Comp. Biochem. Physiol. Vol. 98A, No. 2, pp. 271–280, 1991
Printed in Great Britain

001530

0300-9629/91 $3.00 + 0.00
© 1991 Pergamon Press plc

MALE SEX STEROIDS AND HORMONAL CONTROL OF MALE COURTSHIP BEHAVIOR IN THE YELLOW-BELLIED SLIDER TURTLE, *TRACHEMYS SCRIPTA*

WILLIAM R. GARSTKA,* WILLIAM E. COOPER JR,† KELLY W. WASMUND‡ and JEFFREY E. LOVICH§¶

*Department of Biological Sciences, University of Alabama in Huntsville, Huntsville, AL 35899, U.S.A. Telephone: (205) 895 6260; †Department of Biology, Auburn University at Montgomery, Montgomery, AL 36193, U.S.A.; ‡Research Genetics, Huntsville, AL 35801, U.S.A.; §Savannah River Ecology Laboratory, Drawer E, Aiken, SC 29801, U.S.A.

(*Received* 14 *June* 1990)

Abstract—1. Survey of androgens and estrogens in serum, liver and testes of male yellow-bellied slider turtles, *Trachemys* (=*Pseudemys*) *scripta*, a species exhibiting dissociated gametogenesis age-dependent melanism, revealed the presence of numerous androgen precursors, androgens, androgen metabolites, and estrogens in quantities varying with season, tissue, and male coloration.

2. The most commonly found and abundant androgens in all males were dehydroepiandrosterone (DHEA) and androstenedione, which were present at significantly higher levels in testes than in serum or liver.

3. Epitestosterone was found in the serum and liver of only melanistic males; testosterone was only rarely recovered.

4. Behavioral testing of castrated male turtles implanted with various androgens, as well as intact and sham-operated controls, revealed that melanistic males courted more frequently than nonmelanistic males, and that exogenous testosterone was most effective in inducing courtship behavior in castrated males.

INTRODUCTION

Older male *Trachemys scripta* are distinctive, so much so that they were originally considered to be a separate species. While male *T. scripta* undergo spermatogenesis and exhibit androgen-dependent secondary sexual characters earlier and at a smaller body size than that at which females mature (Cagle, 1948a,b, 1950; Evans 1946, 1952; Gross, 1986), they become melanistic at a body size equivalent to the minimum size at sexual maturity for females (Lovich *et al.*, 1990). In the course of studies attempting to determine the reproductive significance of melanism we discovered a number of biochemical differences between nonmelanistic (younger) and melanistic (older) males (Lovich *et al.*, 1990), including that serum cholesterol was nearly three times higher in nonmelanistic than melanistic males during the April breeding period while April testicular cholesterol was nearly 10 times higher than in melanistic males (Lovich *et al.*, 1990). Because cholesterol is a precursor of the sex steroid hormones, this suggested to us that the pattern of steroid biosynthesis might differ between nonmelanistic and melanistic males and that steroids might be elevated (at least in melanistic males) during the breeding season.

If courtship behavior by melanistic or nonmelanistic male *T. scripta* was found to be stimulated by steroids present at elevated concentrations during the breeding season, this would present a different situation than is seen in garter snakes, the most studied species with dissociated gametogenesis, where neither the testes nor testosterone are seen to affect sexual behavior and where measured androgens are not elevated during the breeding period (Crews, 1984; Crews *et al.*, 1984; Garstka and Crews, 1982).

Recent studies with reptiles (Bourne, 1981, 1984; Bourne and Licht, 1985; Bourne *et al.*, 1985) have shown that the proportional concentrations of various steroids may differ markedly between reptilian and mammalian species, among reptiles, and seasonally (Bourne and Seamark, 1978; Courty and DuFaure, 1980). However, in many previous studies of vertebrates having a dissociation of breeding and gametogenesis, especially the critical studies with garter snakes (Camazine *et al.*, 1980; Crews *et al.*, 1984; Garstka *et al.*, 1982), generalized conclusions regarding androgens and their effect have been based on radioimmunoassays sensitive primarily to testosterone and dihydrotestosterone. This raises the possibility that the dissociated breeding pattern seen in numerous species (Crews, 1984) and attendant general conclusion that steroids are ineffective in stimulating courtship in these species (Crews, 1984; Crews *et al.*, 1984; Crews and Moore, 1986; Garstka *et al.*, 1982) may be an artifact of failure to examine the effective steroids.

Our goals in this present study were (1) to identify the steroids present in serum, liver, and testis of male yellow-bellied slider turtles, (2) to quantify any seasonal variation by examining tissues sampled at times of emergence from hibernation, breeding, and testicular recrudescence, and (3) to test the ability of the important steroids detected to stimulate courtship behavior in castrated males. These data allowed characterization of seasonal changes in steroids and

*To whom reprint requests should be addressed.

examination of the hypotheses that (1) steroids other than testosterone might regulate courtship behavior in male *T. scripta* and (2) that the steroid cycles, courtship intensity, and steroidal stimulation of courtship might differ between age classes of males as evidenced by melanism.

MATERIALS AND METHODS

Animals, housing and maintenance

For steroid determinations, male turtles were collected monthly, except December, from the White Springs Unit of Wheeler National Wildlife Refuge, Limestone County, Alabama. Turtles were collected with drag seines or trammel nets, were killed rapidly by decapitation upon collection, and were exsanguinated. Blood was reserved on ice and allowed to clot at 5°C overnight before the serum was separated. Tissues (liver and gonad) were dissected and reserved on ice until 1:5 homogenates in phosphate-buffered 0.9% saline with 0.01% sodium azide could be prepared. Blood serum and low-speed supernatants of homogenates were stored at −20°C until the assays could be performed.

Turtles for the behavioral experiment were collected from McElmurray's Pond, Aiken County, South Carolina with baited hoop traps. All males were sexually mature (Gibbons *et al.*, 1981), and ranged in size from 136 to 202 mm plastron length ($\bar{X} = 162$ mm). Melanistic males were significantly larger ($\bar{X} = 165$ mm) than nonmelanistic males ($\bar{X} = 157$ mm; $t = 2.23$, df = 54, $P < 0.025$). Females ranged from 175 to 252 mm plastron length, with a mean of 206 mm. These turtles were initially housed in a 15 × 22 m artificial pond at the Savannah River Ecology Laboratory, Savannah River Plant, South Carolina. This pond, and the stock tanks for behavioral assay, were supplied with flow-through water directly from a well, which maintained the water temperature at 18–21°C. Turtles were marked for individual identification by either drilling coded holes in the shell margin or by attachment of a numbered plastic tag (Ferner, 1979). Once per week, turtles were fed a modified Purina Turtle Breeder Chow #35 containing 2% dry-weight stable calcium. Basking sites were provided (Lovich, 1988). Following random assignment to treatment group and any necessary surgical procedures (see below), male and female turtles were housed separately in 2 m diameter stock tanks. Each tank had a sand bottom and 25 cm of water.

