pulchra*, breeding is seasonal but it appears that not all females breed annually (Goldberg & Miller, 1985). In studies examining regulation of female reproduction in a viviparous lizard, plasma P₄ concentrations were positively associated with corpus luteum development, peaking during early pregnancy and declining slowly for the remainder of gestation, in the viviparous, imbricate alligator lizard *Barisia imbricata imbricata* (Martínez-Torres et al., 2003, 2014). Spermiogenesis has been studied in males of this species (Gribbins et al., 2013) although hormonal regulators have not. Xenosaurids, knob-scaled lizards found in Mexico and Guatemala, are all viviparous and give birth in the summer (Pianka & Vitt, 2003; Zamora-Abrego et al., 2007); there is a possibility that neonates receive maternal care following birth through an extended time of association (Lemos-Espinal et al., 1997). The timing of reproductive events associated with conception, development, and birth (e.g., testis volume, follicle volume, embryo size) appear consistent across species (Woolrich-Pina et al., 2014). Some populations are distributed over a wide range of environments and show potential local adaptation in reproductive traits; for example, a population of *Xenosaurus platyceps* invests more in relative litter mass in a high altitude, temperate forest than in a low altitude, tropical forest (Rojas-González et al., 2008). Hormone data related to reproduction are not available.

2.3.5 Anolidae

In *Anolis*, the sole genus in Anolidae, details of the relationship between hormones and reproduction generally are well known. Reproduction is seasonal in most anoles, including the most well-studied members of the genus—the green anole (*Anolis carolinensis*) and the brown anole (*Anolis sagrei*). Males and females transition from an overwintering to a reproductive state during spring, showing increased locomotor and behavioral activity, sex steroid concentrations (T for males; E₂ and P₄ for females), gonadal recrudescence, and ultimately courtship and copulation (Crews, 1980; Jenssen et al., 2001; Jones et al., 1983; Tokarz et al., 1998). Levels of reproductive activity and steroids remain high for the duration of the 4-month breeding season, after which they decline rapidly prior to the transition into fall and overwintering (Jenssen et al., 1996, 1995; Lovern et al., 2001; Lovern & Wade, 2001; Tokarz et al., 1998). Plasma T levels in males are maximal during breeding; even GnRH injections failed to increase concentrations further in a field population of breeding male green anoles (Husak et al., 2009). Increasing temperature along with daylength are important environmental inputs for initiating reproduction (Licht, 1967, 1971, 1973). In the laboratory under high temperatures and long days, breeding can be initiated earlier and maintained for longer than environmental conditions permit in the field; however, females and eventually males will undergo gonadal regression and will stop reproductive activity regardless of photothermal regime (Licht, 1971, 1973; Lovern et al., 2004).

Reproductive activity in anoles is mediated by headbobbing displays—communication signals that are species-specific and stereotyped (Crews, 1980; Jenssen, 1977), similar to the acoustic signals of songbirds. Headbobbing displays involve vertical movement of the head associated with extension of a flexible piece of skin under the throat called a dewlap. In *Anolis carolinensis*, adult males and females possess a common display type repertoire, but the sexes differ substantially in the use of these displays. Male dewlaps are substantially larger than those of females, and they use them more often (Jenssen et al., 2000). During courtship, both males and females headbob, but male displays almost always include dewlap extension, whereas female displays almost always do not (Orrell & Jenssen, 2003). The male continues to approach the female while displaying as courtship progresses. Female receptivity typically is indicated by stationary posture, headbobbing without dewlap extension, and, sometimes, a posture in which the neck is bent forward. The male then grasps the female on the back of the neck as he everts one of his two bilateral and independently controlled hemipenes and intromits. The duration of copulation is variable, lasting anywhere from under 5 min to near 60 min (Greenberg & Noble, 1944; Jenssen & Nunez, 1998).

Sex differences in reproductive behavior, as described earlier, are paralleled by sex and seasonal differences in endogenous steroid levels. Plasma T levels are elevated in males during the breeding season as compared to the non-breeding season (20–25 ng/mL breeding vs <10 ng/mL non-breeding) for both *Anolis carolinensis* (Lovern et al., 2001) and *Anolis sagrei* (Tokarz et al., 1998). These breeding T concentrations are relatively low when compared to values obtained in some other male lizards (200% to >500% higher; cf. Courte & Dufaure, 1982 for *Lacerta vivipara*; Moore, 1986 for *Sceloporus jarrovi*; Andò et al., 1990 for *Podarcis sicula*; Klukowski & Nelson, 1998 for *Sceloporus undulatus*; Wack et al., 2008 for *Phrynosoma cornutum*).

Experiments manipulating steroid exposure have demonstrated that T is critical for stimulating male reproduction in *Anolis carolinensis* and *Anolis sagrei*. Castration or treatment with the antiandrogen cyproterone acetate reduces reproductive activity, and exogenous T prevents or reverses the effect (*Anolis carolinensis*: Adkins & Schlesinger, 1979; Crews et al., 1978; Mason & Adkins, 1976; Rosen & Wade, 2000; Winkler & Wade, 1998; *Anolis sagrei*: Tokarz, 1986, 1987, 1995). In many vertebrates, T is a prohormone for male sexual behaviors, which are facilitated following conversion of T into E₂ by the enzyme aromatase and/or into DHT by the enzyme 5α-reductase (Meisel & Sachs, 1994). In contrast, T conversion into E₂ is not necessary for the expression of male sexual behavior in Anolidae. Treatment of male *Anolis carolinensis* with the aromatase inhibitor fadrozole has no effect on their sexual behavior (Winkler & Wade, 1998). Similarly, exogenously applied E₂ following castration has little or no effect on male sexual behavior in *Anolis carolinensis* (Crews et al., 1978; Mason & Adkins, 1976; Winkler & Wade, 1998) or *Anolis sagrei* (Tokarz, 1986). The data on DHT, however, are more complicated. Adkins and Schlesinger (1979) found that DHT stimulates reproductive behavior in castrated male *Anolis carolinensis* and Tokarz (1986) found the same for *Anolis sagrei*, whereas Crews et al. (1978) found for *Anolis carolinensis* that it is ineffective when given alone in stimulating masculine reproductive behavior, but in combination with E₂ it can promote behavior in some individuals. Systemic inhibition of 5α-reductase indicates that T conversion into DHT can promote full expression of masculine sexual behaviors, but DHT alone does not appear to do so (Rosen & Wade, 2000). Males housed under non-breeding conditions do not exhibit reproductive behavior, even when exogenous androgens are given, although androgen-treated males will extend their dewlaps with increased frequency (O'Bryant & Wade, 2002).

Female anoles ovulate a single follicle at a time, generally alternating between the left and right ovary, leading to the production of multiple, single-egg clutches spaced out over

the course of the breeding season (Smith et al., 1973). In *Anolis carolinensis*, FSH stimulates ovarian growth, vitellogenesis, and follicle recruitment, although even at high doses females still produce just one egg at a time (Jones et al., 1976; Roth & Jones, 1992). Control of ovulation involves sensory input from the ovaries to the hypothalamus, asymmetric amine production in the hypothalamus (dopamine, serotonin, norepinephrine; initially associated with the ovary containing the smaller yolked follicle but switching to the ovary containing the preovulatory follicle a couple of days prior to ovulation) and neurohormonal output that maintains a pattern of alternation (Desan et al., 1992; Jones, Lopez, et al., 1997; Jones, Wapstra, & Swain, 1997). Once ovulated, the egg is fertilized and shelled in the ipsilateral oviduct prior to oviposition (Conner & Crews, 1980). As at any one time it is common to have a corpus luteum present, plasma P₄ levels remain consistently high in the breeding season (across multiple ovulatory cycles) as compared to the nonbreeding season (Jenssen et al., 2001; Jones et al., 1983). In contrast, E₂ varies during the breeding season across the ovulatory cycle (Crews, 1980; Jones et al., 1983). Although plasma T concentrations are a fraction of those seen in males, they do vary in females as well. Plasma T concentrations are higher in breeding females than in nonbreeding females, and they are higher in breeding females specifically when they are likely to be receptive than at other reproductive cycle stages (Lovern et al., 2001; Lovern & Wade, 2001).

Female reproductive behavior is also mediated by gonadal steroids. E₂ clearly is the most potent activator of the behavior for *Anolis carolinensis* (Winkler & Wade, 1998); this effect can be maximized in the presence of P₄ (McNicol & Crews, 1979) and inhibited by administration of an estrogen receptor blocker (Tokarz & Crews, 1980). T also facilitates reproduction in females (Adkins & Schlesinger, 1979), an effect that is at least partially induced by its conversion into E₂ (Winkler & Wade, 1998).

Interestingly, during the breeding season androgens readily facilitate male-like sexual behaviors in gonadectomized female *Anolis carolinensis*, as for males described earlier, and the reproductive behaviors are qualitatively similar in individuals of both sexes when tested with receptive, stimulus females. However, manipulated females consistently display the behaviors at lower frequencies than do males, although results vary across studies as to whether this sex difference in androgen responsiveness reaches statistical significance (Adkins & Schlesinger, 1979; Mason & Adkins, 1976; Winkler & Wade, 1998). Similarly, T implants in juvenile males as well as females can stimulate adult male-typical, breeding season behavior such as dramatically increased display rates, territoriality, and aggressive and courtship-like interactions not otherwise seen in juveniles (Lovern et al., 2001), suggesting high

flexibility of the sexes to respond with male-typical reproductive behaviors when the underlying hormonal regime supports them.

2.3.6 *Carphodactylidae, Diplodactylidae, Eublepharidae, Gekkonidae, Phyllodactylidae, Pygopodidae, and Sphaerodactylidae*

Lizards commonly known as geckos (or legless lizards for the Australian family Pygopodidae, due to their loss of front limbs and vestigial hind limbs (Patchell & Shine, 1986)) are distributed in seven families and number over 2000 species. They are distributed worldwide, though predominantly in desert, tropical, and subtropical regions (Pianka & Vitt, 2003; Uetz et al., 2023). Most but not all are nocturnal, unlike the majority of other lizard taxa, and species range in adult size from under 3 to over 30 cm snout-to-vent length (Pianka & Vitt, 2003). Nearly all gecko species are oviparous with clutch sizes of one or two eggs produced multiple times per season, but three genera from family Diplodactylidae (*Naultinus*, *Hoplodactylus*, and *Rhacodactylus*) are viviparous (Pianka & Vitt, 2003). Reproductive mode is diverse; most reproduce sexually and possess genotypic sex determination, but a few species are parthenogenetic (some species of the Gekkonid genera *Hemidactylus*, *Hemiphyllodactylus*, *Heteronotia*, *Lepidodactylus*, and *Nactus*), and a few exhibit TSD (e.g., *Eublepharis* from the family Eublepharidae and some *Phelsuma* from the family Gekkonidae (Pianka & Vitt, 2003).

Similar to Scincidae, for such a large group of lizards there is very little information on hormones and reproductive cycles, although reproductive cycles more generally have been studied, e.g., for the Gekkonid species *Gekko japonicus* (Ikeuchi, 2004), *Hemidactylus flaviviridis* (Khan & Rai, 2004), and *Phyllodactylus lanei* (Ramírez-Sandoval et al., 2006). However, one species from the family Eublepharidae, the leopard gecko (*Eublepharis macularius*), has become a model organism in lizard reproduction due to its ease of maintenance in captivity and its reproductive mode of TSD (e.g., Crews & Moore, 2005). This species is native to southwestern Asia, although most if not all studies on its reproduction have occurred in laboratories using animals from captive-bred stock. Females are produced at low and high incubation temperatures, whereas mixed sex ratios are produced at intermediate temperatures (female-biased at low-intermediate and male-biased at high-intermediate temperatures) (Crews & Moore, 2005). Adult females lay two-egg clutches (or, rarely, one-egg clutches) repeatedly across the breeding season (LaDage et al., 2008; Rhen et al., 2000). Females readily mate with multiple males both within and between clutches although they can store sperm and continue egg production in the absence of males (LaDage et al., 2008). Most of the work on reproduction in leopard geckos has been on females, specifically

incubation temperature effects on reproductive physiology, morphology, and behavior (see Crews & Moore, 2005; Chapter 1, this volume).

Hormonal changes across the reproductive cycle also have been assessed, however. In females, plasma concentrations of the androgens T and DHT increase markedly during late vitellogenesis and then decline sharply after ovulation; plasma E₂ shows the same pattern and is higher in concentration than T and DHT except during late vitellogenesis when T is nearly twice as high as E₂ (Rhen et al., 2000). Unlike most lizards, plasma P₄ peaks during early vitellogenesis but then declines to previtellogenic levels following ovulation (Rhen et al., 2000); this is opposite to the pattern typically observed. Interestingly, incubation temperature influences plasma steroid concentrations in adult females; females from eggs incubated at high temperatures had higher T and DHT concentrations and lower P₄ concentrations (they did not differ in E₂ concentrations) (Rhen et al., 2000). During juvenile development the pattern is opposite, as juvenile females from eggs incubated at different temperatures did not differ in plasma T or DHT concentrations, but females at high incubation temperatures had lower plasma E₂ concentrations than females incubated at lower incubation temperatures (Rhen et al., 2005). In general, incubation temperature effects on male plasma steroid concentrations were absent.

2.3.7 Chamaeleonidae

Despite the charismatic nature of chameleons and the intense popularity of several species in zoos and the pet trade, comparatively little is known about hormonal influences on reproduction in this fascinating group of over 200 species. The opportunities for interesting hormone-reproduction relationships seem compelling given that, across species, there is considerable diversity in the degree of sexual dimorphism and reproductive lifespan (e.g., Karsten et al., 2008; Pianka & Vitt, 2003). Further, in recent years reproductive behavior has been well-studied for both males and females of several species (e.g., the Madagascan chameleons *Furcifer labordi* and *Furcifer verrucosus* (Karsten, Andriamananjara, et al., 2009; Karsten, Ferguson, et al., 2009) and the veiled chameleon *Chamaeleo calyptratus* (Kelso & Verrell, 2002)). Additionally, dramatic and in some cases sex-atypical behaviors for lizards have been revealed, such as high sexual aggression in female dwarf chameleons, *Bradypodion pumilum* (Stuart-Fox & Whiting, 2005) and active mate guarding by males of recently mated females in the serially polygynous common chameleon, *Chamaeleo chamaeleon* (Cuadrado, 2000, 2001). Most chameleons are oviparous although a few give live birth; across oviparous species, clutch sizes can vary tremendously, from 2 to 4 eggs in smaller species like leaf chameleons of the genus *Brookesia* to 40 or more in

species like *Chamaeleo calyptratus* (Andrews, 2008; Pianka & Vitt, 2003). Finally, our understanding of how to maintain chameleons successfully in captivity has improved considerably (e.g., Andrews, 2008; Ferguson et al., 2002, 1996; Karsten, Andriamananjara, et al., 2009; Karsten, Ferguson, et al., 2009), making them increasingly amenable to laboratory study as well.

One species in which recent progress has been made in understanding hormonal influences on reproduction is the previously mentioned veiled chameleon, *Chamaeleo calyptratus*. Researchers have taken advantage of the availability of this species from commercial suppliers and the use of noninvasive fecal steroid assays to measure gonadal and adrenal steroids in females in relation to reproductive status (Kummrow et al., 2011, 2021; Pimm et al., 2015). Fecal P₄, E₂, T, and even CORT show changes across the female reproductive cycle and between reproductive and nonreproductive time periods, generally being found in higher concentrations in ovulatory females. The techniques as well as the findings of these studies will be helpful in understanding basic hormone-reproduction events in laboratory- and zoo-maintained species, including when reproductive issues arise (e.g., egg binding which can be a common problem in some captive reptiles including *Chamaeleo calyptratus*).

