GENERAL AND COMPARATIVE ENDOCRINOLOGY 99, 204–210 (1995)

Identification of Sex in Hatchling Loggerhead Turtles (*Caretta caretta*) by Analysis of Steroid Concentrations in Chorioallantoic/Amniotic Fluid

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Accepted April 24, 1995

A major difficulty in sea turtle conservation is the inability to nonlethally and noninvasively identify the sex of hatchling sea turtles. Traditional sexing techniques such as plasma sex steroid quantification cannot be applied to hatchlings without sacrificing the hatchlings or utilizing invasive procedures. This paper presents a technique for sexing hatchling sea turtles by analysis of sex steroid concentrations in egg chorioallantoic/amniotic fluid (CAF). Metabolites of estradiol-17β (E) and testosterone (T) in CAF are best expressed as an index or E:T ratio. Chorioallantoic/amniotic fluid E:T ratios for males (0.5 ± 0.1) were significantly lower than those for females (2.2 ± 0.3). When separated by utilizing an E:T ratio of 1.25 as the determinant index value, 27 of 28 hatchlings were designated correctly as males (E:T < 1.25) or females (E:T $\geq$ 1.25). Sex was verified for all hatchlings by gonadal histology. This study shows significant concentrations of T and E metabolites in CAF and plasma of hatchling loggerhead turtles and illustrates the use of a nonlethal, noninvasive method for determining sex, which could be potentially utilized for other endangered reptile and avian species. © 1995 Academic Press, Inc.

In many species of reptiles, the sex of individuals is determined by the temperature of egg incubation, known as temperature-dependent sex determination (reviewed in Bull, 1980). Loggerhead sea turtles (*Caretta caretta*) follow a sex-determining regime in which 100% males are produced at cooler temperatures (~27°), 100% females are produced at warmer temperatures (~31°), and a 1:1 sex ratio is produced at a "pivotal" temperature between the two extremes (~29°) (Mrosovsky, 1988). Researchers have expressed concern about the potentially adverse effects of altered nest incubation temperature caused by environmental (Mrosovsky *et al.*, 1984a,b) and anthropogenic sources (Morreale *et al.*, 1982; Dutton *et al.*, 1985). However, current analyses of sea turtle population dynamics are hindered by the inability to estimate the sex ratio produced at any given beach or geographic region without sacrificing large numbers of hatchlings.

Loggerhead hatchlings are sexually monomorphic and, thus, noninvasive sex identification based on morphometrics (Burke *et al.*, 1994) is not possible. Historically, researchers have been forced to sacrifice sea turtle hatchlings to obtain gonads for histological examination (Mrosovsky, 1988; Horikoshi, 1992), but a nonlethal method for sexing would be preferred. One alternative is analysis of plasma testosterone concentrations, which have been used previously to indicate the sex of immature sea turtles (Owens *et al.*, 1978; Wibbels *et al.*, 1987). However, the removal of an adequate volume of blood necessary for such assay techniques could be lethal to hatchlings. The present research investigates a noninvasive technique for identifying the sex of loggerhead turtle hatchlings. After a hatchling successfully pips and exits the egg, fluid derived from the amnion and allantois remains in the eggshell. This combination of chorioallantoic and amniotic fluids [hereafter refered to as chorioallantoic/amniotic fluid (CAF) for simplicity] contains waste products

0016-6480/95 $12.00
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of the embryo. Since embryonic development requires an environment rich in sex steroids (Bogart, 1987; Bern, 1990), it is hypothesized that the fluid contains sex steroids and their metabolites in differential quantities that can be used to determine the sex of hatchling turtles. This hypothesis is tested by developing a radioimmunoassay analysis of CAF for metabolites of testosterone and estradiol-17β and the results are compared with those of a histological examination of the gonads and plasma sex steroid concentrations.

MATERIALS AND METHODS

Animals

Twenty-eight loggerhead eggs were collected from various beaches in North Carolina and Florida as part of a larger study of loggerhead turtle population genetics. Eggs were collected late in embryonic development, after sex determination had occurred, and placed in an incubator at 31° in moist vermiculite. After pipping, the hatchling was removed from the egg and anesthetized with ketamine. Blood was collected from the heart with a syringe treated with sodium heparin. Gonads were removed and fixed in neutral-buffered formalin, and CAF was collected from the egg. Plasma and CAF were stored at $-72°$ prior to analysis.

Gonad Histology

Gonads were preserved in neutral-buffered formalin. After dehydration in a series of alcohol washes, the fixed tissues were infiltrated in paraffin, sectioned at 8μm, and stained using a modified hematoxylin/eosin procedure (Humason, 1972). Sex designation was based on development of cortical and medullary regions and presence or absence of seminiferous tubules (Yntema and Mrosovsky, 1980). Sex was diagnosed independently by three researchers.

