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Annual Cycles of Steroid Hormone Production, Gonad Development, and Reproductive Behavior in the Atlantic Stingray

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The mating season of the Atlantic stingray (*Dasyatis sabina*), which begins in August and continues through April, is the longest documented for any elasmobranch fish. Despite this protracted mating period, female stingrays ovulate synchronously at the end of the mating season and there is no evidence for sperm storage by females. Thus, the proximate causal factors and ultimate function of this extended preovulatory mating are unknown. Annual cycles of the gonadal steroids testosterone (T), dihydrotestosterone (DHT), 17ß-estradiol (E_2), and progesterone (P_4) were measured for 26 months in a wild estuarine population of Atlantic stingrays to test for associations with their reproductive biology, gametogenesis, and sexual behavior. Serum androgen levels in males showed four phases within an annual cycle: (1) androgen suppression between reproductive seasons (April–July), (2) primary androgen increase during the onset of spermatocyte development (August–October), (3) androgen decrease following maximum testis growth and spermatocyte development (November–December), and (4) secondary androgen increase during the peak of sperm maturation (January–March). Increases in male E_2 and P_4 were correlated with spermatocyte/spermatocyst formation, maximum testis weight, and the primary (but not secondary) androgen surge. We propose that the production of male androgens across the full seven-month preovulatory mating period promotes their aggressive reproductive behavior and drives the protracted mating season of this species. In females, serum T and DHT showed relatively brief increases near ovulation, whereas E_2 and P_4 showed brief increases near both ovulation and parturition. The increase in female androgens near ovulation may increase female aggression when they are impregnable by courting males and enhance their choice of mates. This estuary sample population shows higher absolute steroid levels and distinct differences in temporal cycles compared to another Florida fresh water lake population, but the cause and significance of these differences are unknown. Experiments are needed to confirm that the aggressive and protracted mating behavior is the result of prolonged male androgen production and to determine whether the sustained preovulatory mating serves some function related to female reproduction.

Key Words: androgen; dihydrotestosterone; elasmobranch; estradiol; progesterone; reproduction; sexual behavior; steroid; stingray; testosterone.

The rays and sharks are represented by more than 800 living species and exhibit diverse reproductive adaptations which clearly distinguish them from other extant fish taxa (Wourms, 1977; Wourms and Demski, 1993). Unlike most derived teleosts which have external fertilization, all male rays and sharks have copulatory organs known as claspers. Reproductive behav-

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ior involves the formation of social groups in which the sexes exhibit complex courtship behaviors that culminate in pair copulation, transfer of sperm to the female, and internal fertilization (Klimley, 1980; Tricas, 1980; Tricas and LeFeuvre, 1985; Nordell, 1994; Carrier *et al.,* 1994; Tricas *et al.,* 1995). In oviparous species, such as skates and catsharks, females only briefly retain the embryo before it is deposited on the bottom. In contrast, females of aplacental viviparous species, such as the spiny dogfish (*Squalus acanthias*), which has a gestation period of nearly 2 years, may retain the embryo within the uterus for extended periods of time. Embryos of viviparous species, such as requiem sharks, hammerhead sharks, and many rays, are also retained within the uterus but receive direct nourishment from the mother either by a placenta or by uterine secretions.

This diversity of reproductive adaptations leads to the question of how the temporal cycles of gamete production, courtship behavior, mating, gestation, and parturition are regulated in different elasmobranch taxa. The major events that occur during these many phases of reproduction are controlled by or associated with periodic cycles in sex steroid hormone production. In the oviparous little skate, *Raja erinacea* (Koob *et al.,* 1986), oviparous spotted catshark, *Scyliorhinus canicula* (Sumpter and Dodd, 1979), and aplacental viviparous dogfish, *Squalus acanthias* (Tsang and Callard, 1987), elevated concentrations of estradiol (E_2) are associated with the follicular phase of egg development and often the activity of accessory reproductive structures, such as the shell gland or oviduct. Elevated concentrations of progesterone (P_4) in *S. acanthias* may inhibit vitellogenin synthesis induced by E_2 (Callard *et al.,* 1992) and in *R. erinacea* may regulate ovulation, encapsulation, and oviposition (Koob *et al.,* 1986). In the placental viviparous female bonnethead shark (*Sphyrna tiburo*), both E_2 and testosterone (T) levels are elevated during the mating and preovulatory stages, whereas P_4 is elevated during preovulatory, ovulatory, and postovulatory stages (Manire *et al.,* 1995). Testosterone production is associated with other reproductive events, such as mating and preovulatory development in female bonnethead sharks (Manire *et al.,* 1995), the follicular phase of female skates (Koob *et al.,* 1986) and dogfish (Tsang and Callard, 1987), and spermatogenesis in male sharks (Dobson and Dodd, 1977; Callard *et al.,* 1985, 1989; Manire and Rasmussen, 1997). These studies clearly demonstrate that, whereas steroid actions may be closely related to specific events in the reproductive cycle, the temporal patterns and associated functions of each hormone differ widely among species.

Populations of the Atlantic stingray, *Dasyatis sabina,* in the estuarine waters of central Florida are of particular interest because they engage in the longest mating period reported for any elasmobranch fish (Snelson *et al.,* 1988, 1997; Johnson and Snelson, 1996; Kajiura and Tricas, 1996; Maruska *et al.,* 1996). Furthermore, individuals mate continuously for 7 months prior to female ovulation in late March. Since there is neither evidence for female sperm storage nor arrested development in this species, the protracted mating does not function for fertilization and is hypothesized to possibly serve other reproductive functions (Maruska *et al.,* 1996). This study presents the seasonal cycles of gonadal steroid hormone levels in the serum of a wild stingray population. These data are used to (1) correlate steroid production with specific stages of spermatogenesis, (2) associate relative steroid levels with specific phases of the stingray reproductive cycle, and (3) assess whether the aggressive reproductive behavior shown by male rays in the protracted mating season could be influenced by androgen steroid production.

MATERIALS AND METHODS

Field Collections

This study was conducted on a population of Atlantic stingrays in the southern Banana River on the east coast of central Florida (28.12.5° N, 80.38.3° W) in the same area used by Kajiura and Tricas (1996) and Maruska *et al.* (1996). The collection site is characterized by a shallow sand/sea grass habitat in water <1 m deep. The estuary is a mixture of sea water and local fresh water runoff with annual ranges in salinity from approximately 20 to 30 ppt. Ten mature stingrays of each sex (male >19 cm disk width (DW), female >22 cm DW) were collected on or near the 15th of each month from August 1992 through February 1995. Fish were captured with beach seines or hand nets, transported to shore, and immediately euthanized to re-

duce the influence of stress on blood hormone levels. Blood samples were taken by direct cardiac puncture, transferred to centrifuge tubes, and placed on ice for 3–6 h. Samples were centrifuged at 7000 rpm (approximately 4100*g*) for 5 min. The serum was then collected and stored at −70°C until assayed. Disk width of each specimen was measured to the nearest mm and total body weight (BW) to the nearest g. Gonads (and the associated epigonal organ) were removed, blot dried, weighed to the nearest mg, fixed in a 4% formaldehyde solution, and stored in 50% isopropyl alcohol. Many of the rays used in this study also provided data for other studies on their reproduction and ecology (Kajiura and Tricas, 1996; Maruska *et al.*, 1996). Water temperature data over the duration of this study were obtained from an environmental monitoring station located in a similar shallow water habitat approximately 3 miles to the south of the collection site and maintained by the Florida Department of Environmental Protection.

Radioimmunoassays (RIAs)

Serum concentrations of 17ß-estradiol, testosterone, dihydrotestosterone (DHT), and progesterone were determined by RIA after purification by chromatography on Sephadex LH-20 microcolumns. Serum aliquots of 500 μl were extracted by shaking for 5 min with 5 ml of freshly opened or redistilled diethyl ether. After freezing the aqueous phase in an ethanol/dry ice bath, the organic phase was decanted and brought to dryness under a stream of air. The dried extracts were resolubilized and sequentially chromatographed on two different Sephadex LH columns. On the first column (1.0 g LH-20, elution mixture, hexane:benzene: methanol, 62:20:13 v/v), 17ß-estradiol was separated from estrone and all neutral steroids (Resko *et al.*, 1975). The neutral fraction from the first column was applied to the second column (2.5 g LH-20, elution mixture, hexane:benzene:methanol, 85:15:5 v/v), and the appropriate fractions for progesterone, DHT, and testosterone were collected (Resko *et al.*, 1980). The purified steroids were then estimated by RIA as described previously (Resko *et al.*, 1975; Hess *et al.*, 1981).

