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The Influence of Sex Steroids on the Sexual Size Dimorphism in the Red-Spotted Garter Snake, *Thamnophis sirtalis concinnus*

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The red-spotted garter snake exhibits adult size dimorphism in which females are the larger sex. To understand which hormones may influence differential growth in this species, growth curves and hormone profiles of estradiol-17β (E_2) and testosterone (T) were constructed in male and female neonates. Growth was manipulated via implantation of exogenous hormones and hormone antagonists. Female neonates are heavier or longer beginning at either 20 or 24 weeks of age, respectively. Although low circulating levels of E_2 and T were present in males and females from birth through 15 weeks of age, these levels were not significantly different between the sexes. Differences in the growth curves of the treated and untreated snakes were significant after 24 weeks of age. Antiestrogen produced male-like growth in females but had no effect on males. Antiandrogen had no effect on either males or females. Exogenous T reduced female growth to that observed in males, and E_2 reduced male growth. These results suggest that a basal level of either E_2 or T is sufficient in males to retain typical male growth patterns. Similar endogenous levels of E_2 appear to have growth-promoting effects in females. Endogenous T does not appear to play a role in female growth.

Key Words: sexual size dimorphism, growth, hormone profile, sex steroids, antiandrogen, antiestrogen.

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INTRODUCTION

The study of variation in body size leads to the examination of factors that influence differential growth and development. Variable growth rates may ultimately affect reproductive output, mating success, competition, and survivorship. Consequently, differential growth may have important impacts on the life history and ecology of animals. Understanding adaptive qualities associated with variable growth and development necessitates a comprehension of the physiological and environmental factors involved.

Focusing on proximate mechanisms, investigators attribute sexual size dimorphism to the regulation of growth by gonadal hormones experienced early in life. Testosterone (T) increases growth of males in some mammalian and avian models where adult males are the larger sex (Slob and Van Derr Werff Ten Bosch, 1975; Gentry and Wade, 1976; Gray *et al.*, 1979; Czaja, 1984; Cikos *et al.*, 1992; Peralta *et al.*, 1994) while decreasing growth in others (Swanson, 1967; Bubenik *et al.*, 1975; Fennell and Scanes, 1992). For example, differential growth rates in rats from puberty onward contribute to the larger size of adult males. A rise of circulating androgens in males at a critical period has been shown to play a role in this increased growth (Slob and Van Derr Werff Ten Bosch, 1975). In guinea pigs, the larger size of males stems from significant differences in the activational effects of testosterone on body size (Czaja, 1984). Contrary to the size differ-

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ences observed between male and female rats and guinea pigs, adult female golden hamsters are longer and heavier than males. Gonadectomy of neonatal male golden hamsters increases growth rates to the levels observed in females (Swanson, 1967). Evidence from these investigations suggests that the role of T in males of species where males are the larger sex is to increase growth while decreasing growth in males of species where females are the larger sex.

In reptiles, females grow faster in species exhibiting female-biased sexual size dimorphism, whereas males grow faster in species where adult males are larger (Andrews, 1982). In wild populations, estimates of growth rates are often calculated from seasonal body size distributions obtained by mark and recapture and not from systematic repeated measures taken from birth onward. Therefore, dimorphism in size may reflect differences in age at maturity between the sexes, with the later maturing sex achieving larger body sizes (Kozlowski, 1989). In snakes, there are many examples of males that mature earlier than females (Parker and Plummer, 1987). Knowledge of individual age is crucial to our understanding of sexually dimorphic growth and its impact on the behavior and ecology of animals. This sexual dimorphism in body size in reptiles may contribute to differences in life history traits influencing foraging tactics, competition, and mating behavior, among others (Shine, 1993).

Much of the work associated with hormonal influences on growth has been conducted with mammals, birds, and fish. It has been hypothesized that small male body size in some reptiles may also depend on testicular factors (Crews *et al.,* 1985). These investigators observed that circulating levels of testosterone in male red-sided garter snakes, *Thamnophis sirtalis parietalis,* increase markedly by the 3rd week of life (compared to the 1st and 2nd weeks only). Growth rates of neonatal females exceeded males beginning at approximately 10 weeks of age. Gonadectomy of neonatal male *T. sirtalis parietalis* increases growth rates to levels exhibited by females. Because testosterone is the major androgen of the testis, a correlation has been drawn between its action and differential growth observed in males. However, this has not been tested empirically in the garter snake model.

