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Family, sex and testosterone effects on garter snake behaviour

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To better understand how genes and hormones interact to affect behaviour in nature, I used a factorial design to test for effects of family membership, sex and testosterone level on activity and defensive behaviour of the common garter snake, *Thamnophis sirtalis*. Behaviours (latency to move, defensive strikes, response distance) were scored prior to hormone manipulation (when snakes were 39 days of age), while sham or testosterone-containing implants were in place (190 days), following implant removal and simulated hibernation (284 days), and when snakes were 428 days of age. Family membership had pervasive effects on all three behaviours and on their ontogenetic trajectories, suggesting strong genetic or maternal components to behavioural variation. Sex had a significant effect on the number of defensive strikes; females struck more frequently than males, but ontogenetic trajectories were similar between the sexes. Testosterone manipulation also had an effect on strikes: snakes in the elevated-testosterone treatment group struck less frequently than shams while implants were in place. Sex and treatment effects on latency to move and response distance were lacking. Family*treatment interaction effects were lacking for latency to move and number of defensive strikes but were present for response distance. Possibly, genetic or maternally induced variation in strikes is mediated through variation in circulating hormone levels, whereas variation in response distance is mediated through receptor-level phenomena.

The interplay between genes and hormones is likely to be important in shaping behavioural evolution (e.g. Crews & Moore 1986; Crews 1987; Moore 1991; Ketterson & Nolan 1992). Evidence for such an interplay is accumulating from twin studies and pedigree analyses of humans (e.g. Mendlewicz et al. 1999; An et al. 2000) and strain comparisons and controlled breeding designs in poultry, livestock and laboratory rodents (Bates et al. 1986; Scott & Washburn 1988). Most frequently, these studies have demonstrated high heritability for circulating levels of hormones and hormone-binding proteins. Linkages between heritable variation in hormone levels and phenotypic traits, such as morphology, life history or behaviour, have been found less frequently (e.g. Bates et al. 1986; Gupta & Brush 1998; Otremski et al. 2000). Especially rare are studies integrating genetic and hormonal influences on behaviour in undomesticated species (but see Fairbairn & Roff 1999; Zera 1999; Zera & Huang 1999), perhaps due to differences in the methods of investigation typically used by behavioural geneticists and endocrinologists. Genetic effects are frequently assessed from patterns of variation within and between groups of relatives or from controlled breeding designs (Falconer & MacKay 1996). In contrast, hormonal influences are often investigated through hormone assays and experimental manipulations (hormone implants, ablation of endocrine glands) without regard to relatedness among individuals (Nelson 1995). Fortunately, methods used to investigate genetic and hormonal influences in nature need not be mutually exclusive. By incorporating hormonal assays and manipulations into analyses of variation within and between groups of relatives, behavioural effects of genes and hormones can be studied simultaneously. This paper reports the results of such an investigation of behaviour in the common garter snake, *Thamnophis sirtalis*.

High levels of genetic variation have been found in *Thamnophis* and allied genera for a range of behavioural traits (reviewed in Brodie & Garland 1993; Burghardt & Schwartz 1999). Behaviours related to prey preference, predator avoidance and escape have been particularly well studied, are repeatable between trials and show high levels of constancy over time (Brodie 1993; Brodie & Russell 1999; Herzog & Burghardt 1988). Quantitative genetic analysis provides heritability estimates of 0.37–0.42 for predator avoidance and escape behaviours (Arnold & Bennett 1984; Brodie 1989; Garland 1988). Studies of hormonal influences on garter snake behaviour have focused mostly on courtship behaviour. Steroid hormones appear to play an organizational role in behavioural differentiation between males and females;

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testosterone manipulations early in life (before snakes first enter hibernation) modify expression of sex-typical courtship behaviour later in life (Crews 1985; Moore & Lindzey 1992; Whittier & Tokarz 1992).

Whether testosterone influences nonreproductive behaviours in garter snakes (e.g. behaviours for which genetic data are available) is unknown. However, in some species of garter snakes, predator avoidance behaviour (e.g. defensive strikes) of neonates differs between males and females (Herzog & Burghardt 1986; Scudder & Burghardt 1983), suggesting that gonadal hormones might be involved. In addition, male garter snakes emerge from hibernation earlier than females and actively search for potential mates, whereas females are less active upon emergence. If testosterone plays an organizational role in mate-searching behaviour (as it does in other aspects of courtship, Crews et al. 1985), males and females might show more general differences in activity as well (e.g. propensity to flee, Shine et al. 2000). Behaviours scored in this study include latency to move, a measure of activity level (Herzog & Burghardt 1986) and defensive behaviour and response distance, measures of response to an approaching threat (e.g. Burghardt 1983; Arnold & Bennett 1984; Formanowicz et al. 1990). Thus, this investigation serves as a test for sex differences in nonreproductive behaviours as well as for possible effects of family membership and testosterone level on these behaviours.