2.3.8 Cordylidae

Spinytail lizards and girdled lizards are found predominantly in southern Africa; like the closely related Gerrhosauridae, they are small- to medium-bodied and diurnally active. Some are oviparous and others are viviparous (Pianka & Vitt, 2003; Uetz et al., 2023). Hormones and reproductive cycles have been studied to some extent in the giant girdled lizard, *Smaug* (formerly *Cordylus*) *giganteus*, and the Karoo girdled lizard, *Karusasaurus* (formerly *Cordylus*) *polyzonus*. *Smaug giganteus* give birth in late summer to 1–3 young, but individual females reproduce only biennially or possibly triennially (Van Wyk, 1994). Plasma E₂ and P₄ concentrations follow the typical pattern—E₂ is highest during vitellogenesis and P₄ is highest during gestation; plasma T also was measured but not detected in this study (Van Wyk, 1994). *K. polyzonus*, in contrast to *Smaug giganteus*, breeds annually (Flemming, 1993, 1994). In males, testicular recrudescence is initiated in the fall and is maximal in the spring when mating commences; plasma T as well as AND concentrations exhibit a bimodal profile, peaking at the onset of recrudescence and then again in the spring during maximal recrudescence and mating (Flemming, 1993). In females, plasma E₂ and P₄ values are similar to those reported for *Smaug giganteus* earlier (Flemming, 1994).

Although hormones related to reproductive cycles have not specifically been reported, work on reproductive tactics and male aggression in the Augrabies flat lizard (*Platysaurus broadleyi*) suggests interesting opportunities for future work. This stunning lizard shows dramatic sexual color dimorphism in which males but not females possess green, orange, or yellow front legs, ventral orange or yellow ventral coloration, blue-turquoise flanks, and blue-violet throats that reflect highly in the UV range (Whiting et al., 2003, 2006). As a result of temporally restricted, locally abundant food resources (black fly plumes), these lizards can be found in high-density groups, where mating territories established and defended by males are at a premium (e.g., around 50% of males do not acquire a territory) (Whiting et al., 2003, 2006). Testosterone may influence the development of the UV signal, and animals with an experimental reduction in UV are aggressively challenged by other males more frequently (Stapley & Whiting, 2006; Whiting et al., 2006). Finally, nonterritorial males may mimic females visually to avoid detection by territorial males and thereby gain access to reproductively active females (Whiting et al., 2009).

2.3.9 Crotaphytidae

In collared lizards (*Crotaphytus collaris*), previous research has investigated the role of hormones in affecting reproductive coloration in females (Cooper Jr. & Ferguson, 1972, 1973) and the association between breeding season territoriality and hormones in males (Baird & Hews, 2007). Similar to work with *Holbrookia propinqua* discussed earlier, treatment with exogenous T, P, or especially P₄ following E₂ priming for several days, stimulates development of reproductive coloration in females (Cooper Jr. & Ferguson, 1972, 1973). Plasma P₄ concentration is highest when females are gravid compared to nonreproductive, vitellogenic, or postgravid females (Masson & Guillette Jr., 1987). Although likely, it is unknown whether endogenous plasma T or E₂ profiles peak at times that support a natural role for these hormones in mediating changes in color and behavior across the reproductive cycle in females. Unlike coloration in female *Holbrookia propinqua*, in *Crotaphytus collaris* coloration appears to signal receptivity rather than nonreceptivity (Baird, 2004). In male collared lizards, first-year, nonterritorial subordinates have higher concentrations of plasma T than do older, dominant territory holders (Baird & Hews, 2007). Plasma concentrations of DHT are lower than those for T and not different between subordinates and territory holders.

2.3.10 Dibamidae

Little is known about blind lizards—the 25 species comprising this family of two genera (*Dibamus* of southeastern Asia and *Anelytropsis* of Mexico). These lizards are

fossorial; males have reduced hind limbs only whereas females are limbless, eyes are vestigial, and there is no external ear opening (Pianka & Vitt, 2003). Available data suggest that all members of this family are oviparous with single-egg clutches (Pianka & Vitt, 2003); sex determination is genetic rather than temperature-dependent (Cole & Gans, 1997; Rovatsos et al., 2022). Nothing is known about the endocrinology of reproduction in these species.

2.3.11 Gerrhosauridae

Plated lizards occur in Africa and Madagascar; all are small-to medium-bodied (~5–30 cm adult snout-to-vent length), diurnally active, and oviparous (Pianka & Vitt, 2003; Uetz et al., 2023). Most are terrestrial, although at least one species is arboreal (*Zonosaurus boettgeri*) and one is semiaquatic (*Zonosaurus maximus*) (Pough et al., 2004). Reproductive endocrinology has not been studied in this group, although pheromonal communication has been documented in *Gerrhosaurus nigrolineatus*, in which males can discriminate between other males and females based on cloacal chemical signals (Cooper Jr. & Trauth, 1992).

2.3.12 Helodermatidae

This family is comprised of one genus and five species, the gila monster (*Heloderma suspectum*) and four species of beaded lizard (e.g., the Mexican beaded lizard, *Heloderma horridum*). These are large (>300 mm total length) venomous lizards and until recently thought to be the only extant venomous lizards (Pianka & Vitt, 2003); work on Komodo dragons (*Varanus komodoensis*; Varanidae) indicates that this species is venomous as well (Fry et al., 2009). Gila monsters are oviparous, showing summer gonadal recrudescence, oviductal eggs in the late summer, with oviposition apparently occurring in the fall after which eggs incubate overwinter and hatch in the spring of the following year; Mexican beaded lizards also are oviparous but the timing of reproductive activity appears shifted to the fall and winter (Goldberg & Lowe, 1997). Female-female aggressive behavior in gila monsters has been documented (Schuett et al., 2023); this behavior likely serves important roles in courtship and nest/offspring guarding. One recent study has measured sex steroids in shed skin of the gila monster, finding P₄ and E₂ levels significantly higher in adult females than in adult males, but also, intriguingly, higher T levels in females than in males, too (Gerhard et al., 2020).

2.3.13 Iguanidae

Iguanidae contains 9 genera and 44 species; previously the families Anolidae, Phrynosomatidae, Crotaphytidae, Corytophanidae, Hoplocercidae, Leiocephalidae, Leiosauridae, Liolaemidae, Opluridae, Polychrotidae, and Tropiduridae

were considered subfamilies in this group (Table 1; Uetz et al., 2023). Among iguanids, a variety of hormone-reproduction studies have been performed with the Galápagos marine iguana, *Amblyrhynchus cristatus*. In this lekking species, some males aggressively defend mating areas and court females with headbobbing displays whereas other males do not defend mating areas but rather attempt to intercept females for mating opportunities using a satellite or sneaker-male tactic (Vitousek et al., 2008). Females can be highly aggressive toward males during the mating period after they have copulated, signaling nonreceptivity, and highly aggressive toward other females during the nesting period in competition for limited nesting sites (Rubenstein & Wikelski, 2005). In males, those that defend mating areas have higher plasma T concentrations than do either satellite or sneaker males (Wikelski et al., 2005), there is a positive relationship between headbobbing frequency and copulations, and males that headbobbed more also had higher plasma T concentrations (Vitousek et al., 2008). Interestingly, Vitousek et al. (2008) demonstrated annual variation within males such that hotshots in the first year of the study (i.e., those with high plasma T concentrations and a high number of copulations) were less likely to be hotshots the following year, having lower plasma T concentrations and a lower number of copulations. Similarly, females can be described as facultatively biennial in reproduction, similar to instances of multiennial reproduction discussed in Section 2.1 (Vitousek et al., 2010). In female *Amblyrhynchus cristatus*, plasma T and E₂ are significantly higher during the mating than during the nesting period, with plasma T and likely E₂ peaking around copulation. Plasma P₄ declines across the mating period but then increases sharply and again declines sharply across nesting, reaching minimal concentrations following oviposition (Rubenstein & Wikelski, 2005). Plasma T concentrations in females are over an order of magnitude lower than those of males (cf. Rubenstein & Wikelski, 2005; Vitousek et al., 2008; Wikelski et al., 2005). Long-lived marine iguanas are a fascinating study organism for hormone-reproductive-immune studies given their behavioral and physiological adaptations for living in a marine environment (e.g., Neuman-Lee & French, 2017).

2.3.14 Lacertidae

A considerable amount of work has been done with lacertids, in particular wall lizards of the genus *Podarcis* and the common lizard, *Lacerta vivipara*. Information on hormones and the reproductive cycle also is available for *Psammodromus algirus*, an oviparous lacertid common throughout the Mediterranean.

The Italian wall lizard, *Podarcis sicula*, is an oviparous, seasonal breeder with springtime emergence from overwintering sites followed by courtship and mating in late spring

and early summer. Consistent with this type of reproductive cycle, in males plasma T concentrations are elevated when courtship and breeding are occurring but not at other times of the year; plasma DHT shows a similar, but lower amplitude, seasonal profile (Andò et al., 1992, 1990; Panno et al., 1992). Plasma T shows the same pattern in *Psammodromus algirus* (Díaz et al., 1994). Interestingly, plasma E₂ and P₄ in males are elevated postbreeding in *Podarcis sicula*, suggesting a role for these steroids in males in initiating testicular regression and the onset of the postreproductive period during the late summer (Andò et al., 1992, 1990; Panno et al., 1992). In support of this relationship, experimental injection of the aromatase inhibitor fadrozole during the fall nonreproductive period causes an increase in plasma T, a decrease in plasma E₂, and a resumption of spermatogenesis (Cardone et al., 2002). However, the generality of increased plasma E₂ and P₄ concentrations in males following the breeding season is difficult to evaluate because so few studies measure E₂ or P₄ in males. Recent work in *Podarcis sicula* additionally has begun to explore the potential role of leptin in the regulation of reproductive cycles in males; thus far it appears that, similar to work in mammals, leptin can have a direct effect on the reproductive axis at multiple locations including the gonads. In *Podarcis sicula*, leptin causes a delay in summer regression of the testes (Putti et al., 2009). Less work, both descriptively and experimentally, has been carried out in females. However, work thus far is consistent with what is seen in other oviparous females; plasma E₂ peaks prior to ovulation, and plasma P₄ follows closely behind, peaking after ovulation (Carnevali et al., 1991; Ciarcia et al., 1993). Plasma E₂ and P₄ concentrations show the same pattern in *Psammodromus algirus* (Díaz et al., 1994).

Most but not all populations of the common lizard, *Lacerta vivipara*, are viviparous (Heulin et al., 2008; Uller & Olsson, 2006). Breeding is seasonal, occurring in late spring-early summer, with birth of young occurring toward the end of summer. In males, hormone relationships to the reproductive cycle are the same as for oviparous lacertids; males show peak plasma T and DHT associated with breeding, and the DHT profile mirrors that of T but at much lower amplitude (Courty & Dufaure, 1979, 1980, 1982). The values seen for plasma T—peaking at 200–500 ng/mL—may be the highest for any lizard measured thus far. In females, plasma E₂ is higher during the later stages of vitellogenesis than it is after ovulation, and this is true of females of both viviparous and oviparous populations (Heulin et al., 2008).

2.3.15 Lanthanotidae

This family is thought to be comprised of a single species—the Bornean earless monitor lizard, *Lanthanotus*

borneensis. Very little is known about this rarely encountered, nocturnal, fossorial species (Maisano et al., 2002; Pianka & Vitt, 2003), and no data on hormones or reproduction are available. Yet, and controversially, this species has been acquired by multiple zoos and is found in the illegal pet trade (reviewed in Nijman, 2021). Ethically studying Bornean earless monitor lizards is currently extremely difficult at best.

2.3.16 Phrynosomatidae

Sceloporus is the most widely studied genus of lizards in this family. Fence lizards (*Sceloporus undulatus*) have a reproductive cycle very similar to anoles, with spring emergence from overwintering dormancy followed by increased activity, territoriality, breeding, and oviposition completed in the summer before a fall inactive period preceding winter dormancy. The primary difference is that *Sceloporus undulatus* females lay one or two large clutches of eggs during the reproductive season, whereas anoles lay single-egg clutches. In males, plasma T begins to increase in advance of the breeding season and remains high before declining postbreeding (Klukowski & Nelson, 1998; McKinney & Marion, 1985). A similar pattern is seen in the striped plateau lizard (*Sceloporus virgatus*), a congener that also is oviparous, in which not only T but also DHT, androstenedione (AND), and E₂ attained peak mean concentrations during the breeding season in males; P₄ also was measured in these males but did not differ from breeding to postbreeding (Abell, 1998).

Whether plasma T concentrations are interpreted as maximal (e.g., as for *Anolis carolinensis* following GnRH injections; Husak et al., 2009) or submaximal during breeding may depend on the timing of measurement or population studied. Klukowski and Nelson (1998) studied a field population of northern fence lizards (*Sceloporus undulatus hyacinthinus*) in Indiana, United States, and found that plasma T concentrations in males that were presented with tethered, conspecific intruders for 3–15 min were not elevated compared to values obtained from control males when blood samples were collected 30 min following intrusion. In contrast, Smith and John-Alder (1999) collected males from a population of eastern fence lizards (*Sceloporus undulatus*) in New Jersey and housed them in outdoor enclosures for trials. They found that repeated exposure to intruder males over 9 consecutive days caused elevated plasma T in territorial males after 4 days of exposure to intruders, but that this effect was eliminated by day 10. No differences in plasma T were observed at day 4 or day 10 when females were introduced into the enclosures, and no differences were observed in response to exposure to either sex in trials conducted during the postbreeding season (Smith & John-Alder, 1999). Plasma T concentrations clearly are higher

in these males during the breeding season than during the nonbreeding season, but whether they are maximal is not clear. Such information has implications for hypothesized relationships between T, mating system (higher T predicted in polygynous systems), parental care (higher T predicted when paternal care is absent), and male-male aggression (higher T predicted when male-male aggression is common) as described in the challenge hypothesis (Wingfield et al., 1990).

Female *Sceloporus undulatus* have received less attention than have males, although females of other sceloporine species have been studied in some detail. In *Sceloporus undulatus*, plasma T concentrations are significantly lower in females than in males (Cox et al., 2005), but whether plasma T cycles with reproductive state in females is not known. Plasma P₄ does cycle; as is common in other species, it is higher when females are gravid than when they are nonreproductive, vitellogenic, or postreproductive (Masson & Guillette Jr., 1987). In contrast to males, Abell (1998) found that female *Sceloporus virgatus* did not differ in plasma T, DHT, AND, or E₂ concentrations from breeding to postbreeding, although there was a marginally significant reduction in plasma E₂ postbreeding and, like males, there was no difference in P. Mean values of E₂ were higher in females than in males, P₄ was comparable, and T, DHT, and AND were higher in males (Abell, 1998). Another study of *Sceloporus virgatus* females (Weiss et al., 2002) examined endocrine profiles across the reproductive cycle (rather than from breeding to postbreeding) and found a different pattern. In this study, conducted on females from southeastern AZ, similar to Abell (1998), plasma P₄ and to a lesser extent T increased following vitellogenesis and then declined following oviposition, whereas plasma E₂ was highest during vitellogenesis and declined steadily through the gravid and then postoviposition stages (Weiss et al., 2002). Consistent with these results, in another oviparous congener, the Mexican boulder spiny lizard (*Sceloporus pyrocephalus*), both plasma E₂ and T concentrations were found to be higher during vitellogenesis than when females were gravid (Calisi & Hews, 2007). Overall, plasma T was much lower in female *Sceloporus pyrocephalus* than in males measured in the same study (plasma E₂ was not measured in males; Calisi & Hews, 2007).

Sexually dimorphic, dorsal, and especially ventral color patterns are important components of communication displays during the breeding season in many sceloporine lizards, and the hormonal regulation of this reproductive trait has received considerable attention. In *Sceloporus undulatus*, this sex difference is dependent on plasma T and can be diminished in males by castration and restored in males, as well as masculinized in females, by exogenous T (Cox et al., 2005; Rand, 1992).