Extraction and chromatographic losses were monitored by adding known amounts of tritiated authentic steroids to independent samples of skate serum and processing these samples in parallel with the unknown samples. Recoveries following the chromatography were 75% for 17ß-estradiol, 88% for progesterone, and 72 and 70% for DHT and testosterone, respectively. Water blanks were also processed in parallel with the unknown samples to provide estimates of solvent blank methods (pg/ml) for each steroid as follows: 17β-estradiol, 3.2 $\pm$ 0.4; progesterone, 22 $\pm$ 3.0; DHT, 18.6 $\pm$ 9.8; and testosterone, 3.5 $\pm$ 1.9. Reported values were corrected for both procedural losses and method blanks before correcting for aliquots assayed. Each sample was diluted with 500 μl of ethanol after chromatography and assayed at different volumes ranging from 5 to 200 μl. The reported values are the average concentrations calculated from aliquots whose values fell within the 5–95% binding limits of the appropriate standard curve following linearization with a logit-log transformation. Intraassay coefficients of variation did not exceed 11% for these assays, and the sensitivity limits for the steroids determined in this study were 17β-estradiol, 5; progesterone, 10; DHT, 5; and testosterone, 5 pg/tube, respectively. All four steroid hormones demonstrated complete parallelism in the RIAs compared to the standards, supporting our assumption that the purification procedure eliminated any major interfering substances. Similar procedures have been followed and documented for a number of studies of serum steroid hormones in elasmobranchs (Snelson *et al.*, 1997; Manire and Rasmussen, 1997; Rasmussen *et al.*, 1999).

Histology

Histological analysis of spermatogenic activity was performed on male rays to correlate tissue development with steroid production. The gonad tissue and preparation methods used in this study were taken from the previously published work of Maruska *et al.* (1996). Briefly, a central section from a single testis was taken from each male, dehydrated, blocked in paraffin, sectioned, and stained with hematoxylin and eosin. Sections were analyzed under a compound scope and classified into distinct stages according to spermatogenic development. Seven distinct gametogenic stages are described for this species: Stage I contains the testicular appendage of the lobe along with primary spermatogonia, Stage II contains spermatocysts with layers of spermatogonial cells and an associated

basement membrane, Stage III shows spermatocysts that contain early spermatocytes, Stage IV shows spermatids within the spermatocysts following the second meiotic division, Stage V contains immature spermatozoa that are unorganized within the spermatocysts, Stage VI contains mature sperm that are organized into packets, and Stage VII contains flattened spermatocysts and degenerating spermatogonia. The contribution of each stage within each testis was estimated as the product of the average cross section area for each stage and gonad weight. This simple estimate is known as the absolute spermatogenic production (ASP) and accounts for differences in gonad weight among samples (Maruska *et al.*, 1996).

Statistical Analyses

One important feature of periodic sampling is that individual fish are not synchronous in their steroid production (especially during transition periods) and this can result in monthly data sets that are not normally distributed. When monthly samples could not be normalized by transformation, we used nonparametric statistics to test for differences in monthly sample population steroid levels across the 26-month study period. Differences in sample medians were tested using the Kruskal–Wallis ANOVA followed by Dunn's method for pairwise comparisons. One *a priori* goal of this study was to identify the onset of steroid production at the end of summer. For this analysis, we used monthly subsets in which data were tested by parametric ANOVA and the Student–Newman–Keuls (SNK) pairwise comparisons method. Associations between steroid concentrations and histological stages of gametogenesis were determined using Pearson's correlation and linear regression. Although many monthly data samples were not normally distributed, we also report means and standard errors in Tables 1 and 3 for comparison with other published studies on this and other species.

RESULTS

A total of 496 Atlantic stingrays were collected over the continuous 26-month sampling period for use in the steroid assays. Mature male rays ($n = 248$) ranged in size from 20.0 to 29.8 cm disk width ($\bar{x} = 24.2 \pm 1.1$ SD cm DW) and total mass from 435 to 970 g ($\bar{x} = 704.2 \pm 100.6$ SD g BW). Mature female rays ($n = 248$) were larger in size and ranged from 21.0 to 32.5 cm ($\bar{x} = 27.3 \pm 1.90$ SD cm DW) and 448 to 1820 g in total body mass ($\bar{x} = 1020.7 \pm 14.3$ SD g BW).

The temperature of the shallow waters of the Indian River Estuary near the study site showed annual variations that were associated with day length and season (Fig. 1A). Day length at the study site latitude reaches a maximum of 13.9 h in June and a minimum of 10.5 h in December. Maximum summer water temperatures of 33.0 and 31.6°C were recorded in July of 1993 and 1994, respectively. After the summer peak, water temperature gradually dropped to winter minima during the months of December to February 1992 (19.2°C) and January 1993 (15.0°C). The shallow waters of the estuary showed only a weak vertical temperature stratification which rarely spanned more than 1°C.

Male Serum Steroid Levels

Androgen steroid concentrations in male rays showed a pronounced, repetitive seasonal cycling over the 26-month sampling period. The maximum T concentration among all males was 17,270 pg/ml for a 24.1-cm-DW, 648-g-BW individual captured in October 1992 (Table 1). The lowest T concentration recorded was 10 pg/ml for several males collected in May and June 1993. Sample population levels of male serum T were at the lowest levels (median $\leq$ 400 pg/ml) from April to July 1993 and April to June 1994 (Fig. 1B). These spring and early summer T minima were followed by increases in T concentration during August 1993 (Kruskal–Wallis ANOVA on April–October 1993, Dunn's test, $df = 6, P < 0.05$) and July 1994 (Kruskal–Wallis ANOVA test on April–September 1994, Dunn's test, $df = 5, P < 0.05$). In 1993, T levels continued to increase through the fall months until they reached a maximum median concentration of 8480 pg/ml in October. Serum T levels then rapidly dropped to one-quarter of their peak concentration (median = 2155 pg/ml) followed by a second peak level of 5475 pg/ml in January and 7405 pg/ml in February 1994 (Kruskal–Wallis ANOVA, SNK Pairwise Multiple Comparisons test, $df = 7, P < 0.05$). Male T levels then declined in the following months to