The current study was conducted to investigate sexually dimorphic growth in the red-spotted garter snake, *Thamnophis sirtalis concinnus.* This species exhibits adult sexual size dimorphism such that females are the larger sex. To gain an understanding of which endogenous gonadal hormones may influence neonatal growth in this species, we constructed growth curves and hormone profiles of estradiol-17β (E_2) and T of individuals of known ages. Often, researchers exploring the effects of gonadal hormones on growth remove the gonads, thereby removing a wide array of factors. To examine the effects of specific gonadal hormones on growth, we experimentally tested the effects of exogenous hormone and also blocked the activity of endogenous hormone on growth with the use of estrogen and androgen antagonists.

MATERIALS AND METHODS

Animal Collection and Husbandry

Gravid *T. sirtalis concinnus* were collected in June and July for 3 consecutive years beginning in 1995. Adult females were collected from various sites throughout Benton County, Oregon. All adult animals were returned to their respective field sites shortly after parturition (generally throughout August and early September).

We identified the gender of individual newborns by inspection of the urogenital opening and eversion of hemipenes. At birth, all neonates were given individual numbers by clipping the ventral scutes with a scleral punch, measured from snout-to-vent (SVL) to the nearest millimeter and weighed to the nearest tenth of a gram. Over the course of these investigations, neonates were randomly housed in 10-gallon glass aquaria with a paper substrate (Animal Specialties, Hubbard, OR) and measured once every 4 weeks. Animals born in the laboratory in 1995 were maintained at 20 $\pm$ 2°, with an additional midday increase from 28 to 33° provided to individual aquaria by a 25-W incandescent light bulb. Air temperature for animals born in 1997 ranged from 17 to 28°, with the same midday increase provided in 1995. The daily light–dark cycle was 14L:10D. Relative humidity ranged from 55 to 65%. Individuals were offered mosquitofish (*Gambusia* spp.) and earthworms (*Lumbricus* spp.) once weekly ad libitum and were provided a constant water supply. Care was taken such that all

animals were individually offered food and were observed to eat.

Hormone Profile

Blood samples were obtained from randomly chosen neonates such that samples from 10 individual males and 10 individual females were obtained each week beginning at 0 weeks (birth) and continuing for 15 weeks. Approximately 40 to 80 μl of blood was collected from the caudal vein. Samples were centrifuged for 3 min at 3000 rpm, the plasma was separated, and both the red blood cells and the plasma were stored at −80°. All samples were obtained within 2 min between 0900 and 1300 h. Plasma levels of estradiol-17β and testosterone were measured by radioimmunoassay following chromatographic separation on celite columns as described by Wingfield and Farner (1975) and modified by Moore *et al.* (2000). High-pressure liquid chromatography (HPLC) conducted by Carl Schreck and Grant Fiest (Oregon State University) provided additional validation of hormone levels.

The lower limit of detection was 0.3 ng/ml for all assays. The intra- and interassay coefficient of variation for all assays was 10%. There was no statistical difference between assigning 0.0 or 0.15 ng/ml for samples falling below the detectable limit (0.3 ng/ml) for either the T assay or the E_2 assay. Given that 0.0 ng/ml is most likely unrealistic, nondetectable levels were given a value of 0.15 ng/ml (one-half the detectable limit).

Hormone Manipulation

All animals were hypothermically anesthetized on wet ice (Crews *et al.*, 1985). A total of 30 male neonates received a 5-mm Silastic capsule (0.76 mm ID × 1.65 mm OD, Dow Corning, Midland, MI) containing 3 mm of packed crystalline E_2, T, antiandrogen (cyproterone acetate), antiestrogen (tamoxifen), or a blank capsule at 1 or 10 weeks of age. Females received crystalline T, E_2, antiandrogen, antiestrogen, or a blank capsule at 1 or 10 weeks of age. Blood samples were obtained from the caudal vein of 10 individuals representing each treatment group at the end of the study. Radioimmunoassay of these final samples was used to validate the effectiveness of the implants. Levels of T and E_2 in 36-week-old controls were analyzed along with the hormone profile mentioned above.