Radioimmunoassay

Plasma samples from neonatal loggerhead turtle hatchlings were analyzed for estradiol-17β and testosterone using radioimmunoassay (RIA) procedures. Samples (50 μl) were extracted twice with 5 ml diethyl ether prior to RIA analysis. Each sample was analyzed in duplicate for both estradiol-17β and testosterone and corrected for extraction efficiencies of 90 ± 3.2 and 82 ± 2.5%, respectively. Standard curves were prepared in buffer with known amounts of radioinert estradiol-17β or testosterone (1, 5, 10, 25, 50, 100, 250, 500, and 1000 pg). The minimum concentration distinguishable from zero was 5.7 pg/ml for estradiol-17β and 7.3 pg/ml for testosterone. Cross-reactivities of the estradiol-17β antiserum, produced by this laboratory, with other steroids were 11.2% for estrone, 1.7% for estriol, <1.0% for estradiol-17α and androstenedione, and <0.1% for all other steroids examined. Cross-reactivities of the testosterone antiserum (purchased from ICN Biomedicals, Inc; 07-189016) with other steroids were 18.75% for 5α-dihydrotestosterone, 3.0% for 5α-androstenediol, <1.0% for androstenedione, and <0.1% for all other steroids examined. A pooled sample (approximately 160 pg estradiol-17β/ml and 120 pg testosterone/ml) was assayed serially in 10-, 20-, 30-, 40-, and 50-μl volumes (final volume of 50 μl with charcoal-stripped plasma). The resulting inhibition curves were parallel to the respective standard curve, with the tests for homogeneity of regression indicating that the curves did not differ. Further characterization of the assays involved measurement of known amounts (1, 2, 5, 10, 25, 50, 100, 250, and 500 pg) of estradiol-17β or testosterone in 50 μl charcoal-stripped plasma [for estradiol-17β: $Y = 4.12 + 1.04X$, $R^2 = 0.9063$; for testosterone: $Y = -5.31 + 1.15X$, $R^2 = 0.8841$; Y = amount of estradiol-17β or testosterone measured (pg); X = amount of estradiol-17β or testosterone added (pg)]. Interassay and intraassay coefficients of variation were 8.7 and 10.1%, respectively, for plasma estradiol-17β and 7.3 and 9.5%, respectively, for plasma testosterone.

CAF samples from loggerhead turtle eggs were also analyzed for estradiol-17β and testosterone immunoactivity using similar RIA procedures. For each determination, samples (100 μl) were initially solubilized in 2 ml citrate buffer (0.5 *M*, pH 5.0) for 6 hr at room temperature. The solubilization step was neccessary because utilization of material from a direct extraction of CAF resulted in assay conditions which negatively affected antibody binding and resulted in an assay procedure which we were unable to validate. This solubilization procedure is also effective in isolating steroids in free and conjugated forms (Gross, 1992). Additionally, the acidity of the aqueous phase has been shown previously to result in the significant hydrolysis of conjugated steroids (Gross, 1992). However, CAF steroid conjugates were not identified in this study. Therefore, the results are indicated as immunoreactivity with estradiol-17β- and testosterone-specific antisera. Following solubilization, samples were centrifuged at 1000*g*, and the supernatant (0.5 ml) was extracted twice with 5 ml diethyl ether prior to RIA analysis. Each sample was analyzed in duplicate for estradiol-17β and testosterone immunoreactivity and corrected for respective solubilization/extraction efficiencies of 84 ± 5.8 and 74 ±4.9%. Standard curves were prepared as described for plasma RIA analyses. The minimum concentration distinguishable from zero was 8.1 pg/ml for estradiol-17β and 9.8 pg/ml for testosterone. A pooled sample (solubilized material; approximately 110 pg estradiol/ml and 100 pg testosterone/ml) was assayed serially in 100-, 200-, 300-, 400-, and 500-μl volumes (final volume of 0.5 ml with charcoal-stripped solubilized CAF). The resulting inhibition curves were parallel to the respective standard curves, with the test for homogeneity of regression indicating that the curves did not differ. Further characterization of the assays involved measurement of known amounts (1, 2, 5, 10, 25, 50, 100, 250, and 500 pg) of estradiol-17β or testosterone in 0.5 ml charcoal-stripped solubilized fluid [for estradiol-17β: $Y = -7.52 + 1.12X$, $R^2 = 0.8235$; for testosterone: $Y = 7.11 + 0.91X$, Y = amount of estradiol-17β or testosterone measured (pg); X = amount of estradiol-17β or testosterone added (pg)]. Interassay and intraassay coefficients of variation were 9.7 and 11.6%, respectively, for estradiol-17β and 10.1 and 9.8%, respectively, for testosterone.