As discussed in Section 1.1.2, the genus *Sceloporus* is comprised of both oviparous and viviparous species, and viviparity has evolved independently multiple times within this genus alone (Pianka & Vitt, 2003). *Sceloporus jarrovi*, the mountain spiny lizard, is a viviparous species that has been studied in some detail. This species breeds in the fall, after which gonads regress in both sexes; females are pregnant over winter and spring and subsequently give birth in early summer, prior to initiating gonadal recrudescence and mating behavior once again (Moore, 1986; Ruby, 1978). Aside from the seasonal shift in timing of reproduction, hormone-reproduction patterns in *Sceloporus jarrovi* are similar to those found in oviparous congeners and other spring-summer breeding lizards. Males have peak plasma T concentrations in the fall breeding season compared to other times of year, but no changes in plasma P₄ (detectable throughout the year) or plasma E₂ (not detectable in any sample) (Moore, 1986). Consistent with a role for T in maintaining reproductive behavior, castration diminishes behaviors related to both territory maintenance and courtship (Moore, 1987). Females, like males, show peak plasma T concentrations in the fall breeding season but unlike males, plasma E₂ also peaks at this time (Moore, 1986; Woodley & Moore, 1999a). Plasma P₄ concentrations in females are higher during pregnancy than at other times of the year (Moore, 1986). Plasma T concentrations are approximately an order of magnitude higher in males than in females; the reverse is true for plasma E₂, and plasma P₄ concentrations are approximately equivalent between the sexes (Moore, 1986). In one of the few studies to experimentally manipulate sex steroids in a field population of females, Woodley and Moore (1999b) demonstrated a likely role for plasma E₂ and possibly to a lesser extent plasma T in promoting female territorial aggression. In females as in males of this species, territories are set up in the summer prior to breeding and maintained through the breeding season and thus are important for successful reproduction (Ruby, 1978; Woodley & Moore, 1999b).

Other Phrynosomatid lizards for which there is information on hormones and reproduction include tree lizards (*Urosaurus*), side-blotched lizards (*Uta*), keeled earless lizards (*Holbrookia propinqua*), and horned lizards (*Phrynosoma*). *Urosaurus ornatus* and *Uta stansburiana* have been particularly useful models for examining the relationships between hormones, environment, and specifically the alternative reproductive phenotypes expressed by males of these species (*Urosaurus ornatus*: Knapp et al., 2003; Moore et al., 1998; reviewed in Crews & Moore, 2005; *Uta stansburiana*: Mills et al., 2008; Sinervo et al., 2000). Collectively this work has revealed, not surprisingly, that hormones such as T are important for reproduction and associated behaviors. More intriguingly, this work has revealed

that the ways in which hormones like T influence reproduction can be extremely variable. For example, plasma T concentration may differ between adult male morphs (*Uta*) or it may not (*Urosaurus*), and morphs may differ in their T (and other hormone) responses to social and environmental stimuli (*Uta* and *Urosaurus*). Similarly, surprising relationships among T, aggressive behavior, and ecomorph type have been found in *Anolis* species assemblages on different Caribbean islands (Husak & Lovern, 2014).

Work with keeled earless lizards, *Holbrookia propinqua*, has focused on females and the relationship between reproductive coloration, hormones, and behavior in females (Cooper Jr. & Crews, 1987, 1988). In this species, the development of bright coloration signals nonreceptivity; bright females reject male courtship whereas plain females do not. Females with bright reproductive coloration have higher plasma concentrations of P, androgens, and E₂ than do plain females, associated with the later stages of follicle growth, ovulation, and shelling of eggs (Cooper Jr. & Crews, 1988). Furthermore, treatment with exogenous P₄ or T stimulates this color development along with the associated aggressive, rejection behavior of females toward males; treatment with E₂ partially stimulates color development but does not produce rejection behavior (Cooper Jr. & Crews, 1987). In comparison to females, male *Holbrookia propinqua* have much higher plasma T concentrations and plasma P₄ concentrations that are roughly equivalent to plain females but substantially lower than those for bright females (Cooper Jr. & Crews, 1988).

Plasma steroids have been measured across the reproductive season for both males and females in free-ranging Texas horned lizards, *Phrynosoma cornutum* (Wack et al., 2008). Males have higher plasma T as well as, intriguingly, plasma E₂ concentrations during the breeding than nonbreeding season but show no difference in plasma P₄ across seasons. Similarly, females have higher plasma E₂ and marginally significantly higher plasma T during the breeding season prior to gravidity, after which concentrations of both steroids decline. Male plasma T is over an order of magnitude higher than female plasma T; plasma E₂ and P₄ actually are comparable between the sexes. The results for plasma T in males and plasma E₂ in females parallel those documented for the San Diego coast horned lizard, *Phrynosoma coronatum blainvilliei* (plasma T was not measured in females, plasma E₂ was not measured in males, and plasma P₄ was not measured in either sex; Alberts et al., 2004).

2.3.17 Scincidae

This is the largest lizard family, with over 150 genera and 1700 species. Skinks form a cosmopolitan group, occurring

on all continents except Antarctica as well as on many oceanic islands. Coupled with their diversity of daily activity rhythms (diurnal and nocturnal), habitats (desert to rainforest; terrestrial, arboreal, fossorial, semiaquatic niches), diet (insectivory/carnivory to herbivory), and body size and morphology (under 4 cm snout-vent length for adults of the genus *Menetia* to over 40 cm snout-vent length for the Solomon Island skink, *Corucia zebra*, along with varying degrees of limb reduction or even absence), it is difficult to stereotype a “typical” skink (Pianka & Vitt, 2003).

Reproductive mode and ecology have been studied in some detail in Scincidae, revealing oviparous and viviparous species, some of which breed annually and some of which breed multiennially, and some of which time breeding with the wet season, some with the dry season, and some can initiate breeding year-round (e.g., Edwards et al., 2002; James & Shine, 1985). Among viviparous species, most show complete lecithotrophy in which eggs contain all the nutrients that will be provided to offspring prior to live birth, whereas others like the Brazilian skink *Mabuya heathi* have highly developed placentae through which nutrients are transferred from mother to embryo (Blackburn et al., 1984). Populations of the same species can even vary in reproductive mode from oviparity to viviparity as is the case for some Australian skinks (Pianka & Vitt, 2003). Although skink reproduction has been studied in several species, their reproductive endocrinology has been particularly underdescribed when the diversity of skinks is considered. Hormones and reproduction have been studied in species of *Niveoscincus* and *Tiliqua* in detail, with limited information available for *Eumeces* and *Oligosoma* as well. (Jones, 2017 has a thorough review of insights we have gained from studies of hormones and reproductive cycles in Australian skinks.)

As discussed in Section 2.2, Tasmanian skinks (*N. ocellatus*) are viviparous and have a primary breeding period in the austral fall when plasma T of males is highest and is associated with peak testis volume and spermatogenesis but dissociated from ovulation, fertilization, and pregnancy in females, which occur the following spring (Jones, Lopez, et al., 1997; Jones, Wapstra, & Swain, 1997). Plasma T generally is low throughout the rest of the year, including the austral spring when some individuals mate again (Jones, Lopez, et al., 1997; Jones, Wapstra, & Swain, 1997). In females, similar to other studies reviewed earlier, plasma E₂ is highest during vitellogenesis whereas plasma P is highest during gestation, although both steroids are detectable throughout the reproductive cycle (Jones, Lopez, et al., 1997; Jones, Wapstra, & Swain, 1997). A similar reproductive pattern is seen in another viviparous Tasmanian skink, *N. metallicus* (Jones & Swain, 1996; Swain & Jones, 1994), including elevated plasma P₄ concentrations during gestation but not sooner (Bennett &

Jones, 2002). In *N. microlepidotus*, reproduction is biennial largely due to a gestation period lasting a year or more (Girling et al., 2002). In this species, plasma P₄ is high during gestation, but it also shows a significant peak upon emergence from hibernation prior to ovulation, earlier in the reproductive cycle than is seen in *N. ocellatus*, *N. metallicus*, and most other vertebrate females. Girling et al. (2002) suggest that high P₄ at emergence could be a mechanism for preventing additional follicle development during a gestation-only year. Plasma E₂ is high during vitellogenesis as would be expected, but a secondary peak is seen during the latter stages of gestation and several months before vitellogenesis is initiated (Girling et al., 2002). Reproductive timing and its relationship to hormone profiles can be variable, even within a genus. In the case of *N. microlepidotus*, the hormone profiles for plasma P₄ and E₂ suggest that each hormone shows a similar pattern annually but with different amplitudes in alternating years in response to, or to allow for, an extended reproductive cycle.

Plasma steroids also have been measured across the reproductive cycle of males and females of another viviparous species, blue-tongued skinks (*T. nigrolutea*). In males, plasma T, E₂, and to a lesser extent P₄ are higher during the breeding than nonbreeding season (Edwards & Jones, 2001a, 2001b). This is similar to the androgen profiles found across the annual cycle in the congener *T. rugosa* (Bourne et al., 1986). Elevated E₂ and/or P₄ for males have been reported in a few other studies as discussed earlier (e.g., *Sceloporus virgatus*, *Phrynosoma cornutum*) as well as the reverse case in which E₂ and P₄ are elevated in postbreeding males (*Podarcis sicula*); either these endocrine profiles are uncommon for males or, more likely, especially for E₂, not measured often enough to know how common they may be. Female blue-tongued skinks are multiennial, such that during any breeding season, some females are reproductively active and some are quiescent (Edwards & Jones, 2001a, 2001b). Reproductively active females show a rise in plasma E₂ and T leading up to and during the mating and ovulatory periods, followed by a decline after ovulation; plasma P₄ increases around the time of mating and ovulation and peaks during gestation (Edwards & Jones, 2001a, 2001b). Interestingly, reproductively quiescent females show a similar but muted pattern for plasma E₂ and T, while plasma P₄ in quiescent females remains basal throughout the year (Edwards & Jones, 2001a, 2001b). This suggests that factors other than reproductive competence (e.g., environmental factors) contribute to annual steroid profiles and/or that reproductively quiescent females retain some possibility to reproduce if conditions change during a quiescent year. Plasma P₄ also has been measured in females of the oviparous great plains skink, *Eumeces obsoletus* (Masson & Guillette Jr., 1987) and females of the viviparous

McCann's skink, *Oligosoma maccanni* (Holmes & Cree, 2006). In both species, plasma P₄ is highest during gravidity/gestation.

2.3.18 *Shinisauridae*

This family is comprised of one semiaquatic species found in southeastern China and northeastern Vietnam, the crocodile lizard, *Shinisaurus crocodilurus*. This species is long-lived and viviparous, like Xenosauridae, the knob-scaled lizards with which *Shinisaurus crocodilurus* was historically grouped prior to elevation to independent family status (Pianka & Vitt, 2003). *Shinisaurus crocodilurus* is an endangered species due to habitat loss and degradation, although captive breeding and husbandry is widely carried out due to the popularity of this species in zoos and in the pet trade (Pianka & Vitt, 2003). Recently the *Shinisaurus crocodilurus* genome was sequenced (Xie, Liang, Chen, et al., 2022), which should allow for the advance of our understanding of this unique species' biology and evolutionary history (e.g., Xie, Liang, Li, et al., 2022).

2.3.19 *Teiidae*

Whiptail lizards have been studied extensively and have yielded tremendous insight into hormone-reproduction relationships. There are multiple parthenogenetic species, but the best studied is the desert-grassland whiptail, *Aspidoscelis* (formerly *Cnemidophorus*) *uniparens*, a triploid, all-female species that arose from the hybridization of the little striped whiptail, *Aspidoscelis* (*C.*) *inornatus* and, most likely, the rusty rumped whiptail, *Aspidoscelis* (*Cnemidophorus*) *burti* (reviewed in Crews & Moore, 2005; Dias & Crews, 2008; Godwin & Crews, 2002). All species are oviparous, regardless of whether reproduction is sexual or parthenogenetic.

In the sexually reproducing *Aspidoscelis inornatus*, males have high plasma T and especially DHT concentrations when reproductively active, detectable but not significantly different plasma P₄ concentrations across the breeding season, and plasma E₂ was not detected (Moore & Crews, 1986). Castration diminishes male courtship and copulatory behavior, and implants of T, DHT, and P₄ (alone or synergistically with T) restore these behaviors in previously castrated males (Lindzey & Crews, 1986; Sakata et al., 2003). In females, plasma E₂ is highest before ovulation, and plasma P₄ is highest after ovulation and before oviposition; plasma androgens were not detectable (Moore & Crews, 1986). In both males and females, the presence of the opposite sex is important for stimulating gonadal recrudescence (Lindzey & Crews, 1988). In the parthenogenetic *Aspidoscelis uniparens*, there is no opposite sex to stimulate behavior, rather individuals alternate in expressing male-typical mounting behavior and female-typical receptive

behavior of the ancestral, sexual species. As for *Aspidoscelis inornatus*, plasma E₂ concentration is higher prior to ovulation and plasma P₄ concentration is higher following ovulation in *Aspidoscelis uniparens* (Moore, Whittier, & Crews, 1985). Thus, this endocrine profile is conserved, but the response to it has changed. Prior to ovulation (high E₂, low P₄), individuals are more likely to express female-typical receptive behavior during pseudosexual interactions; following ovulation and until oviposition (low E₂, high P₄), individuals are more likely to express male-typical mounting behavior during pseudosexual interactions (Moore, Whittier, Billy, & Crews, 1985). As seen for *Aspidoscelis inornatus*, experimentally elevated plasma T or P₄ (alone or in synergism with T) promotes male-typical mounting behavior in *Aspidoscelis uniparens* as well (Sakata et al., 2003). This sensitivity to T is despite the fact that plasma T is undetectable throughout the breeding season in *Aspidoscelis uniparens* (Crews et al., 1986). Furthermore, although male and female *Aspidoscelis inornatus* express sex differences in brain regions associated with reproduction and typical of other vertebrates—males have a larger anterior hypothalamus-preoptic area (AH-POA) and females have a larger ventromedial region of the hypothalamus (VMH)—in the all-female *Aspidoscelis uniparens*, both brain regions are comparable in size to those of female *Aspidoscelis inornatus* regardless of reproductive state (Wade & Crews, 1991). Thus, like endocrine profiles, size relationships in brain regions associated with reproduction are conserved. Recent work by Dias and Crews (2008) suggests that the brain neurotransmitter serotonin links hormones and behavior in *Aspidoscelis uniparens*, as in many other taxa, by playing an inhibitory role in the expression of reproductive behavior. Serotonin presence in the AH-POA suppresses mounting whereas in the VMH it suppresses receptivity.

2.3.20 *Varanidae*

This family is comprised of one genus (*Varanus*) and nearly 90 species of monitor lizards. Varanids are long-lived; geographically widespread across Africa, Asia, and Australia, exceptionally diverse in habitats (desert to swamp, arboreal to terrestrial to aquatic); and highly variable in adult body size (e.g., the pygmy monitor, *Varanus brevicaudus*, weighs <20g as an adult whereas the Komodo dragon, *V. komodoensis*, can weigh 150kg) (Pianka & Vitt, 2003). All are oviparous, and at least two species appear to show facultative parthenogenesis, the Argus monitor lizard *V. panoptes* (Lenk et al., 2005) and the Komodo dragon *V. komodoensis* (Watts et al., 2006). Clutch size can be highly variable within and between species (e.g., de Buffrénil & Rimbot-Baly, 1999), but generally reproduction occurs during warm, dry months, oviposition occurs shortly thereafter, and incubation duration (~90–270 days

across species) matches hatching to the wet season and/or prey abundance (Jacob & Ramaswami, 1976; Phillips & Millar, 1998; Wikramanayake & Dryden, 1988). Extensive data on clutch size, seasonality, frequency, incubation, and hatching for multiple species in captivity are available (Mendyk, 2012). Plasma T and total estrogens have been measured in the white-throated savannah monitor, *V. albigularis* (Phillips & Millar, 1998). Plasma T concentration is higher in males than in females, but it peaks in both sexes associated with mating. Plasma estrogens also peak at this time in females (they were not measured in males).

2.3.21 Xantusiidae

Lizards in this family commonly are known as night lizards, and they are found in North and Central America and Cuba. They are small in body size (typically 0.5 cm snout-to-vent length or less), long-lived (around 10 years for some species), and viviparous (Pianka & Vitt, 2003). Although known as night lizards, they can be active during the day, too, but tend to be difficult to detect, moving about in rock crevices, under leaf litter and other organic ground matter (Pianka & Vitt, 2003). Xantusiids mate in the fall, with gestation and birth occurring late summer or early fall of the following year (Aguilar Cortés et al., 1990; Pianka & Vitt, 2003; Ramírez-Bautista et al., 2008). Reproductive hormone profiles have not been published for any lizard in this family, although one study found that exogenous P₄ did not reduce vitellogenin production stimulated by exogenous E₂ in females, but it did reduce ovarian weight and follicle size, presumably by inhibiting vitellogenin uptake (Yaron & Widzer, 1978).