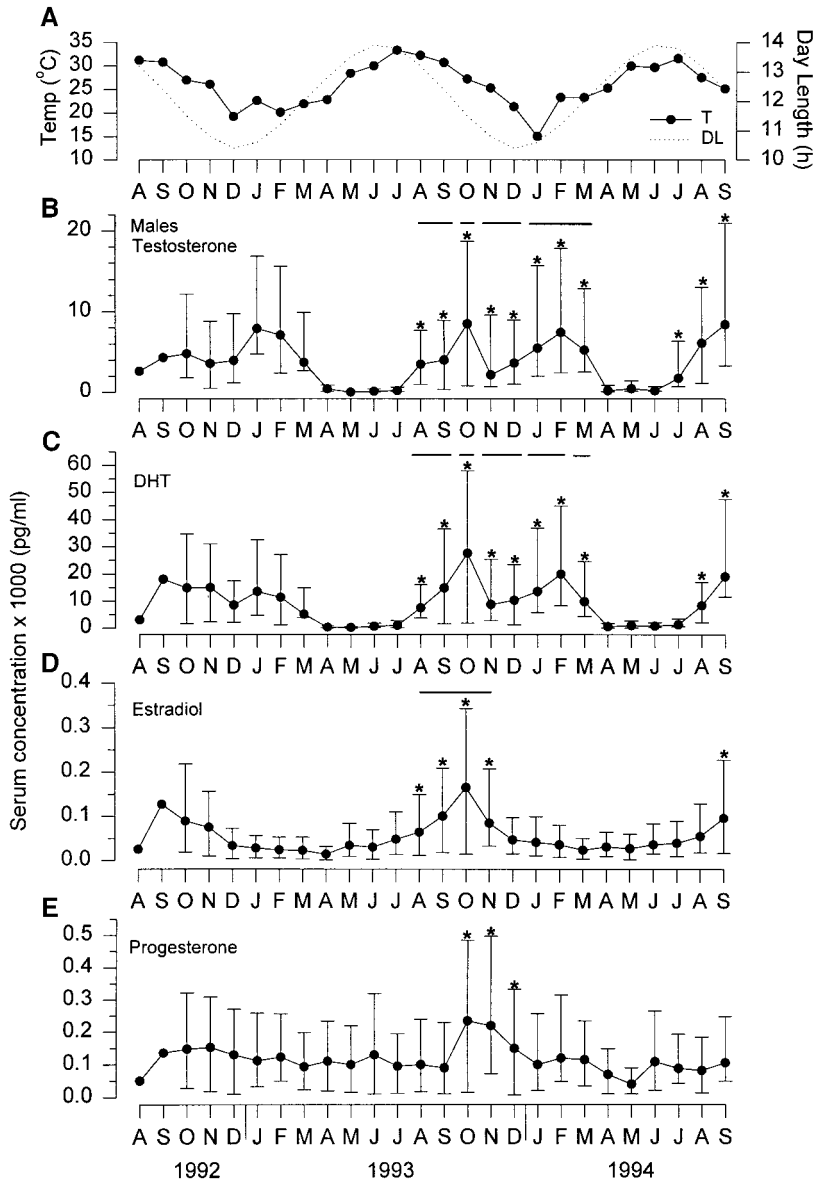

FIG. 1. Serum steroid hormone concentrations (median, 25th, and 75th percentiles) in male Atlantic stingrays, *Dasyatis sabina*, collected from the Banana River estuary between August 1992 and September 1994. (A) The monthly water temperature (T) and day length (DL) profiles across the sampling period. Maximum day length (13.5 h) and annual temperatures (>30°C) occurred during the months of June and July, respectively. Shortest day length (10.5 h) occurs in December and lowest water temperatures were recorded during December 1992 and February 1994. (B) Testosterone (T) and (C) dihydrotestosterone (DHT) levels in the sample population show a periodic cycling that begins in August and has a major peak in October and a second minor peak in January–March. Note the close covariation in changes in T and DHT. Bars above T and DHT levels show which months differ and support the existence of bimodal peaks during the period of androgen surge. (D) Estradiol shows a broad, monophasic cycle that peaks in October. (E) Progesterone is detectable in the serum throughout the year and peaks in October 1993 along with the other steroids. $*P < 0.05$.

minimum levels in April 1994. This pattern of a second winter peak in T was also evident during January and February 1993 but we could not test for differences because of small sample sizes.

Serum concentrations of male DHT followed a similar pattern of seasonal variation that closely tracked that of T (Fig. 1C). A maximum DHT concentration of 60,480 pg/ml was recorded from the same animal

TABLE 1
Serum Hormone Concentrations for Estuarine Male Atlantic Stingrays, *Dasyatis sabina*

Sample date	*n*	E_2	P_4	T	DHT
August 1992	1	25	50	2620	2980
September 1992	1	127	136	4298	18000
October 1992	13	97.7 ± 8.8 (52, 89, 146)	166.7 ± 19.9 (97, 148, 367)	5656.9 ± 1158.2 (2440, 4780, 17270)	21324.6 ± 4090.2 (11840, 14780, 60480)
November 1992	9	73.9 ± 6.1 (45, 75, 103)	160.8 ± 16.0 (112, 153, 257)	4634.4 ± 814.7 (2380, 3560, 10150)	15516.7 ± 1350.1 (10110, 14940, 23290)
December 1992	8	33.1 ± 3.7 (14, 33.5, 46)	133.8 ± 10.9 (89, 130, 197)	5387.5 ± 1693.9 (1480, 3935, 16440)	8330.0 ± 1675.4 (1900, 8445, 18330)
January 1993	11	47.9 ± 22.2 (11, 28, 268)	113.8 ± 13.2 (46, 112, 178)	6173.6 ± 1142.8 (400, 7880, 10320)	12905.5 ± 2440.3 (230, 13430, 24400)
February 1993	10	26.1 ± 4.0 (11, 24, 58)	109.7 ± 10.8 (63, 123, 161)	7121.8 ± 1176.2 (1190, 7080, 15930)	12457.3 ± 1644.8 (1530, 11310, 21060)
March 1993	10	24.3 ± 2.5 (13, 22.5, 38)	93.4 ± 10.0 (53, 93.5, 155)	3783.0 ± 947.5 (280, 3695, 8090)	5396.0 ± 1406.9 (270, 5070, 11100)
April 1993	10	14.1 ± 1.6 (5, 14, 22)	110.3 ± 7.3 (79, 110.5, 150)	402.0 ± 55.8 (140, 400, 710)	373.0 ± 68.8 (90, 310, 780)
May 1993	11	37.2 ± 5.6 (9, 34, 72)	179.1 ± 66.1 (10, 100, 770)	197.3 ± 164.9 (10, 10, 1840)	312.7 ± 63.5 (100, 230, 810)
June 1993	13	34.4 ± 3.0 (22, 30, 56)	165.4 ± 22.5 (90, 130, 380)	233.1 ± 83.0 (10, 110, 1100)	807.7 ± 159.6 (220, 660, 2300)
July 1993	10	48.6 ± 5.3 (24, 48, 77)	92.0 ± 6.1 (50, 95, 120)	296.0 ± 87.7 (50, 205, 900)	1162.0 ± 193.0 (240, 1035, 2100)
August 1993	10	65.3 ± 7.4 (21, 64, 94)	112.0 ± 11.0 (70, 100, 160)	3406.0 ± 457.7 (940, 3480, 5760)	6221.0 ± 878.2 (1830, 7450, 8920)
September 1993	10	98.1 ± 11.7 (40, 100.5, 170)	109.0 ± 19.2 (20, 90, 240)	4468.0 ± 481.7 (2680, 3985, 7130)	16497.0 ± 2119.0 (7900, 14755, 28500)
October 1993	10	158.8 ± 9.1 (105, 165.5, 196)	225.0 ± 12.9 (150, 235, 280)	9328.4 ± 771.8 (7160, 8480, 14800)	27063.1 ± 1709.5 (14588, 27550, 34653)
November 1993	10	91.9 ± 14.4 (40, 84.5, 163)	207.0 ± 24.9 (100, 220, 290)	3935.0 ± 1035.9 (210, 2155, 8300)	11091.0 ± 2632.8 (420, 8665, 25700)
December 1993	10	41.5 ± 5.1 (10, 46.5, 67)	153.0 ± 12.5 (60, 150, 200)	3749.0 ± 600.4 (350, 3610, 6370)	10324.0 ± 1506.2 (750, 10240, 17940)
January 1994	10	42.3 ± 4.8 (23, 41, 60)	124.0 ± 17.5 (70, 100, 210)	6735.0 ± 1268.7 (2360, 5475, 13380)	16162.0 ± 3231.4 (4860, 13495, 34570)
February 1994	10	36.8 ± 3.4 (22, 35.5, 56)	137.0 ± 21.9 (60, 120, 250)	7442.0 ± 1239.9 (1780, 7405, 13670)	17923.0 ± 3032.7 (3400, 19875, 30970)
March 1994	10	24.8 ± 2.3 (16, 23.5, 42)	156.0 ± 58.7 (60, 115, 680)	5325.0 ± 1078.2 (410, 5240, 11760)	10678.0 ± 2195.9 (950, 9755, 22920)
April 1994	11	30.6 ± 4.7 (10, 31, 60)	70.0 ± 4.7 (50, 70, 100)	805.5 ± 435.9 (10, 200, 4940)	2102.7 ± 1062.5 (220, 570, 11610)
May 1994	11	29.3 ± 2.4 (18, 27, 45)	44.6 ± 8.5 (10, 40, 100)	714.6 ± 177.1 (100, 450, 1840)	1318.2 ± 195.8 (750, 960, 2770)
June 1994	10	34.0 ± 4.8 (10, 36, 52)	116.7 ± 24.5 (10, 109, 275)	320.9 ± 88.4 (67, 217, 840)	1063.2 ± 229.6 (345, 822, 2450)
July 1994	10	46.2 ± 7.1 (25, 39.5, 91)	77.4 ± 12.8 (10, 89, 125)	2875.5 ± 686.6 (967, 1750.5, 6300)	1510.8 ± 317.7 (410, 1282, 3588)
August 1994	8	56.4 ± 8.8 (25, 55, 92)	93.0 ± 13.8 (50, 82.5, 160)	5692.5 ± 637.1 (2690, 6112, 7569)	7145.4 ± 871.8 (1900, 8264, 9110)
September 1994	10	102.1 ± 11.1 (45, 96, 151)	114.0 ± 22.0 (40, 106, 234)	8177.8 ± 1680.4 (236, 8404.5, 15890)	18108.5 ± 4191.6 (265, 18980, 34249)

Note. Values are mean ± standard error, with minimum, median, and maximum in parentheses. All steroid data are expressed in pg/ml. *n*, number of individuals sampled; E_2, 17β-estradiol; P_4, progesterone; T, testosterone; DHT, dihydrotestosterone.

captured in October 1992 that also had the highest measured T. The minimum DHT concentration of 90 pg/ml was from a 25.2-cm-DW, 778-g-BW male captured in April 1993. Sample population DHT levels were lowest in the months of April, May, and June of each year with monthly median levels between 230 and 960 pg/ml. Increases in DHT levels were detected in August 1993 (Kruskal–Wallis ANOVA on April–October 1993, Dunn's test, $df = 6$, $P < 0.05$) and August 1994 (Kruskal–Wallis ANOVA on May–September 1994, Dunn's test, $df = 4$, $P < 0.05$). The August 1993 rise was followed by dual peaks in October and January–February as seen for T (Kruskal–Wallis ANOVA on August 1993–May 1994, Dunn's test, $df = 7$, $P < 0.05$). The concentration of DHT in males was on average approximately 2.3 times that of T and is seen in the correlation plot in Fig. 2A. Thus, the production of DHT is closely associated with that of T, with both androgens showing bimodal peaks in October and January–February.