Statistical Analyses

Concentrations of steroids were determined from raw data using commercial software from Beckman (ImmunoFit EIA/RIA program; Fullerton, CA). Statistics were performed in Statview (Abacus Concepts, Inc. Berkeley, CA, 1992). All comparisons were tested with a *t* test, with significance indicated at $P < 0.05$. Estrogen/testosterone ratios were log transformed prior to statistical analysis, but actual ratio values are reported.

RESULTS

Sex Designation

Histological examination revealed that 17 hatchlings were male and 11 were female. Histological examination of the ovaries and testes revealed normal structures consistent with the age of the animals examined. Females had ovaries characterized by a surface epithelium composed of squamous cells overlying an ovarian cortex having many primary follicles. The ovarian follicles at this stage consisted of a single or double layer of granulosa cells surrounding a single oocyte having a distinct nucleus. The testes exhibited a histological appearance characterized by the presence of well-formed seminiferous tubules lined with germ cells, at various stages of development, and Sertoli cells. Mitotic activity was noted in many of the germ cells. Overall, sex designation was agreed upon in all cases, and no sexually ambiguous individuals were noted.

Endocrine Analyses

Table 1 summarizes the radioimmunoassay results. Male hatchlings had significantly higher ($P < 0.05$) concentrations of testosterone (T) than females for both plasma and CAF. Similarly, female hatchlings had significantly higher ($P < 0.05$) concentrations of estradiol-17β (E) than males for both plasma and CAF. Concentrations of T and E did not differ significantly between plasma and CAF within each sex. When E:T ratios were examined as a single index, ratios for male hatchlings were significantly lower ($P < 0.05$) than ratios for female hatchlings for both plasma and CAF (Figs. 1 and 2). In both plasma and CAF, neither estradiol-17β nor testosterone concentrations individually were able to predict hatchling sex with any great degree of accuracy. Plasma E concentrations estimated sex correctly for 65% of the males and 91% of the females, for an overall accuracy of 75%. Similarly, plasma T concentrations estimated sex correctly for 53% of the males and 100% of the females, for an overall accuracy of 71%. CAF concentrations of E were similarly accurate for sex estimation in 65% of the males and 82% of the females, for an overall accuracy of 71%, whereas CAF T concentra-

TABLE 1

MEAN ESTRADIOL-17β (E), TESTOSTERONE (T), AND E:T RATIOS (±SE) FOR PLASMA AND CHORIOALLANTOIC/AMNIOTIC FLUIDS (CAF) FROM FEMALE AND MALE HATCHLING LOGGERHEAD TURTLES

	N	Estradiol-17β (pg/ml)	Testosterone (pg/ml)	E:T ratio
Plasma				
Females	11	198 ± 44	76 ± 13	2.7 ± 0.4
Males	17	106 ± 15	215 ± 38	0.60 ± 0.1
CAF				
Females	11	201 ± 26	104 ± 16	2.2 ± 0.3
Males	17	98 ± 12	226 ± 25	0.50 ± 0.1

Note. Mean estradiol-17β (E), testosterone (T), and E:T ratios each differed ($P < 0.05$) between males and females in both plasma and CAF. Plasma E and T concentrations estimated sex correctly with 75 and 71% accuracy, respectively. CAF E and T concentrations estimated sex correctly with 71 and 75% accuracy, respectively. For both plasma and CAF, E:T ratios provided the best technique for designating sex correctly, identifying 27 of 28 hatchlings, for a 96% accuracy.

