Changes in Plasma Testosterone, Estrogen and Progesterone Concentrations Throughout the Annual Reproductive Cycle in Male Viviparous Blue-tongued Skinks, *Tiliqua nigrolutea*, (Scincidae), in Tasmania

ASHLEY EDWARDS AND SUSAN M. JONES

School of Zoology, University of Tasmania, GPO Box 252C-05, Hobart, Tasmania, 7001, Australia

email: Ashley.Edwards@utas.edu.au

Key Words: estrogen, progesterone, reproduction, skink, testosterone, testis, *Tiliqua nigrolutea*
Abstract.— Few published studies have detailed comprehensively the correlations between plasma steroid hormone peaks and the timing of reproductive events in male squamate reptiles. We examined the patterns of plasma testosterone (T), estrogen (E) and progesterone (P4) concentrations in males of the viviparous blue-tongued skink, Tiliqua nigrolutea, throughout the annual cycle. Plasma T concentrations varied through the annual cycle, peaking at 10.9 ± 3.00 ng ml\(^{-1}\) during spermiogenesis, coincident with agonistic male–male interactions, but falling prior to the mating period. Mean plasma T concentrations were basal (2 – 3 ng ml\(^{-1}\)) during reproductive quiescence. Mean plasma E concentrations were significantly elevated (778.0 ± 120.00 pg ml\(^{-1}\)) during the mating period, but basal (<300 pg ml\(^{-1}\)) both before and after mating. Mean plasma P4 concentrations peaked during the mating period (1.1 ± 0.17 ng ml\(^{-1}\)) and declined significantly after mating. We propose a potential role for E and P4 in the stimulation of male reproductive behavior during the mating period.
INTRODUCTION

The gonadal steroids which control reproduction in reptiles are assumed to be testosterone (T), progesterone (P4), and 17β-estradiol (E2) (Kime, 1987). However, there are few comprehensive published descriptions of the annual profiles of steroid concentrations, particularly E2 and P4, in the plasma of male reptiles. Most studies of reproduction in male squamates have described simply the annual cycle of hypertrophy and regression of reproductive organs (Sanyal and Prasad, 1967; Nilson, 1980; Krohmer and Aldridge, 1985; Flemming, 1993a; Shea, 1993; Aldridge and Brown, 1995), or have combined such information with descriptions of cycles of plasma androgen concentrations only (Arslan et al., 1978; Courty and Dufaure, 1979; 1980; Johnson et al., 1982; McKinney and Marion, 1985; Bourne et al., 1986; Moore, 1986; Flemming, 1993b; Swain and Jones, 1994; Schuett et al., 1997). Thus, in many cases, supposition about the roles of these gonadal steroids in the regulation of reptilian reproduction is based on analogy with mammals rather than experimental evidence (Ozon, 1972; Wiebe, 1985; Chieffi and Pierantoni, 1987).

Elevated plasma androgens have been implicated in the stimulation of reproductive behavior in male vertebrates, including reptiles (Crews, 1975; Crews et al., 1978; Lance, 1984; Lindzey and Crews, 1986; Moore, 1987). However, male mating behavior in the garter snake *Thamnophis sirtalis parietalis* is at least partially (Crews, 1991), if not completely (Mendonca et al., 1996), independent of androgens. Several studies have suggested that in male reptiles, estrogens may influence reproductive behavior through aromatisation of androgens in the brain. Aromatase activity has been detected in the brains of the turtle *Chrysemys picta* (Callard et al., 1977) and males of the
lizard Podarcis sicula sicula (Gobbetti et al., 1994). In the lizard A. carolinensis, exogenous E2 more reliably reinstates reproductive behavior in male castrates than T (Crews and Morgentaler, 1979). However, aromatisation of T to E2 does not appear to be necessary for the induction of the reproductive behaviors that occur coincident with increases in plasma testosterone in male A. sagrei (Tokarz, 1986).