The levels of E_2 in the blood of males also showed a distinct periodic cycling but only a single prominent annual peak (Fig. 1D). The maximum E_2 concentration of 268 pg/ml was recorded from a 24.0-cm-DW, 720-g-BW male captured in January 1993. The lowest E_2 concentration of 5 pg/ml came from a 24.9-cm-DW, 657-g-BW male collected in April 1993. Serum E_2 concentrations of the sample population in 1993 were lowest during April 1993 (median = 14 pg/ml), increased in August, and peaked in October at a median of 166 pg/ml (ANOVA on April–December 1993, SNK test, $df = 8$, $P < 0.05$). However, in the following year, males showed more variation in E_2 levels, and the first increase in E_2 concentration was detected in the month of September (Kruskal–Wallis ANOVA on March–September 1994, Dunn's test, $df = 6$, $P < 0.05$).

Although male E_2 concentration coincides with the October 1993 peak in androgen levels, it does not appear to sustain a robust production throughout the entire androgen steroid cycle. The association between T and E_2 concentrations during the two T peaks for the 1993–1994 reproductive season are shown for 90 males in Fig. 2B. During the first surge in T from August through November, the elevation in E_2 concentration in individual males covaried strongly ($r = 0.78$, Linear regression (in pg/ml): $E_2 = 46.1 + 0.011$ T, $P < 0.001$). In contrast, the association of E_2 and T concentrations during the second T surge period from December through March was much weaker ($r = 0.44$), with low E_2 levels that rarely exceeded 60 pg/ml (Linear regression (in pg/ml): $E_2 = 26.3 + 0.002$ T, $P < 0.005$). Thus, males appear to have a peak E_2 level that is highly associated with the surge in T during October but not during the second surge of T during January and February.

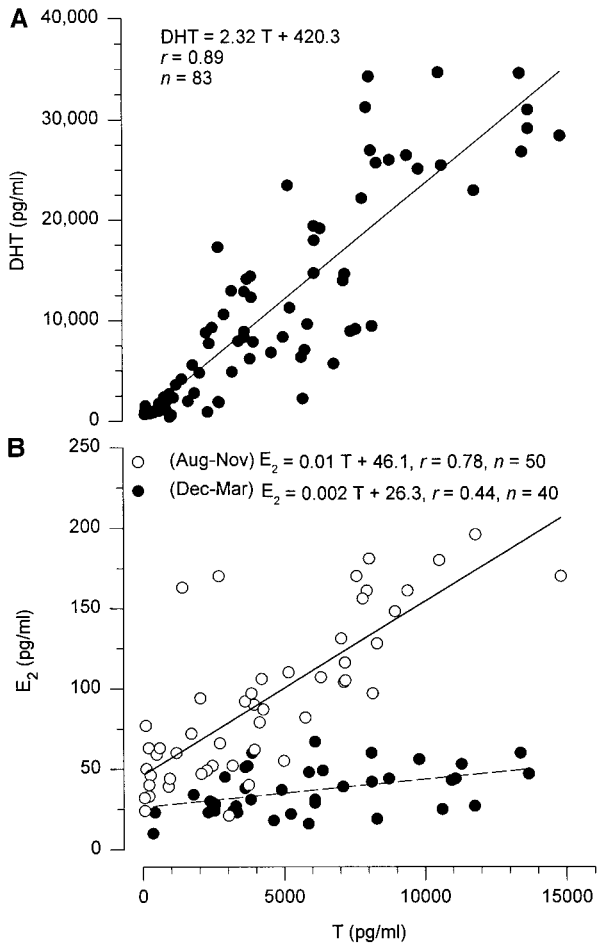

FIG. 2. The association between serum concentrations of androgens and estradiol for male Atlantic stingrays, *Dasyatis sabina*, collected from the Banana River estuary from August 1993 to March 1994. (A) Relationship between dihydrotestosterone (DHT) and testosterone (T) shows that these androgens covary in concentration throughout the year. Note that DHT concentration is on average approximately 2.3 times greater than T. (B) Relationship between estradiol (E_2) and T shows that productions covary at different rates across the year. Note that E_2 production is approximately 5 times greater during the early phase of gonad development (August–November) compared to the remainder of the reproductive season (December–March). Linear regressions, correlation coefficients, and sample sizes (n) are shown.

TABLE 2
Histological Stages of Testicular Development and Associated Changes in Serum Steroid Levels in the Atlantic Stingray, *Dasyatis sabina*

Stage	Description	Characteristics	Relative activity associated with steroid production
I	Primary zone	Loosely organized germ cells mixed with spermatogonia and putative Sertoli cells	Precedes October T, P_4, and E_2 peaks and coincides with second T peak in January
II	Early spermatocysts	Dividing spermatogonia and Sertoli cells organize into membrane-bound spermatocysts with a hollow lumen	Coincident with peak T, P_4, and E_2 production in October but only weakly with second T peak
III	Spermatocytes	Sertoli cells enlarge and migrate to periphery of spermatocyst; spermatogonia form meiotic primary and secondary spermatocytes	Strongly associated with T, P_4, and E_2 peaks in October but weakly with second T peak
IV	Spermatids	Spermatids develop from secondary spermatocytes, grow, and fill spermatocyst lumen	Onset lags beginning of T, P_4, and E_2 production in fall but is maximum before second T peak
V	Immature sperm	Spermiogenesis occurs and immature sperm associate with Sertoli cells	Falls within boundaries of T production
VI	Mature spermatocysts	Sperm mature and form spermatozeugmata that are associated with a single Sertoli cell that ruptures when sperm are released	Falls within boundaries of T production
VII	Degenerate zone	Empty spermatocysts and Sertoli cells degenerate	Most active during second T peak

Note. Stage descriptions modified from Maruska *et al.* (1996).

Monthly median serum levels of P_4 were >40 pg / ml for the entire study (Fig. 1E). In addition, there was a relatively high variability in P_4 among individuals for each month that obscured detection of any periodicity. However, elevated levels of P_4 were detected during October, November, and December 1993 above levels in July and August (Kruskal–Wallis ANOVA on July–December 1993, Dunn's test, $df = 5$, $P < 0.05$). This P_4 elevation was coincident with the October peaks for T, DHT, and E_2.

Steroid Production and Testis Development

Spermatocysts develop in the stingray testis and exhibit a series of histologically identifiable gametogenic stages, as reported by Maruska *et al.* (1996); these are summarized in Table 2. These stages begin with spermatogonia (SI), the organization of spermatocytes into membrane-bound spermatocysts (SII–SIV), spermiogenesis (SV–SVI), and spermatocyst degeneration (SVII). The histological stages of gonads were examined in relation to individual steroid hormone levels for 83 males collected during the period of July 1993 to September 1994, and the proportion by weight was estimated for stages SII–SVI. Figure 3 shows the mean monthly plot for each of these gametogenic stages and associated male steroid levels. The formation of spermatogonia and early Sertoli cells (SI) begins during the summer months but shows the strongest growth during January–March. The summer and fall activity of SI is closely tied to the rapid development of spermatocysts (SII and SIII) and the associated Sertoli cell growth and migration. This activity coincides with the onset of T, E_2, and P_4 production and subsequent October steroid peaks. However, note that the robust development of spermatocysts seen in October is greatly reduced during the second surge of T during January–March. Spermatid production (SIV) begins in September, peaks in December, and then rapidly subsides. This stage is also present during the decrease of E_2, P_4, and the first T surge but subsides before the second T peak. Immature (SV) and mature (SVI) sperm stages involve spermiogenesis, the association of spermatozoa with large Sertoli cells, and fall within the broad period of T production. The presence of empty spermatocysts and degeneration of Sertoli cells (SVII) occurs primarily in January–March during the second surge in T.

Female Serum Steroid Levels

Androgen steroid concentrations in female stingrays also showed periodic cycles in production (Figs.