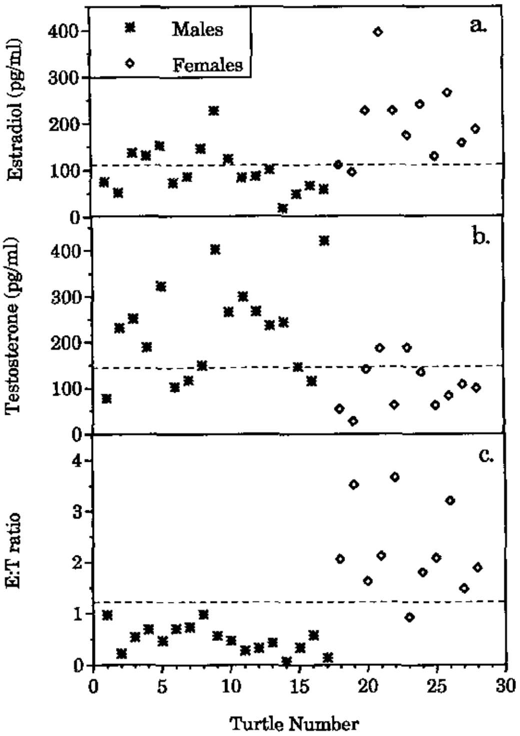

FIG. 1. (a) Plasma estradiol-17β (E), (b) testosterone (T), and (c) E:T ratios for individual hatchling loggerhead turtles. The dotted lines represent the best statistical separations between males and females. Individually, plasma E and T concentrations estimated sex correctly with 75 and 71% accuracy, respectively. E:T ratios as an index provided the best technique for designating sex correctly, identifying 27 of 28 hatchlings, for a 96% accuracy.

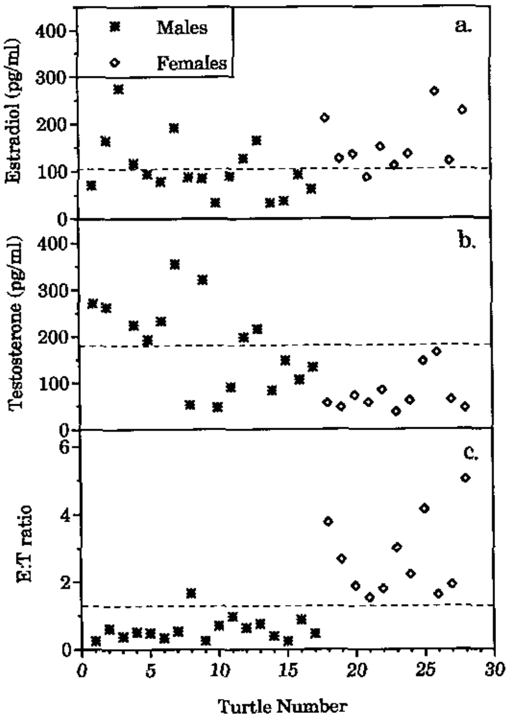

FIG. 2. (a) Chorioallantoic/amniotic fluid (CAF) estradiol-17β (E), (b) testosterone (T), and (c) E:T ratios for individual hatchling loggerhead turtles. The dotted lines represent the best statistical separations between males and females. Individually, CAF E and T concentrations estimated sex correctly with 71 and 75% accuracy, respectively. E:T ratios provided the best technique for designating sex correctly, identifying 27 of 28 hatchlings, for a 96% accuracy.

tions estimated sex correctly for 71% of the males and 82% of the females, for an overall accuracy of 75%. Both plasma and CAF E:T ratios were more accurate as an index for sex identification, with a correct sex estimation for 94% of the males and 100% of the females for plasma and an accuracy of 100% for males and 91% for females for chorioallantoic fluid. Overall, for both plasma and CAF, E:T ratios provided the best technique for designating sex correctly, identifying 27 of 28 hatchlings, for a 96% accuracy.

Whereas the E:T ratio index accurately identified the sex of 27 of 28 individuals when a determinant index value of 1.25 was utilized (see Figs. 1 and 2), it must be noted that the values for estradiol-17β and testosterone from CAF most likely include significant levels of metabolites for both steroids. These steroids are normally excreted as conjugated metabolites (Gross, 1992). Therefore, results from the analyses of chorioallantoic fluids are indicated as metabolites of estradiol and testosterone since identification of specific metabolites was neither practical nor necessary for these experimental efforts.

DISCUSSION

T and E and/or their metabolites were detected in the plasma and egg CAF for loggerhead turtle hatchlings. As previously shown for immature loggerhead turtles (Wibbels *et al.*, 1987), plasma T concentrations were higher in males and E concentrations were higher in females. CAF and plasma displayed similar patterns, and the concentrations of T and E did not differ between plasma and CAF within each sex. This suggests that both CAF and plasma can be utilized to determine the sex of hatchling loggerhead sea turtles. However, the use of CAF provides a relatively simple, noninvasive technique that poses no detriment to the hatchling.

We attempted to predict the sex of hatchlings with E concentrations, T concentrations, or E:T ratios. Concentrations of T or E alone were unable to predict the sex of hatchlings at an acceptable level of accuracy. However, female hatchlings had a significantly higher mean E:T ratio in plasma and egg CAF than males, enabling accurate designation of the sex in 27 of 28 hatchlings when an E:T ratio of 1.25 was used as the distinguishing index between sexes. Plasma and CAF were equally effective in predicting sex using the E:T ratios. Future studies can reliably use CAF analysis to determine the sex of hatchling loggerhead turtles.