Hormone assay

Samples had to be pooled to obtain sufficient volume to complete the extraction and assay. Each pooled sample consisted of 3 ml each of serum, testis homogenate supernatant, or liver homogenate supernatant of the same three males; each pool was thus 9.0 ml. Four pooled samples for nonmelanistic males were made for the emergence-from-hibernation period (January and February), two for the breeding period (April), and five during testicular recrudescence (August–November). For melanistic males one pooled sample was made for the emergence period, two for breeding, and five for recrudescence.

Solvent extractions of the steroids were made using a variation of the method of Chattoraj (1982). Briefly, each 9-ml sample with 5α-cholestane (a non-biological steroid) added as an internal standard was extracted twice with 25-ml volumes of diethyl ether and the ether volumes pooled. The ether extracts were partitioned into acidic and neutral/basic fractions. 1 N NaOH was used to recover a phenolic (acidic) fraction. The phenolic extract was then made acidic with 1 N HCl and extracted with ether. Both the phenolic ether fraction (containing estrogens) and the non-phenolic ether fraction (containing androgens) were washed several times with dH_2O until the pH of the water wash was neutral. The ether extracts were dried with anhydrous Na_2SO_4 and filtered into tubes with teflon-lined screw caps. Next, 5α-cholestane was quantitatively added to the estrogen extract. The solvent was evaporated under N_2.

Each of the extracts (an estrogen and an androgen extract for each pooled sample) was treated with trimethylchlorosilane and hexamethyldisilazane in chloroform at 60°C for 60 min to produce volatile trimethylsilylether derivatives of the relatively non-volatile steroids (Chattoraj, 1982). Solvent and excess reactants were evaporated under N_2. Only those samples to be assayed on a day were silane treated that day.

To the dried silyl residue, 50 μl of tetrahydrofuran was added and 2 μl were injected onto a 30-m fused silica capillary column (1 μm ID) in a Hewlett-Packard 5890 Gas Chromatograph. A temperature program of 225°C for 1 min, then 5°C/min to 280°C for 15 min was used to elute the steroid derivatives. An analog strip as well as digitized column retention times and peak areas were recorded. Identification of steroids was made by comparison of relative retention times to the 5α-cholestane internal standard. A mixed extracted androgen or estrogen standard was run with each daily batch to assure uniformity of relative retention times for identification and to provide relative peak areas for each lot of extracted samples for quantification of the steroids.

Steroid standards for comparison included: (1) androgen precursors: pregnenolone (5-pregnen-3β-ol-20-one), progesterone (4-pregnen-3,20-dione), 17α-hydroxypregnenolone (5-pregnen-3β,17α-diol-20-one), 17α-hydroxyprogesterone (4-pregnen-17α-ol-3,20-dione), as well as 20α-dihydroxyprogesterone (=20-hydroxyprogesterone; 4-pregnen-20α-ol-3-one; see Bourne, 1984), (2) androgens: dehydroepiandrosterone (=DHEA; 5-androsten-3β-ol-17-one), androstenedione (4-androsten-3,17-dione), testosterone (4-androsten-17β-ol-3-one), epitestosterone (=cistestosterone; 4-androsten-17α-ol-3-one; see Bourne *et al.*, 1985), and dihydrotestosterone (=DHT; 5α-androstan-17β-ol-3-one), (3) androgen metabolites: etiocholanolone (5β-androstan-3α-ol-17-one), androsterone (5α-androstan-3α-ol-17-one), and epiandrosterone (5α-androstan-3β-ol-17-one), and (4) estrogens: 17β-estradiol (1,3,5(10)-estratrien-3,17β-diol), estriol (1,3,5(10)-estratrien-3,16α,17β-triol), estrone (1,3,5-(10)-estratrien-3-ol-17-one, epiestradiol (1,3,5(10)-estratrien-3,17α-diol), 16α-hydroxyestrone (1,3,5(10)-estratrien-3,16α-diol-17-one), 2-hydroxyestrone (1,3,5(10)-estratrien-2,3-diol-17-one), 16-epiestriol (1,3,5(10)-estratrien-3,16β,17β-triol), and 17-epiestriol (1,3,5(10)-estratrien-3,16α,17α-triol). Terminology used here is that of the source of our hormone standards, Steraloids Inc.

The assay conditions used were able to separate these androgens and estrogens. While the list of tested steroids is not exhaustive it does sample the major pathways of androgen and estrogen biosynthetic activity (see Fig. 1).

Surgery and hormone treatment

Groups of male turtles were castrated or sham-operated under Brevital anaesthesia (Wang *et al.*, 1977; Camazine *et al.*, 1981; Lawson and Garstka, 1985). An area of about 1.5 × 1.5 cm was opened on each side of the carapace lateral to the dorsal attachment site of the peritoneal musculature using a small circular saw. The muscle layer of the peritoneal wall was teased apart and the testes either removed or tugged. The muscle was sutured and the shell replaced and covered with epoxy sealant.

Silastic capsules for hormone treatment were made of 2.0-cm lengths of silastic tubing (2.3 mm OD 1.0 mm ID) with 1.5 cm of packed hormone filling (Dziuk and Cook, 1965). Capsules were implanted intraperitoneally during surgery. Several males treated with epitestosterone received additional implants anterior and lateral to the hind limb beneath skin and connective tissue and alongside peritoneal muscle and lymphatics.