3 CONCLUSIONS AND FUTURE DIRECTIONS

Despite advances in knowledge since the first edition of this chapter (Lovern, 2011), hormones and their relationship to reproductive cycles have been well-described for relatively few lizard species, representing a minority of families. In part this is due to the fact that, just as in 2011, many lizard species are difficult to access, find, and study for myriad reasons including environmental, geographic, and political. The data we do possess on hormones and reproductive cycles in lizards lead to several conclusions and point to fruitful areas for future study.

First, the wealth of information available for genera such as *Podarcis*, *Lacerta*, *Cnemidophorus*, *Anolis*, *Sceloporus*, *Calotes*, *Niveoscincus*, and *Eublepharis* and reviewed earlier gives us a firm foundation for understanding hormones and reproductive cycles in lizards. From a review of available data, it is clear that gonadal steroid profiles

across the reproductive cycle and their hypothalamic-pituitary regulation have remained generally consistent across lizard species regardless of reproductive mode (e.g., sexual vs asexual reproduction, oviparity vs viviparity, genotypic vs temperature-dependent sex determination). Roles for E₂ and P₄ in females and T in males in initiating and maintaining reproductive condition furthermore are similar to those seen in mammalian species. Additionally, work on androgen roles in females and E₂ and P₄ roles in males demonstrate some potentially novel or at minimum not widely recognized steroid effects on reproduction. More studies that characterize all of these steroids in both males and females will be useful.

Second, in conjunction with work on the model species in the genera listed earlier, additional targeted work on under-represented lizard taxa would yield a tremendous comparative perspective. For example, there are taxa such as Chamaeleonidae and Gekkonidae that contain hundreds of lizard species and for which there is good information on reproduction and behavior but no data on underlying endocrine regulation. Chameleons in particular generate widespread interest and appeal, and they exhibit considerable diversity in sexual dimorphism, lifespan, and reproductive strategies and modes, providing excellent justification and preliminary data for tests of hormonal effects. There are also individual, well-studied species within taxa that have otherwise received little attention but seem to be prime targets for productive research. For example, the Augrabies flat lizard (Cordylidae: *Platysaurus broadleyi*) has dramatic sexual color dimorphism, high density mating territories, and multiple reproductive strategies in males, but less work on female reproductive strategies and potential seasonal and strategy-based hormonal correlates in either sex. Finally, there also are atypical lizard taxa for which almost nothing is known and therefore any contribution would be valuable. One example of an area in need of attention is the hormonal underpinnings of the ecologically similar but phylogenetically very distant worm lizards (Amphisbaenidae, Tropidophidae, Bipedidae, Blanidae, Cadeidae, and Rhineuridae); glass, alligator, and legless lizards (Anguidae and Diploglossidae); and blind lizards (Dibamidae). These groups apparently have experienced similar selection pressures leading to convergent evolution on limb reduction or loss and various degrees of fossoriality, although reproductive mode has not converged as some show oviparity and some viviparity, with long reproductive lifespans and generally few offspring per clutch/litter (Pianka & Vitt, 2003). Another example is the crocodile lizard (*Shinisaurus crocodilurus*), the only species in family Shinisauridae. This lizard is long-lived, viviparous, and exhibits low fecundity. It is also threatened due to loss of necessary habitat, but popular and widespread among zoos and in the pet trade, making laboratory, if not field, investigations feasible.

Third, efforts to monitor and protect lizard populations are worthy for biodiversity as well as practical considerations. Increasingly, physiological and neuroendocrine approaches are being used to address conservation goals by informing both causes and consequences of population change or decline as well as providing useful tools for assessment of the effectiveness of actions taken. Given documented effects of endocrine disruptors, habitat alteration or loss, invasion of nonnative species, and climate change as reviewed in Section 1.1.3, there is ample reason to initiate new as well as to continue existing monitoring programs of lizard populations. Monitoring of reproductive cycles and hormonal regulation in particular allow for an integrative assessment of individual responses to environmental inputs. Thus, documenting “baseline” population conditions will greatly inform us whether changes in environmental inputs lead to changes in reproduction and if so, to what extent endocrine modulation is involved.

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Chapter 12

Hormones and reproductive cycles in snakes

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ABBREVIATIONS

APOA	anterior preoptic area of hypothalamus
AVT	arginine vasotocin
BCI	body condition index
CORT	corticosterone
DHT	dihydrotestosterone
E2	17 beta estradiol
ER	estrogen receptor
EDC	endocrine-disrupting chemical
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
GC	glucocorticoid
GnRH	gonadotropin-releasing hormone
GnIH	gonadotropin-inhibitory hormone
GTH	gonadotropic hormone
HPA axis	hypothalamic-pituitary-adrenal axis
HPG axis	hypothalamic-pituitary-gonadal axis
LH	luteinizing hormone
LTD	low-temperature dormancy
NS	nucleus sphericus
P4	progesterone
PGF _{2α}	prostaglandin F2 _{alpha}
PR	progesterone receptor
T	testosterone
T3 and T4	triiodothyronine and thyroxine
TSH	thyroid-stimulating hormone
VMH	ventromedial hypothalamus

1 INTRODUCTION

Although there have been a fair number of studies conducted on snake reproduction, they have predominantly been conducted on a relatively small number of temperate species. This chapter focuses on current knowledge of the hormonal regulation of reproduction in snakes. Because so many studies have been published on hormonal regulation of various aspects of reproduction in snakes, especially the red-sided garter snake (*Thamnophis sirtalis parietalis*), we attempt to provide a broad overview of the

effects of hormones on reproduction in snakes, focusing on seminal studies and referring to review articles when possible. First, we describe what is known about the relationship between sex steroids and reproduction in snakes. In this section, we discuss *T. s. parietalis* separately from all other snake species. Although such a distinction is not phylogenetically justified, it enables us to provide a thorough presentation of the well-studied species without biasing the review of the reproductive endocrinology of snakes in general with data from a single species. We then discuss the limited literature on hypothalamic hormones, gonadotropins, hormone receptors, binding globulins, and neuroendocrinology for all snakes. We follow this with a discussion of environmental, behavioral, and nonreproductive physiological influences on hormones and reproduction in snakes and conclude with conservation implications and suggestions for future research.

2 REPRODUCTIVE CYCLES IN SNAKES

Several attempts have been made to describe/categorize snake reproductive cycles. Classification schemes typically describe the temporal association of spermatogenesis with mating and female reproductive events. Saint Girons (1982), for example, reviewed the reproductive cycles of male snakes in relation to female cycles and climate. In doing so, he described four patterns of male reproduction (i.e., prenuptial/aestival, postnuptial/vernal, continuous, and mixed), and highlighted that these four types of male reproduction in snakes should be viewed as noteworthy points along a cline from distinctly seasonal to continuous and from associated to dissociated. In the over four decades since Saint Girons (1982), it has become increasingly appreciated that the diversity of reproductive strategies exhibited by snakes defies discrete categorization into a handful of strategies. Recently, Aldridge et al. (2020) suggested that the focus on the season (i.e., aestival vs vernal) in the categories proposed by Saint Girons (1982) distracts from

important differences in reproductive strategy, and we agree. We propose that snake reproductive cycles are best characterized by using the terms prenuptial, postnuptial, and continuous to describe spermatogenesis relative to mating. Prenuptial spermatogenesis describes species where seasonal spermatogenesis occurs immediately prior to or coincident with mating, and postnuptial spermatogenesis describes a scenario where spermatogenesis is paused and sperm are stored in the male reproductive tract for a period prior to mating. Continuous spermatogenesis describes aseasonal species in which spermatogenesis occurs year-round. The terms dissociated and associated (Crews, 1976) can be used to describe the production of sex steroids in relation to gametogenesis and or mating. Using these terms, any combination of spermatogenic pattern (pre vs. postnuptial), hormone secretion (associated vs dissociated), and season can be used to describe the reproductive cycle of a population. For example, a population with summer spermatogenesis, spring mating, and elevated androgens in the summer can be described as having postnuptial-associated spermatogenesis and dissociated spring mating. By accommodating combinations of season, spermatogenesis, and hormone secretion, we feel that this standardized classification scheme provides flexibility and enables the description of the diversity of snake reproductive strategies, and we use it throughout this chapter to describe reproductive cycles. We also, again, stress that these categorizations should be considered notable points along a continuum.

Reproductive cycles in female snakes are less variable than in males. Many species exhibit less-than-annual reproduction, in that not all adult females reproduce each year (Duvall et al., 1992). In most species studied, the timing of reproductive events is such that neonates are born or hatched when resources are abundant and climatic conditions are favorable. Typically, ovulation occurs in late spring or early summer, and hatching or parturition occurs in summer or early fall (Saint Girons, 1982). Of course, exceptions exist, especially in the tropics, which have minimal or even lack annual temperature cycles. Regardless, marked seasonality may nonetheless exist in tropical snake populations where annual patterns in precipitation can impose selection on the timing of reproductive events (e.g., Brown & Shine, 2006; Wilson et al., 2006; Mathies, 2011). However, given the great diversity of tropical snakes, further studies are necessary to better understand breeding cycle trends in this critical biome.

Whereas the timing of reproduction is most frequently related to obvious annual temperature or precipitation cycles, other factors including altitude, phylogeny, foraging mode, body size, and body condition may also play critical roles in regulating the timing of reproduction (Dunham et al., 1988; Vitt, 1992). To fully understand reproductive

timing, it is essential to understand reproductive physiology, especially the relationships between hormones and reproductive events (e.g., mating, spermatogenesis, vitellogenesis, gestation, parturition). Snakes are notoriously secretive animals, but advances in techniques for following individual snakes in the wild (e.g., radiotelemetry), as well as in methods of quantifying hormones (e.g., radioimmunoassay) in the 1970s and 1980s allowed researchers to begin examining the hormonal regulation of reproduction in snakes. Some of the earliest studies examined snake gonadotropins (GTHs) and sex steroid hormones, in general confirming that the structure and function of these hormones in snakes are similar to those of their better-studied mammalian counterparts. In particular, *T. s. parietalis* emerged as a model organism for studies of hormonal regulation of reproduction in snakes (Krohmer, 2004), largely because a population in Manitoba, Canada that dens communally by the thousands provided accessibility to large numbers of snakes for both free-ranging and laboratory experiments. Most of our knowledge of advanced topics in this field comes from studies of *T. s. parietalis*. These studies have provided extremely valuable insights into the hormonal regulation of reproduction in snakes, due to the ease of sampling multiple individuals, as well as their ready display of mating behaviors in captivity. However, the extremely high latitude and the unique mating behavior of this population call into question the applicability of these results to snakes in general. More recent studies by several laboratories have provided data on the hormonal regulation of reproduction in other snake species. These comparative data have proven quite valuable, but the universality of existing knowledge on snake reproductive physiology remains uncertain as these additional studies still disproportionately represent temperate species, especially viperids.

2.1 Steroids and reproduction in snakes other than *T. s. parietalis*

Studies of plasma steroid hormone concentrations in free-ranging snakes (other than *T. s. parietalis*) have mostly been conducted on vipers, with several studies on colubrids and hydrophiids. These studies typically examined seasonal variation in hormone concentrations in relation to reproductive events, including mating, spermatogenesis, vitellogenesis, and gestation. Since females of many viperid species mate on a less-than-annual basis, it is possible to collect blood samples for hormone quantification simultaneously from nonreproductive and reproductive females in a single population. This is advantageous for studying the relationship between hormones and reproduction because differences in hormone concentrations between these groups can more likely be attributed to reproduction (although correlated variables such as body condition may contribute to such

differences). In addition, intraspecific and interspecific variation in the association between mating and follicular activity allows hormonal changes responsible for mating behaviors to be at least partially uncoupled from those responsible for physiological events.

2.1.1 Females

In general, studies of the relationship between steroid hormones and reproduction in female snakes show that plasma 17β -estradiol (E_2), and in some studies, testosterone (T), is elevated during vitellogenesis, reflecting the role of E_2 in stimulating the production of vitellogenin by the liver (Ho et al., 1982). Plasma progesterone (P_4) is often elevated during gestation in viviparous snakes, reflecting its role in the maintenance of pregnancy (Mead et al., 1981; reviewed in Custodia-Lora & Callard, 2002). Taylor et al. (2004) described the following patterns in female western diamond-backed rattlesnakes (*Crotalus atrox*). Plasma concentrations of E_2 , T, and P_4 were low in nonreproductive females throughout the year. In reproductive females, E_2 is elevated from March through June, especially in April and May. This timing coincides with vitellogenesis in *C. atrox* (Taylor & DeNardo, 2005) and many other temperate species. Plasma T concentrations are low throughout the year in reproductive females, with a slight elevation in spring that may be due to the production of E_2 (T being a precursor to E_2 in steroid biosynthesis). Increased plasma T during vitellogenesis is observed in the Chinese cobra (*Naja naja*, Bona-Gallo et al., 1980), and three species of snakes that inhabit marine environments to varying extents (*Acrochordus granulatus*, *Cerberus rhynchos*, *Laticauda colubrina*; Gorman et al., 1981). In pygmy rattlesnakes (*Sistrurus miliarius*), E_2 is elevated in individuals during the spring coincident with vitellogenesis; however, snakes with secondary follicles often have baseline E_2 well before ovulation occurs in the population, suggesting that E_2 -induced vitellogenesis is either completed before ovulation or may be pulsatile in some species (Lind et al., 2020). Saint Girons et al. (1993) found that female aspic vipers (*Vipera aspis*) displaying mating behaviors had significantly higher plasma dihydrotestosterone (DHT) concentrations, but not T concentrations, than females that were not displaying mating behaviors; however, sample size was very low in this analysis. In viviparous *C. atrox*, P_4 is elevated in reproductive females in May through August, and peaks in June–July, corresponding with the gestation period (Taylor et al., 2004). In the oviparous *N. naja*, P_4 concentrations similarly are elevated from ovulation until oviposition (Bona-Gallo et al., 1980). In *C. atrox*, plasma corticosterone (CORT) concentrations are more variable annually than the other steroid hormones but increase dramatically in reproductive females in July and August, during late gestation. Plasma CORT and P_4 concentrations

return to baseline by September (after parturition) in reproductive females (Taylor et al., 2004). Similarly, pregnant *S. miliarius* exhibits a steady rise in plasma CORT during gestation with a sharp drop immediately following parturition (Lind et al., 2020). In early pregnancy, *S. miliarius* exhibits no differences in either baseline or stress-induced CORT compared to nonreproductive snakes (Lind et al., 2023). Both baseline and stress-induced CORT rise during late pregnancy in *V. aspis* (Lorioux et al., 2016), and a rise in baseline CORT late in gravidity has also been observed in oviparous Children's pythons, *Antaresia childreni* (Brusch et al., 2020). The exact role of CORT during pregnancy/gravidity is equivocal. Some authors have suggested that the rise in CORT immediately preceding birth may serve as a cue for parturition in viviparous snakes (Taylor et al., 2004). Alternatively, the rise in CORT may be the result of increased energetic constraints experienced during late compared to early pregnancy (Lorioux et al., 2016). These two hypotheses are not mutually exclusive and should be tested in future studies.

