Age and Litter Effects on Testosterone Levels in Young Water Snakes

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To test whether a pulse of testosterone reported in young male garter snakes also occurs in related snakes, testosterone levels were measured in 18 male water snakes (*Nerodia sipedon*) belonging to four litters at 5–6, 16–17, 29–30, and 250–267 days of age. Testosterone levels were uniformly low over the first 30 days of life but increased significantly by 250–267 days of age; there were also significant differences among litters in levels of this hormone by that time.

EXPERIMENTAL investigations of growth of the garter snake, *Thamnophis sirtalis*, suggest that testosterone plays a role in the postnatal development of sex differences in body size and head dimensions (Crews et al., 1985; Shine and Crews, 1988). In this species, females attain larger body size and larger head size relative to body size than do males. Reduction of testosterone levels in male garter snakes via castration results in higher (femalelike) growth rates in body size and relative head dimensions than are seen in males that undergo castration plus testosterone supplementation or that undergo sham manipulation (Crews et al., 1985; Shine and Crews, 1988). Furthermore, high circulating levels of testosterone have been reported in 2–3-week-old males and are hypothesized to play an organizational role in establishing sexually distinct growth trajectories (Crews et al., 1985).

Evidence for high levels of testosterone in young garter snakes comes from radioimmunoassay of blood samples from 4–5-day-old males (n = 8), in which levels averaged 1.03 ng/ml, and 13–20-day-old males (n = 3), in which levels averaged 64.46 ng/ml (Crews et al., 1985, table 1). These levels are surprisingly high, approaching (4–5-day-old males) or exceeding (13–20-day-old males) those typically seen in adult male garter snakes (1–18 ng/ml; Weil, 1985; Crews et al., 1987; Krohmer et al., 1987). To test whether elevated neonatal testosterone levels occur among males of other species that exhibit the garter snake pattern of sexual dimorphism, we assayed testosterone in young water snakes, *Nerodia sipedon*. Because water snakes are larger at birth than are garter snakes (4–6 g vs 1–2 g, King et al., 1999), we were able to collect blood samples nondestructively and repeatedly, allowing us to monitor changes in testosterone levels within individuals over time. In addition, because the water snakes used in our study were members of four litters, we were able to test for possible family differences in testosterone levels.

MATERIALS AND METHODS

Four gravid female *N. sipedon* were collected in Ottawa County, Ohio, on 8 August 1995, and maintained in the laboratory until parturition between 21 August and 8 September, 1995. Females and neonates (n = 48; 6–17 per litter) were housed individually in a room maintained at 26–28 C with a 12:12 L:D photoperiod. Females and neonates had continuous access to fresh water and were offered fish to eat twice per week. Blood samples (100–300 μl) were collected from caudal blood vessels using a heparinized syringe (Bush and Smeller, 1978) when neonates were 5–6 days old, 16–17 days old, 29–30 days old, and 250–267 days old. [These neonates were also used in an experimental analysis of the effect of prey size on growth in relative head dimensions between the third and fourth bleedings; Queral-Regil and King, 1998.] Blood samples were centrifuged and the plasma fraction was frozen for hormone analysis following completion of all four bleedings. Testosterone levels were determined by radioimmunoassay as described by Camazine et al. (1980) and Terranova (1981). Testosterone antibody was provided by G. Niswender (Colorado State University). This antibody shows high cross-reactivity with 5 alpha-dihydrotestosterone (58%) and lower cross-reactivity to androstenedione (2%; G. Niswender, pers. comm.). However, because dihydrotestosterone accounts for a relatively small proportion of total androgens (Mason and Crews, 1985; Crews et al., 1985), our assays provide a reasonably accurate estimate of testosterone levels. Briefly, steroids were ether-extracted from plasma (50–200 μl diluted to 1 ml with distilled water) and standards (1.95–500 pg testosterone in 100 μl methanol) and added to a radio-immunoassay containing ^{3}H testosterone (approximately 5000 cpm; New England Nuclear NET-370) and testosterone antibody (1:60,000 dilution). Samples and standards were incubated overnight at 4 C, after which bound was separated from unbound steroid with a