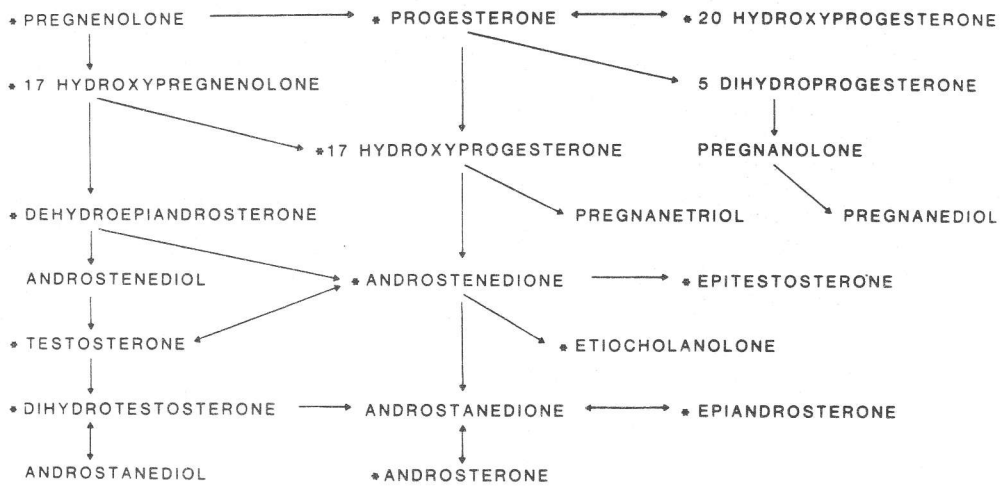

Fig. 1. Steroid hormone metabolic pathway—synthesis and breakdown of androgens. Trivial names are used; see text (Materials and Methods—Hormone assay) for alternative trivial names and chemical names of the steroids. Pathways are after Eik-Nes (1969), Feder (1981), and Freedman and Freedman (1970). Each steroid analysed is indicated with an asterisk. Conversion of androgens to estrogens is not shown. However, testosterone is aromatized to 17β-estradiol, androstenedione to estrone, and dehydroepiandrosterone to estriol (Callard *et al.*, 1977, 1978; Feder, 1981; Grodin *et al.*, 1973; Hemsell *et al.*, 1974).

Behavioral testing regimen and design

Eight treatment groups of male *T. scripta* were used: (1) sham-operated controls, $n = 5$; (2) castrated controls, $n = 5$; (3) intact controls, $n = 5$; (4) castrated and cholesterol-treated controls, $n = 5$; (5) castrated and testosterone-treated, $n = 10$; (6) castrated and epitestosterone-treated, $n = 10$; (7) castrated and androstenedione-treated, $n = 5$; (8) castrated and dehydroepiandrosterone-treated, $n = 10$. During the 1-month period after surgery and before behavioral testing, two epitestosterone-treated males died as did two testosterone-treated, one androstenedione-treated, one intact, and two cholesterol-treated males.

Courtship tests were conducted between 22 September and 6 October 1987. While no fall matings have been observed in the Alabama population sampled (no evidence of cloacal sperm in 36 females sampled September and October 1986; Garstka, pers. observ.), they have been occasionally observed in other populations under natural and laboratory conditions, including in the South Carolina population (Cagle, 1950; Jackson and Davis, 1972; Lovich *et al.*, 1990; Moll and Legler, 1971).

Courtship tests were independent: each male was tested once with one female and no female was used as a stimulus more than once. All stimulus females used were reproductively mature (greater than 160 mm plastron length; Cagle, 1948a,b, 1950; Gibbons *et al.*, 1981; Gross, 1986). For each test, the test male and stimulus female were placed into a 2.1-m diameter stock tank at approximately 0830 hr. They were observed from an elevated blind from 0900–1000 hr and from 1600–1700 hr; preliminary observations revealed little social activity at mid-day. After the test both were removed and the test tank was allowed to flush overnight with the flow-through water supply.

Courtship was scored in the following manner, based on both literature (Cagle, 1950) and personal observations: 0 = no courtship, 1 = trailing—the male swims after the female, 2 = orientation—the male swims in front of the female and faces her, swimming backwards, 3 = titillation—the male rapidly waves his foreclaws in front of the female's head, 4 = positioning—the male moves behind and dorsal to the female, 5 = mounting or attempting to mount—the male attempts to swing his tail under that of the female, and 6 = intromission. Morning and afternoon tests were given separate scores, the higher of which was used in the statistical analyses.

Statistical analyses

Statistical tests for steroid assays were non-parametric due to wide departure from normality produced by a high frequency of nondetectable steroid levels. Frequency differences were examined using Fisher's Exact Test. Ordinal data were tested using the Mann–Whitney U for independent groups and Friedman Two-Way ANOVA for repeated measures which included multiple comparisons of pooled samples across tissues (Hollander and Wolfe, 1973).

Fisher's Exact Test was used to calculate probabilities associated with results of courtship trials (Siegel, 1956). All tests were 1-tailed because androgens were predicted to increase the intensity of courtship. A Mann–Whitney U Test on courtship intensity was conducted using rank courtship scores for the combined DHEA and androstenedione-treated groups versus combined intact and sham-operated groups. No other such tests were done because only in this case were results likely to differ from those of Fisher's Exact Tests.

RESULTS

1. *Steroid analyses*

Numerous androgen precursors, androgens, androgen metabolites, and estrogens were detected in quantities varying with season, tissue, and male coloration (Tables 1–4). Among androgen precursors, pregnenolone was detected in only two of the nine pooled samples from the emergence and breeding periods (one each) and only in serum, but in seven of the 10 pooled samples from recrudescence, distributed among serum, testis, and liver (Table 1). Thus, pregnenolone was present more frequently during recrudescence ($P = 0.05$). No other differences in pregnenolone among groups, times, or tissues approached significance. Progesterone was detected in all tissues in most pools in all seasons. Its concentration differed among tissues ($\chi^2_r = 7.45$, df = 2, $P < 0.05$, 2-tailed), the only significant difference being a higher level in the testes than in serum ($P < 0.05$). The only significant seasonal difference in progesterone concentrations was that it was lower in

Table 1. Androgen precursors recovered from the tissues of male yellow-bellied slider turtles, *Trachemys scripta*

	Pregnenolone	Progesterone	17α-hydroxy pregnenolone	17α-hydroxy progesterone	20α-dihydroxy progesterone
Serum (ng/ml):					
Emergence:					
nonmelanistic	6	112	ND	66	ND
melanistic	ND	12	ND	ND	ND
Breeding:					
nonmelanistic	39	213	ND	ND	ND
melanistic	ND	24	26	ND	ND
Recrudescence:					
nonmelanistic	42	142	ND	511	ND
melanistic	7	47	ND	23	ND
Testes (ng/g):					
Emergence:					
nonmelanistic	ND	682	ND	7730	ND
melanistic	ND	408	ND	12,960	ND
Breeding:					
nonmelanistic	ND	2454	ND	48,100	ND
melanistic	ND	298	ND	2360	2
Recrudescence:					
nonmelanistic	534	842	ND	7800	ND
melanistic	95	444	294	470	ND
Liver (ng/g):					
Emergence:					
nonmelanistic	ND	170	ND	330	ND
melanistic	ND	170	ND	ND	ND
Breeding:					
nonmelanistic	ND	39	ND	ND	ND
melanistic	ND	475	ND	2155	ND
Recrudescence:					
nonmelanistic	ND	118	ND	ND	ND
melanistic	ND	178	ND	ND	ND

Steroids were extracted from blood serum or tissue homogenates pooled from three males. Volatile trimethylsilyl ether derivatives were prepared and analysed by gas chromatography. Steroids were identified by relative retention time by comparison with 5α-cholestane, the internal standard. Quantification was by digitzed peak areas relative to a known amount of 5α-cholestane. Results are expressed as ng/ml serum and ng/g wet wt tissue. Values shown are mean values of the pooled samples run. ND indicates none detected.