Garter snakes have several logistical attributes that make them well suited for the work described here. Among the most important of these is large family size (averaging 15.6 and ranging from 6 to 31 among 28 captive-born families; R. B. King, unpublished data), which allows for an experimental design in which sex, hormone and family effects can be tested simultaneously. Whereas previous studies have attempted to avoid the potentially confounding effects of family membership on responses to hormone manipulation (Crews 1985; Crews et al. 1985; Shine & Crews 1988), they have not explicitly tested for family effects. Another attribute of garter snakes is that hormonal manipulations can be carried out easily via hormone-containing implants (e.g. Crews 1985; Crews et al. 1985). Finally, the social behaviour of garter snakes consists primarily of aggregative and reproductive behaviours; intraspecific aggression appears to be lacking (Burghardt 1983; Gillingham 1987; Ford & Burghardt 1993). Thus, androgen levels are unlikely to be modified by aggressive interactions as may occur in territorial animals.

The effects of hormones on behaviour typically result from a cascade of events and genetic variation might occur at any point in this cascade. The effect of testosterone starts with an internal or external environmental stimulus triggering the production of releasing hormones by the hypothalamus, the releasing hormones trigger release of gonadotropins by the anterior pituitary and these subsequently trigger secretion of testosterone. Testosterone travels to receptors in the central nervous system where it is involved in regulation of gene transcription which ultimately affects behaviour and morphology (Hadley 1984). The task of detecting genetic variation is made easier by dividing these events into two groups: factors that influence the level of circulating hormones (e.g. responsiveness to environmental stimuli, responsiveness to releasing hormones, rate of secretion, androgen binding proteins in the plasma, hormone half-life), and factors that influence an organism's response to a given level of hormone (receptor- and postreceptor-level phenomena, including receptor density, receptor affinity, efficiency of conversion of hormone to an active form and neuroendocrine interactions). Two research strategies for detecting genetic variation in hormone effects are then evident. One strategy is to test for genetic variation in levels of circulating hormones. A second strategy is to manipulate hormone levels and test for genetic variation in behavioural responses.

I used this second strategy in the research described here. Offspring born to wild-caught females were divided into three groups, a sham-manipulated group and two hormone-manipulated groups. In one hormone-manipulated group, testosterone levels were elevated via testosterone-containing implants. In the other, testosterone levels were functionally reduced via implants containing flutamide, which binds competitively with testosterone receptors, thus blocking testosterone's action (Liao et al. 1974; Neumann et al. 1977; Alexandre & Balthazart 1987). The goal of these manipulations was to mask individual variation in testosterone levels so that differences in receptor-level phenomena (e.g. receptor density, receptor affinity) might be detected. Comparisons between sham- and hormone-manipulated siblings provide a test for an effect of testosterone manipulation, whereas comparisons of treatment effects among litters provide a test for possibly heritable variation in response to hormone manipulation.

Table 1. Year and site of collection of gravid female garter snakes producing litters included in this study

	Year			
Site*	1994	1995	1996	Total
East Harbor	6	2	1	9
Winous Point	0	5	3	8
Middle Bass Island	1	3	1	5
Rattlesnake Island	2	1	0	3
Sugar Island	0	0	2	2
Total	9	11	7	27

*See Figure 2 in Lawson & King (1996) for locations of study sites.