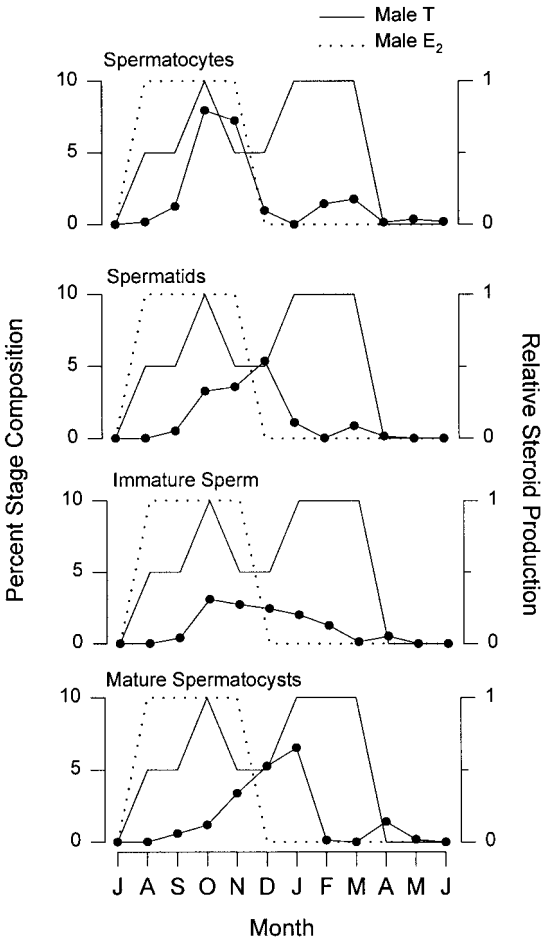

FIG. 3. Stages of testes development and changes in steroid levels in male Atlantic stingrays, *Dasyatis sabina*, collected from the Banana River estuary from July 1993 to June 1994. Four histological stages of spermatogenesis (SIII–SVI of Maruska *et al.*, 1996) are expressed as percentage of total gonad weight for each monthly sample (closed circles). Steroid concentrations for T and E_2 are expressed as differences among months as determined by statistical analyses for this period in Fig. 1 and are expressed in relative values. Note that the October peak in T is coincident with peak activity of primary and secondary spermatocytes, whereas the second January–March peak in T is coincident with a relatively brief activity of spermatocytes and peak of sperm maturation. E_2 production is associated only with the first production of spermatocytes.

4B and 4C). Females had a relatively low, sustained median T level that did not exceed 140 pg/ml ($\bar{x}$ = 200 pg/ml) for most of the study (Table 3, Fig. 4B). Monthly median T concentrations showed only one detectable spike at 1102 pg/ml during the month of March 1994 (Kruskal–Wallis ANOVA, $df = 25$, $P < 0.05$), with the highest T concentration of 10,373 pg/ml recorded from a 28.0-cm-DW, 946-g-BW individual. However, when monthly samples were compared for the period from December 1992 to May 1993, increases of much lower magnitude were also detected in February and March 1993 (Kruskal–Wallis ANOVA, $df = 5$, $P < 0.05$). This interyear variability in T surges may be due to its brief duration relative to the 4-week sampling interval. A similar annual increase in DHT was seen for females during March–April 1993 and March 1994 (Kruskal–Wallis ANOVA for September–April 1993, $df = 5$, $P < 0.05$), but as for T were brief and of low magnitude compared to males (Fig. 4C). Despite the relatively low androgen levels in most females, the production of DHT was closely associated with that of T, as found for males. Figure 5 shows the relationship between serum levels of DHT and T for individual females collected throughout the study. The high correlation coefficient ($r = 0.97$) indicates that the female blood concentrations of T and DHT covary. Also, note that the regression coefficient ($\beta = 2.47$) is similar to that for males, with the average DHT concentration in females approximately 2.5 times that of T.

Female serum estradiol had sustained levels of 50–250 pg/ml across most months of the study but were exceeded by two annual surges. Two prominent peaks in E_2 were identified during July 1993 and July–August 1994, and a second increase was also seen in March of 1993 and 1994 (Kruskal–Wallis ANOVA, Dunn's test, $df = 25$, $P < 0.05$) (Fig. 4D). Similar to that observed for T, the relatively low magnitude of E_2 recorded during March 1993 compared to March 1994 may be the result of a brief E_2 spike that occurred between sampling periods.

Female progesterone showed sustained monthly levels between 50 and 100 pg/ml across the entire study with at least four periods of elevation (Fig. 4E). A nonparametric analysis of variance across all months of the study revealed P_4 surges in July 1993, July 1994, and March 1994 (Kruskal–Wallis ANOVA, $df = 25$, $P < 0.05$). Because Dunn's test could not compare ranks for the February–March 1993 period when the entire data set was analyzed, we separately examined for a change in P_4 during December 1992–April 1993. During this time period, P_4 increased in February 1993 (ANOVA, $df = 4$, $F = 9.11$, $P < 0.0001$; SNK Multiple Comparisons Test, $P < 0.05$). Similar to the other female steroids, the relatively low

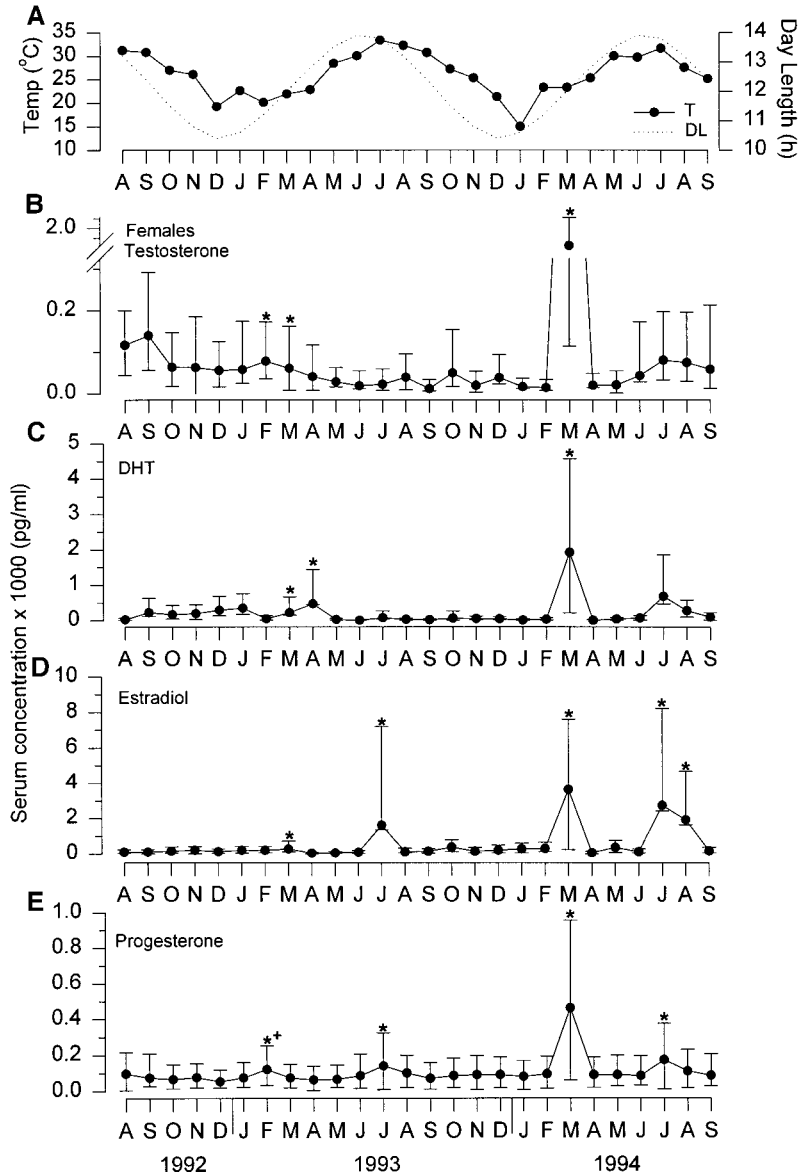

FIG. 4. Serum steroid hormone concentrations (median, 25th, and 75th percentiles) in female Atlantic stingrays, *Dasyatis sabina,* collected from the Banana River estuary between August 1992 and September 1994. (A) Monthly water temperature and day length profiles across the sampling period as shown in Fig. 1A. (B) Female testosterone and (C) dihydrotestosterone (DHT) levels show a periodic cycling that peaks in February, March, or April. Note the close covariation in androgen levels, especially during 1994. (D) Estradiol levels show two annual surges during March and July of each year and occur during ovulation and parturition, respectively. (E) Progesterone shows coincident peaks with E_2 in February–March and July of each year. All four steroids in females show an annual surge in February–March, whereas E_2 and P_4 show a second annual surge in July. * $P < 0.05$ as determined by nonparametric analysis in A, B, and C of the entire 26-month data set and Dunn's multiple comparison test. *$^+$ $P < 0.05$ as determined by parametric ANOVA and SNK paired comparisons test in D for the months of December 1992–April 1993.

magnitude of this P_4 increase may be the result of our regular midmonth sampling that missed a brief surge in this hormone. These findings indicate that female P_4 levels increase near ovulation in the spring and near parturition in the summer.