Table 2. Androgens detected in blood serum, testis, and liver of male yellow-bellied slider turtles, *Trachemys scripta*

	Dehydroepi-androsterone	Androstenedione	Testosterone	Epitestosterone	Dihydrotes-tosterone
Serum (ng/ml):					
Emergence:					
nonmelanistic	205	40	12	ND	ND
melanistic	100	ND	ND	88	ND
Breeding:					
nonmelanistic	1830	84	ND	ND	ND
melanistic	1216	6	ND	370	ND
Recrudescence:					
nonmelanistic	1529	62	ND	ND	ND
melanistic	105	ND	ND	ND	ND
Testes (ng/g):					
Emergence:					
nonmelanistic	41,280	1024	372	ND	682
melanistic	59,180	1189	ND	ND	ND
Breeding:					
nonmelanistic	245,900	17,590	ND	ND	818
melanistic	27,740	28	ND	ND	ND
Recrudescence:					
nonmelanistic	23,920	1404	394	ND	ND
melanistic	2519	51	ND	ND	ND
Liver (ng/g):					
Emergence:					
nonmelanistic	3983	679	ND	104	ND
melanistic	1719	ND	ND	ND	ND
Breeding:					
nonmelanistic	4180	113	ND	ND	ND
melanistic	4096	545	ND	2155	ND
Recrudescence:					
nonmelanistic	885	14	ND	ND	115
melanistic	328	2	ND	20	46

Steroids were extracted, analysed, identified, and quantified as in Table 1. The same pooled samples were used to identify androgens.

Table 3. Androgen metabolites identified in the blood serum, testis, and liver of male yellow-bellied slider turtles, *Trachemys scripta*

	Etiocholanolone	Androsterone	Epiandrosterone	Dihydrotestosterone
Serum (ng/ml):				
Emergence:				
nonmelanistic	34	191	ND	ND
melanistic	ND	ND	ND	ND
Breeding:				
nonmelanistic	335	1056	ND	ND
melanistic	14	10	3	ND
Recrudescence:				
nonmelanistic	307	69	ND	ND
melanistic	11	ND	ND	ND
Testes (ng/g):				
Emergence:				
nonmelanistic	8938	11,040	ND	682
melanistic	2411	ND	ND	ND
Breeding:				
nonmelanistic	11,618	16,690	ND	818
melanistic	229	ND	ND	ND
Recrudescence:				
nonmelanistic	3069	10,994	506	ND
melanistic	55	ND	ND	ND
Liver (ng/g):				
Emergence:				
nonmelanistic	984	1087	28	ND
melanistic	160	ND	ND	ND
Breeding:				
nonmelanistic	39	2021	ND	ND
melanistic	1595	ND	ND	ND
Recrudescence:				
nonmelanistic	331	1456	20	115
melanistic	47	ND	ND	46

Androgen metabolites were extracted, analysed, identified, and quantified as in Table 1. The same pooled samples of the same males were used in these assays as in Tables 1 and 2.

the liver during recrudescence than for the combined pools from emergence and breeding seasons (U = 0; $n = 5, 6$; $P < 0.036$, 2-tailed). Concentrations of the remaining important androgen precursor, 17α-hydroxyprogesterone, differed among tissues ($\chi^2_r = 14.00$, df = 2, $P < 0.005$, 2-tailed). Testis concentrations were significantly greater than those in liver ($P < 0.005$) and serum ($P < 0.025$). Concentrations were undetectable in all but a few liver and serum pools (2 each). 17α-hydroxypregnenolone and 20α-dihydroxyprogesterone (Bourne, 1984) were only detected once each.

The androgens DHEA, androstenedione, testosterone, epitestosterone, and DHT were detected (Table 2). Among these, DHEA was present most consistently and at the highest concentrations. Concentrations of this steroid varied among tissues ($\chi^2_r = 29.37$, df = 2, $P < 0.001$). Levels were significantly greater in the testes than in serum ($P < 0.001$) and liver ($P < 0.005$). Concentrations in liver and serum did not differ. Androstenedione showed no consistent significant seasonal trend in all tissues, but was significantly more concentrated in serum during the breeding season than during recrudescence (Fisher $P = 0.0256$, 1-tailed) and was less likely to be detectable in the other tissues during recrudescence than at other times, but not significantly so. The concentration of androstenedione varied among tissues, being greatest in the testes ($\chi^2_r = 23.41$, df = 2, $P < 0.001$). The testis concentrations, although substantially greater, were not significantly greater than those in serum and liver by the stringent multiple comparison tests used. However, testis concentration exceeded that in serum according to the Sign Test ($P = 0.05$). Surprisingly, testosterone was detectable in only three pooled samples, two at emergence and one during recrudescence. Testosterone was absent during the breeding season; breeding is dissociated from high testosterone levels. Epitestosterone was present in three serum pools, one at emergence and two during the breeding season. It was also detected in two liver pools during emergence and two during recrudescence. No epitestosterone was detected in the testes. DHT was found only in testis and liver, being detected in the testis in two pools during emergence and one during breeding, and in the liver in two pools during recrudescence.

Table 4. Estrone recovery from blood serum, testis, and liver of male yellow-bellied slider turtles, *Trachemys scripta*

	Serum (ng/ml)	Testis (ng/g)	Liver (ng/g)
Emergence:			
nonmelanistic	74	1142	102
melanistic	ND	2106	ND
Breeding:			
nonmelanistic	12	2210	ND
melanistic	1	4963	150
Recrudescence:			
nonmelanistic	82	1102	207
melanistic	34	167	116

The same pooled samples of the same males as in Tables 1, 2, and 3 were used here.

Androgen metabolites observed were etiocholanolone, androsterone, epiandrosterone, and DHT (Table 3). Etiocholanolone occurred in most pools in all tissues at all seasons; it showed no significant overall seasonal or tissue differences. Androsterone levels varied significantly among tissues ($\chi^2 = 10.04$, df = 2, $P < 0.01$). The only significant pairwise

comparison was a greater concentration of androsterone in testis than in serum ($P < 0.005$). There were no differences in androsterone levels among seasons for the entire data set. Results for DHT are presented above.