An increasing number of published studies suggest that P4 may also be important in stimulating reproductive behavior in some male squamates (Lindzey and Crews, 1988; 1992; Young et al., 1991; Witt et al., 1994). Exogenous P4 has been shown to stimulate sexual behaviors in males of the lizard Cnemidophorus inornatus (Lindzey and Crews, 1986). This finding is contrary to the usual in male vertebrates; typically P4 inhibits male sexual behaviors (Moore and Lindzey, 1992).

Given these conflicting reports, it is surprising that annual changes in plasma concentrations of P4 or estrogen (E) in male reptiles have not been documented in more species; such information is vital for us to better understand the hormonal control of reproduction in reptiles. Saint Girons et al. (1993) provided one of the few published studies in which the timing of behaviors associated with reproduction is correlated with changes in concentrations of all three primary gonadal steroids in the plasma of a male squamate reptile. They reported significant annual variation in plasma T, E2 and P4 concentrations in male Vipera aspis, correlated with the timing of physiological and behavioral events (Saint Girons et al., 1993). However, more studies of this nature are required.

Tiliqua nigrolutea is a large, viviparous skink distributed throughout southeastern Australia (Cogger, 1992). Adult males can range from 25–29 cm snout–vent length
(SVL) and weigh between 300 and 450 g, with females somewhat larger and heavier. In Tasmania, where this study was conducted, *T. nigrolutea* occurs in low altitude heath, savanna woodland and dry sclerophyll forest in the cool temperate regions of the state (Rawlinson, 1974). Presented here is a comprehensive examination of annual cycles of plasma T, E and P4 concentrations in male *T. nigrolutea*, correlated with the timing of agonistic and mating behaviors.
MATERIALS AND METHODS

Animals. — Lizards were captured opportunistically by hand throughout southeastern Tasmania from Sep – Jan. Males were distinguished from females by their relatively broader heads (our unpublished data) and an examination of the cloacal opening for the musculature of the hemipenes. Animals were housed in roofed outdoor enclosures 1.9 x 3.4 x 2.1 m; these were wire-fronted, allowing access to UV light and a natural photoperiod. The direct sunlight and a 120 W floodlight globe as an additional heat source at the front of each cage provided a temperature gradient across which the lizards could thermoregulate during their active season of the austral spring to mid-autumn (Sept - Apr). We provided bark and leaf litter in which the animals could hide. Mixed-sex groups of approximately five animals (one male per group) were maintained in these cages. Additional males were held separately in similar, but smaller cages, to prevent agonistic interactions and their possible effects on plasma hormone concentrations. The lizards were maintained on a varied diet of fresh fruits, live snails and tinned catfood, provided two to three times weekly. Water was available ad libitum. The number of captive male animals varied from 12–19 over the period of the study.

Blood sampling. — Blood samples were collected at monthly intervals. Samples were taken routinely between 0930 and 1230 without anaesthesia from the caudal artery, using a heparinised syringe. Samples were held on ice until centrifuging at 6400 rpm and plasma was stored frozen at -20 C until analysis. Up to 1 ml of blood was taken from each animal, although some samples were much smaller and occasionally no blood was obtained. Twelve samples were collected from each of 10 animals for the measurement
of plasma T and eight animals for the measurement of P4 concentrations and nine samples were obtained from each of nine animals for the measurement of E.