Statistical Analysis

Two-way analysis of variance (ANOVA) was used to determine the significance of age and sex on gonadal hormones. Repeated-measures ANOVA (SAS Institute) was used to determine significant differences in the slope of log-transformed body mass and SVL growth curves and significant effects of sex.

RESULTS

Growth 1995

Slope of the body mass curves of males and females did not differ between birth and 20 weeks (body mass: $F = 1.08$, $P = 0.317$). Slope of the SVL curves of males and females did not differ between birth and 24 weeks (SVL: $F = 1.94$, $P = 0.132$). However, both curves differed significantly between the sexes from 20 weeks (body mass) or 24 weeks (SVL) through the end of the study (48 weeks) (body mass: $F = 4.12$, $P = 0.01$, SVL: $F = 3.99$, $P = 0.012$) (Fig. 1).

Hormone Profiles

Testosterone levels of individual males and females ranged from 0.15 to 1.0 ng/ml over the entire profile. Age did not have a significant effect on circulating levels of testosterone in males or females (males: $F = 1.08$, $P = 0.391$, females: $F = 1.16$, $P = 0.327$). Likewise, E_2 levels in both sexes ranged from 0.15 to 1.2 ng/ml from birth through 15 weeks and at the 36th week. There was no significant effect of age on E_2 in either sex (males: $F = 0.44$, $P = 0.963$, females: $F = 0.32$, $P = 0.121$). There was no significant difference in the mean values of T or E_2 between the sexes ($F = 2.07$, $P = 0.152$) (Fig. 2).

Hormonally Manipulated Growth

Within-treatment comparisons indicated that there was no age effect associated with the timing of implantation and growth. Therefore, all analyses of hor-

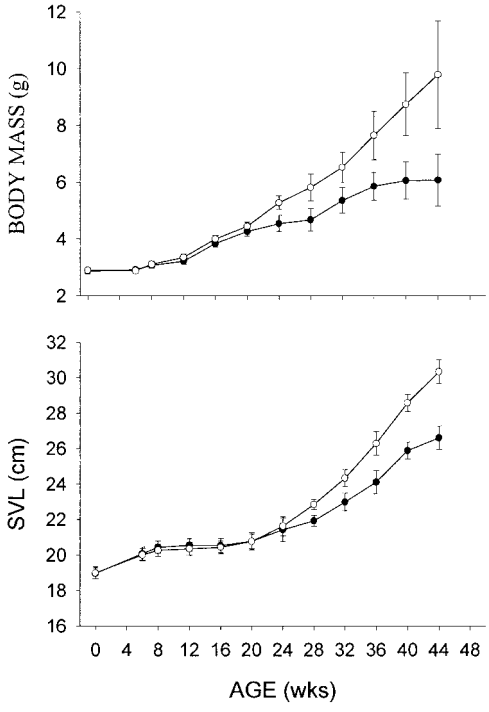

FIG. 1. Growth of neonatal red-spotted garter snakes in 1995. Body size is presented as means ± *SE*. Open circles represent females (n = 20). Closed circles represent males (n = 19). Females are larger than males after 24 weeks.

mone treatment effects were based on combined age groups (the 1- and 10-week post-birth implants). In addition, there were no significant differences in the body mass curves between the treatment groups, so the following analyses focus on changes in SVL or skeletal growth. All but 1 of the males implanted with testosterone died before the end of the study, as did all of the females implanted with estradiol. These animals and those that did not survive the 36-week study period were removed from the analysis. Hormone analysis of blood samples taken from animals at the end of the study revealed that mean (± *SE*) plasma levels of T and E_2 delivered by implants were 18.62 ± 1.25 and 22.45 ± 2.27 ng/ml, respectively.