Two of the estrogens studied, estrone and 17β-estradiol, were detected, but estrone was found more frequently and at higher concentrations (Table 4). Estrone concentrations varied significantly among tissues ($\chi^2 = 15.50$, df = 2, $P < 0.001$). Testis concentrations of estrone were significantly greater than those in liver and serum (both $P < 0.005$), but those of liver and serum did not differ. In all three tissues, estrone concentrations differed significantly with seasons. Estrone levels were higher in the testes during the combined emergence and breeding seasons than during recrudescence (U = 11; $n = 8$, 11; $P < 0.02$, 2-tailed). In the serum and liver, estrone levels were higher during recrudescence than during the combined earlier periods (U = 19; $n = 8$, 11; $P < 0.05$ and U = 18; $n = 8$, 11; $P < 0.05$, respectively; both 2-tailed). 17β-estradiol was detected in only three pools.

Melanistic and nonmelanistic males differed in tissue concentrations of certain androgen precursors, androgens, androgen metabolites, and estrogens. Among androgen precursors, melanistic males differed from nonmelanistic males in patterns of progesterone and 17α-hydroxyprogesterone concentrations. Melanistic males had higher liver concentrations of progesterone during the combined emergence and breeding periods than during recrudescence (U = 0; $n = 5$, 6; $P = 0.036$, 2-tailed). Concentrations of 17α-hydroxyprogesterone were greater in the testes of nonmelanistic than of melanistic males for the entire study (U = 18; $n = 8$, 11; $P = 0.05$, 2-tailed) and during recrudescence (U = 3; $n = 5$; $P = 0.05$, 2-tailed).

Melanistic and nonmelanistic males differed in the levels and distribution of three androgens: DHEA, androstenedione, and epitestosterone. Concentrations of DHEA were greater in melanistic males during combined emergence and breeding than during recrudescence, in the testes and liver (each U = 7; $n = 5$, 6; $P = 0.036$, 2-tailed). During recrudescence, DHEA levels were significantly greater among nonmelanistic than melanistic males in all three tissues combined (U = 18; $n = 8$, 11; $P < 0.05$, 2-tailed) and in the testis (U = 0; $n = 5$, 5; $P < 0.008$, 2-tailed) and liver (U = 2; $n = 5$, 5; $P < 0.032$, 2-tailed). In the serum, a similar, but not significant, difference was observed (U = 4; $n = 5$, 5; $P = 0.096$, 2-tailed). Androstenedione concentrations were significantly greater in nonmelanistic than melanistic males in the serum (U = 7; $n = 8$, 11; $P < 0.002$, 2-tailed) and testis (U = 10; $n = 8$, 11; $P < 0.02$, 2-tailed) but not in the liver. Epitestosterone was detected in a significantly higher frequency of melanistic than nonmelanistic males during the combined emergence and breeding seasons (Fisher $P = 0.024$, 2-tailed). Also, this androgen was detected more frequently among melanistic males during the combined emergence and breeding period than during recrudescence (Fisher $P = 0.036$, 2-tailed).

Melanistic and nonmelanistic males differed in certain respects in levels of the androgen metabolites etiocholanolone and androsterone. Nonmelanistic males had greater serum and liver concentrations of etiocholanolone during recrudescence than did melanistic males (Fisher $P = 0.048$, 2-tailed). Nonmelanistic males also had higher levels for the same comparison in testes, but the difference was not significant (U = 4; $n = 5$, 5; $P = 0.096$, 2-tailed). Additionally, serum etiocholanolone concentration was higher at all times in nonmelanistic than melanistic males (U = 9; $n = 8$, 11; $P < 0.01$). Androsterone was at slightly higher concentration in nonmelanistic males in all tissues than in melanistic males (serum: U = 3; $n = 8$, 11; $P < 0.001$; testis, liver: U = 0, $n = 8$, 11; $P < 0.001$ for each). Androsterone was undetectable among melanistic males except in one serum pool sampled during the breeding season. However, it was detected in all pools from nonmelanistic males except in one serum pool during recrudescence. Androsterone was also significantly more concentrated in nonmelanistic than in melanistic males in each of the tissues in the combined emergence and breeding samples (U = 0; $n = 3$, 6; $P = 0.024$ for each tissue) as well as during recrudescence (U = 0; $n = 5$, 5; $P = 0.008$ for each tissue). Among nonmelanistic males, androsterone levels varied significantly among tissues during emergence ($\chi^2_r = 8.00$, df = 2, $P = 0.009$, 2-tailed), being greater in the testis than in the liver ($P < 0.05$, 2-tailed). Androsterone levels also varied substantially among tissues in nonmelanistic males during recrudescence, but not significantly ($\chi^2_r = 6.20$, df = 2, $P = 0.10$, 2-tailed). Although pairwise comparisons are not usually made without a significant main effect, a large difference between testis and serum levels suggests that an exception might be appropriate. Considered separately, testis levels were significantly higher than serum levels ($P < 0.02$, 2-tailed).

The only notable difference in estrogen concentrations between melanistic and nonmelanistic males was a higher testicular estrone level in nonmelanistic than in melanistic males during recrudescence, but this difference was not significant (U = 4; $n = 5$, 5; $P = 0.096$, 2-tailed).

2. *Behavioral studies*

Results of the behavioral studies are summarized in Table 5. Males that were castrated or castrated and treated with cholesterol did not court, whereas several males in the combined intact and sham-operated groups did. However, with the small sample sizes and low frequencies of courtship by intact males, this difference did not attain significance ($P = 0.124$).

Testosterone significantly increased the frequency of male courtship behavior. Testosterone-treated males courted more frequently than combined castrates and cholesterol-treated males ($P = 0.013$). Epitestosterone, the only androgen found at a substantial level in breeding season males, did not stimulate courtship; there was no difference in frequency of courtship between the combined castrated control groups and the epitestosterone-treated group ($P > 0.10$). Neither DHEA nor androstenedione significantly affected male courtship behavior. Although at least one male treated with each of these hormones courted, neither courtship frequencies nor intensities for their combined treatment groups differed from

Table 5. Courtship behavior of male yellow-bellied slider turtles, *Trachemys scripta*

	Treatment Groups							
	Intact	Sham-operated	Castrated	Castrated/cholesterol	Castrated/testosterone	Castrated/epitestosterone	Castrated/DHEA	Castrated/androstenedione
Number of melanistic males tested	2	2	5	2	5	4	2	3
Number of nonmelanistic males tested	2	3	0	1	3	4	5	1
Number of melanistic males courting	1	2	0	0	4	0	0	1
Courtship scores of melanistic males	3	3, 3	0	0	2, 2, 2, 3	0	0	3
Number of nonmelanistic males courting	0	0	0	0	1	0	3	0
Courtship scores of nonmelanistic males	0	0	0	0	2	0	1, 2, 2	0

Courtship was scored using an ordinal system of 0 = no courtship to 6 = intromission (see Materials and Methods for full description and justification). Each test was independent: each male was tested once with two observation periods, the higher score of which was used.

those for males of the combined control groups (Fisher $P = 0.170$; z score corrected for ties for Mann–Whitney Test was $z = -1.55$, $P = 0.06$).