**Radioimmunoassays.**— Analytical reagent grade isoctane, hexane and ethanol were purchased from Biolab Scientific Pty. Ltd. (Victoria, Aust.). Scintillation fluid (Ecolite +) came from ICN (Costa Mesa, CA.). [1,2,6,7-³H]-Testosterone (spec. act. 100 Ci/mmol) and [1,2,6,7-³H]-P4 (spec. act. 80-110 Ci/mmol) were purchased from Amersham Life Sciences (UK). Testosterone antiserum was a gift from A. J. Bradley (details in Bradley, 1990). Plasma T concentrations were assayed by a modification to the radioimmunoassay of Castro et al. (1974) as detailed in Swain and Jones (1994). Inter- and intraassay coefficients of variation for the testosterone assay were < 10% and 6%, respectively (Swain and Jones, 1994). Progesterone antiserum was from J. Malecki (details published in McDonald et al., 1988). The P4 radioimmunoassay method was described in Jones and Rose (1992) with a minor modification for this study: P4 was eluted from the columns in 3 ml isoctane. Intra- and interassay coefficients of variation for the P4 assay were 12.1% and 8.4% respectively. All T and P4 assay samples were measured as outlined in Jones and Rose (1992). Plasma E was measured using Spectria coated-tube radioimmunoassay kits as in Jones and Swain (1996). Cross-reactivities for the E2 antiserum are: E2, 100%; estrone (E1), 1.16%; estriol, 0.45%; T and P4, <0.001%. Intra- and interassay coefficients of variation for this assay were 13% and 8%, respectively. The limit of detection for all three assays was 10 pg authentic steroid. Assays were validated using *T. nigrolutea* plasma (T and E assays) or pooled skink plasma (P4 assay): in all cases serial dilutions of plasma ran parallel to the standard curves.
Statistics.— Mean monthly plasma hormone concentrations were compared by repeated measures analysis of variance ((M)ANOVA) using SYSTAT 8.0 (Wilkinson et al., 1998). A significance level of $\alpha = 0.05$ was used throughout. All data were log transformed prior to analysis to satisfy assumptions of normality and homogeneity of variance and all values are presented as mean ± 1 standard error (SE). The original data sets were reduced to include only those individuals for which samples from all (or most) sample periods were available. Occasional missing data points were assigned the mean value for animals in the same sample period, although no more than one such value was assigned to any individual or any sample period (Mundry, 1999; D. Ratkowsky, pers. comm.). *A posteriori* Student t tests were conducted for each hormone profile, as the precise timing of these events was unknown before samples were collected. The periods inspected were either the animals’ emergence from hibernation or the mating period. Visual examination of completed hormone profiles and concurrent behavioral observations of the captive sample population was used to identify relevant successive pairs of sample sets.
RESULTS

Plasma steroid concentrations.— Mean monthly plasma T concentrations in male *T. nigrolutea* from November 1995 to October 1996 are shown in Fig. 1. A distinct unimodal annual cycle was evident, with uniformity between males in both the timing of the seasonal plasma T pattern and the magnitude of plasma T concentrations. Mean plasma T concentration varied significantly throughout the annual cycle ((M) ANOVA: $F_{(9,11)} = 12.504, P = 0.000$). Plasma T concentrations decreased significantly ($t = 3.097, P = 0.013$) from $8.8 \pm 0.79$ ng ml$^{-1}$ during the mating period (Nov) to $5.3 \pm 0.62$ ng ml$^{-1}$ in early summer (Dec) when post-mating male lizards were reproductively quiescent. Plasma T concentrations were basal (approx. 1–3 ng ml$^{-1}$) from mid–summer to mid–winter (Jan–Jul), before increasing significantly ($t = -2.450, P = 0.037$) to $8.0 \pm 3.12$ ng ml$^{-1}$ in late winter (Aug), when male lizards emerged from hibernation, about four weeks earlier than females. Mean plasma T concentration peaked at $10.9 \pm 3.05$ ng ml$^{-1}$ in mid spring (Oct), coincident with the completion of spermiogenesis and the observation of the onset of agonistic male-male interactions. There was no correlation between peak plasma T concentration (Oct) and SVL.