METHODS

General

I obtained study animals by collecting gravid females in the wild (Ottawa Co., Ohio, U.S.A.) and maintaining them in captivity until parturition. Females were collected in late May and early June 1994, 1995 and 1996 (Table 1) and housed individually until parturition. Following parturition in late July–early August, I classified

Table 2. Timing of behavioural tests, hormone manipulation, simulated hibernation and collection of blood samples from neonatal garter snakes

	Age (days)		
	Mean	Minimum	Maximum
Behaviour scored*	39	34	51
Implants inserted	109	106	112
Behaviour scored*	190	187	195
Blood samples drawn	195	190	201
Implants removed	196	190	203
Entry into hibernation	213	208	218
Emergence from hibernation	283	276	289
Behaviour scored1	284	278	290
Blood samples drawn	318	314	323
Behaviour scored*	428	425	434
Blood samples drawn	437	428	446

*Ages shown refer to the start of behavioural testing.

neonates by sex by everting the hemipenes of males, then measured them and placed them in individual cages for captive maintenance. Fresh water was available continuously and food (large earthworms for gravid females, small earthworms or earthworm pieces for neonates) was provided three times a week. The room in which snakes were housed was maintained at about 26°C and 50% RH with a 12:12 h light:dark photic cycle. A heating cable running under one end of the cages provided gravid females with a thermal gradient ranging from room temperature to 35°C.

I divided litters containing at least four males and four females into sham- and hormone-manipulation treatments. Most families were divided into sham- and elevated-testosterone treatment groups, or sham- and flutamide-treatment groups, but a few large families were divided into sham-, elevated-testosterone and flutamide-treatment groups. Within families, at least two males and two females were assigned to each treatment. I scored neonates for three behaviours at 39 days of age (Table 2). Implants were inserted when snakes were 109 days of age and behaviours were scored again when snakes were 190 days of age, after implants had been in place for 81 days. Implants were then removed and snakes were placed in simulated hibernation (7°C, 0:24 h LD photic cycle) for 70 days. Behaviours were scored again following removal from hibernation when snakes were 284 days of age. A final set of behavioural scores was obtained when snakes were 428 days of age. The schedule of hormone manipulation and hibernation used here approximately parallels that used by Crews (1985) in his analysis of garter snake courtship behaviour. The schedule of behavioural tests was designed to detect (1) family and sex effects on behaviour soon after birth (39 days), (2) activational effects of testosterone on behaviour (190 days), and (3) organizational effects of testosterone on behaviour following emergence from hibernation (284 days) or later in life prior to adulthood (428 days) (in nature, garter snakes reach adulthood at 2–3 years of age, Rossman et al. 1996). To document the effectiveness of implants in manipulating testosterone levels and the time course of hormone manipulation, I collected blood samples from neonates at 195 days of age (while implants were in place), 318 days of age and 437 days of age. I also scored neonates for a set of morphological characters that were analysed separately (unpublished data). Upon completion of the study, surviving animals were returned to the wild or maintained in captivity for use in captive breeding.

Hormone Manipulation and Assay

Hormone levels were manipulated using subcutaneous implants. Implants consisted of 7-mm lengths of empty silastic tubing (0.62 mm inner diameter × 1.2 mm outer diameter, Baxter T5715-3), tubing filled with 4–5 mm of crystalline testosterone (Sigma T-1500), or tubing filled with 4–5 mm of flutamide (Sigma F-9397). Implants were sealed with silicon adhesive and inserted at mid-body through a small lateral incision 2–3 scale rows above the venter (following Crews 1985; Crews et al. 1985). Prior to implantation, snakes were immobilized via hypothermia. Ethanol was used to clean the skin prior to implantation. Cyanoacrylate adhesive (Nexaband Liquid, Abbott Laboratories Abbott Park, Illinois, U.S.A.) and cloth first-aid tape was used to seal incisions.

Blood samples (100–300 μl) were collected from caudal blood vessels of unanaesthetized snakes using a heparinized syringe (Bush & Smeller 1978). Blood samples were centrifuged and the plasma fraction was frozen for hormone analysis following completion of bleed collection from all snakes. Testosterone levels were determined by radioimmunoassay as described by King et al. (2000). 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) added to a radioimmunoassay containing ^{3}H testosterone (ca. 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 and unbound steroid was separated using a charcoal-dextran suspension. After centrifugation, the supernatant from each sample was added to vials containing scintillation cocktail (Bio-Safe II, Research Products International, Mt Prospect, Illinois, U.S.A.), and counted in a Beckman liquid scintillation counter. Testosterone levels were determined using a logit–log curve-fitting programme. Testosterone levels at 195, 318 and 437 days were determined in separate assays. Intra-assay variability was 10%, interassay variabitity was 13%, and assay sensitivity ranged from 3 to 3327 pg per sample at 195 days, 5–1275 pg per sample at 318 days, and 6–2065 pg per sample at 437 days. The testosterone antibody used here (provided by G. Niswender, Colorado State University), had high cross-reactivity with 5-alpha-dihydrotestosterone (58%) and lower cross-reactivity with androstenedione (2%). However, because dihydrotestosterone accounts for a relatively small proportion of total androgens in garter snakes (Crews et al. 1985; Mason & Crews, 1985), the assay provides a reasonably accurate estimate of testosterone levels.