Melanism appears to affect courtship behavior. In normal males, i.e. in the combined intact and sham-operated controls, melanistic males were significantly more likely to court than were nonmelanistic males ($P = 0.048$). A similar difference did not occur in the combined data for hormone-treated groups in which one or more males courted ($P > 0.10$), but the relative frequency of courtship by melanistic males differed somewhat among androgens. Among testosterone-treated males, nearly the same proportions of melanistic and nonmelanistic turtles courted as in the combined control groups ($P > 0.25$ each). A somewhat different pattern occurred in the combined DHEA- and androstenedione-treated groups, in which only one of five melanistic males and three of six nonmelanistic males courted. Although the differences are not significant ($P > 0.05$), they hint that melanistic and nonmelanistic males might respond differently to various androgen treatments. For the DHEA-treated group a higher proportion of nonmelanistic males courted than for the combined controls; this difference is suggestive, but not significant ($P = 0.083$).

DISCUSSION

1. *Steroids*

High levels of androgen precursors are expected to occur during recrudescence, and were observed in the case of pregnenolone. With progesterone, concentrations were lower in the liver, but not in the serum or testes, during recrudescence, suggesting that progesterone may be more efficiently converted to other steroids in the testes during recrudescence. The testis was presumably a major source and/or binder of progesterone and 17α-hydroxyprogesterone, both of which were more concentrated in the testes than in other tissues. The findings that progesterone and 17α-hydroxyprogesterone were present on emergence from hibernation and during breeding were expected because these steroids occur early in the biosynthetic path leading to the androgens (refer to Fig. 1).

Although numerous androgens were detected, the pattern of concentrations was surprising. DHEA, the androgen most prevalent and present in the highest concentrations, was presumably of testicular origin or was efficiently taken up by the testes as indicated by its relatively high concentration there. The same applies to androstenedione. The more frequent presence of androstenedione in serum during breeding than during recrudescence is only marginally significant, but is consistent with findings in the cobra (Tam and Phillips, 1969). Its biological importance, if any, is unclear. That DHT levels were low or undectable at all times agrees with the findings of several workers on other reptiles (Callard, 1967; Camazine *et al.*, 1980; Lofts and Choy, 1971; Tam and Phillips, 1969).

The absence of detectable testosterone in most males was both unexpected and troubling, especially given its reported presence in other emydid turtles (e.g. Silva *et al.*, 1984; Licht *et al.*, 1985). Possible explanations for this discrepancy are: (1) that *T. scripta* differs from its relatives in this respect,

(2) that *in vivo* there is very rapid turnover of testosterone, (3) that biosynthesis may lead to alternative isomeric forms such as epitestosterone, (4) that our assays are incorrect, or (5) that previous reports are misleading. There are difficulties with any single assay system. Neither our assays nor those of most previous workers were confirmed with alternative test methods; we used GLC exclusively, relying on retention times of silyl ether derivatives to identify unknowns, while others have used either radioimmunoassay or recovery of labeled hormones in *in vitro* systems treated with radioactive precursors. RIA can be misleading because of cross-reactivity unless highly specific antibodies are used (as by Wiebe *et al.*, 1988). GLC can be misleading as well, especially if either unextracted or otherwise unprocessed samples are used. Clearly, the best and most reliable analysis would include confirmation of results by an entirely different chemical method—in our case mass spectrometry or specific RIA.

If our results are accurate, a possible explanation for the low levels of testosterone appears in our estrogen assays. While our methods allowed us to identify eight estrogens, only estrone was identified regularly; male slider turtles exhibited substantial levels of estrone but not 17β-estradiol, which is usually considered the most common vertebrate estrogen (Hadley, 1984; van Tienhoven, 1983). 17β-estradiol is the aromatization product of testosterone while estrone is aromatized androstenedione (Callard *et al.*, 1977, 1978; Feder, 1981). 17β-estradiol can also be synthesized from estrone, and in mammals most estrone is so converted (Hadley, 1984; Feder, 1981). The enzyme to convert estrone to estradiol (17β-oldehydrogenase) is the same enzyme that converts androstenedione to testosterone (Feder, 1981). Therefore, a deficiency of that single enzyme in male *T. scripta* would be consistent with both the lack of testosterone and 17β-estradiol and the presence of estrone in our results.

Elevated tissue concentrations of steroids during the emergence and breeding periods prior to recrudescence could be due to adrenal steroids that might then be taken up by the testes and acted upon by testicular biosynthetic enzymes (as described by Eik-Nes, 1969) or aromatized peripherally to estrone (Callard *et al.*, 1977, 1978; Grodin *et al.*, 1973; Hemsell *et al.*, 1974) which in turn might be taken up from the blood by testicular binding proteins. In the male cobra (*Naja naja*), which also exhibits dissociated gametogenesis, the testes are small but the adrenals enlarge during the breeding season (Lofts *et al.*, 1971; Tam and Phillips, 1969). Any conclusions with regard to the role of the adrenal must await our further studies of adrenal suppression and stimulation.

2. *Attempts at hormonal induction of courtship behavior*

No hormones reliably induced intense courtship in male *T. scripta*. These findings could have been a result of either a male effect or a function of female quality. We made no attempt to control female stimulus quality; proximal stimuli necessary for intense courtship and copulation are unknown in these turtles and may have been lacking in the present study. If female attractivity or behavior are under endocrine control and are important elements stimulating or maintaining male courtship behavior, our experiments may have given only a minimal indication of androgenic effects. Further, we purposely chose the fall of the year for our tests. While courtship has been described in the fall in this species little is known of its significance or extent. We felt that if we could reliably induce courtship in the fall we would have a more robust effect.