Female *V. aspis* in France has the same seasonal mating patterns as *C. atrox* (spring vitellogenesis and mid to late summer parturition) and also show elevated E_2 during spring (Saint Girons et al., 1993). Interestingly, reports of plasma P_4 concentrations in reproductive *V. aspis* are inconsistent. Saint Girons et al. (1993) concluded that plasma P_4 is highly variable among snakes, with no clear difference between reproductive and nonreproductive females. However, in other studies, plasma P_4 concentrations of reproductive female *V. aspis* are elevated in May through August (gestation), decrease in September (after parturition), and rise again during winter (Bonnet et al., 2001; Naulleau & Fleury, 1990). Bonnet et al. (2001) hypothesized that the winter increase in P_4 , which is especially marked in postreproductive snakes, may act to block vitellogenesis in seasonally emaciated snakes, as P_4 may inhibit hepatic synthesis of vitellogenin (Callard et al., 1992, 1994). In Chinese green tree vipers, *Trimeresurus stejnegeri*, E_2 concentrations are slightly elevated during vitellogenesis and P_4 concentrations are dramatically elevated during gestation (Tsai & Tu, 2001). Almeida-Santos et al. (2004) found similar relationships between sex steroid concentrations and reproductive states in female Neotropical rattlesnakes (*Crotalus durissus terrificus*) in southeastern Brazil. In this species, E_2 peaks during vitellogenesis (winter) and P_4 is high during gestation (late winter, spring, and summer), whereas both hormones are low throughout the year in nonreproductive females.

2.1.2 Males

As with females, the intraspecific and interspecific variation in the number of mating periods per year and timing of the mating periods relative to gonadal activity among temperate

male snakes provides the potential opportunity to uncouple the relationship between T and breeding behavior from that of T and other physiological events, including spermatogenesis. Several studies have shown that T is elevated during the breeding season(s), providing a strong indication that T stimulates reproductive behaviors in male viperids.

In viperid species with a single annual breeding season, plasma T concentrations are high coincident with spermatogenesis and either coincident with or preceding breeding behaviors. In the cottonmouth (*Agkistrodon piscivorus*), T peaks in late summer, at the same time that males show spermiogenic activity, hypertrophy of the sexual segment of the kidney, and breeding behavior (Graham et al., 2008; Johnson et al., 1982; Zaidan et al., 2003). Similarly, the black-tailed rattlesnake (*Crotalus molossus*) has a single mating season (late summer) and shows a single annual peak in T concentrations during this time (Schuett et al., 2005). The Chinese cobra (*N. naja*) and European adder (*Vipera berus*) show a similarly coordinated single peak in T at the same time as spermiogenesis and mating, although these occur in the spring rather than late summer (Bona-Gallo et al., 1980; Loftis et al., 1966; Naulleau & Fleury, 1984). In some species with a single late-summer mating season (e.g., timber rattlesnakes, *Crotalus horridus*) T steadily rises throughout summer (presumably coincident with spermatogenesis), but declines sharply during the peak month where mating is observed (Aug; Lind & Beaupre, 2014, 2015). Similarly, in subtropical *S. miliaris*, peak mating activity is observed in September and October but continues through February (Lind et al., 2018). Male T steadily rises through the summer months and peaks in August, immediately preceding the mating season. Plasma T then steadily declines to baseline over the extended 5–6 month mating season. Males sampled while engaging in reproductive associations with females had higher T compared to the general population, and this relationship held over the course of the mating season. In this sense, both *C. horridus* and *S. miliaris* exhibit a mating strategy described as prenuptial and, at least partially, dissociated with respect to peak T, although histological studies are necessary to confirm the timing of spermatogenesis.

Many snake species show two annual mating seasons, usually in spring and fall (separated by summer and winter periods of reproductive inactivity). In Saharan sand vipers (*Cerastes vipera*), active spermatogenesis with associated elevations in plasma testosterone occurs in both the fall and spring, while mating occurs in spring (Sivan et al., 2012). Common water snakes (*Nerodia sipedon*), rough greensnakes (*Opheodrys aestivus*), *C. atrox*, Mojave rattlesnakes (*Crotalus scutulatus*), and *V. aspis* breed in both spring and fall, and males show elevated T in both of these seasons (Aldridge et al., 1990; Naulleau et al., 1987; Saint Girons et al., 1993; Schuett et al., 2002, 2005; Taylor et al., 2004; Weil & Aldridge, 1981). *C. atrox* and *C. scutulatus*

show bimodal peaks in circulating DHT at the same time as the peaks in T (Schuett et al., 2002, 2005), although DHT concentrations are much lower than T concentrations. In a laboratory study, T concentrations of copperheads (*Agkistrodon contortrix*) peak in spring and late summer, corresponding to their breeding seasons (Schuett, Fernandez, et al., 1997). These species show the postnuptial pattern of spermatogenesis: T concentrations are high during spermatogenesis and mating in the late summer and fall, but are also high during the spring when mating is occurring in the absence of spermatogenesis. Testosterone concentrations are lowest in summer when the breeding activity does not occur. In the Sonoran Desert, *C. atrox* sampled while basking during the winter when the species is mostly inactive exhibit intermediate T concentrations between those of summer (lowest) and of the spring and fall breeding seasons (Schuett et al., 2006). The reason for elevated T concentrations in winter is unknown, but it is possible that basking behavior during mild winter days prepares the snakes for the abrupt onset of the spring mating season or that there is low winter metabolic clearance of steroids produced in the fall, as has been suggested in *T. sirtalis* (Krohmer & Lutterschmidt, 2011).

Interestingly, absolute plasma steroid hormone concentrations of many viperids appear to be much higher than those of most other snakes and other vertebrates. For example, peak T concentrations in *A. contortrix* and rattlesnakes are in the range of 100 ng/mL (Schuett et al., 2002, 2005; Schuett, Harlow, et al., 1997; Taylor et al., 2004), which is 1–3 orders of magnitude higher than concentrations observed in many other snakes. However, the closely related cottonmouth (*A. piscivorus*) has much lower plasma T concentrations than *A. contortrix* (Graham et al., 2008; Johnson et al., 1982; Zaidan et al., 2003), and the congeneric *C. molossus*, *C. horridus*, and eastern diamondback rattlesnakes (*C. adamanteus*) have substantially lower steroid concentrations than *C. atrox* or northern Pacific rattlesnakes (*Crotalus oreganus*) (Hoss et al., 2011; Lind & Beaupre, 2014; Lind et al., 2010; Lutterschmidt et al., 2009; Schuett et al., 2005). *S. miliaris* exhibits T concentrations intermediate between these extremes (Lind et al., 2018). Even within species, large variation in absolute T concentration has been reported. For example, Lind and Beaupre (2014) report plasma T concentrations from an Arkansas population of *C. horridus* that are nearly an order of magnitude higher than concentrations reported in a population from Pennsylvania (Lutterschmidt et al., 2009). The reasons for, and implications of, differences in absolute steroid concentrations in snakes, whether they be related to assay methodology, phylogeny, or ecophysiology/adaptation are unknown and should be investigated.

Studies on species with bimodal mating seasons, where spermatogenesis and mating are uncoupled in at least one season, suggest that T is responsible for mating behaviors,

since T is elevated during both mating periods regardless of whether the testes are spermatogenically active or regressed (e.g., Lind et al., 2010; Taylor et al., 2004). Some exceptions are evident: male *V. aspis* sometimes has very low androgen concentrations during mating (Naulleau et al., 1987; Saint Girons et al., 1993).

Most research on steroid hormones in snakes has focused on terrestrial snakes from temperate regions, in which reproduction is highly seasonal. Noteworthy exceptions, however, deserve comment. Gorman et al. (1981) described the seasonal steroid hormone concentrations in relation to reproductive events in three semi-marine to marine snakes (*A. granulatus*, *C. rhynchops*, and *L. colubrina*) from the Philippines. These three species show varying degrees of seasonality in their reproductive patterns. *A. granulatus* is the most seasonal, with mating activity, spermatogenesis, and peak T concentrations occurring in the fall, shortly after vitellogenesis occurs in females. In contrast, *C. rhynchops*, and especially *L. colubrina* show spermatogenesis throughout the year. Although spermatogenesis and T concentrations in *C. rhynchops* peak in the fall, there is evidence for year-round gonadal activity. In *L. colubrina*, males show no seasonal trends in spermatogenic activity or T concentrations. These data suggest that species inhabiting more thermally constant environments, such as the ocean and the tropics, may show year-round reproduction. However, the gonadal cycles of many tropical snakes are highly seasonal (Brown & Shine, 2006). The same is likely true for hormonal cycles. For example, in Central American boa constrictors (*Boa imperator*), male T is strongly linked to annual wet-dry cycles (Holding et al., 2014).

2.2 Steroids and reproduction in red-sided garter snakes (*T. sirtalis parietalis*)

2.2.1 Females

Unlike our limited knowledge regarding steroids and reproduction in other species of snakes, studies of *T. s. parietalis* go beyond a basic correlational examination between plasma hormone concentrations and the reproductive cycle. Studies of the reproductive endocrinology of *T. s. parietalis* have employed manipulative designs to more closely examine their reproductive endocrinology. Female *T. s. parietalis* exhibits a rather unusual relationship between reproductive events and hormones in comparison to other species of snakes. Notably, they do not show elevated P₄ during gestation (Whittier, Mason, & Crews, 1987). Like many other snakes studied, plasma T is elevated during vitellogenesis (Whittier, Mason, & Crews, 1987). Spring mating occurs when E₂ concentrations are low (Garstka et al., 1982), but Mendonça and Crews (1996) have shown through ovariectomy and hormone replacement therapy that even low E₂ concentrations appear to be important in making female snakes attractive and receptive to males.

Additionally, the physical act of mating induces a surge in E₂ in females, but plasma E₂ concentrations are not necessarily elevated during vitellogenesis (Garstka et al., 1985; Mendonça & Crews, 1990; Whittier & Crews, 1989; Whittier, Mason, & Crews, 1987). Copulation, but not courtship, stimulates the release of prostaglandin F_{2α} (PGF_{2α}) by female *T. s. parietalis* (Whittier & Crews, 1989). It was hypothesized that this prostaglandin, possibly along with the mating-induced surge in plasma E₂, causes females to become unreceptive and unattractive to males. This decline in receptivity is affected by cloacal distention during mating, which transmits a neural signal to the brain, and the decline in attractivity is mediated possibly by a pheromone present in copulatory plugs deposited by males (Mendonça & Crews, 2001).

Whittier (1992) examined the effects of steroid hormones on ovarian and liver tissue in the adult female *T. s. parietalis*. Treatment of nonreproductive females with E₂ stimulates an increase in oviductal mass and oviductal cell size but does not affect ovarian mass. 17β-estradiol also stimulates an increase in liver mass and hepatocyte size and number. Treatment with T and DHT stimulates an increase in oviductal mass and oviductal cell size and a slight increase in hepatocyte size and number. These data suggest that E₂ and androgens, even when not aromatizable (i.e., DHT), can affect the oviduct and to a lesser extent the liver of female *T. s. parietalis*.

2.2.2 Males

Male *T. s. parietalis* emerges from communal hibernacula in spring and initiates courtship behavior that lasts several weeks (Crews & Garstka, 1982). Hawley and Aleksiuk (1975, 1976) showed that increased temperatures experienced during spring stimulate reproductive behaviors. The onset of male courtship behavior seems to be independent of T and, instead, dependent on warming after low-temperature dormancy (LTD) emergence (Hawley & Aleksiuk, 1975, 1976). In fact, males that have been castrated for as long as 3 years will still court females after emergence from LTD. Krohmer and Crews (1987a) suggest a possible role for the anterior preoptic area of the hypothalamus (APOA) in the impacts of temperature on courtship, as lesions to this area disrupted both courtship behavior and thermoregulation. During spring mating, males utilize sperm produced the previous summer and stored through the winter in the vasa deferentia (Krohmer et al., 1987); according to the paradigms described earlier, they therefore exhibit a postnuptial, dissociated reproductive tactic (Crews, 1976). However, studies suggest that *T. s. parietalis* also mate in the late summer and fall (Mendonça & Crews, 1989; Whittier & Crews, 1989), and more recent studies demonstrate that (1) T is elevated in fall and remains elevated throughout winter dormancy (Krohmer & Lutterschmidt, 2011), and (2) blocking

aromatase activity during winter dormancy eliminates male courtship behavior (Krohmer, 2020).

It was initially reported that plasma androgen concentrations are low in the spring when mating occurs (Camazine et al., 1980), again highlighting the dissociated pattern of reproduction in this species. However, later studies demonstrated that androgen concentrations are in fact elevated upon emergence from LTD but drop rapidly after emergence (Krohmer et al., 1987; Moore et al., 2000). In accordance with this, the sexual segment of the kidney, which is known to be stimulated by androgens in squamate reptiles (Bishop, 1959), was hypertrophied in the late summer and the spring but regressed during the summer when androgen concentrations are low (Krohmer et al., 1987). Since there is now evidence for both spring and late summer mating, and elevated T during both fall and emergence from LTD in spring, this species does not exhibit a strictly dissociated pattern of reproduction. However, mating behavior is not actively dependent upon T in male *T. s. parietalis* (Camazine et al., 1980; Crews, 1984). Courtship behavior is hypothesized to be independent of androgens because castration, adrenalectomy, or hypophysectomy fails to prevent courtship behavior in males, whereas treatment with hypothalamic hormones, GTHs, arginine vasotocin, or sex steroid hormones does not stimulate courtship behavior in adult males (Crews et al., 1984; Garstka et al., 1982). It is possible that prior exposure to androgens organizes brain regions involved in reproduction, and then low temperatures in winter followed by a spring warming activate reproductive behaviors. In support of this hypothesis, T implants elicit courtship behavior in neonatal and yearling, but not adult, males and females (Crews, 1985).

Counterintuitively, recent studies demonstrate that male courtship behavior is dependent upon neural aromatase activity during winter LTD, suggesting an important role for sex steroids in the control of male mating behaviors (Krohmer, 2020). The hypotheses that male mating behavior is independent of sex steroids and that mating behavior is dependent upon aromatase activity are mutually exclusive. These and other testable and contradictory hypotheses, which have emerged in the literature on the hormonal control of reproductive behavior in *T. sirtalis*, need to be addressed in future studies.

2.3 Hypothalamic hormones, gonadotropins, hormone receptors, binding globulins, and steroidogenic gene expression in snakes

2.3.1 Hypothalamic hormones and gonadotropins

Data on the structure and distribution of gonadotropin-releasing hormone (GnRH) in snakes are scarce and

contradictory. Licht et al. (1984) found that chicken and mammalian GnRH failed to stimulate gonadotropic hormone (GTH) release in Chinese cobras (*N. naja*). However, other studies have provided evidence that snakes possess a hormone resembling either mammalian or chicken GnRH. Nozaki et al. (1984) found cell bodies in the hypothalamus and hippocampus of Japanese rat snakes (*Elaphe climacophora*) that were immunoreactive for mammalian GnRH. Sherwood and Whittier (1988) used high-performance liquid chromatography to show that *T. s. parietalis* has a single form of GnRH that most closely resembles cGnRH-I. Smith et al. (1997) examined the distribution of GnRH in the brains of male *T. s. parietalis* and confirmed that this species has a GnRH resembling cGnRH-I, with GnRH-like immunoreactivity distributed in many brain regions.

Only a single study has measured concentrations of gonadotropic hormones GTHs in snakes. Bona-Gallo et al. (1980) found that plasma GTH concentrations (detected using a radioimmunoassay with a labeled cobra GTH of unspecified structure) peaked in male *N. naja* during winter when testicular recrudescence begins. In females, plasma GTH concentrations also peaked in winter but showed a second peak during spring vitellogenesis. In general, the relationship between GTH concentrations, steroid hormone secretion, and events in the reproductive cycle is not straightforward in this species.