Differences in the SVL growth curves of the treated and untreated snakes were significant beginning after 24 weeks (F = 3.63, $P < 0.001$) (Figs. 3 and 4). Growth curves of male and female controls had significantly different slopes (F = 5.17, $P < 0.001$). Antiestrogen reduced female growth such that it did not differ significantly from that of control males (F = 0.18, P = 0.678) but was significantly lower than that of control females (F = 4.87, $P < 0.001$) (Fig. 4). Growth of females treated with exogenous T was significantly less than that of control females (F = 4.21, $P < 0.0001$) but did not differ significantly from that of control males (F = 1.91, P = 0.1918) (Fig. 4). Growth curves of antiandrogen- and antiestrogen-treated males were not significantly different from that of control males (antiandrogen: F = 2.95, P = 0.1063, antiestrogen: F = 1.39, P = 0.3279). However, males treated with E_2 showed significantly reduced growth (F = 18.53, P = 0.0051) (Fig. 4). Growth of females treated with antiandrogen was not significantly different from that of control females (F = 2.01, P = 0.4239). See Table 1 for a summary of these results.

DISCUSSION

Female skeletal growth was significantly greater than that of males after the 24th week of life. These

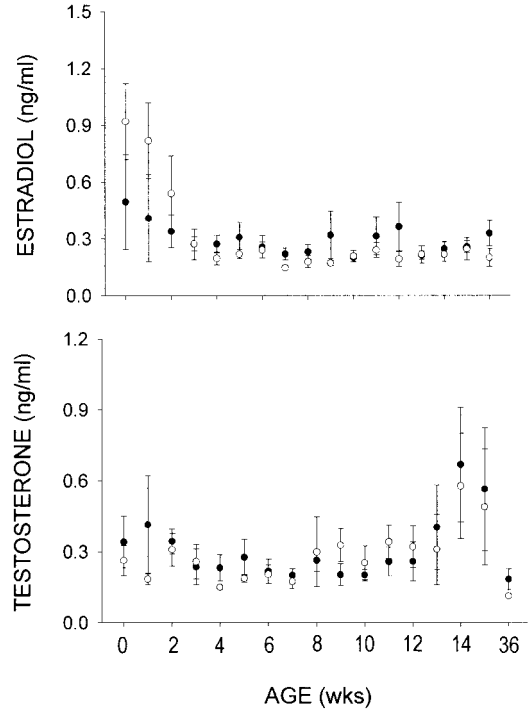

FIG. 2. Circulating levels of endogenous E_2 and T in intact neonatal red-spotted garter snakes from birth to 15 weeks and at the 36th week. Values are means ± *SE*. Males are represented by closed circles (n = 10 for each week). Females are represented by open circles (n = 10 for each week).

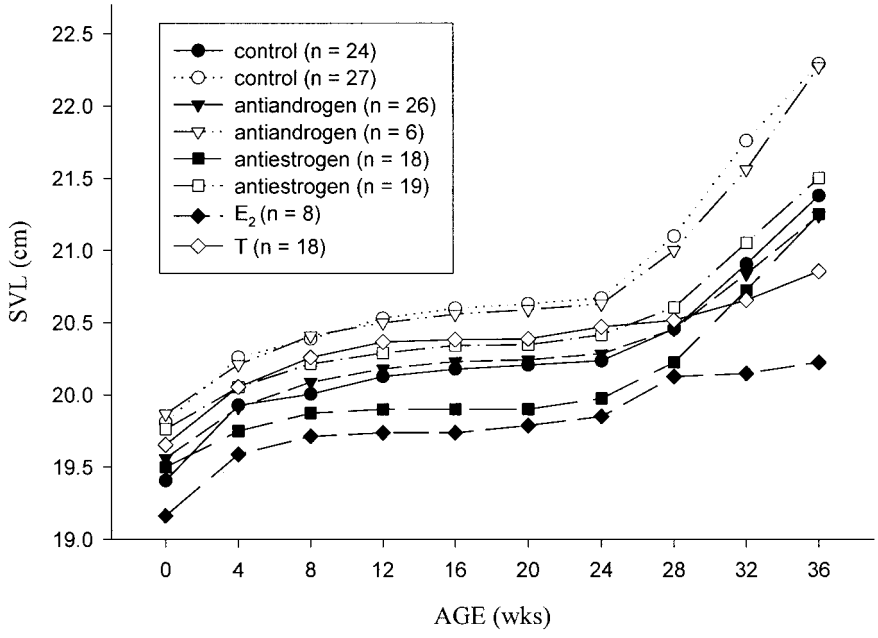

FIG. 3. Growth of hormonally manipulated neonatal red-spotted garter snakes. There are no significant differences in skeletal growth (SVL) between birth and 24 weeks of age. Overall growth of control and antiandrogen females was significantly greater than that of antiestrogen females and all male groups. E_2 significantly reduced growth of males after 24 weeks. Open circles represent females. Closed circles represent males. Standard error bars are left off for clarity.