Based on the finding of high concentrations of epitestosterone in male lizards (Bourne *et al.*, 1985), we hypothesized that this androgen might be present in male *T. scripta*. This would have explained the difficulty some other workers have had in using traditional androgens to induce sexual behavior in some reptiles, especially those having dissociated gametogenesis. We found epitestosterone in substantial concentrations in the serum of melanistic males during the breeding season, but the concentration was much lower than that of DHEA. Further, epitestosterone did not affect courtship of male *T. scripta*; it has no known function. Only testosterone significantly increased the frequency of courtship.

The increased courtship induced by testosterone seems paradoxical given that we did not detect it in our breeding season samples. This may simply indicate that testosterone is not a primary hormone normally stimulating courtship but that the turtles have a very high sensitivity to it. Alternatively, it may be that blood, testis, and liver levels are low because of high turnover and efficient binding to target tissues. Testosterone also stimulates courtship in male musk turtles, *Sternotherus odoratus* under some environmental conditions (Mendonça, 1987), suggesting that androgens may have important effects on courtship in male turtles but do not elicit strong courtship in the absence of appropriate physical and social stimuli, probably including reproductively active females.

3. *Relationship of male melanism to hormones and behavior*

Males of the two colour patterns differed both hormonally and behaviorally. The pronounced differences between melanistic and nonmelanistic males in the relative concentrations and seasonal cycles of androgen precursors, androgens, and androgen metabolites demonstrate that steroid production and utilization by these male groups differ profoundly. When sampling animals from wild populations it is thus very important to note age in addition to reproductive condition and time of year. Nonmelanistic males had higher levels of several steroids, in part due to differences during recrudescence. The higher 17α-hydroxyprogesterone and DHEA levels suggest higher activity in nonmelanistic males of the early stages in the biosynthesis of androgens and account for their higher levels of androstenedione and of androgen metabolites. The only androgen for which melanistic males had higher levels, epitestosterone, was present at low concentrations and only during emergence and breeding. Its source is unknown. These numerous differences suggest that steroidal regulation of breeding might be different in nonmelanistic and melanistic males.

Further evidence supporting a difference in steroidal reproductive mechanisms is provided by seasonal differences in steroid production between melanistic and nonmelanistic males. The finding among melanistic males of higher levels of two androgens, DHEA in the liver and epitestosterone in liver and testes during emergence and breeding than during recrudescence is expected for a species with dissociated gametogenesis if either of these androgens is important to breeding.

The finding that melanistic males courted more frequently than nonmelanistic males is consistent with findings that in many species larger, older males mate more frequently than do smaller, younger males. Because there were no copulations or indications of female choice favoring melanistic male *T. scripta* it is not clear whether melanistic males have any advantage over nonmelanistic males in obtaining mates. Our data do not reveal any androgen that both elicits courtship and is significantly more concentrated in melanistic males. Nevertheless, the combined higher frequency of courtship and elevated steroid levels, particularly of DHEA and androstenedione, suggest that the differences in courtship between melanistic and nonmelanistic males could be steroidally mediated.

4. *Conclusions and implications for studies of dissociated gametogenesis*

We are left with but a fragmentary understanding of the control of courtship behavior in *T. scripta*. It appears that sex steroid hormones may play a role, but we have not demonstrated unequivocally that they normally do. Given our minimal understanding of the roles of hormones in turtle reproductive behavior, it is premature to state that the hormones which were not effective or were only minimally effective are unimportant. While it is apparent that a range of androgenic steroids are present in these turtles and may affect their sexual behavior, further study is needed to determine their functions and significance.

Acknowledgements—Support for this work was provided by contract DE-AC-76SR00819 between the U.S. Department of Energy and the University of Georgia through its Savannah River Ecology Laboratory (SREL). Support was also provided by grants 2-66104 and 2-66145 from the University of Alabama Research Administration to WRG. WEC was supported as a visiting faculty research participant at SREL. We thank Gary Meffe for providing laboratory space at SREL for the surgery and Whit Gibbons and Justin Congdon, also of SREL, for discussions of the work. We also thank the refuge managers at Wheeler National Wildlife Refuge, Decatur, Alabama for permission to collect turtles.

REFERENCES

Bourne A. R. (1981) Blood metabolites of injected [14C] progesterone in the lizard *Tiliqua rugosa*. *Comp. Biochem. Physiol.* **70B,** 661–664.

Bourne A. R. (1984) Occurrence of 20β-hydroxysteroid oxidoreductase activity in the kidney of the lizard *Tiliqua rugosa*. *Comp. Biochem. Physiol.* **77B,** 221–222.

Bourne A. R. and Licht P. (1985) Steroid biosynthesis in turtle testes. *Comp. Biochem. Physiol.* **81B,** 793–796.

Bourne A. R. and Seamark R. F. (1978) Seasonal variation in steroid biosynthesis by the testis of the lizard *Tiliqua rugosa*. *Comp. Biochem. Physiol.* **59B,** 363–367.

Bourne A. R., Taylor J. L. and Watson T. G. (1985) Identification of epitestosterone in the plasma and testis of the lizard *Tiliqua rugosa*. *Gen. Comp. Endocrinol.* **58,** 394–401.

Cagle F. R. (1948a) Sexual maturity in the male turtle, *Pseudemys scripta troostii*. *Copeia* **1948,** 108–111.

Cagle F. R. (1948b) The growth of turtles in Lake Glendale, Illinois. *Copeia* **1948,** 197–203.

Cagle F. R. (1950) The life history of the slider turtle, *Pseudemys scripta troostii* (Holbrook). *Ecol. Mogogr.* **20,** 33–54.

Callard G. V., Petro Z. and Ryan K. J. (1977) Identification of aromatase in the reptilian brain. *Endocrinology* **100,** 1214–1218.

Callard G. V., Petro Z. and Ryan K. J. (1978) Phylogenetic distribution of aromatase and other androgen converting enzymes in the central nervous system. *Endocrinology* **103,** 2283–2290.

Callard I. P. (1967) Testicular steroid synthesis in the snake *Natrix sipedon pictiventris*. *J. Endocrinol.* **37,** 105–106.

Camazine B., Garstka W. R., Tokarz R. R. and Crews D. (1980) Effects of castration and androgen replacement on male courtship behavior in the red-sided garter snake (*Thamnophis sirtalis parietalis*). *Horm. Behav.* **14,** 358–372.

Camazine B., Garstka W. R. and Crews D. (1981) Techniques for gonadectomizing snakes. *Copeia* **1981,** 884–886.

Chattoraj S. C. (1982) Endocrine function. In *Fundamentals of Clinical Chemistry* (Edited by Tietz N. W.), pp. 699–823. Saunders, Philadelphia.

