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Heritable variation in testosterone levels in male garter snakes (*Thamnophis sirtalis*)

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Abstract

Circulating testosterone levels were measured at 195, 318 and 437 days of age in 76 male garter snakes (*Thamnophis sirtalis*) born to 26 wild-caught females. Testosterone levels increased significantly with increasing mass at all three ages and differed significantly among families at 318 and 437 days. 'Full-sib' estimates of heritability were near one at 318 and 437 days but these estimates may be inflated by maternal effects, dominance and epistasis. Evidence of heritable variation in circulating testosterone levels suggests that the evolution of traits affected by this hormone (reproductive, territorial and agonistic behaviour; body size and shape) might be mediated through changes in this hormonal control mechanism.

Key words: behaviour, heritability, morphology, snakes, testosterone, *Thamnophis sirtalis*

INTRODUCTION

Steroid hormones such as testosterone influence a variety of phenotypic traits of animals, including reproductive, territorial, and agonistic behaviour, plus body size and shape. Thus, the evolution of these phenotypic traits may be mediated through steroid hormone pathways. One step in establishing such a linkage is to demonstrate that there is heritable variation in hormonal control mechanisms. Historically, documentation that such mechanisms have a genetic basis has involved twin studies and pedigree analyses of humans or strain comparisons and controlled breeding designs in poultry, livestock and laboratory rodents (e.g. Bates *et al*., 1986; Meikle, Stringham, Woodward & West, 1986; Meikle, Stringham, Woodward & Bishop, 1988; Meikle, Stringham, Woodward & Nelson, 1988; Scott & Washburn, 1988; Jaquish *et al*., 1997; Zarazaga *et al*., 1998; Mendlewicz *et al*., 1999; An *et al*., 2000). Evidence that natural populations harbour the sort of genetic variation in hormonal control mechanisms that might be acted on by natural selection or other evolutionary processes is rare (but see Fairbairn & Roff, 1999; Zera, 1999; Zera & Huang, 1999). Here, evidence is presented for heritable variation in testosterone levels in male garter snakes *Thamnophis sirtalis* and the implications of such variation for the evolution of traits influenced by steroid hormones are discussed.

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METHODS

Subjects were male offspring born to 26 wild-caught gravid female garter snakes (2–4 offspring per female) and reared in captivity (King & Turmo, 1997; King, 2002). Offspring were housed individually, had continuous access to fresh water, and were fed earthworms 3 times/week. The room in which offspring were housed was maintained at 24–26 °C and about 50% relative humidity with a 12:12 L:D photocycle. These offspring were part of a larger investigation of genetic and hormonal effects on behaviour and morphology and were assigned to sham, elevated-testosterone, or flutamide treatment groups at random within sexes and families (King, 2002). The analysis presented here concerns only sham-manipulated animals. These animals were implanted with empty subcutaneous silastic capsules when 108–197 days old and underwent simulated hibernation when 213–283 days old (King, 2002). Ten snakes died during the experiment and thus, sample size decreased from 76 (26 families) to 66 (24 families).

Blood samples (*c*. 200–500 μl) were drawn from caudal vessels when snakes were 195, 318 and 437 days old. Each sample was collected into a sterile disposable 1 ml syringe with 27 gauge needle into which *c*. 0.05 ml heparin (600 units per ml) had been drawn and flushed to prevent coagulation. Snakes were restrained in 1 hand by positioning the head between 2 fingers, wrapping the snake around the hand, and pulling the tail taut between thumb and finger. Using the other hand, the needle was inserted mid-ventrally from posterior to anterior at a 45° angle and *c*. 10 scale rows posterior to the