Studies have indirectly assessed the actions of GTHs by evaluating the effects of mammalian GTHs on androgen secretion and testicular development in snakes. Both mammalian follicle-stimulating hormone (FSH) and luteinizing hormone (LH) stimulate gametogenesis, steroid production, and ovulation in snakes (reviewed in Licht, 1974). Tsui and Licht (1977) showed that LH and FSH from various species of vertebrates, including other reptiles, stimulated androgenesis in *C. atrox* and *T. s. parietalis*, but to varying degrees (depending both on the source species of the GTH and the species being tested). This study also suggested that snake GTHs may be structurally divergent from those of many other vertebrates because snake GTHs failed to elicit androgen production in turtle gonads. Garstka et al. (1985) showed that ovine FSH stimulated hepatic yolk synthesis and yolk deposition into eggs of *T. s. parietalis*. Krohmer (1986) found evidence that ovine FSH stimulates testicular development (e.g., increased testis mass, seminiferous tubule diameters, and epithelial cell size), and ovine LH stimulates androgenesis in immature northern water snakes (*N. sipedon*). Interestingly, however, these effects may depend on season, as bovine LH (but not FSH) stimulates androgenesis in the spring, and bovine FSH (but not LH) stimulates androgenesis in the summer and fall in adult water snakes (Weil, 1982). Alternatively, ovine and bovine GTHs may be divergent enough in structure to elicit different responses in water snakes, or the differences in the

two studies may have to do with the maturity of the snakes. Studies utilizing radioiodinated human FSH showed that this hormone binds readily to snake gonads and that LH may act at the same binding site as FSH (Licht et al., 1977, 1979; Licht & Midgley, 1976, 1977). Collectively, these studies raised the question as to whether snakes actually have two GTHs, or a single GTH with the actions attributed collectively to FSH and LH in mammals. Although this question has yet to be answered definitively, a study using tissues from the lancehead (*Bothrops jararaca*) successfully cloned the snake FSH receptor (FSHR) but failed to identify a receptor in the LH receptor family (Bluhm et al., 2004). Similarly, an FSH beta gene has been annotated in the genomes of *T. sirtalis* (NCBI reference sequence NW_013658033.1) the tiger rattlesnake, *Crotalus tigris* (NCBI reference sequence NW_024097195.1), and the corn snake, *Pantherophis guttatus* (NCBI reference sequence NW_023010720.1). None of these annotated snake genomes indicate the presence of an LH gene or receptor. It is therefore possible that snakes have a single GTH that stimulates gametogenesis, steroid production, and ovulation, or conversely that snake LH and the LH receptor are distinct from mammalian and avian homologs in structure.

In summary, although ongoing work in other vertebrate groups continues to push the field of reproductive neuroendocrinology forward, snakes have, with a few notable exceptions, been left out of such progress. Our current understanding is that snakes express GnRH-I and its receptor (Winters et al., 2022), but may have a single gonadotropin. Other advances in the field, including the identification of the role of gonadotropin-inhibitory hormone (GnIH) in vertebrate reproduction (including squamates, Singh et al., 2008) and elucidation of the fact that both GnRH and GnIH may act as paracrine signals produced by the gonads that play important roles in coordinating spermatogenesis and steroidogenesis independently of pituitary gonadotropin signaling (McGuire & Bentley, 2010a, 2010b; Meccariello et al., 2014), have important implications for neuroendocrine integration. However, these mechanisms have not been investigated in snakes. An understanding of such important nuances of neuroendocrine regulation of snake reproduction has the potential to explain conflicting experimental outcomes of early studies of the neuroendocrine regulation of reproduction in snakes (e.g., effects of castration, hypophysectomy, and aromatase on reproduction in *T. s. parietalis*).

2.3.2 Steroid receptors

Like studies of GTH receptors, studies of steroid hormone receptors and binding proteins in snakes are limited. Kleis-San Francisco and Callard (1986) identified the P₄ receptor (PR) in the oviduct of *Nerodia* sp. The presence of PRs in the oviducts of this viviparous snake may reflect

its role in inhibition of oviductal motility during pregnancy (Callard et al., 1992). Riley and Callard (1988) described a high-affinity, nuclear estrogen receptor (ER) in the liver of female *Nerodia* sp., reflecting the role of this hormone in vitellogenesis in snakes. In a phylogenetic study of ER receptor affinity, the Okinawa habu (*Protobothrops flavoviridis*) and the Japanese four-lined ratsnake (*Elaphe quadrivirgata*) were demonstrated to have the highest E₂ sensitivity of all vertebrate taxa studied to date (Katsu et al., 2010; Yatsu et al., 2016). However, the functional significance of high ER affinity in these snakes is unknown. More recently, Ashton et al. (2018) examined seasonal variation in the mRNA expression of estrogen receptor alpha in *T. s. parietalis*. Estrogen receptor expression was not correlated with sex or E₂ levels but did exhibit seasonal variation in the liver. Specifically, females exhibited higher mRNA expression in the fall when E₂ levels were low. The authors hypothesize that increased fall expression in females may prime energy storage pathways in preparation for spring vitellogenesis.

2.3.3 Steroid binding proteins and steroidogenic enzymes

Only a few studies exist on steroid hormone-binding proteins (SHBP) in snakes. Riley et al. (1988) identified an SHBP in *Nerodia* sp. with a broad binding specificity, binding to E₂, P₄, T, DHT, and CORT with medium-high affinity and limited capacity. In a phylogenetic analysis, Desantis et al. (2013) identified a lower proportion of GCs bound to binding globulins in mud snakes (*Farancia abacura*) compared to most other vertebrates. The potential for advanced molecular tools (e.g., transcriptomics and genomic sequencing) to transform our understanding of the role of protein receptors and binding globulins in snake reproductive cycles cannot be overstated.

The seasonal expression of steroidogenic enzymes has received little attention in the literature on snake reproduction. A recent study by Lincoln et al. (2023) examined the gonadal expression of genes for key steroidogenic enzymes (StAR, HAS17b3, Cyp17a1, and aromatase) in *T. s. parietalis*. Counterintuitively, expression of StAR, a rate-limiting enzyme in steroidogenesis, was highest in males during the spring mating season when the gonads are regressed and T is either low or declining. StAR expression could be increased to upregulate the synthesis of a nonsex steroid (e.g., CORT); however, expression was measured in the gonad and expression of Cyp17a1, an enzyme involved in the production of T precursors, was also high in the spring (although not significantly different from other seasons). Given that gonadal steroidogenic activity was associated with breeding in this classic dissociated mating system, the authors state that their results “further call into question the associated-dissociated binary when describing hormone-behavior patterns in vertebrate

reproduction." We agree and again stress that seasonal reproductive cycles in snakes should be considered as points along a continuum from associated to dissociated.

2.4 The neuroendocrinology of reproduction in snakes

Most of our current knowledge of the neuroendocrinology of reproduction in snakes comes from studies on *T. s. parietalis*. This species has a distribution of sex-steroid-responsive brain neurons similar to other vertebrates (Crews & Silver, 1985). Researchers have utilized histological techniques, hormone implants, lesioning, and surgical removal techniques to study the roles of the anterior preoptic area of the hypothalamus (APOA), the ventromedial hypothalamus (VMH), the nucleus sphericus (NS, or amygdala), the septum, and the pineal gland (see Section 3.1) in regulation of reproduction in *T. s. parietalis* and a few other species.

Crews et al. (1993) found evidence for sexual dimorphism in two brain regions in laboratory-housed *T. s. parietalis*: hibernating females have a smaller APOA than hibernating males, and females prevented from hibernating have a smaller NS than males prevented from hibernating. Otherwise, there are no sexual dimorphisms in the brain regions studied. Males show no seasonal or hormone implant-induced variation in APOA, VMH, or NS volume, whereas females show significantly smaller APOA and VMH regions during LTD than during the rest of the year. Implants of E₂ stimulated an increase in the size of the APOA of females (Crews et al., 1993).

The APOA is integral in the coordination of stimuli and sexual behaviors in this species (Friedman & Crews, 1985a, 1985b; Krohmer & Crews, 1987a), which is typical of vertebrates in general (Crews & Silver, 1985; Panzica et al., 1996). Lesions to the APOA in male *T. s. parietalis* at various times in the annual cycle abolish courtship behavior (Friedman & Crews, 1985a, 1985b; Krohmer & Crews, 1987a). These lesions also disrupt thermoregulatory behavior (Krohmer & Crews, 1987a), and since exposure to cold winter temperatures followed by heating in spring is essential to the activation of courtship behavior in males (Hawley & Aleksiuk, 1975, 1976), it is probable that the APOA integrates temperature cues with efferent networks important in courtship behavior.

The NS and the septum appear to play inhibitory roles in courtship behavior in male *T. s. parietalis*, as lesions to these areas facilitate courtship behavior in males (Krohmer & Crews, 1987b). Lesions to the NS, but not the septum, lead to a significant increase in circulating androgens, and lesions to both areas increase the number of sexual granules in the sexual segment of the kidney (Krohmer & Crews, 1987b).

The enzyme aromatase is responsible for converting T to estrogens, and it is E₂ rather than T that actually stimulates many male sexual behaviors in vertebrates (Roselli et al., 1985; Vagell & McGinnis, 1997). Therefore, the distribution of aromatase in the male brain can be informative about areas of the brain important in sexual behavior. Krohmer et al. (2002) found evidence for aromatase immunoreactivity throughout the brain of *T. s. parietalis*, but it was highly concentrated only in the APOA, the NS, and the septum. Because these are the brain areas associated with courtship behavior, local conversion of androgens to E₂ may be important in the activation of sexual behavior. Indeed, recent studies demonstrate a causal link between neural aromatase and male mating behavior (Krohmer, 2020). Again, these are surprising findings considering that studies have repeatedly shown that mating behavior in male *T. s. parietalis* is not dependent on androgens (Camazine et al., 1980; Crews, 1984; Crews et al., 1984; Garstka et al., 1982).

3 INFLUENCES ON THE REPRODUCTIVE CYCLE

3.1 Environmental influences

Environmental stimuli are critical to seasonal reproduction in most animals. In mammals and birds, photoperiod is the primary proximate factor that regulates reproductive activity (reviewed in Dawson et al., 2001). However, in snakes and other reptiles, temperature appears to be the primary cue that controls reproductive activity, although experimental studies are, again, disproportionately focused on high-latitude *T. s. parietalis*, and there are likely species where other environmental cues are used to coordinate snake reproductive cycles. In *T. s. parietalis*, the duration of exposure to cold temperatures is critical for stimulating reproduction, whereas photoperiod has inconsistent effects (reviewed in Whittier, Mason, Crews, & Licht, 1987).

Although the relationship between temperature and reproduction has been known for decades, the hormonal mechanisms by which the interaction operates remain unclear. Candidate hormonal mechanisms linking temperature and reproduction include sex steroids, melatonin, and thyroid hormones. Below we summarize what we know about these mechanisms in snakes.

Recent work on *T. s. parietalis* has demonstrated that GnRH-expressing neurons in the medial preoptic area of the hypothalamus are sensitive to temperature. Specifically, low-temperature winter dormancy increased immunoreactive cell number and size. These findings are consistent with the hypothesis that neuroendocrine GnRH signaling is involved in previously established trans-seasonal effects of steroids on male reproductive behavior (Lutterschmidt et al., 2022). In a study by Krohmer and Jurkovic (2020),

exposure to both LTD and exogenous sex steroids increased dendritic spine density in the APOA. The authors suggest that LTD and sex steroids work in concert to organize/entrain spring mating behavior in males.

In *N. naja*, LTD reduces plasma concentrations of numerous hormones, and concentrations return to elevated levels in spring (El-Deib, 2005). Thyroid hormone warrants specific attention as a potential regulator between temperature and reproduction. There is a direct relationship between temperature and iodine uptake by the thyroid (Turner & Tipton, 1972a), and cold temperatures can reduce target tissue responses to thyroid hormones (Turner & Tipton, 1972b). As in other taxa, thyroxine (T_4) increases tissue metabolism in snakes (Thapliyal et al., 1975), but it may also have a direct effect on reproduction. In the most thorough study of T_4 and reproduction in snakes, Bona-Gallo et al. (1980) demonstrated a pronounced annual T_4 cycle with plasma concentrations lowest in winter and substantially elevated during late spring. In males, plasma T_4 concentrations were elevated when testes mass and plasma T concentrations peaked in May, but plasma T_4 concentrations did not actually peak until June, which led the authors to suggest that T_4 may play a role in testis regression. In females, T_4 concentrations peak at the height of ovarian activity (May).

Similarly, in male *V. berus*, circulating concentrations of T_4 are greatest during the period of sexual activity (L. Kelley, reported in Garstka et al., 1982). However, acutely treating sexually inactive male *T. s. parietalis* with either T_4 or triiodothyronine (T_3) led to no changes in reproductive activity within 24 h (Garstka et al., 1982).

Interestingly, thyroid-stimulating hormone (TSH) treatment of hypophysectomized male glossy snakes (*Arizona elegans*) restored spermatogenesis (Chiu & Lynn, 1971), but the mechanism of this effect was not determined, and the effect may simply be a result of cross-reactivity of TSH with GTH receptors. Recent work in *T. s. parietalis* has elucidated experimental links among temperature, the thyroid axis, melatonin, and reproduction. Whereas 6 weeks of LTD reduces TSH-immunoreactive (TSH-ir) cell numbers in the brain, treatment with the melatonin precursor 5-hydroxytryptophan eliminates the effect of LTD on TSH-ir cell number and affects male courtship behavior (Winters et al., 2022). The authors conclude that the thyroid axis is temperature sensitive and responsive to melatonin.

The pineal gland also deserves specific attention as a potential mediator between environmental conditions and reproduction (Pang et al., 1998; Tamarkin et al., 1985). In reptiles, the pineal gland, through the secretion of melatonin, communicates changes in photoperiod and environmental temperature to the body. In turn, melatonin affects circadian cycles, thermoregulation, and reproductive

activity (reviewed in Tosini, 1997). In *T. s. parietalis*, melatonin shows a diel cycle with elevated plasma concentrations during scotophase; however, during LTD in total darkness, plasma melatonin is undetectable (Mendonça et al., 1995). Within hours of emergence, circulating melatonin concentrations peak. In the diamondback water snake (*Nerodia rhombifer*), cool temperatures reduce plasma melatonin concentrations and remove the difference in plasma concentration between samples taken during the scotophase and those taken during the photophase despite controlling photoperiod at 12:12 L:D (Tilden & Hutchison, 1993).

Regarding melatonin's effect on reproductive activity, pinealectomy disrupts gonadal development and regression in Indian checkered water snakes (*Fowlea (Natrix) piscator*, Haldar & Pandey, 1989). Seasonal melatonin cycles vary among *T. sirtalis* populations with differing climate conditions and courting cycles, although the relationship between the interpopulation variations in melatonin and courtship is unclear (Lutterschmidt & Mason, 2008). Males that court during the spring have typical melatonin diel cycles (with increased concentrations during the scotophase) whereas males that do not court have reversed diel cycles (Mendonça et al., 1996a). Pinealectomizing males prior to winter LTD reduces courtship behavior the following spring (Crews et al., 1988; Mendonça et al., 1996a; Nelson et al., 1987). Furthermore, treatment with exogenous melatonin inhibits courtship behavior (Lutterschmidt et al., 2004). Treatment with a melatonin receptor antagonist, luzindole, increased GnRH immunoreactive cell number in male *T. s. parietalis* exposed to 6 weeks of LTD, suggesting that GnRH signaling is sensitive to melatonin (Winters et al., 2022). Furthermore, treatment with the melatonin precursor, 5-hydroxytryptophan, eliminates the effect of LTD on TSH-ir cell number and affects male courtship behavior (Winters et al., 2022). Together, these results demonstrate that gonadal development and courtship behavior depend on diel cycles of melatonin. However, pinealectomy in spring does not alter existing courtship behavior, suggesting that melatonin cycles are critical for providing the initial stimulus for courtship but not for modulating courtship once initiated (Mendonça et al., 1996b).

3.2 Social influences

Although descriptive studies on seasonal hormonal cycles in snakes have been conducted in numerous species, it is remarkable that studies that manipulate sex steroid profiles are almost completely limited to studies of *T. s. parietalis*. As a result, knowledge of how specific reproductive behavior components (e.g., attractivity, courtship, receptivity, copulation) are regulated and how these events influence the hormonal milieu of the individuals experiencing them comes almost exclusively from this species.

3.2.1 Attractiveness, courtship and copulation

Courting by males is enhanced by the presence of other males (Joy & Crews, 1985). In females, attractiveness is not affected by ovariectomy during either the fall or the LTD period preceding emergence but is abolished when females are ovariectomized in the spring prior to emergence. Receptivity, on the other hand, is inhibited by ovariectomy at any of the three time points. Receptivity was reinstated in ovariectomized females that were given exogenous E₂ 1 hour prior to emergence, but not in females treated approximately 1 week after emergence (Mendonça & Crews, 1996). It is important to reiterate that the seasonal hormonal patterns of other snake species can differ greatly from that of *T. s. parietalis* (see earlier discussion), and thus the hormonal and social effects on reproductive behavior in this discussion, which is based predominantly on *T. s. parietalis*, should not be broadly applied to snakes in general.