It has been suggested that the ratio of estrogens to androgens, and not the absolute concentrations of the hormones, may mediate gonadal sex differentiation in reptiles with temperature-dependent sex determination (Bogart, 1987). Our data provide support for this hypothesis. T and E plasma concentrations, for example, vary among individuals to the extent that some males have higher E concentrations than do many females, and several females have higher testosterone concentrations than do many males. The E:T ratios for these individuals, however, show distinct patterns between males and females.

The measured concentrations of sex steroids in the plasma of hatchlings are higher than those reported for immature loggerhead turtles (Wibbels *et al.,* 1987). Although this could be due to assay differences, the high values are most likely a true indication of the increased concentration of sex steroids. Previous analyses of E and T concentrations for plasma from immature loggerhead sea turtles (Gregory, 1994), which utilized the assay procedures as indicated in this paper, were similar to those reported previously by Wibbels *et al.* (1987). Sex determination in a freshwater turtle (*Trachemys scripta*) occurs during the second trimester of embryonic development, but sexual differentiation of the gonads continues until, and possibly beyond, hatching (Wibbels *et al.,* 1991). The differentiation of gonads is stimulated in part by sex steroids, with testosterone supporting testis development and estrogens supporting ovarian formation. Therefore, we propose that sex steroid production in loggerhead turtles occurs in periodic waves, with the first surge occurring during gonad development and later surges occurring after sexual maturity during periods of reproductive activity. This hypothesis is supported by several lines of evidence. Gonadal aromatase activity, which is responsible for the conversion of androgens to estrogens, is increased considerably in late embryonic development of leatherback turtles (*Dermochelys coriacea;* Desvages *et al.,* 1993) and saltwater crocodiles (*Crocodylus porosus;* Smith and Joss, 1994) incubated at female-producing temperatures. Also, *T. scripta* embryos possess hydroxysteroid dehydrogenase (a steroidogenic enzyme responsible for the conversion of pregnenolone to progesterone) activity immediately prior to, during, and after gonadal differentiation (Thomas *et al.,* 1992). Although the gonads exhibit biochemically active steroidogenic enzymes, the gonadal–adrenal–kidney complex may not be the primary source of steroid production in embryos (Thomas *et al.,* 1992; White and Thomas, 1992). Further research into the source, timing, and magnitude of steroid production in embryonic turtles is needed to more fully understand temperature-dependent sex determination.

In addition to containing the nitrogenous waste products of the embryo, the chorioallantoic fluid serves as a depot for metabolized or native hormones produced by the embryo. The embryo either must dispose of excess circulating hormones or succumb to receptor desensitization and/or malformations. Catecholamines in avian eggs provide an excellent example. Catecholamine excess causes malformations in chicken embryos (Sarasa and Climent, 1987; Shields *et al.,* 1990), and to avoid such malformations, avian embryos dispose of excess catecholamines in the allantoic fluid (Epple *et al.,* 1992). Steroid hormones are also deposited in allantoic fluid. Bovine fetal allantoic fluid contains T, androstenedione, E, and estrone (Dominguez *et al.*, 1990), and estrone concentrations in the allantoic fluid of fetal pigs mimic estrone concentrations in the maternal plasma (Knight and Kukoly, 1990). The present study demonstrates that sex steroids are deposited as waste into the chorioallantoic/amniotic fluid of chelonian embryos. This deposition may be an evolutionarily conserved trait among amniote embryos.

The sex steroids in egg CAF were successfully used to determine the sex of individual loggerhead turtles. This technique can enable researchers to nonlethally and noninvasively determine the sex of large numbers of hatchling turtles, thereby promoting conservation through an increased understanding of sea turtle population dynamics. Steroid analyses of CAF can also be used to monitor the effects of human alterations (such as beach nourishment and environmental contamination) on the sex ratios of hatchling sea turtles. Future studies should focus on similar nonlethal, noninvasive sexing techniques for other endangered reptile and avian species.

ACKNOWLEDGMENTS

We thank E. Possardt, B. Schroeder, A. Foley, and R. Mezich for arranging egg collections. Brian Bowen graciously allowed us to collect samples from turtles used in his genetic studies. The technical assistance of You-Xiang Qui and John Matter in histology preparation and examination is much appreciated as well as the generous use of histology equipment from the laboratory of L. J. Guillette. All animal care was in full compliance with the University of Florida Institutional Animal Care and Use Committee guidelines. Research was conducted with appropriate state and federal permits.

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