cloaca until a vertebra was reached, then drawn back slightly. Blood samples were collected in late morning and early afternoon and sampling typically took 5 min or less per snake. Plasma and red blood cell fractions were separated by centrifugation and plasma was frozen for radioimmunoassay of testosterone levels as described previously (King, Cline & Hubbard, 2000; King, 2002). Samples collected from snakes of a given age were assayed together with intra-assay variation of 10.3% and inter-assay variation of 13.2%. The lower and upper limits of detectability (defined as ratios of bound to unbound testosterone of 95% and 5%, respectively) were 3.3–3227.4 pg/sample at 195 days, 4.7–1274.9 pg/sample at 318 days, and 6.1–2064.7 pg/sample at 437 days. Plasma volume used for assays ranged from 100 to 300 µl (because plasma volume varied among samples, limits of detectability in pg/ml also varied). Before analysis, testosterone levels were transformed by taking natural logarithms to meet more closely assumptions of normality and equality of variances. Samples having ratios of bound to unbound testosterone of $< 5\%$ or $> 95\%$ were assigned a level equal to the upper or lower detectable limit of that assay ($n = 14$ of 76 samples from 195 days of age, 19 of 70 samples from 318 days, 10 of 66 samples from 437 days).

Analysis of covariance (ANCOVA) with family as factor and mass as covariate was used to test for family differences in testosterone levels at 195, 318 and 437 days. $MS_{among\ families}$ and MS_{error} from ANCOVA were used to compute total and among-family variance components. Heritability was computed as $2{*}\sigma^2_{among\ families}/(\sigma^2_{among\ families} + \sigma^2_{error})$ (computational formulas for variance components, heritability, and standard errors can be found in Becker, 1992: 52–54). This method provides an estimate of what Brodie & Garland (1994) refer to as 'full-sib' heritability and assumes that littermates are full sibs and that maternal effects do not influence the trait of interest. This method may underestimate narrow-sense heritability (h^2_n, the proportion of variation in phenotype attributable to additive genetic variance) if multiple paternity occurs within litters and may overestimate h^2_n if maternal effects are present (Brodie & Garland, 1993).

RESULTS

Testosterone levels varied from 9 to 4379 pg/ml (mean = 88.7 pg/ml, back-transformed from natural logarithms) at 195 days, 31–5456 pg/ml (mean = 96.1 pg/ml) at 318 days, and 43–13 765 pg/ml (mean = 1183.2 pg/ml) at 437 days. The slope of the relationship between mass and testosterone level did not differ among families at 195 days (ANCOVA: $F_{25,24} = 1.92$, $P = 0.058$), 318 days ($F_{22,24} = 1.64$, $P = 0.119$), or 437 days ($F_{22,20} = 1.77$, $P = 0.099$). Testosterone levels increased significantly with increasing mass at 195 days, 318 days and 437 days (Table 1, Fig. 1). Testosterone levels did not differ significantly among families at 195 days but did differ significantly at 318 days and 437 days (Table 1, Fig. 1).

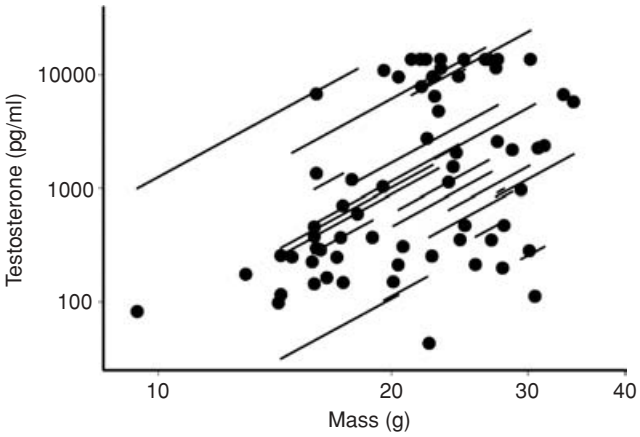

Fig. 1. Effects of family membership and body size (mass, g) on testosterone levels in male garter snakes at 437 days of age. Points represent individual garter snakes, separate lines represent families. Length of lines corresponds to the range of variation in mass within families.