Whereas environmental stimuli tend to regulate the seasonality of reproduction, social cues often fine-tune the timing of reproduction. Snakes are mostly solitary animals, and thus locating a mate is not a foregone conclusion. To facilitate mate acquisition, it has been demonstrated in many species that females produce pheromones that enable males to locate them (LeMaster et al., 2001). Methyl ketones secreted from the skin of the female's dorsum are also critical to female attractiveness and induce intensive courtship by males (reviewed in Mason, 1993; Mason et al., 1989). These sexual signals are highly specific and thus can contribute both to species isolation (Ford & O'Bleness, 1986; Mason, 1993; Shine et al., 2002, 2004) and population isolation (LeMaster & Mason, 2003), as well as enable males to distinguish female body size (LeMaster & Mason, 2002), distinguish female mating history (O'Donnell et al., 2004), and orient copulatory posture (Shine, O'Connor, & Mason, 2000). Additionally, male snakes possess skin lipids that inhibit female pheromone-induced courtship by other males (Mason et al., 1989).

Brown tree snakes (*Boiga irregularis*) are atypical in that both males and females show courtship behavior (Greene & Mason, 2000). In this species, females use a combination of an attractiveness pheromone and an inhibitory pheromone, the latter of which is present in cloacal secretions, to regulate courtship by males; however, cloacal secretions from males or females do not inhibit female courtship (Greene & Mason, 2003).

The act of copulation leads to numerous physiological changes. Because sperm have been delivered during copulation, future copulations are, theoretically, of less value to the female and would negatively affect the reproductive success of the first male (Levine et al., 2020; Uller & Olsson, 2008). The ejaculate of male *T. s. parietalis* contains secretory granules from the renal sex segment that

combine with cloacal secretions from the female to form a gelatinous copulatory plug at the oviductal-cloacal junction. Copulatory plugs are relatively rare among snakes and may reflect costs associated with high-density mating aggregations (reviewed in Uribe et al., 1998). The copulatory plug provides a temporary physical barrier against future matings. Inhibition of attractiveness may be the result of chemical cues in the semen, as females smeared on the back with semen, but not ones smeared with a copulatory plug, failed to elicit courting responses from males (Shine, Olsson, & Mason, 2000). Although the seminal chemical cues have not yet been identified, it has been proposed that PGF_{2α} may play a role. PGF_{2α} is present in the semen of other vertebrates. Female plasma concentrations of PGF_{2α} are elevated immediately after copulation in *T. s. parietalis*, and injection of PGF_{2α} decreases attractiveness and receptivity in unmated females. Mechanical stimulation of the reproductive tract can stimulate endogenous release of prostaglandins, and thus the female may provide an alternate or additional source of PGF_{2α} (reviewed in Mendonça & Crews, 2001). Regardless of whether PGF_{2α} is involved, physical stimulation of the cloaca is important in altering attractiveness and receptivity at copulation in *T. s. parietalis* (reviewed in Mendonça & Crews, 2001). Additional work has demonstrated that sensory input from the cloaca at copulation alters patterns of metabolism in the VMH, an area of the brain often associated with female sexual behavior (Mendonça et al., 2003).

In addition to playing a role in altering attractiveness and receptivity, physical cues during mating stimulate the progression of reproductive activity in the ovary. Shortly after copulation, female *T. s. parietalis* have a surge in plasma E₂ and undergo ovarian recrudescence. These responses can be prevented by the removal of cloacal sensation via spinal transection or the application of local anesthetic prior to mating (Mendonça & Crews, 1990). Similarly, female blood pythons (*Python curtus*) will not undergo vitellogenesis without the presence of, and likely copulation by, a male (DeNardo & Autumn, 2001). It is intriguing that, at least in these two distantly related species, males are involved in promoting early follicular growth despite the fact that sperm is not needed for fertilization until weeks later at ovulation. However, some female *T. s. parietalis* undergo ovarian recrudescence without mating in spring, at least in some years (reviewed in Mendonça & Crews, 1990).

3.2.2 Male-male interactions

The predominant social influences on reproduction are a result of male-female interactions, but male-male interactions can also alter reproductive activity. Encounters between two males can lead to agonistic interactions. Male-male combat is widespread among snakes (Shine,

2003), with the combat of viperid snakes being the best documented. In *V. berus*, chemical signals from the female elicit courtship by males, whereas other skin-derived chemical cues from the male appear to induce combat behavior (Andrén, 1986). Among crotaline snakes, combat is very ritualistic. In *A. contortrix*, individuals that lose combats not only flee the site, but courtship remains inhibited for at least 24 h (Schuett, 1996). These combat losers have increased plasma CORT concentrations, which may provide a mechanistic link between combat loss and courtship suppression (Schuett, 1996; Schuett & Grober, 2000).

An interesting male-male interaction exists in the unique mating system of *T. s. parietalis*. This species shows no male-male aggression, but some males act as female mimics that exhibit altered courtship behavior and are attractive to other males (Mason & Crews, 1985). The significance of this reproductive strategy is unclear. It was initially postulated that female mimicry is a relatively permanent alternative reproductive tactic and may serve as a distraction to other males present in the mating ball and thus enhance the mating success of the mimic (Mason & Crews, 1985). Later, Shine, Harlow, LeMaster, et al. (2000) describe the scenario where female mimicry is a transient stage of recently emerged males since they have poor locomotory performance and thus would not be competitive against other males in accessing copulations. By being female mimics, newly emerged snakes would not waste energy on fruitless courting and they would also induce other males to waste energy by courting mimics (Shine, Harlow, LeMaster, et al., 2000). Regardless of the ultimate strategy, the female mimicry phenomenon, including both the inhibition of courtship behavior and the attractiveness of female mimics to other males, is driven by male production of a pheromone similar to that of females (Mason & Crews, 1985; Shine, Harlow, LeMaster, et al., 2000). The regulatory mechanism for the production of this pheromone is not fully understood, but it may involve T since she-males have higher plasma T concentrations than do normal males (Mason & Crews, 1985). Female attractiveness is driven in part by the distribution of methyl ketones in the skin lipid milieu. Treatment of male snakes with E₂ results in a female-typical shift in skin methyl ketones and increased attractivity (Parker & Mason, 2012). This effect has also been documented in *B. irregularis* (Parker et al., 2018). Interestingly, and in contrast to previous descriptive reports demonstrating elevated T in female mimics, castrated males produce female sex pheromones and are actively courted by males (Parker & Mason, 2014). Furthermore, exogenous T reduces attractivity, and treatment with the aromatase inhibitor fadrozole both reduces courtship of females by males by 50% in field assays and results in downregulation in skin methyl ketones associated with female attractivity

(Rucker & Parker, 2022). Early studies proposing that female mimicry is a permanent alternative reproductive strategy (e.g., Mason & Crews, 1985) examined snakes that had recently emerged and therefore may either have low T or high T held over from winter LTD in combination with high aromatase activity (Krohmer, 2020; Krohmer & Lutterschmidt, 2011). Either state could, in theory, result in a female-typical skin lipid milieu, and the transient female mimic hypothesis is plausible given what we know about the effects of LTD on aromatase and the effect of aromatase on behavior and attractivity (Krohmer, 2020; Rucker & Parker, 2022; Winters et al., 2022).

3.2.3 Mating aggregations

Due to the relatively secretive and solitary nature of most snake species, reports of mating aggregations or “mating balls,” which consist of multiple males simultaneously courting a single female, are relatively uncommon among snakes. As with much of snake reproductive biology, mating balls are best documented in *T. s. parietalis* where mating balls that commonly consist of 20–30 males courting a single female form upon female emergence from overwintering (Gregory, 1974). Although males in a mating ball may attempt to displace each other to improve their position, they show no direct aggression toward each other (Joy & Crews, 1985). Although natural mating balls in this species have been widely observed, manipulative studies of mating balls are limited. However, Joy and Crews (1985) used controlled experiments to find that male *T. s. parietalis* courted females more actively when there was a mating ball compared to when males were alone with her and that there was a positive correlation between the number of additional males present and the intensity of courtship by the test male.

Mating balls have also been documented in numerous other species, across multiple families including Acrochordidae (e.g., Shine, 1986), Boidae (e.g., Bertona & Chiaravaglio, 2003; Rivas & Burghardt, 2001), Colubridae (e.g., Gregory, 1974; Mushinsky, 1979; Herrington, 1989; Madsen & Shine, 1993), Elapidae (e.g., Shetty & Shine, 2002), Lamprophiidae (e.g., Fukuyama et al., 2022), and Pythonidae (e.g., Brusch et al., 2019; Doody et al., 2009; Shine & Fitzgerald, 1995). Across much of the phylogenetic diversity of snakes, there appears to be a dichotomy relating male reproductive behavior to sexual size dimorphism. Aggressive male-male combat has been documented in 124 snake species, and there is a high correlation between male-male combat and sexual dimorphism where the male is the larger of the sexes (Shine, 1978, 1994). In contrast, although the data are much more limited, documented occurrences of mating balls have been exclusively limited to species where females are the larger sex. This dichotomy is not driven by distant phylogenetic relationships. Within the mostly Madagascan subfamily Pseudoxyrhophiinae,

male *Leioheterodon madagascariensis* and *L. modestus* are larger than females and these species engage in male-male combat, whereas nonaggressive mating balls have been documented in *Dromicodryas bernieri* and *D. quadrilineatus*, which both show female-larger sexual size dimorphism (Fukuyama et al., 2022). Even at a finer phylogenetic scale, the carpet python (*Morelia spilota variegata*) shows male-larger sexual size dimorphism and aggressive male-male combat, whereas the diamond python (*M. s. spilota*), a subspecies with a distribution just to the south of *M. s. variegata*, shows female-larger sexual size dimorphism and mating ball behavior without any male-male aggression (Shine & Fitzgerald, 1995).

Interestingly, despite mating balls being associated with female-larger species, larger males within a mating ball have greater success at achieving copulation than do smaller males (Madsen & Shine, 1993; Luiselli, 1996; Shine, Olsson, Moore, et al., 2000; see also Joy & Crews, 1988). Rivas and Burghardt (2001) suggest that, despite larger males having greater reproductive success than smaller males, female-larger size dimorphism persists as a result of stabilizing selection in males where being larger is beneficial in males up until a point where they approach female size, which can lead to them being mistaken as females by other males. Support for the idea that males can be mistaken for females merely because of size comes from observations of smaller males courting large males (Noble, 1937; Rivas & Burghardt, 2001).

The dichotomy in male reproductive behavior between male-male combat and mating balls appears broadly across the phylogenetic tree of snakes. Although ultimate mechanisms for this dichotomy have been discussed, little is known in regard to the proximate mechanisms regulating variation in these behaviors both across reproductive strategies and within a given strategy. Therefore, studies exploring possible hormonal involvement in influencing these behaviors would provide valuable insight into the relative importance of visual, physical, and chemical mechanisms of regulation of male reproductive strategies among snakes.

3.2.4 Parental care

Hormones also play a key role in the regulation of both maternal and paternal care of offspring in vertebrates. In snakes, the attendance of offspring has been observed primarily in viviparous crotaline snakes (Greene et al., 2002) but also in an oviparous python (Alexander, 2018). In viperids, mothers often attend offspring until their first shed. Attending mothers alter their antipredator behaviors during the period of attendance (Greene et al., 2002). Several studies have since attempted to characterize the role of steroids and posterior pituitary peptides in the maternal care of offspring. A study on *A. piscivorus* attempted to describe

how the removal of a litter postbirth affects temporal patterns in CORT, T, P₄, and E₂. The presence or absence of a litter did not alter reproductive steroids; however, females in the removal group exhibited a steady increase in CORT postbirth, and females allowed to attend offspring exhibited a sharp spike in CORT coincident with the timing of neonatal ecdysis (Hoss et al., 2014). In postparturient *S. miliaris*, plasma CORT was not related to spatial patterns observed between mothers and offspring (Lind, Ramis, et al., 2017). Unfortunately, no manipulative experimental studies have examined the role of steroid hormones in parental attendance of offspring in snakes.

Posterior pituitary neuropeptides play an important role in maternal care in vertebrates. In mammals, the hormones oxytocin and arginine vasopressin mediate maternal and paternal care. The reptilian homolog of arginine vasopressin, arginine vasotocin (AVT), has been implicated in social behaviors in reptiles and amphibians (Wilczynski et al., 2017). In a study on maternal care of offspring in *S. miliaris*, blockade of the vasotocin receptor disrupted the relationship between maternal post parturient body condition and the tightness of association between mothers and offspring (Lind, Birk, et al., 2017). However, receptor blockade is an insufficient method to clearly establish the role of a hormone in any particular behavior, and in a follow-up study, exogenous administration of AVT did not alter the tightness of mother-offspring associations in *S. miliaris* (Lind, Ramis, et al., 2017). Although promising preliminary studies have been conducted, the endocrine regulation of offspring attendance in snakes remains a relatively unexplored frontier.

Although the attendance of offspring is taxonomically quite limited in snakes, female attendance of her egg clutch, while not common, is more widespread among snakes (Stahlschmidt & DeNardo, 2010). Encircling of the eggs by the female provides thermal and hydric benefits to the developing embryos (Stahlschmidt et al., 2008; Stahlschmidt & DeNardo, 2009), and postoviposition females have a strong drive to encircle their eggs to the point that female *A. childreni* will encircle the eggs of a conspecific, a pseudoclutch made from the shells of a conspecific, and, to some extent, a grouping of stones that are similar in shape to a clutch of eggs (Brashears & DeNardo, 2012). Despite the importance of this behavior to egg survival and the internal drive of the females to provide it, no studies have examined the mechanisms underlying it.

3.3 Physiological influences

3.3.1 Body condition and energetics

Beyond environmental and social influences, reproductive activity is also influenced by nonreproductive aspects of the individual. Because of the high energy demand

associated with reproduction, body condition is especially important in influencing the reproductive activity of snakes. Many snakes use a capital breeding strategy rather than one of income breeding (reviewed in Gregory, 2006). So, in capital breeders, reproductive activity is based on energy reserves more so than current food intake (Drent & Daan, 1980). In capital breeders, females need to attain a minimum threshold of body condition prior to committing to a reproductive event, although this threshold may vary over the years (Madsen & Shine, 1999; Naulleau & Bonnet, 1996).

The relationship between body condition and reproductive activity is well established; however, little is known about the hormonal regulation of this relationship in snakes. In *V. aspis*, females in poor body condition have low circulating E₂ concentrations and are not sexually receptive, but once a female is beyond a critical threshold of body reserves she exhibits elevated plasma E₂ concentrations and is receptive (Aubret et al., 2002). Males, however, do not show a threshold response, as all males had detectable plasma T concentrations and displayed courtship behavior, but both plasma T and courtship behavior increased proportionately with body condition (Aubret et al., 2002). Similarly, in a field study of *C. horridus*, male T was positively correlated with body condition index (BCI) and metrics of male time energy allocation during the breeding season (Lind & Beaupre, 2015). In *S. miliarius*, T is positively correlated with BCI and this relationship holds outside of the breeding season when the gonads are presumably quiescent, T is low and no mating behavior is observed in the population (Lind et al., 2018, 2019). BCI is also negatively related to CORT in *S. miliarius*, and it is possible that the positive relationship between BCI and T may be mediated by CORT. A review of the literature by Lind et al. (2018) highlights that many, but not all, snakes studied to date exhibit a negative relationship between BCI and CORT and that positive correlations between BCI and T are also common. Neither negative relationships between T and BCI nor positive relationships between BCI and CORT have been reported in snakes to our knowledge. These results highlight that there must be some mechanism informing the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes of the energetic state of the individual.