Courty Y. and DuFaure J. P. (1980) Levels of testosterone, dihydrotestosterone, and androstenedione in the plasma and testis of a lizard (*Lacerta vivipara* Jacquin) during the annual cycle. *Gen. Comp. Endocrinol.* **42,** 325–333.

Crews D. (1984) Gamete production, sex hormone secretion, and mating behavior uncoupled. *Horm. Behav.* **18,** 22–28.

Crews D., Camazine B., Diamond M., Mason R., Tokarz R. R. and Garstka W. R. (1984) Hormonal independence of courtship behavior in the male garter snake. *Horm. Behav.* **18,** 29–41.

Crews D. and Moore M. C. (1986) Evolution of mechanisms controlling mating behavior. *Science* **231,** 121–125.

Dziuk P. J. and Cook B. (1965) Passage of steroids through silicone rubber. *Endocrinology* **78,** 208–211.

Eik-Nes K. B. (1969) Patterns of steroidogenesis in the vertebrate gonads. *Gen. Comp. Endocrinol.* Suppl. **2,** 87–100.

Evans L. T. (1946) Endocrine effects upon the claws of immature turtles, *Pseudemys elegans*. *Anat. Rec.* **94,** 64.

Evans L. T. (1952) Endocrine relationships in turtles II. Claw growth in the slider, *Pseudemys scripta troostii*. *Anat. Rec.* **112,** 251–263.

Feder H. H. (1981) Essentials of steroid structure, nomenclature, reactions, biosynthesis, and measurements. In *Neuroendocrinology of Reproduction* (Edited by Adler N. T.), pp. 19–64. Plenum Press, New York.

Ferner J. W. (1979) A review of marking techniques for amphibians and reptiles. *S.S.A.R. Herpetol. Circul.* **9,** 31.

Freedman M. A. and Freedman S. N. (1970) *Introduction to Steroid Biochemistry and its Clinical Applications*. Harper and Row, New York.

Garstka W. R., Camazine B. and Crews D. (1982) Interactions of behavior and physiology during the annual reproductive cycle of the red-sided garter snake (*Thamnophis sirtalis parietalis*). *Herpetologica* **38,** 104–123.

Garstka W. R. and Crews D. (1982) Female control of male reproductive function in a Mexican snake. *Science* **217,** 1159–1160.

Gibbons J. W., Semlitsch R. D., Greene J. L. and Schubauer J. B. (1981) Variation in age and size of maturity of the slider turtle (*Pseudemys scripta*). *Amer. Natural.* **117,** 841–845.

Grodin J. M., Siiteri P. K. and MacDonald P. C. (1973) Source of estrogen in postmenopausal women. *J. Clin. Endocrinol. Metab.* **36,** 207–214.

Gross M. (1986) Gametogenesis and the reproductive function of the kidney in the male slider turtle, *Pseudemys scripta*. MS thesis. Univ. Ala. in Huntsville.

Hadley M. E. (1984) *Endocrinology*. Prentice Hall. Englewood Cliffs.

Hemsell D. L., Grodin J. M., Brenner P. F., Siiteri P. K. and MacDonald P. C. (1974) Plasma precursors of estrogen II. Correlation of the extent of conversion of plasma androstenedione to estrone with age. *J. Clin. Endocrinol. Metab.* **38,** 476–479.

Hollander M. and Wolfe D. A. (1973) *Nonparametric Statistical Methods*. John Wiley, New York.

Jackson C. G. and Davis J. D. (1972) Courtship display behavior of *Chrysemys concinna suwanniensis*. *Copeia* **1972,** 385–387.

Lawson G. O. and Garstka W. R. (1985) Castration of turtles, *Pseudemys scripta*. *J. Ala. Acad. Sci.* **56,** 89 (abstr).

Licht, P., Breitenbach G. L. and Congdon J. D. (1985) Seasonal cycles in testicular activity, gonadotropin, and thyroxine in the painted turtle, *Chrysemys picta*, under natural conditions. *Gen. Comp. Endocrinol.* **59,** 130–139.

Lofts B. and Choy Y. L. (1971) Steroid synthesis by the seminiferous tubules of the snake *Naja naja*. *Gen. Comp. Endocrinol.* **17,** 588–591.

Lofts B., Phillips J. G. and Tam W. H. (1971) Seasonal changes in the histology of the adrenal gland of the cobra, *Naja naja*. *Gen. Comp. Endocrinol.* **16,** 121–131.

Lovich J. E. (1988) Aggressive basking behavior in eastern painted turtles (*Chrysemys picta picta*). *Herpetologica* **44,** 197–202.

Lovich J. E., McCoy C. J. and Garstka W. R. (1990) The development and significance of melanism in the slider turtle. In *Life History and Ecology of the Slider Turtle* (Edited by Gibbons J. W.), pp. 233–254. U.S. National Museum, Washington.

Mendonça M. T. (1987) Timing of reproductive behaviour in male musk turtles, *Sternotherus odoratus*: effects of photoperiod, temperature, and testosterone. *Anim. Behav.* **35,** 1002–1014.

Moll E. O. and Legler J. M. (1971) The life history of a neotropical slider turtle, *Pseudemys scripta* (Schoepff) in Panama. *Bull. Los Angeles County Mus. Nat. Hist. Sci.* **11,** 1–102.

Mount R. H. (1975) *The Reptiles and Amphibians of Alabama*. Auburn Univ. Agric. Expt. Station, Auburn.

Siegel S. (1956) *Nonparametric Statistics for the Behavioral Sciences*. McGraw-Hill, New York.

Silva A. M. R., Moraes G. S. and Wasserman G. F. (1984) Seasonal variations of testicular morphology and plasma levels of testosterone in the turtle *Chrysemys dorbigni*. *Comp. Biochem. Physiol.* **78A,** 153–157.

Tam W. H. and Phillips J. G. (1969) Seasonal changes in the *in-vitro* production of testicular androgens by the cobra (*Naja naja* Linn). *Gen. Comp. Endocrinol.* **13,** 117–125.

van Tienhoven A. (1983) *Reproductive Physiology of Vertebrates*. Second Edition. Cornell University Press, Ithaca.

Wang R-T., Kubie J. L. and Halpern M. (1977) Brevital Sodium: an effective anaesthetic agent for performing surgery on small reptiles. *Copeia* **1977,** 738–743.

Wiebe R. H., Williams L. E., Abee C. R., Yeoman R. R. and Diamond E. J. (1988) Seasonal changes in serum dehydroepiandrosterone, androstenedione, and testosterone levels in the squirrel monkey (*Saimiri boliviensis boliviensis*). *Am. J. Primatol.* **14,** 285–291.