Table 1. Analysis of covariance and heritability estimates of testosterone levels in male garter snakes at 195, 318, and 437 days of age

Source	d.f.	MS	F	P	h^2 (95% CI)
195 days					
Mass	1	13.03	8.28	0.006	
Family	25	2.09	1.33	0.195	0.20 (−0.24, 0.76)
Error	49	1.57			
318 days					
Mass	1	7.06	11.33	0.002	
Family	22	3.34	5.36	< 0.001	1.18 (0.72, 1.56)
Error	46	0.62			
437 days					
Mass	1	18.36	13.38	0.001	
Family	22	4.66	3.40	< 0.001	0.91 (0.38, 1.39)
Error	42	1.37			

To test for positive correlations among testosterone levels at 195, 318 and 437 days, elevations of the relationship between mass and testosterone level were computed for each family. These elevations provide a size-independent measure of level within families. Significant positive correlations in testosterone level were found between 195 and 318 days (Pearson's $R = 0.397$, d.f. = 22, one-tailed $P = 0.031$) and 318 and 437 days (Pearson's $R = 0.515$, d.f. = 22, one-tailed $P = 0.006$) but not 195 and 437 days (Pearson's $R = 0.016$, d.f. = 22, one-tailed $P = 0.472$)

Heritability of testosterone level did not differ significantly from zero at 195 days but was significantly greater than zero at 318 days and at 437 days (Table 1). Numerical estimates of heritability at 318 and 437 days were large, equalling 1.18 and 0.91, respectively. Repeating these analyses with only those animals whose testosterone levels fell within the range of detectability led to identical conclusions regarding the significance of

family membership and body size effects and produced heritability estimates of similar magnitude (results not shown).

DISCUSSION

This study demonstrates that in young male garter snakes, testosterone levels differ significantly among families. Body size also differs significantly among families (R. B. King, unpublished data), but by including mass as a covariate in our analyses, differences in testosterone levels among families are evident independent of differences in body size. Family effects, such as those on testosterone reported here, have been found consistently for a wide range of morphological, physiological and behavioural traits of natricine snakes (garter snakes and their allies) (reviewed by Brodie & Garland, 1993; Burghardt & Schwartz, 1999; see also King, 1997; Burghardt, Layne & Konigsberg, 2000). Patterns of variation within and among families have been used previously to estimate heritability via full-sib analysis as used here (Brodie & Garland, 1993). As noted above, such estimates assume that litters consist of full-sibs and that maternal effects are negligible. Evidence is mounting that in some natricines, including *T. sirtalis*, multiple paternity within litters is commonplace (Gibson & Falls, 1975; Schwartz, McCracken & Burghardt, 1989; Barry, Weatherhead & Phillipp, 1992; McCracken, Burghardt & Houts, 1999; Prosser, 1999; Albright, 2001; Garner *et al*., 2002). By itself, multiple paternity should lead to underestimates of h^2 using full-sib analysis. King, Milstead *et al*. (2001) have taken advantage of the occurrence of multiple paternity within litters to explore the possibility that maternal effects also contribute to among-family variation. Based on an analysis of four litters each sired by two males (eight sireships total), it seems that maternal effects may inflate estimates of h^2 obtained using full-sib analysis. The nature of these maternal effects remains unexplored and may include both maternal environmental effects (e.g. effects of the common uterine environment shared by littermates) and maternal genetic effects (effects of maternal genotype on offspring phenotype). Dominance, epistasis and common post-natal environmental effects can also inflate full-sib estimates of heritability (Brodie & Garland, 1993). Given these possibilities, caution is needed in the interpretation of the heritability estimates reported here, especially given that some estimates approach (437 days) or exceed (318 days) the theoretical maximum of 1 (Table 1).