As with the plasma T profile, the annual pattern of changing E concentrations in the plasma of male *T. nigrolutea* varied significantly throughout the reproductive cycle (Fig. 2) ((M)ANOVA: $F_{(7,8)} = 6.267, P = 0.000$). Mean plasma E2 concentration was basal (<300 pg ml$^{-1}$) in emergent animals in early spring (Sept), but became significantly elevated to $460.8 \pm 55.41$ pg ml$^{-1}$ from mid spring (Oct) ($t = -6.721, P = 0.000$), when spermatogenesis had been completed, through to late spring and early summer (Nov–Dec), when mating was observed, peaking at $778.0 \pm 120.99$ ng ml$^{-1}$ in December and
dropped sharply to 396.9 ± 53.44 pg ml\(^{-1}\) by mid summer (Feb), when males were reproductively quiescent. Concentrations remained low (approximately 300 pg ml\(^{-1}\)) until males began hibernating in late autumn (May).

Mean monthly plasma P4 concentrations in male *T. nigrolutea* from November 1995 to October 1996 were low but above the limit of detection of the assay throughout the year, and varied significantly over time (Fig. 3) ((M)ANOVA: \(F_{(8,11)} = 4.556, P = 0.002\)). Plasma P4 concentration peaked during the mating period (Nov) at 1.1 ± 0.17 ng ml\(^{-1}\) and fell significantly to 0.8 ± 0.09 ng ml\(^{-1}\) by early summer (Dec) (\(t = 4.334, P = 0.002\)). There was a further significant decline to 0.3 ± 0.08 ng ml\(^{-1}\) in mid-winter (Jun) (\(t = 2.458, P = 0.039\)) but mean plasma P4 concentration rose significantly (\(t = -2.94, P = 0.019\)) when males emerged at the end of winter (Aug).
DISCUSSION

Male reptiles commonly display an annual pattern of plasma T concentrations with low concentrations during the early stages of spermatogenesis and a peak during spermiogenesis, corresponding with peak testicular hypertrophy (Lance, 1984). Male *Tiliqua nigrolutea* exhibit this typical pattern: plasma T concentrations are low during autumn (Mar–Apr) when spermatogenesis commences, but rise during mid–spring (Oct), peaking at $10.9 \pm 3.05 \text{ ng ml}^{-1}$ and declining through the second half of the mating period. This pattern closely resembles that of the plasma T cycle of *T. rugosa* (Bourne and Seamark, 1975), although peak plasma T concentrations (approximately 40 ng ml$^{-1}$) are higher in *T. rugosa*. However, the major androgen in *T. rugosa* is epitestosterone (epiT), not found in *T. nigrolutea* (Bourne et al., 1985), and epiT is present in much higher concentrations (approximately 140 ng ml$^{-1}$) than T in the blood (Bourne et al., 1986).

Among reptiles, much variation occurs in the magnitude of the T peaks between species. Plasma T peaks at 80 -100 ng ml$^{-1}$ in the snake, *Agkistrodon contortix*, (Schuett et al., 1997) and $51.7 \pm 1.6 \text{ ng ml}^{-1}$ in the viviparous lizard, *Niveoscincus metallicus* (Swain and Jones, 1994), but only approximately 3 ng ml$^{-1}$ in the snake *A. piscivorus* (Johnson et al., 1982).

Circulating concentrations of E also follow an annual pattern in male *T. nigrolutea*. Plasma E concentrations are elevated during the mating period (Oct – Dec) but drop rapidly to basal concentrations for the rest of the active season. This implies a role for E in the induction of sexual behaviors. However, the relatively high plasma E concentrations detected suggest we may be measuring one or more alternative estrogens in addition to E2 (V. Lance, pers. comm.). Additionally, E2 is not synthesised from
pregnenolone (P5) by the testes or from T by peripheral tissues in vitro (Edwards, 1999). Given that plasma T declines during the mating period in male *T. nigrolutea*, we suggest that an alternative estrogen may be responsible for stimulating mating behavior in males of this species. Cross-reactivity of the kit antiserum used in this study with other common estrogens (E1 and E3) is low, but no information is provided by manufacturers about cross-reactivity at the six position of the steroid nucleus, which is modified in the generation of the antiserum. Whittier and Hess (1992) reported the cross-reactivity of the polar steroids 6α- and 6β-hydroxyestradiol (6α- and 6β-OH-E2) with an E2 kit antiserum in another squamate reptile, the garter snake, *T. s. parietalis*. A presently unidentified steroid more polar than E2 has been isolated from in vitro incubations using *T. nigrolutea* testicular tissue, but it is less polar than 6α- and 6β-OH-E2 (Edwards, 1999).