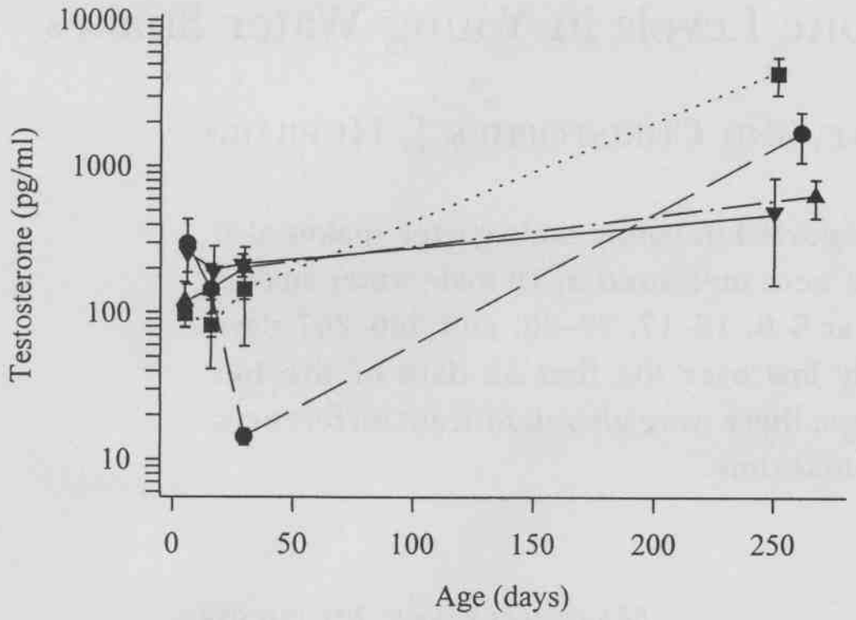

Fig. 1. Changes in testosterone levels with age in young water snakes. Shown are means and standard errors for males from four families (n = 4–6 per families). Separate symbols and line styles distinguish families. Testosterone levels were measured in pg/ml and are plotted on a log scale.

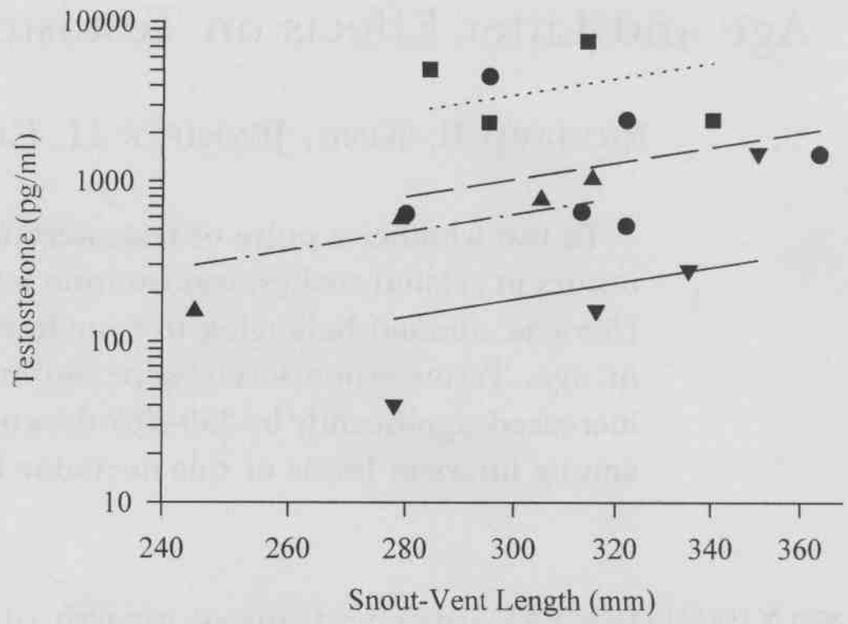

Fig. 2. Relationship between snout–vent length and testosterone levels in 250–267-day-old male water snakes belonging to four families. Testosterone levels were measured in pg/ml, and snout–vent length was measured in millimeters; both variables are plotted on log scales. Separate symbols and line styles distinguish families as in Figure 1 (squares are associated with the dotted line, circles with the dashed line, upward pointing triangles with the dot-dashed line, and downward pointing triangles with the solid line. Regression lines are drawn to reflect differences in intercept but not in slope (see text).

charcoal-dextran suspension. After centrifugation, the supernatant from each sample was added to vials containing scintillation cocktail (Bio-Safe II, Research Products International) and counted in a Beckman liquid scintillation counter.

Testosterone levels were determined for 18 male water snakes at each of four ages using a logit-log curve-fitting program. Testosterone levels reported here were determined in a single assay with an intraassay variability of 10% and a sensitivity range of 15.6–500 pg/tube. Data obtained were used to test (1) whether testosterone levels varied among families or with age (from 5–6 days to 250–267 days) and (2) whether testosterone levels varied among families or with body size at 250–267 days. Repeated-measures analysis of variance was used to test for change in testosterone level with age. Because testosterone was undetectable (less than 15.6 pg) in some samples (three 5–6-day-old males, five 16–17-day-old males, seven 29–30-day-old males), data were analyzed twice. In one analysis, samples with undetectable testosterone levels were assigned a value of 0 pg/ml; in the other analysis, these samples were assigned the minimum detectable value of 15.6 pg/tube. Results were qualitatively similar in the two analyses and only the latter analysis is reported here. Analysis of covariance was used to test for an effect of body size and family membership on testosterone levels at 250–267 days of age. Body size (natural log transform of snout–vent length) was measured when snakes were 176–194 days of age. In all analyses, testosterone levels were transformed by taking natural logarithms to better meet assumptions of normality and equality of variance.