In utero hormonal effects on behaviour and morphology are well known in litter-bearing mammals (e.g. vom Saal, 1989; Clark, Karpiuk & Galef, 1993; Drickamer, 1996). These effects arise as a result of prenatal production of steroid hormones by developing foetuses and their influence can span multiple generations (Clark *et al*., 1993). In litter-bearing mammals, *in utero* hormonal effects are most evident between adjacent foetuses (e.g. on females developing between two males). More generalized effects of *in utero* hormonal conditions are suggested by a significant correlation between litter sex ratio and garter snake subcaudal scalation, a sexually dimorphic trait (Osypka & Arnold, 1999). However, this effect was seen in only one of two populations studied. Whether such *in utero* effects also influence post-natal offspring hormone profiles has not been investigated.

Testosterone levels in young male garter snakes increase with increasing body size. Snakes included in this analysis were comparable in size to sub-adults and small adults, ranging from 213 to 366 mm snout–vent length (SVL, mean = 288.5 mm) and 5.0–16.8 g (mean = 10.57 g) at 195 days, 224–370 mm SVL (mean = 302.1 mm) and 5.9–20.9 g (mean = 11.69 g) at 309 days, and 276–440 mm SVL (mean = 369.9 mm) and 9.4–34.3 g (mean = 22.01 g) at 438 days. Males reach sexual maturity at an estimated SVL of 360 mm and maximum male SVL is 700 mm in the population from which these snakes were derived (King, 1988). Testosterone levels reported here are lower than those seen during a pulse of high testosterone that occurs within a few days of birth in *T. sirtalis* (24 540–122 490 pg/ml; Crews *et al*., 1985: table 1). However, this pulse is not evident in another natricine snake (King, Cline *et al*., 2000). By 437 days of age, testosterone levels reported here (43–13 765 pg/ml) approach those reported elsewhere for adult male garter snakes (1400–72 000 pg/ml: Weil, 1985: table 1).

Steroid hormones influence a number of sexually dimorphic morphological and behavioural traits in garter snakes. Testes removal and testosterone replacement experiments demonstrate that the smaller body size and smaller relative head dimensions (controlling for body size) of male garter snakes result from an inhibitory effect of testosterone on growth (Crews *et al*., 1985; Shine & Crews, 1988). Defensive behaviour (number of strikes at a stimulus) also differs between the sexes (females strike more frequently than males) and changes in response to hormone manipulation (snakes treated with elevated testosterone strike less frequently than shams) (King, 2002). However, evidence that individual variation in testosterone level is correlated with variation in behaviour or morphology is equivocal (R. B. King, 2002, pers. obs.).

Results described here suggest that one way morphological and behavioural traits may evolve is through genetic changes in levels of circulating hormones (e.g. via changes in responsiveness to environmental stimuli or releasing hormones, rate of hormone secretion, amount and affinity of androgen-binding hormones, and hormone half-life). Genetic variation in hormone receptor and post-receptor phenomena (receptor density and affinity, conversion of hormone to an active form, neuroendocrine interactions) would provide an alternative pathway for the evolution of hormonally controlled morphological and behavioural traits. Further investigation of linkages among apparent genetic variation in morphology and behaviour and hormonal control mechanisms are needed. Behavioural and morphological traits vary geographically in natricine snakes (Herzog & Schwartz, 1990; Grudzien *et al*., 1992; King, 1997;

Queral-Regil, 1998; Burghardt & Schwartz, 1999; Bittner 2000). In addition, the degree of sexual dimorphism in body size and head dimensions varies among populations of *T. sirtalis* and among species of natricine snakes (Shine & Crews, 1988; Shine, 1991; Krause, Burghardt & Gillingham, 2003). Numerous studies suggest a heritable basis to variation in these traits (Arnold, 1981*a*,*b*, 1988; Arnold & Bennett, 1984; Garland, 1988; Brodie, 1989, 1993; Dohm & Garland, 1993; King, 1997; Burghardt *et al*., 2000; Albright, 2001; reviewed in Brodie & Garland, 1993; Burghardt & Schwartz, 1999), but the proximate mechanism by which this genetic variation is expressed remains largely univestigated.

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