Behavioural Tests

I scored three different measures of garter snake behaviour, latency to move, defensive behaviour and response distance. Each behaviour was scored on two consecutive days and averaged across days for analysis. Behavioural tests were conducted in an environmental room maintained at 22°C. Latency to move and defensive behaviour (total number of strikes at a stationary and a moving stimulus) were scored sequentially in a carpeted 75-cm diameter arena. I measured latency to move by placing a snake in the centre of the arena under an 8-cm diameter opaque cover for 2 min, raising the cover from behind one-way glass, and recording the time elapsed until the snake moved its head outside an 11-cm diameter circle marked on the carpet. After 30 s, I recorded the number of strikes at a stationary stimulus. I held the stimulus (my finger) about 2 cm in front of the snake's head and recorded the number of strikes in a 1-min interval. After another 30 s, I recorded the number of strikes at a moving stimulus. In this test I wiggled my finger rapidly from side to side, and again, recorded the number of strikes in 1 min (strikes at a stationary stimulus and strikes at a moving stimulus follow Herzog & Burghardt 1986; see also King & Turmo 1997). I summed the number of strikes at the stationary stimulus and at the moving stimulus for analysis.

I recorded response distance beginning on the day following completion of the other behavioural measures. For this test, I placed a snake under an 8-cm diameter opaque cover in a compartment (10 × 10 cm) at one end of an arena (125 × 10 cm). A stylized predator (a white paper silhouette of a bird's head measuring 4 cm wide × 8 cm high with beak and eyes marked in black) was positioned at the other end of the arena. Plexiglas and a removable partition separated the snake from the rest of the arena. After 2 min, I lifted the cover and removed the opaque partition and then moved the predator from side to side and towards the snake in 10-cm increments using a long rod from behind one-way glass. I recorded the distance at which the snake first responded (e.g. by orienting towards the threat or fleeing rearward). Snakes that showed no change in behaviour were given a score of -10. Thus, snakes that responded to a distant stimulus had high response distance scores and snakes that failed to respond or responded only to a nearby stimulus had low scores.

Behavioural tests were conducted blind to sex and treatment of snakes and to previous behavioural scores. To meet assumptions of normality and equality of variance more closely in the analyses described below, I transformed latency to move using natural logarithms, the number of strikes by adding one and computing the square root, and the response distance by adding 10 and dividing by 110 (converting response distance to a proportion) and then computing the arcsine of the square root.

Analysis

I tested for sex, family and treatment effects using analysis of variance (ANOVA) of behaviour at 39 days of age and repeated measures multivariate analysis of variance (MANOVA) of behaviour over the entire course of the experiment (O'Brien & Kaiser 1985; Potvin et al. 1990; von Ende 1993). In these analyses, scores for a given behaviour at different ages were dependent variables; sex, family and treatment were between-subjects factors; and time (MANOVA only) was a within-subjects factor. MANOVA identifies two general sources of variation: 'between-subjects' and 'within-subjects' effects. Between-subjects effects (main effects of and interactions among the between-subjects factors) reflect differences in a given response variable among factor levels over the entire course of an experiment and can arise during an experiment or from pre-existing differences present at the start of an experiment. In the present study, between-subjects effects were useful in identifying overall effects of sex and family. In contrast, within-subjects effects (main effects of within-subjects factors and interactions among within-subjects and between-subjects factors) reflect differences in how the score of a given response variable changes over time. Within-subjects effects, if significant, provide unambiguous evidence for a treatment effect on behaviour.