Results

There were significant time and time-by-family interaction effects on testosterone levels in male water snakes (Time: $F_{3,12} = 24.3$, $P < 0.001$; Time-by-Family: $F_{9,12} = 4.2$, $P = 0.001$). Testosterone levels were consistently low when males were 5–6 days old, 16–17 days old, and 29–30 days old and increased markedly by the time males were 250–267 days old (Fig. 1). However, the degree to which testosterone levels increased varied among families (Fig. 1). Analysis of covariance of testosterone levels at 250–267 days revealed that larger males had higher testosterone levels ($F_{1,13} = 4.99$, $P = 0.044$), that the slope of this relationship was similar among families ($F_{3,10} = 2.67$, $P = 0.104$, common slope = 3.68), but that the intercept of this relationship varied among families ($F_{3,13} = 8.06$, $P = 0.003$; Fig. 2). Conclusions regarding changes in testosterone levels over time and differences in testosterone levels among families were unaffected by natural log transformation. However, testosterone levels were uncorrelated with snout–vent length at 250–267 days when untransformed variables were analyzed ($F_{1,13} = 0.01$, $P = 0.919$), suggesting caution in the interpretation of this relationship.

Discussion

The results presented here suggest that young male water snakes do not exhibit elevated testosterone levels early in life. Testosterone levels showed no detectable increase over the first 30 days of life and fell well below those reported in adult male snakes. Why elevated testosterone levels might be present in young garter snakes (Crews et al., 1985) but not in young water snakes (this study) is unclear. Garter snakes and water snakes have a close phylogenetic affiliation (Lawson, 1987) and show similar patterns of sexual dimorphism as adults (females attain larger body size and relative head dimensions in both; Shine, 1991). However, the age at which testosterone levels first increase may differ between species, perhaps occurring later in *N. sipedon* than in *T. sirtalis*. Significant sexual dimorphism is present at birth in *T. sirtalis*, suggesting that early exposure to testosterone (even in utero exposure) might set sex-specific growth trajectories that persist into adulthood (Shine and Crews, 1988). In contrast, significant sexual dimorphism is apparently not present at birth in water snakes (King et al., 1999) and sex-specific growth trajectories may be established later in life.

Testosterone levels did increase by the time male water snakes reached an age of 250–267 days, averaging nearly 900 pg/ml (Fig. 1). These levels approach the lower end of testosterone levels seen in free-ranging adult male garter snakes (1000–18,000 pg/ml; Weil, 1985; Crews et al., 1987; Krohmer et al., 1987) but fall short of those seen in four adult male water snakes (36,000–58,000 pg/ml; RBK, unpubl. data). Male *N. sipedon* reach adulthood at an age of 2–3 years and at a size of about 430 mm snout–vent length (SVL; King, 1986). Males in this study averaged just 308 mm SVL and 16.5 g when measured at 176–194 days of age, suggesting that testosterone levels begin to increase before the onset of sexual maturity in male *N. sipedon*. Significant sexual dimorphism in body size and relative head dimensions is also evident prior to the onset of sexual maturity. Among the snakes included in this study, females exceeded males by 10% in SVL, 26% in mass, and 4% in jaw length at 176–194 days of age (Queral-Regil and King, 1998). If testosterone functions in the establishment of sex-specific growth trajectories, testosterone levels in males must increase well before 176 days of age. Alternatively, sex-specific growth trajectories may be established by some other mechanism. Additional data on testosterone levels in neonatal and juvenile garter snakes and water snakes, and in species showing other patterns of sexual dimorphism as adults (e.g., Shine, 1993; King, 1997), would be of interest.

Testosterone levels at 250–267 days of age differed significantly among males born to different females (Fig. 2). Males in one family showed no increase in testosterone levels from 30 to 250–267 days of age, whereas males in the other three families showed varying degrees of increase over this period. These family differences in testosterone levels cannot be attributed to differences in body size; body sizes overlap among families (Fig. 2), and SVL was included as a covariate in testing for an effect of family membership. Family differences are of interest from an evolutionary perspective because they suggest the presence of genetic variation in the amount or timing of testosterone production. Given the effects that hormones such as testosterone can have on behavioral and morphological traits, genetic variation in hormonal control mechanisms may be of evolutionary importance (e.g., Crews and Moore, 1986; Moore, 1991; Ketterson and Nolan, 1992). Genetic variation in hormonal control mechanisms has been demonstrated in domestic animals (Shire, 1979), but such demonstrations are generally lacking for undomesticated animals (but see Zera and Zhang, 1995). Alternatively, family differences in testosterone levels observed here may be nongenetic in origin, perhaps arising from characteristics of the common uterine environment shared by siblings. Potentially, these alternatives could be distinguished through controlled breeding experiments or by experimentally manipulating uterine conditions.

Acknowledgments

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