Steroid Cycling, Temperature, and Day Length

Monthly temperature and day length profiles for the study site are displayed along with male and female serum steroid concentrations (Figs. 1A and

TABLE 3
Serum Hormone Concentrations for Estuarine Female Atlantic Stingrays, *Dasyatis sabina*

Sample date	*n*	E_2	P_4	T	DHT
August 1992	3	96.3 ± 34.1	109.0 ± 17.7	144.7 ± 76.6	33.3 ± 19.2
		(36, 99, 154)	(87, 96, 144)	(28, 117, 289)	(5, 25, 70)
September 1992	9	113.7 ± 14.1	90.4 ± 14.8	977.6 ± 858.0	2410.1 ± 2174.5
		(35, 106, 195)	(43, 74, 156)	(35, 140, 7840)	(62, 222, 19800)
October 1992	8	168.5 ± 32.8	71.3 ± 11.3	72.5 ± 13.6	207.0 ± 44.9
		(38, 148, 288)	(33, 65.5, 128)	(34, 64, 147)	(62, 167, 416)
November 1992	3	203.0 ± 6.5	64.0 ± 12.5	102.7 ± 39.7	213.7 ± 46.2
		(196, 197, 216)	(39, 76, 77)	(63, 63, 182)	(146, 193, 302)
December 1992	8	136.5 ± 25.9	54.1 ± 8.4	56.6 ± 8.5	310.3 ± 82.6
		(63, 121, 302)	(26, 52.5, 96)	(25, 56, 95)	(31, 287.5, 689)
January 1993	9	189.1 ± 13.9	72.3 ± 7.6	81.7 ± 21.8	628.8 ± 327.2
		(137, 195, 248)	(37, 74, 109)	(14, 58, 202)	(77, 347, 3204)
February 1993	9	182.6 ± 18.7	114.1 ± 10.1	86.2 ± 17.1	72.8 ± 26.7
		(104, 194, 269)	(77, 121, 151)	(34, 78, 180)	(5, 47, 266)
March 1993	11	378.5 ± 92.5	67.3 ± 5.7	76.3 ± 10.6	267.6 ± 67.4
		(107, 268, 1118)	(34, 73, 101)	(34, 61, 152)	(31, 218, 641)
April 1993	11	39.6 ± 4.3	68.2 ± 6.5	61.0 ± 11.5	1126.4 ± 559.5
		(20, 36, 43)	(41, 62, 76)	(31, 41, 59.5)	(5, 470, 969.5)
May 1993	10	61.3 ± 16.4	66.0 ± 7.2	26.3 ± 5.3	25.2 ± 5.5
		(6, 45.5, 164)	(40, 65, 110)	5, 28.5, 51)	(5, 24, 59)
June 1993	10	93.9 ± 13.2	92.0 ± 11.1	46.9 ± 28.2	10.0 ± 3.4
		(53, 83, 202)	(40, 85, 140)	(5, 19, 298)	(5, 5, 35)
July 1993	10	3024.3 ± 1057.7	159.0 ± 13.7	38.6 ± 13.0	156.2 ± 62.8
		(112, 1612, 9666)	(110, 140, 250)	(7, 22.5, 136)	(5, 73.5, 621)
August 1993	10	475.9 ± 315.6	96.0 ± 5.8	45.6 ± 7.9	58.9 ± 26.5
		(23, 102.5, 3252)	(80, 100, 140)	(18, 39.5, 103)	(8, 32.5, 291)
September 1993	10	137.3 ± 15.9	77.0 ± 9.4	28.3 ± 15.8	55.8 ± 38.2
		(67, 136.5, 242)	(40, 70, 140)	(5, 12, 169)	(5, 20, 398)
October 1993	10	367.5 ± 53.4	99.0 ± 16.6	137.7 ± 73.0	157.9 ± 62.8
		(163, 371, 643)	(50, 85, 220)	(19, 50, 780)	(40, 66, 610)
November 1993	10	166.9 ± 36.3	101.0 ± 11.9	23.5 ± 4.3	53.5 ± 10.9
		(48, 140, 401)	(50, 90, 170)	(5, 19.5, 44)	(5, 54, 124)
December 1993	10	218.4 ± 31.7	95.0 ± 14.0	48.7 ± 17.7	50.5 ± 15.1
		(63, 201, 403)	(30, 90, 190)	(5, 38.5, 194)	(5, 46.5, 173)
January 1994	10	271.2 ± 42.6	84.0 ± 9.8	161.1 ± 147.7	25.7 ± 7.3
		(19, 269, 508)	(40, 80, 160)	(5, 16.5, 1,490)	(5, 13, 67)
February 1994	10	303.8 ± 64.6	91.0 ± 6.2	32.3 ± 17.6	32.5 ± 4.7
		(6, 291.5, 636)	(50, 95, 120)	(5, 14.5, 187)	(6, 35.5, 52)
March 1994	10	3795.3 ± 197.8	463.0 ± 25.3	1491.2 ± 309.5	2992.6 ± 862.4
		(3145, 3656.5, 5013)	(360, 465, 630)	(651, 1102, 3913)	(1303, 1924, 10373)
April 1994	9	60.1 ± 4.9	86.7 ± 7.8	21.6 ± 5.5	15.1 ± 5.7
		(28, 60, 76)	(50, 90, 120)	(5, 20, 53)	(4, 5, 55)
May 1994	9	333.2 ± 48.1	93.3 ± 11.6	31.0 ± 10.7	46.3 ± 10.1
		(22, 361, 529)	(60, 90, 150)	(5, 21, 112)	(23, 40, 123)
June 1994	10	117.6 ± 20.2	83.3 ± 10.9	780.4 ± 708.3	68.3 ± 8.2
		(40, 109.5, 193)	(40, 85.5, 130)	(5, 43.5, 7150)	(30, 70, 111)
July 1994	10	2894.6 ± 805.1	178.3 ± 17.0	81.4 ± 15.2	58224 ± 56214
		(150, 2750, 6160)	(100, 173.5, 290)	(10, 80.5, 150)	(26, 681.5, 564000)
August 1994	9	1632.3 ± 400.3	105.6 ± 10.6	98.4 ± 27.0	276.7 ± 55.2
		(75, 1933, 2988)	(40, 111, 145)	(23, 75, 275)	(64, 276, 554)
September 1994	10	175.3 ± 25.0	89.5 ± 10.6	90.4 ± 22.1	110.6 ± 18.8
		(75, 172, 332)	(50, 87, 132)	(5, 59, 200)	(34, 95.5, 239)

Note. Values are mean ± standard error, with minimum, median, and maximum in parentheses. All steroid data are expressed in pg/ml. *n*, number of individuals sampled; E_2, 17β-estradiol; P_4, progesterone; T, testosterone; DHT, dihydrotestosterone.

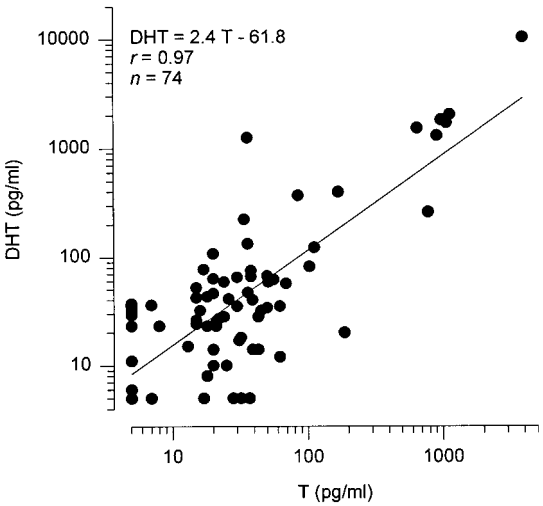

FIG. 5. The association between serum concentrations of testosterone (T) and dihydrotestosterone (DHT) for female Atlantic stingrays, *Dasyatis sabina,* collected from the Banana River estuary. Concentrations of these two androgen steroids covary across the year. Linear regression, correlation coefficient, and sample size (*n*) are shown.