Males of other lizards exhibit annual cycles of E2 production, elevated plasma E2 does not always coincide with mating. Saint Girons et al. (1993) demonstrated that sexually active male *Vipera aspis* have high plasma T and low E2 during the mating period, with E2 being elevated to approximately 520 pg ml⁻¹ in non-mating males. In male *Podarcis s. sicula* plasma E2 concentrations increase in the post-reproductive refractory period to about 1.5 ng ml⁻¹ (Ando et al., 1992). Intracranial implants of E2 in castrated male *A. carolinensis* restore sexual behaviors (Crews and Morgentaler, 1979), but later studies on anoles suggest that the expression of reproductive behavior in *Anolis* species is under the direct control of androgens (Adkins-Regen, 1981; Tokarz, 1986).

We also examined plasma concentrations of P4 in male *T. nigrolutea*. Plasma P4 concentrations showed a seasonal pattern in male *T. nigrolutea*: mean concentrations were low throughout the year with a small but significant elevation prior to emergence
This significant change observed in male plasma P4 concentrations may be, simply, a function of an overall increase in metabolism as a result of higher temperatures during the animals’ active season, such as has been documented in other ectothermic vertebrates (Kime, 1979; 1987; Kime and Hyder, 1983). However, P4 has been shown to stimulate male reproductive behavior in some lizards (Lindzey and Crews, 1986; 1988; 1992; Young et al., 1991; Moore and Lindzey, 1992; Witt et al., 1994) acting as a progestin rather than through conversion to other steroids (Moore and Lindzey, 1992). Plasma P4 concentrations have also been measured in males of the lizard P. s. sicula, in which there was a post-reproductive increase which peaked at approximately 8 ng ml⁻¹ (Ando et al., 1992). The lack of a pronounced annual pattern in T. nigrolutea, however, implies that P4 may not have a primary role in the induction of reproductive behaviors in males of this species.

Bourne et al. (1986) suggested that in Tiliqua rugosa long-term captivity may negatively affect the expression of reproductive behavior and depress plasma steroid concentrations; captive male T. rugosa do not display a seasonal plasma androgen cycle (Watson et al., 1987). All males of T. nigrolutea used in our study, however, continued to cycle normally in comparison with opportunistically sighted and wild-caught individuals in the timing and magnitude of plasma steroid peaks, the timing of regular skin moults and in the expression of agonistic and mating behaviors. There was no significant difference in mean plasma T concentration in November between males in the captive population and eight wild individuals that were opportunistically captured at that time (Edwards and Jones, unpubl. data). Any stress caused by handling and blood sampling is unlikely to have had a significant impact on plasma steroid concentrations.
Kreger and Mench (1993) considered the impact of handling and restraint on *Tiliqua scincoides* and found no significant chronic effect on plasma concentrations of the stress steroid hormone, corticosterone (B). Additionally, Moore et al. (1991) observed that the effects of acute handling stress in the lizard *Urosaurus ornatus* are rapidly dissipated. *Tiliqua nigrolutea* is a placid animal that adapts quickly to captive life. Our animals were housed under conditions of natural temperature and photoperiod with only an additional heat source provided for basking, and underwent a normal hibernation. We are confident that the mean plasma steroid concentrations reported here reflect those in wild populations.