differences were not directly attributable to differential levels of endogenous gonadal hormones experienced after birth. However, antiestrogen administration to females reduced growth to male levels. Exogenous T had similar effects, suggesting a growth-promoting effect of estrogens and a growth-suppressing effect of T in females. Addition of E_2 significantly reduced male growth compared to that of untreated males, whereas additional T proved lethal. In addition, removal of the effects of endogenous T did not change male or female growth. Considering these findings along with those of Crews *et al.* (1985), where castrated males (removal of endogenous T and E_2) grew like untreated females, suggests that male neonatal *T. sirtalis concinnus* need endogenous levels of either T or E_2 to maintain the male condition (Table 1). These findings are in agreement with those in some mammals and fish where gonadal steroids have differential effects between the sexes (Jannson *et al.*, 1985; Painson *et al.*, 1992; Holloway and Leatherland, 1997; Zou *et al.*, 1997).

One major difference between this study and that by Crews *et al.* (1985) is the level of endogenous T found in males in that study at 3 weeks of age. Male *T. sirtalis parietalis* had approximately 65 ng / ml T as opposed to roughly 0.3 ng / ml T at that same period in the current study. These hormone levels persist in *T. sirtalis concinnus* from ontogeny until 15 weeks, are at this level at 36 weeks of age, and are similar to those found in 8-month-old snapping turtles, *Chelydra serpentina* (Rhen *et al.*, 1996). Unlike our study, males of this turtle species exhibit significantly greater levels of T than do females (Rhen *et al.*, 1996). Investigation of hormone levels at critical points of growth in other sexually dimorphic reptilian species would be highly informative.

The most likely action of antiandrogens and antiestrogens is through competition with natural hormones for the receptor sites in the target cells (Rastogi and Chieffi, 1975). However, response of target tissues to sex steroids, antiestrogens, and antiandrogens are dose dependent (Rastogi and Chieffi, 1975) and can be complex. Tamoxifen, in particular, has exhibited differential effects associated with different target tissues (Sato *et al.*, 1996). Therefore, the antiestrogen tamoxifen may have negative effects on female growth separate from its role as an estrogen antagonist.

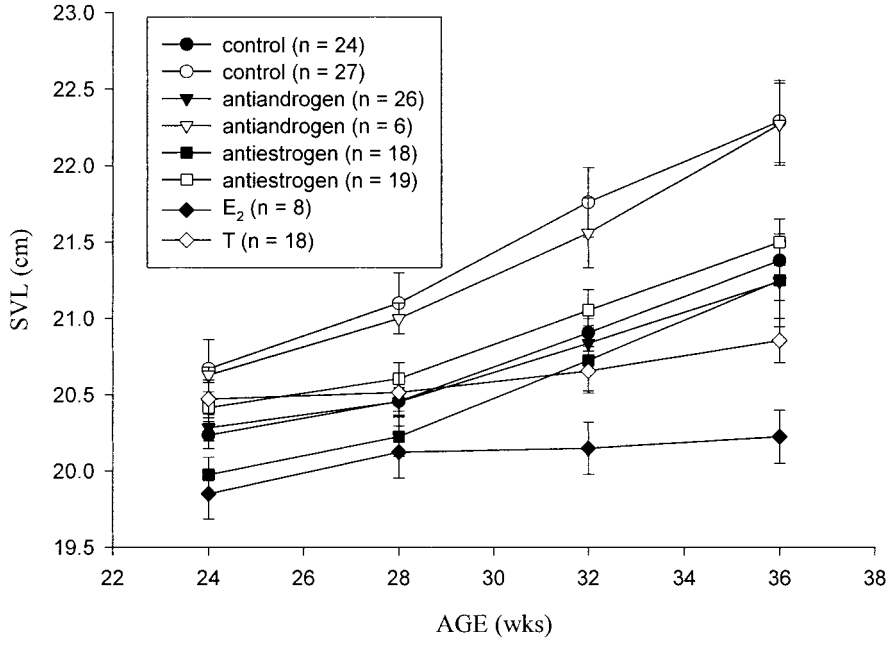

FIG. 4. Growth of hormonally manipulated neonatal red-spotted garter snakes between 24 and 36 weeks of age. Antiandrogen had no effect on male or female growth. Antiestrogen reduced female growth but had no effect on males. Body size is represented by mean SVL ± *SE*. Open circles represent females. Closed circles represent males.