4A). Monthly mean water temperatures, which showed a cycle pattern similar to that of day length, were less defined and often lagged light levels by about 1 month. In males, the period of peak temperature and day length precedes the August elevation of steroid production, whereas the seasonal minima coincide with the second androgen peak. In females, annual maximum day lengths and temperatures were associated with relatively brief increases in P_4 and E_2 during parturition. In contrast, day length and temperature minima preceded ovulation (and the brief spikes recorded for all steroids) by 1–2 months.

DISCUSSION

Reproductive Activity

The Atlantic stingray is widely distributed in marine, estuarine, and adjacent freshwater river systems of the southeastern coastal areas of North America, and its reproductive biology is among the best studied of the batoid elasmobranch fishes. The annual chronology of reproductive behaviors, gonad activity, and embryonic development in relation to steroid cycling for our study population of *D. sabina* is summarized in Fig. 6. Histological evidence for testes recrudescence is first seen when spermatocytes begin meiosis in late July or early August, organize into spermatocysts, and begin to produce mature sperm in September (Fig. 3; Maruska *et al.,* 1996). The testes reach maximum mass as spermatocytes proliferate during the month of October. Peak sperm production occurs in January with a second less-pronounced spermatogenic stage in spring. After the major spermatogenic activity subsides, sperm for mating are supplied by stores in the seminal vesicles until the end of April (Maruska *et al.,* 1996).

Oogenesis in female *D. sabina* involves enlargement of 2–4 ova that begins in October and extends through spring (Maruska *et al.,* 1996). This is coincident with the onset of female mating activity and the appearance

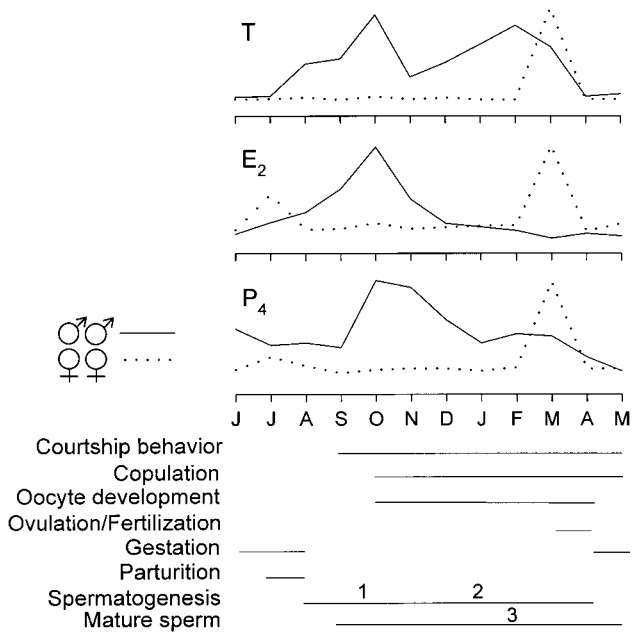

FIG. 6. Summary diagram of the 1993 sex steroid cycles and the associated reproductive events for the study population of Atlantic stingrays, *Dasyatis sabina.* As male testosterone (T) increases in August, males initiate the mating season 1 month later with biting and courting of females in September. Copulation begins in October when male T, estradiol (E_2), and progesterone (P_4) peak and continues throughout the period of elevated T until the end of April. Periods of maximum gonad size (1), maximum mature sperm presence in testes (2), and maximum seminal vesicle diameter (3) are indicated, as reported by Maruska *et al.* (1996). Spikes in female T, E_2, and P_4 are associated with ovulation in March, whereas E_2 and P_4 are also associated with parturition in July.

of fresh mating wounds (Kajiura *et al.,* 2000). Sperm are found throughout the length of the female reproductive tract for a consecutive 8-month period (October through May), and there is no evidence for sperm storage or postfertilization arrested development (Lewis, 1982; Snelson *et al.,* 1987; Maruska *et al.,* 1996). Females ovulate synchronously, probably within a 1- to 2-week period near the end of March, with all eggs fertilized by mid-April. Gestation, which involves aplacental viviparous development of the young, continues for about 4 months, with parturition in late July or August.

This reproductive timing observed in our estuarine *D. sabina* study population is in general agreement with that in other populations in Georgia estuaries (Schwartz and Dahlberg, 1978), in the northern Indian River Lagoon estuary (Snelson *et al.,* 1988), and in the freshwater Lake Monroe, Florida (Johnson and Snelson, 1996). As found in the present study, sperm are stored in the seminal vesicles after testicular gametogenesis and used for copulation during spring months. These studies indicate that different subpopulations of *D. sabina* exhibit similar timing of gametogenic features, protracted mating, gestation, and parturition across a wide geographical range.

Male Steroid Production and Associated Gonad Development Stages

This study demonstrates a clear annual pattern of androgen steroid levels, especially androgens, that are associated with different phases of spermatogenesis. Based upon the steroid cycling and gonadal development in male stingrays, four phases of the androgen cycle are identified.

Androgen suppression. During the months of April–July, there was no evidence of reproductive activity in the field population. Gross features and histological structures in the gonads were quiescent. Male serum androgens and other measured steroids were essentially undetectable.

Primary androgen increase. Increases in serum T were detected in August coincident with the earliest histological evidence for spermatocyte formation. Primary spermatocytes, which are contained within membrane-bound spermatocysts, undergo a nonsynchronous first meiotic division and are surrounded by a peripheral layer of Sertoli cells (Maruska *et al.,* 1996). This arrangement (developmental stages SII and SIII of Maruska *et al.,* 1996) is similar to the Zone II organization of testicular gametogenesis described for *Squalus acanthias* (Callard *et al.,* 1985). Our finding of a close association between spermatocyst development and T production in the stingray is consistent with *in vitro* experiments that demonstrate T production by the Sertoli cells of the spermatocysts (Du Bois *et al.,* 1989; Cuevas and Callard, 1989). Within the protracted period of stingray androgen production are two distinct peaks in T. The initial rise and October peak in T occurs during maximum spermatocyte density and the proliferation of the presumed steroidogenic Sertoli cells. This October peak is also associated with maximum gonad size but is followed by a decrease in testes mass after formation of spermatocysts (Maruska *et al.,* 1996; Johnson and Snelson, 1996).

Androgen decrease. The androgen peak was followed by a decrease in testis size, number of spermatocytes, and levels of serum T and DHT from November to December (Figs. 3 and 6). However, male rays were still producing sperm and actively mating in the field population.

Secondary androgen increase. The brief decrease in serum androgens was followed by a second increase in androgen levels that was longer in duration (January–March) but statistically indistinguishable in magnitude from the October T peak. Like the first rise in T, this second peak was also associated with the development of spermatocysts that was of relatively short duration (Fig. 3). Thus, the dual annual peaks in male androgen production do not appear to be correlated with increased gonad size but rather with spermatocyte formation, the associated Sertoli cell steroid production, and sperm maturation.

The elevation of serum DHT in male stingrays from August through March closely follows the production of T. The close association of these two androgens is also evident in the bimodal peaks of serum DHT in October and February. One major difference in the expression of these two androgens is that serum DHT levels exceed those of T by about two- to threefold. DHT is a direct product of T metabolism in the presence of 5α-reductase, which is known to occur in the gonads and brain. In other vertebrates, DHT is known to provide inhibitory feedback to the gonadotropin releasing hormone (GnRH) production in the hypothalamic region of the brain and it regulates sexual

behavior (Sheridan, 1979). However, while target sites for DHT are likely to exist in the diencephalon, the sites of androgen receptors in the elasmobranch brain that regulate male reproduction remain to be identified. It is also important to consider the possible action of other androgens not measured in this study, such as 11-ketotestosterone, which is the most active form in teleosts (Borg, 1994) and was recently reported in the bonnethead shark, *S. tiburo* (Manire *et al.,* 1999).

One major difference from the temporal pattern of androgen cycling in our study population is seen in the freshwater Atlantic stingray breeding population in Lake Monroe, Florida (Snelson *et al.,* 1997). Males at that site did not exhibit a detectable increase in serum androgens between May and December but did so only from January to March. Thus, Lake Monroe males do not show the primary androgen rise that occurred in August–October for 3 consecutive years in our study population but only the secondary androgen rise (Figs. 1B and 1C). This difference is not due to the lack of gonad tissue recrudescence in the Lake Monroe population, since there is a prominent increase in gonad size that also peaks in October (Johnson and Snelson, 1996). Rather, these differences are likely related to differential steroidogenic activities of spermatocytes or Sertoli cells in the gonad tissue, but this remains to be confirmed through histological analysis of male gonads from the freshwater population.