The hormone leptin might be considered an important link between energy state and reproduction. Leptin occurs in a diverse group of vertebrates and is known, at least in mammals, to be involved in the energy stores-reproduction relationship (reviewed in Paolucci et al., 2006) and might play a similar role in snakes. Recent work in *T. s. parietalis* supports this hypothesis. Exogenous mammal leptin increases reproductive behavior in aphagic males and females (Wilson et al., 2021). The increasing availability of molecular tools for measuring and manipulating peptide hormones in nonmodel vertebrates promises great potential

for future studies to establish clear links between energetic status and allocation decisions in snakes.

In addition to body reserves influencing reproduction, many snake species show size-dependent fecundity with larger females producing a greater clutch mass (Ford & Seigel, 1989; Madsen & Shine, 1994; see also Lourdais et al., 2002). This relationship can lead to sexual size dimorphism with females being larger than males. Many factors including age, food intake, and possibly hormones can affect growth rate and body size (reviewed in Taylor & DeNardo, 2005) and thus influence sexual size dimorphism. There is some evidence that, in *T. sirtalis* where females are larger than males, estrogens promote growth whereas T suppresses it (Crews et al., 1985; Lerner & Mason, 2001).

3.3.2 Stress

Stress is also known to have a dramatic effect on reproduction in reptiles, including snakes (reviewed in Moore & Jessop, 2003; Chapter 7, this volume). Stress may negatively influence reproduction either through direct or indirect effects of elevated CORT on reproductive hormones or other components of the reproductive system (Wingfield et al., 1998). In *B. irregularis*, capture and placement in outdoor enclosures under ambient conditions terminate spermatogenesis and development of the sexual segment of the kidney (Aldridge & Arackal, 2005). Plasma steroids were not assessed in this study; however, trapping and confinement of *B. irregularis* leads to an increase in plasma CORT concentrations (Mathies et al., 2001). Furthermore, elevated plasma CORT was thought to explain, at least in part, low reproductive activity in a free-living population of *B. irregularis* on Guam (Moore et al., 2005). Apparently, however, *B. irregularis* can acclimate to captivity, as individuals held for a long term in captivity are reproductively active and have low plasma CORT concentrations (Moore et al., 2005).

The susceptibility of an animal to stress can vary seasonally. Unlike *B. irregularis*, which has an extended breeding season, *T. s. parietalis* in Manitoba, Canada, has an extremely short reproductive period. During the non-breeding summer, males respond to capture stress with elevated CORT and reduced T. However, during the primary reproductive period in spring and the secondary mating and pre-LTD period in fall, the hormonal response to stress is variable but, in general, reduced (Moore et al., 2001; Lutterschmidt & Mason, 2005; see also Moore et al., 2000). In the conspecific red-spotted garter snake (*T. s. cinnicus*), which has an extended breeding season in Oregon, capture during the reproductive season induces the classical stress response of increasing CORT while decreasing T (Lutterschmidt et al., 2004; Moore et al., 2001). Interestingly, in the one study in which capture induced an elevated CORT response in the Manitoba population of *T. s.*

parietalis during the breeding season, there was no effect of stress on courtship behavior (Moore et al., 2000). However, in separate studies, exogenous treatment with CORT inhibits courting (Lutterschmidt et al., 2004; Moore et al., 2001). Although inconsistencies exist, the overall conclusion from these studies is that, like other vertebrates, snakes have a classical stress response, but the hormonal and or behavioral response may be attenuated during the reproductive season for populations in which reproductive opportunities are limited. Surprisingly, the shift from nonresponsiveness to responsiveness to capture stress can be abrupt, as the classical hormonal response to capture stress is intact in male *T. s. parietalis* that are dispersing from mating sites to feeding sites (Cease et al., 2007).

Analyzing the effects of CORT as a modulator for the stress response (and thus an inhibitor of reproduction) is complicated by CORT's most basic function as an energy mobilization hormone. As such, elevated CORT may augment reproduction (reviewed in Moore & Jessop, 2003). In male *T. s. parietalis*, baseline plasma CORT concentrations are elevated during the spring breeding season (Moore et al., 2001), and it is thought that this elevated CORT facilitates reproduction by mobilizing much-needed energy stores to sustain costly courtship activity during this period of aphagia (Moore & Jessop, 2003). Treatment with a glucocorticoid inhibitor, metyrapone, resulted in snakes preferring food cues to reproductive cues in a Y maze trial. Metyrapone-treated snakes also exhibited an increased number of neuropeptide Y immunoreactive cells in the reptilian homologs of the hippocampus and amygdala (Lutterschmidt & Maine, 2014). In contrast to *T. s. parietalis*, baseline CORT in free-living male *S. miliaris* is lowest during peak spermatogenesis and the onset of the mating season and elevated during the quiescent winter months. Although CORT is elevated in pregnant compared to nonreproductive females in the summer, elevated concentrations never approach the high baseline CORT concentrations observed in the winter months (Lind et al., 2020). In an experimental study, exogenous CORT does not alter plasma T in free-ranging southern Pacific rattlesnakes (*Crotalus helleri*; Claunch et al., 2017). However, CORT and T are negatively correlated in multiple snake species (Lind et al., 2018). The conflicting findings of past studies aimed at identifying clear roles for CORT in snakes, and vertebrates in general, may result from unappreciated nuances in receptor expression and affinity. Future studies should investigate the importance of glucocorticoid receptor expression in the coordination of snake reproductive cycles.

3.4 Embryonic influences

Nearly all studies of snake reproductive endocrinology have examined the relationships among behavior, physiological

state, and hormones in adults, but it is important to at least briefly consider the embryonic snake. Many reptiles, including lizards, are known to have environmental sex determination in which environmental conditions, particularly temperature, influence sex by altering embryonic hormone exposure. Environmental sex determination has not been identified in any snake (reviewed in Janzen & Paukstis, 1991). However, exposure of embryos to steroids may alter other embryonic traits. Corticosterone treatment of gravid female western terrestrial garter snakes (*Thamnophis elegans*) led to reduced embryonic and early offspring survivorship as well as changes in morphological and behavioral characteristics of the offspring (Robert et al., 2009). Also in *T. elegans*, prenatal sex ratio affects subcaudal scale counts of female offspring in one of two populations (Osypka & Arnold, 2000), and elevated baseline CORT during pregnancy was associated with smaller offspring that grew faster during the first 3 months of life (Palacios et al., 2023). Although limited, these studies together suggest that embryonic snakes are susceptible to the influences of steroids.

4 HORMONES, ENVIRONMENT, AND CONSERVATION

Snake populations are increasingly threatened by habitat loss, climate change, pollution, and disease (Cox et al., 2022). Although a thorough review of such threats is beyond the scope of this chapter, it is important to highlight the fact that each of these threats may impact populations by altering endocrine processes that translate environmental cues into fitness-related physiological and behavioral responses. An improved understanding of both the effect of environmental perturbations on hormones and the role of hormones in coordinating reproductive cycles can be used to predict and identify threats and inform conservation efforts.

Although the effects of a warming climate on reproductive phenology are well documented in plants and animals, including some snake species (e.g., Rugiero et al., 2013; Turner & Maclean, 2022), the impacts of warming on life history trait expression and fitness are currently unclear. Warming temperatures may seem advantageous to vertebrate ectotherms (e.g., Üveges et al., 2016). However, warming winter temperatures may impose energetic costs and affect reproductive processes that are stimulated by LTD (reviewed in Moss & MacLeod, 2022). Current predictions also indicate that climate change will increase the severity and duration of drought in some regions. Given that many reptiles live in drought-prone areas and precipitation patterns can influence reproductive activity and offspring survival, it is important to understand the effects of water limitation on reproduction in snakes. In a field experiment in northern Pacific rattlesnakes (*C. oreganus*),

Capehart et al. (2016) demonstrated that experimental hydration had a significant positive effect on female reproduction but did not affect either baseline or stress-induced CORT. However, reproduction and dehydration have compounding effects on CORT in *A. childreni* housed in the laboratory (Brusch et al., 2020). Similarly, water deprivation increases maternal CORT in viviparous *V. aspis*, and dehydration enhances growth rates in their offspring (Dupoué et al., 2016). Further, simultaneous treatment of *V. aspis* with dehydration and a simulated heat wave additively increased baseline CORT and mass loss in nonreproductive individuals (Dezetter et al., 2022). Increasing global temperatures and altered patterns of precipitation may alter hormonally mediated reproductive processes, and it is important that research continues to investigate how climate change will impact the endocrine physiology of free-living snakes, particularly as it pertains to reproduction.

The increasing spread of disease also threatens global snake populations. For example, ophidiomycosis (or snake fungal disease) has recently been identified as a threat to snake biodiversity (Lorch et al., 2016). The disease is caused by a keratinophilic skin fungus, *Ophidiomyces ophiodiicola*, and afflicts diverse snake taxa (Allender et al., 2015; Lorch et al., 2015). Recent work in an afflicted population of *S. miliaris* has demonstrated how knowledge of seasonal reproductive ecology and physiology can be used to identify potential sublethal impacts of infection on individuals and populations. Lind et al. (2018, 2019) demonstrated that natural infection is associated with increased CORT and the suppression of sex steroids in both males and females. Specifically, infected males had lower T during summer spermatogenesis and the fall mating season compared to uninfected males, suggesting that infection reduced male reproductive investment. Similarly, infected females were less likely to exhibit elevated E₂ indicative of vitellogenesis in the spring, and infected females were also less likely to enter annual reproductive bouts.

Endocrine-disrupting contaminants (EDCs) can bioaccumulate in snakes (e.g., Tashiro et al., 2021; Wu et al., 2020) and have the potential to negatively impact aquatic and semiaquatic snake populations. Research regarding the impact of EDCs on snakes, specifically, is limited. Pregnant female *N. sipedon* fed fish dosed with the widely used herbicide atrazine exhibited disrupted E₂ production and altered litter characteristics, including stillbirth ratio, sex ratio, and offspring scale symmetry (Neuman-Lee et al., 2014). Beyond this single study, the potential threat that EDCs represent to global snake populations is unknown.

Employing endocrine tools to characterize sublethal impacts of climate change, disease, and other anthropogenic disturbances requires a basic understanding of (1) the reproductive ecology of the population of interest, (2) how the

environment impacts endocrine signaling, and (3) the role of hormones in mediating fitness-related reproductive processes. This chapter provides a foundation for ongoing and future work targeted at using endocrine tools in conservation and management.

5 FUTURE DIRECTIONS TO AUGMENT EXISTING KNOWLEDGE

As snakes are a distinct suborder of vertebrates with over 4000 species, it is important that we obtain a thorough understanding of their reproductive endocrinology. It is imperative that we distinguish traits that are relatively conserved among snakes from those that may be unique to individual species or small groups of species. Thus, we are in dire need of data from a greater diversity of snake species. Although progress has been made over the past decade, the reproductive endocrinology of snakes remains grossly biased (and possibly misrepresented) by data from a single species, *T. s. parietalis*. Studies of *T. s. parietalis* have led the way in our understanding of snake reproductive endocrinology, and such leadership in the field must continue. However, evaluating large and diverse taxonomic groups based on the study of a single species is dangerous. Applying findings based on studies of *T. s. parietalis* to snakes, in general, may be especially problematic given that *T. s. parietalis* has numerous traits that are atypical of most snakes, including high latitude, cold climate distribution, and mating system in which reproduction entails massive mating balls formed immediately at female emergence. The bias regarding snake study species goes beyond *T. s. parietalis* in that data from other species come disproportionately from temperate species, particularly viperids. Although modest progress has been made in recent years, there remains a great need for studies of the reproductive endocrinology of tropical snakes and snakes other than vipers and colubrids (e.g., Scolecophidia, basal Alethinocephidia, and the speciose Elapidae).

Beyond being a relatively large taxonomic group, snakes show diverse reproductive strategies, and this diversity provides a unique opportunity for comparative studies to better understand the reproductive endocrinology of vertebrates in general. Data collected thus far strongly indicate that snakes utilize an HPG hormonal axis similar to that of other vertebrates, at least on a broad scale. However, the diversity of reproductive tactics within the squamates (lizards and snakes) is unmatched by other major terrestrial taxonomic groups. Although the group is ancestrally oviparous, viviparity has evolved over 115 times (Blackburn, 2015) and occurs in approximately 20% of squamates (Shine, 1985). Additionally, some squamates, including snakes, are facultatively parthenogenetic, with the ability to produce offspring either sexually or asexually (Booth & Schuett, 2016). The

integration of environment into hormonal cues that coordinate facultative parthenogenesis in female snakes is completely unknown, and this is a potentially valuable avenue of future research. Similarly valuable, but less extreme physiologically, are the variations in the timing and frequency of reproduction among snakes. Specific environmental cues (e.g., increasing temperatures after a cool period) are thought to be critical in regulating snake reproductive cycles. However, some species can have multiple reproductive events in a given year and under different environmental conditions. The reproductive events may be limited to multiple mating periods (e.g., as described earlier for many rattlesnakes) or entail the production of multiple clutches. Multiple clutch production is typically limited to captive snakes (Ford & Seigel, 2006, although it has been documented in wild common keelbacks, *Tropidonophis mairii*, Brown & Shine, 2002). This does not negate the value in using that potential to understand reproductive stimuli in snakes. Similarly, the diversity of male mating tactics, including male-male combat and mating balls, provides ample opportunity for comparative approaches and the investigation of the proximate hormonal mechanisms that underlie reproductive tactics.

To fully examine the details of snake reproductive endocrinology, it is imperative that studies move away from the predominant use of descriptive studies and utilize manipulative experiments. Only in *T. s. parietalis* have a considerable number of manipulative studies been conducted to better understand the causal relationships among reproductive behavior, gonad function, and hormones. Potential manipulative approaches are numerous and range from hormone modulation (e.g., organ removal, hormone treatment, hormone antagonist treatment) to modification of organism state (e.g., energy balance, water balance, stress, function of senses) to environmental manipulation (e.g., temperature, photoperiod, humidity, rainfall, pheromonal cues, social structure). Conducting such experiments in both the laboratory where variables can be more tightly controlled, and in the field where conditions are more natural will be essential.

It is clear that more attention needs to be focused on the structure and function of peptides involved in the HPG axis. This includes sequencing of genes for GTHs and GnRH in diverse snakes and the use of molecular tools to characterize the existence and importance of GnIH. Since its discovery in quail (Tsutsui et al., 2000), GnIH has been identified in multiple species of fishes, birds, and mammals (reviewed in Ubuka et al., 2008). GnIH has also recently been found in turtles and lizards (Tsutsui & Ubuka, 2021). GnIH is potentially an important regulator of reproduction, and thus a thorough understanding of the regulation of reproduction in snakes is likely impossible without the consideration of GnIH.

Numerous other hormones are worthy of study to assess their roles in snake reproduction. Hormones such as prolactin (Ng et al., 1990), arginine vasotocin (Silveira et al., 1992), and growth hormone (Ng et al., 1993) have been identified in at least one snake species, yet their potential roles in reproduction have received little attention. Beyond putative sequences derived from the limited snake genomes available, the structure and function of hormones such as leptin and relaxin have yet to be characterized in snakes. However, these hormones may have important impacts on the reproductive cycles of snakes (e.g., Wilson et al., 2021) that will need to be explored in future studies.

One final area of study that has received considerable attention in other taxa, especially birds, is that of maternal transfer of sex steroids to offspring via the yolk. Since initially described by Schwabl (1993), maternal steroid transfer has been shown to have dramatic effects on offspring phenotype (reviewed in Radder & Shine, 2007), although the mechanisms of transfer between the female and the yolk as well as the yolk and the embryo are poorly understood (Moore & Johnston, 2008). Despite this broad attention, the demonstrated biological importance of yolk transfer has yet to be explored in snakes (Palacios et al., 2023).

Since the publication of the previous volume of this chapter in 2011, much progress has been made. However, many of the gaps highlighted over 10 years remain to be addressed. Regardless, contributions by numerous investigators have expanded our understanding of the hormonal control of snake reproductive cycles over the last several decades. It is essential to build on this foundation, as doing so will not only provide valuable insight into the evolution of reproductive tactics in vertebrates, but will provide tools and frameworks to better characterize, predict, and manage threats to snake biodiversity.

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