Further experimental studies are required to elucidate the hormonal control of reproductive behaviors in males of *T. nigrolutea* and other squamates as there appear to be differences between species in the plasma steroid hormones that are elevated during mating. This study has addressed the lack of published work in which data on annual steroid hormone profiles and the timing of coincident reproductive events are available.

**Acknowledgments.**— This research was funded by a School of Zoology, University of Tasmania postgraduate research allowance to A.E. We thank A.J. Bradley for generous gifts of antisera and D. Ratkowsky for statistical advice. This research was carried out under University of Tasmania Animal Ethics approval number 95046.
FIGURE LEGENDS

FIG. 3.1. Changes in mean monthly plasma testosterone concentrations in male *T. nigrolutea* throughout the annual reproductive cycle. Sampling was from November 1995 to October 1996. Values are means ± 1 standard error, N = 10.

FIG. 3.2. Changes in mean monthly plasma estrogen concentrations in male *T. nigrolutea* throughout the annual reproductive cycle. Sampling was from September 1996 to May 1997. Values are means ± 1 standard error, N = 8.

FIG. 3.3. Changes in mean monthly plasma progesterone concentrations in male *T. nigrolutea* throughout the annual reproductive cycle. Sampling was from November 1995 to October 1996. Values are means ± 1 standard error, N = 9.
LITERATURE CITED

ADKINS-REGAN E. 1981. Hormone specificity, androgen metabolism and social

ALDRIDGE R. D., and BROWN W. S. 1995. Male reproductive cycle, age at maturity, and
cost of reproduction in the timber rattlesnake (*Crotalus horridus*). *J. Herpetol.*
29:399-407.

ANDO S., CIARCIA G., PANNO M. L., IMBROGNO E., TARANTINO G., BUFFONE M.,
BERALDI E., ANGELINI F., and BOTTE V. 1992. Sex steroids in the plasma and testis
during the reproductive cycle of the lizard *Podarcis s. sicula* Raf. *Gen. Comp.

rhythm in the spiny-tailed lizard, *Uromastix hardwicki*. *Gen. Comp. Physiol.* 36:16-
22.

BOURNE A. R., and SEAMARK R. F. 1975. Seasonal changes in 17β-hydroxysteroids in
the plasma of a male lizard (*Tiliqua rugosa*). *Comp. Biochem. Physiol.* 50B:535-
536.

in the plasma and testis of the lizard *Tiliqua (Trachydosaurus) rugosa*. *Gen. Comp.
Endocrinol.* 58:394-401.

BOURNE A. R., TAYLOR J. L., and WATSON T. G. 1986a. Annual cycles of plasma and
testicular androgens in the lizard *Tiliqua (Trachydosaurus) rugosa*. *Gen. Comp.
Endocrinol.* 61:278-286.
BRADLEY A. J. 1990. Failure of glucocorticoid feedback during breeding in the male re- 
tailed phascogale *Phascogale calura* (Marsupialia: Dasyuridae). *J. Steroid 

CALLARD G. V., PETRO Z., and RYAN K. J. 1977. Identification of aromatase in the 


Norris and R. E. Jones, (eds.), Hormones and Reproduction in Fishes, Amphibians 


COURTY Y., and DUFASURE J. P. 1979. Levels of testosterone in the plasma and testis of 
the viviparous lizard (*Lacerta vivipara* Jacquin) during the annual cycle. *Gen. Comp. 

COURTY Y., and DUFASURE J. P. 1980. Levels of testosterone, dihydrotestosterone, and 
androstenedione in the plasma and testis of a lizard (*Lacerta vivipara* Jacquin) 


CREWS D. 1991. Trans-seasonal action of androgen in the control of spring courtship 

CREWS D. and MORGENTALER A. 1979. Effects of intracranial implantation of oestradiol 
and dihydrotestosterone on the sexual behaviour of the lizard *Anolis carolinensis*. *J. 
Endocrinol.* 82:373-381.