Male stingrays in the present study show a distinct periodic increase in serum E_2 that begins in August, peaks in October, and ends by December (Fig. 1D). The elevation of E_2 closely follows testis growth and the first surge in T but not the second increase from January to March (Fig. 2B). Callard *et al.* (1985) showed that aromatase activity is highest in the primary and secondary spermatocytes in the testis of the spiny dogfish, *S. acanthias.* Thus, the coincidence of E_2 with the first T surge is not surprising since this is the time of peak spermatocyte activity. Furthermore, the continued decline in E_2 during the second surge in T indicates that aromatase activity may wane as spermatocytes mature. This could be due to decreased production of aromatase in meiotic spermatocytes or possibly the production of an aromatase inhibitor during January–March. The production of E_2 in male stingrays may serve a parahormonal function in the gonad and feedback control of pituitary gonadotropes or GnRH release in the Atlantic stingray hypothalamus (Forlano *et al.,* 2000).

The fresh water Atlantic stingray population in Lake Monroe (Snelson *et al.,* 1997) shows an annual pattern of E_2 production that also appears to differ substantially from that of our estuarine study population. During the period of low E_2 production (April–July), Lake Monroe males show sustained minimum estradiol levels of only about 10 pg/ml. In contrast, the period of low E_2 in our estuary population, as measured over a broader period from December to July, showed monthly median concentrations between 10 and 50 pg/ml (Fig. 1D). The surge in E_2 for Lake Monroe rays began in August, peaked at around 45 pg/ml in October and November, and then rapidly decreased. The timing of the E_2 surge appears to be similar among populations, but the median E_2 peak for the Banana River estuary stingrays in October 1993 was 166 pg/ml or about triple that reported for the Lake Monroe population. Whereas these differences in measured maxima of E_2 might be explained by short-term peaks that were missed by the sampling interval used by Snelson *et al.* (1997), estradiol production in the Lake Monroe population appears to occur at a lower level throughout the year.

Of all male steroids measured in this study, the production of P_4 showed the least clear pattern of annual cycling. Progesterone levels for the male rays ranged from 100 to 200 pg/ml over most of the study. The only detected increase in P_4 levels occurred in October–December 1993. Snelson *et al.* (1997) reported that P_4 levels varied irregularly over the course of their study, with an elevation in November and a drop in December followed by a second increase in January and February. P_4 is known to be a precursor for T production in many vertebrates. Its correlation with increased production of T in these two sample populations would be expected if it serves as the substrate for androgen production. However, a function for the prolonged elevation of P_4 at other times of the year is unclear. During these times, P_4 may function in the negative feedback loop to the pituitary or hypothalamus for inhibition of GnRH release, but sites of P_4 action in the elasmobranch brain remain to be identified.

Female Steroid Production and Associated Gonad Development Stages

Female stingrays show brief seasonal cycles in serum steroid hormone levels. Female monthly median T levels were below 100 pg/ml for most of the study, with brief increases being detected only during winter and early spring of both years (February and March 1993, February 1994). These surges in T were accompanied by a clear elevation in serum DHT (March and April 1993, March 1994) that was also brief and of relatively low magnitude compared to that of males. These findings are in distinct contrast to those of Lake Monroe females, which showed no elevation in T associated with a major reproductive event (Snelson *et al.,* 1997). However, as found in the current study, the DHT peaks from Lake Monroe females occurred during ovulation but differed by having a second DHT peak during mid-June. The second T peak was suggested to possibly function in the histotroph nourishment of embryos or some other development process in the uterus (Snelson *et al.,* 1997). The lack of a second summer androgen surge in the present study may represent physiological differences between the populations. Alternatively, it is possible that the appearance of the summer androgen surge is of such brief duration that, by chance, it was not detected in our monthly sampling schedule.

The elevation of serum E_2 in females was associated with two distinct reproductive events. The first surge in E_2 occurred during the month of March when oocytes undergo final maturation. E_2 is known to promote synthesis of vitellogenin in the liver and to stimulate the uptake of vitellogenin by the elasmobranch oocyte (Craik, 1978; review in Callard *et al.,* 1991). Elevated E_2 during the preovulatory period also occurs in the Lake Monroe population of *D. sabina* (Snelson *et al.,* 1997), the bonnethead shark, *S. tiburo* (Manire *et al.,* 1995), and the little skate, *R. erinacea* (Koob *et al.,* 1986). The lack of elevated E_2 during the full period of oocyte vitellogenesis indicates that the spring surge of E_2 may function in late oocyte vitellogenesis and possibly ovulation. The second observed surge in female E_2 occurred during the summer months of July and August and overlaps late embryonic development and parturition. However, this late summer E_2 surge in our estuarine sample population contrasts with the midsummer peak of E_2 in the Lake Monroe population of *D. sabina,* which preceded parturition by more than one month (Snelson *et al.,* 1997). In the Lake Monroe population, the E_2 correlation was proposed to be involved with the transition from yolk-dependent to histotroph-dependent embryonic nutrition rather than parturition. Clearly, more investigation is needed to determine the function of estradiol and possibly androgens during gestation in the stingray. Furthermore, the many differences in gonadal steroid concentrations and cycling among the freshwater and estuarine populations need to be examined in relation to different osmotic or other possible environmental influences (Guillette *et al.,* 1996).

Elevated levels of female E_2 were associated with elevated T only during late winter or early spring, and we are unable to confirm such an association during the summer. The surge in female E_2 during the spring months occurs when oocytes are of maximum size prior to ovulation. Tsang and Callard (1992) showed that, in the spiny dogfish, *S. acanthias,* it is the granulosa and thecal cells of the oocyte follicle that have the capacity to synthesize both T and E_2. While oocyte follicles may be responsible for the spring E_2 production, the question of E_2 production during parturition is less clear because we could not demonstrate a coincident increase in summer T. The simplest explanation is that our nonparametric analyses were not sensitive enough to detect a real difference that could be seen graphically in 1993 and 1994.

Increases in female P_4 levels occurred at important phases of the reproductive cycle. Elevations of P_4 that were coincident with elevated E_2 and androgens were detected in February 1993 and March 1994 near ovulation. A second ephemeral pulse of P_4 occurred during July of 1993 and 1994 and is clearly associated with parturition. Similar peaks and reproductive timing of female P_4 were also reported for Lake Monroe stingrays by Snelson *et al.* (1997). The preovulatory production of E_2 in association with P_4 in female rays indicates a possible interaction between these hormones, such as receptor priming as occurs in female rats. However, whether P_4 affects female stingray reproductive behavior, induces ovulation, or is directly related to the parturition event remains to be experimentally demonstrated.

Androgen Steroids and Associated Stingray Reproductive Behaviors

The prolonged systemic elevation of androgen hormones in male stingrays may directly influence the protracted reproductive activity seen in the population. The reproductive behavior of most elasmobranch fishes involves a period of aggressive social interactions among males and females (Tricas, 1980; Tricas and LeFeuvre, 1985) that can last many weeks or months (Babel, 1967; Carrier *et al.,* 1994; Nordell, 1994; Maruska *et al.,* 1996). Aggressive interactions in *D. sabina* start in late August or early September when males begin to chase, bite, and nip at the fins of both males and females (Kajiura *et al.,* 2000). During the prolonged mating season, males often follow females with their snout close to the female vent and subsequently bite the female body and fins. This aggressive reproductive behavior is coincident with, and may be caused by, the seasonal elevation of serum androgens. We propose that the production of androgen steroids initiates, promotes, and sustains the aggressive sexual behavior of male rays and ultimately drives the protracted mating season in this species.

Increases in serum androgens in female rays during ovulation may affect reproductive behavior in addition to feedback control of gonadotropin secretion in the diencephalon. For example, brief spikes of androgen steroids could increase female aggression and function to enhance mate choice by females. Since females do not store sperm, the males that copulate with females nearest the time of ovulation will most likely fertilize the female's eggs. The increased serum androgen concentrations in females during the end of March may enhance female aggression and their ability to flee persistent males during the time of ovulation and subsequent impregnation. This may ultimately influence the paternity of their young by rejection of less desirable males.

In conclusion, we have shown that the annual cycling of gonadal steroids in the stingray is closely associated with specific phases of gametogenesis and reproductive events in the sample population. The close association between the mating season and the androgen expression in males indicates the need for experiments to demonstrate whether the aggressive and protracted 7-month mating season is truly driven by male androgen production. In addition, any action of the protracted mating activity upon the reproductive state of female stingrays, such as a neuroendocrine reflex for ovarian development (per Maruska *et al.,* 1996), remains to be investigated.

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