All of the E_2-treated females and, similar to the findings of Crews *et al.* (1985), many of the E_2-treated males (females in the Crews *et al.* study were not implanted with E_2) died before the end of the experiment. In addition, all of the males implanted with T and a considerable number of the females failed to survive. This suggests that levels of these hormones exceeded some maximal threshold, resulting in deleterious effects (usually death).

Administration of a high dose of T reduces growth in female rats that is assumed to be caused by the aromatization of T to E_2 (Gentry and Wade, 1976; Gray *et al.*, 1979). Addition of exogenous T in male garter snakes could be converted to E_2. This would explain the reduced growth rates observed early in the growth profile of male *T. sirtalis concinnus* but does not provide an explanation for the failure of T-implanted males to survive.

A number of investigators have explored interactions between gonadal hormones and growth hormone (GH). Growth hormone is known to promote muscle and skeletal growth in animals by directly acting on target tissues as well as indirectly through insulin-like growth factors (IGFs) (Florini *et al.*, 1996). Administration of exogenous E_2 increases GH levels in rainbow trout (Holloway and Leatherland, 1997) and female goldfish (Zou *et al.*, 1997). These GH-promoting effects of E_2 are similar to those found in rat and human cell cultures (Slootweg *et al.*, 1997). In gonadectomized rats, replacement of E_2 increases GH hormone secretion in females (Painson *et al.*, 1992). Similar results are found in gonadectomized males given T (Jannson *et al.*, 1985; Jansson and Frohman, 1987). Exposure to the gonadal steroid of the opposite sex reverses these effects (Jannson *et al.*, 1985; Painson *et al.*, 1992). In general, it appears that E_2 can have growth-

TABLE 1
Summary of Growth in Hormonally Manipulated Neonatal Red-Spotted Garter Snakes

Treatment	Male results	Female results
e	M	M
E	< M	x
T	x	M
t	M	F
Gonadectomized[a]	F	?

Note. E, T = exogenous hormone; e, t = antiestrogen, antiandrogen; M = male-like growth; F = female-like growth; < = less than; x = deceased.

[a] From Crews *et al.* (1985).

promoting effects but that these effects may be different between the sexes. The measurement of GH in neonatal garter snakes would be enlightening. However, we know of no GH assays for reptiles.

There appear to be three distinct stages within the 1st year of growth in the red-spotted garter snake. Stage 1 occurs in the 1st postnatal month, during which neonates exhibit nondifferential growth (Fig. 3). This growth has been attributed to metabolism of yolk reserves (Fitch, 1965; Platt, 1969; Clark, 1970; Ewert, 1979). During stage 2 (4–24 weeks), growth rates of males and females are highly reduced but remain undifferentiated. This period coincides with the first season of life previous to and during winter dormancy. Individuals subjected to artificial winter dormancy in the laboratory (4°, 0L:24D, and denied food) and those held at 20°, 12L:12D, and provided food did not exhibit significant differences in growth rates during this second stage (Lerner, unpublished data). Thus, reduction in growth occurs regardless of hormone treatment or food provision. Differential growth occurs during the third stage after the time coinciding with winter dormancy in nature. It would appear that neonatal growth in these animals, at least during the first several months, is physiologically fixed.

Our study has shown that although basal levels of endogenous E_2 and T are not different, they differentially affect growth of male and female red-spotted garter snakes. Endogenous E_2 is necessary for female-like growth. Endogenous E_2 or T is sufficient for the relatively reduced growth in males. Male-like growth can be induced in females by addition of exogenous T. We speculate that these mechanisms may operate through differential feedback effects of sex steroids on the secretion of growth hormone. Analysis of growth in ovariectomized females would further define the role of these sex steroids in mediating sexual dimorphism.

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