I conducted analyses using SPSS 10.0 statistical software. All ANOVA and MANOVA made use of type III sums of squares. Pillai's trace was used in MANOVA significance testing. The assumption of equality of variance was tested using Levene's test. Where this assumption was not met, spread-versus-level plots were examined to ensure that there was no systematic relationship between group means and variances. Examination of the frequency distribution of residuals revealed no marked departures from normality. Each behaviour was tested separately (see Results for evidence of the absence of any strong correlation among behaviours). Because some mortality occurred over the course of the investigation, sample size was maximized over each time interval by conducting separate analyses of behaviour at 39–190 days, 39–284 days and 39–428 days. For analyses of behaviour at 39–284 days and 39–428 days, sample size criteria were relaxed from two to one offspring per sex per treatment. This precluded testing the highest-order interaction in these analyses but this interaction was consistently nonsignificant at 39 days of age (see Results) and over 39–190 days. Comparison of analyses using the original versus relaxed sample size criteria revealed no qualitative difference in the conclusions reached (analyses not shown). For analyses of behaviour at 39–284 days and 39–428 days, reverse Helmert contrasts were used to identify over which time intervals significant changes in behaviour occurred. Except as noted below, results were qualitatively similar for analyses of behaviour at 39–190 days, 39–284 days and 39–428 days and so for simplicity only analyses of the entire 39–428 day interval are presented here. Observed effect size (η^2, computed by SPSS as $SS_{\text{among groups}}/SS_{\text{error}}$) and observed power (the likelihood of committing a type II error given the observed effect size) were computed for all analyses. Observed power is necessarily low when observed effect size is small. For this reason, minimum detectable effect size (η^2_{min}) was also estimated ($\eta^2_{\text{min}}=df_h*F/(df_h*F+df_e)$, where df_h and df_e refer

Table 3. *P* values from repeated measures multivariate analysis of variance of behaviour in sham- versus flutamide-treated garter snakes

Source	*df*	*F*	*P*	η^2	η^2_{min}	Power
Latency to move						
Time	3,42	8.63	<**0.001**	0.381	0.169	0.990
Time*family	15,132	1.68	0.062	0.160	0.166	0.891
Time*sex	3,42	1.25	0.303	0.082	0.169	0.310
Time*treatment	3,42	0.89	0.456	0.059	0.169	0.227
Time*family*sex	15,132	0.55	0.908	0.059	0.166	0.339
Time*family*treatment	15,132	0.40	0.978	0.043	0.166	0.241
Time*sex*treatment	3,42	0.59	0.628	0.040	0.169	0.161
Number of strikes						
Time	3,42	24.09	<**0.001**	0.632	0.169	1.000
Time*family	15,132	3.63	<**0.001**	0.292	0.166	0.999
Time*sex	3,42	0.25	0.863	0.017	0.169	0.093
Time*treatment	3,42	2.88	**0.047**	0.170	0.169	0.646
Time*family*sex	15,132	1.184	0.291	0.119	0.166	0.721
Time*family*treatment	15,132	1.446	0.135	0.141	0.166	0.825
Time*sex*treatment	3,42	0.563	0.642	0.039	0.169	0.156
Response distance						
Time	3,42	4.97	**0.005**	0.262	0.169	0.886
Time*family	15,132	1.93	**0.025**	0.180	0.166	0.937
Time*sex	3,42	2.17	0.106	0.134	0.169	0.513
Time*treatment	3,42	0.43	0.730	0.030	0.169	0.129
Time*family*sex	15,132	0.50	0.938	0.054	0.166	0.305
Time*family*treatment	15,132	1.20	0.277	0.120	0.166	0.729
Time*sex*treatment	3,42	1.08	0.367	0.072	0.169	0.274

Because the primary interest of this analysis is in treatment effects on changes in behaviour over time (i.e. the time*treatment interaction), only within-subjects sources of variation are shown. *P* values less than 0.05 are shown in bold.

to hypothesis and error degrees of freedom and *F* is the corresponding critical value for α=0.05, Stevens 1992, page 177). Effect sizes η^2=0.01, 0.06 and 0.14 correspond to small, medium and large effect sizes (Cohen 1988; Stevens 1992).

I initially compared sham- and flutamide-treated snakes to determine whether these two groups might be pooled in order to increase sample size in subsequent analyses. The general absence of time*treatment interaction effects while implants were in place (see Results), together with the observation that testosterone levels were uniformly low in all but the elevated-testosterone treatment snakes, suggested that the use of flutamide to functionally reduce testosterone levels was unnecessary. Therefore, I pooled sham- and flutamide-treated snakes and conducted two subsequent sets of analyses. The first involved tests for family and sex effects on behaviour in the pooled sham- and flutamide-treatment groups and the second involved tests for treatment effects between the elevated-testosterone treatment group and the pooled sham- and flutamide-treatment groups. Comparison of analyses in which sham- and flutamide-treatment snakes were not pooled with those in which these treatments were pooled revealed no qualitative difference in the conclusions reached (analyses not shown).

RESULTS

Repeatability

Repeatability was computed as the intraclass correlation between measures of each behaviour across successive days. Repeatability was highest when snakes were tested at 39 days of age (r_I=0.62, 0.78 and 0.30 for latency to move, strikes and response distance (*N*=377 animals from 26 families). Repeatability at 190, 284 and 428 days of age ranged from 0.43 to 0.56 for latency to move, 0.65–0.73 for strikes and 0.09–0.19 for response distance (*N*=213, 176 and 165 sham-treated animals from 24 families at 190, 284 and 428 days, respectively).

Flutamide-treatment Effects

Ninety-three offspring (43 males, 50 females) belonging to seven families were divided into sham- and flutamide-treatment groups (*N*=50 and 43, respectively). MANOVA revealed no significant within-subjects effects of treatment (i.e. no time*treatment, time*family*treatment, time*sex*treatment, time*family*sex*treatment effects) on latency to move or response distance over any time interval (Table 3). MANOVA revealed a significant time*treatment effect on strikes over the entire experiment (Table 3, 39–428 days; *P*=0.047). However, this source of variation was far from significant over 39–190 days (*P*=0.745) or 39–284 days (*P*=0.958). Furthermore, reverse-Helmert contrasts revealed that this time*treatment effect arose only during the 284–428 day interval (190 versus 39 days: $F_{1,44}$=0.003, *P*=0.960; 284 days versus previous times: $F_{1,44}$=0.028, *P*=0.867; 428 versus previous times: $F_{1,44}$=11.922, *P*=0.006). Observed

power of tests for within-subjects effects involving treatment was sometimes low and minimum detectable effect size was large (η^2>0.14) over the entire experiment (Table 3). However, analysis over 39–190 days had power sufficient to detect a medium time*treatment effect size (η^2=0.058). Analysis of covariance, with family and treatment as factors and ln(snout–vent length) as a covariate, revealed that ln(testosterone) did not differ between males belonging to sham- and flutamide-treatment groups while implants were in place (195 days: $F_{1,38}$=0.191, P=0.665, η^2=0.005, observed power=0.071, minimum η^2=0.098) or following implant removal (318 days: $F_{1,37}$=0.038, P=0.847, η^2=0.001, observed power=0.054, minimum η^2=0.100; 437 days: $F_{1,32}$= 0.003, P=0.957, η^2<0.001, observed power=0.050, minimum η^2=0.115). Given these results, sham- and flutamide-treatment groups were pooled for the analyses that follow.

Family and Sex Effects

Three hundred and seventy-seven offspring (188 males, 187 females) belonging to 26 families were used to test for sex and family effects on behaviour prior to hormone manipulation. ANOVA revealed highly significant family effects on all three behaviours (Table 4). In addition, there was a highly significant effect of sex on strikes; females struck more frequently than males. No significant family*sex interactions were present. Power was sufficient to detect small sex effects and medium to large family*sex effects (Table 4). Consistent with ANOVA results, MANOVA revealed significant between-subjects effects of family on all three behaviours and of sex on strikes over the entire experiment (Table 4). There were significant within-subjects effects of time and of family (i.e. a time*family interaction) on all three behaviours, indicating that behavioural scores changed over time and that the pattern of change differed among families (Table 4, Fig. 1). Reverse-Helmert contrasts indicated that significant time effects were present over all intervals except when contrasting latency at 428 days with previous times and when contrasting strikes at 190 days with 39 days (Table 4). Latency decreased slightly between 39 and 190 days and increased following emergence from simulated hibernation (Fig. 1). Strikes remained constant from 39 to 190 days, decreased following emergence from simulated hibernation (284 days) and then increased by 428 days (Fig. 1). Response distance decreased from 39 to 190 to 284 days and then increased by 428 days (Fig. 1). Reverse-Helmert contrasts revealed that, with one exception, time*family effects were significant for all three behaviours over all time intervals (Table 4). The one exception was the contrast of response distance at 428 days with previous times. Time*family effects are evident in profile plots (Fig. 1): some families showed increases while other families showed decreases in a given behaviour over a given time interval. The time*sex interaction was consistently nonsignificant, indicating that changes in the behaviour of males and females occurred in parallel (Table 4, Fig. 1) (power was sufficient to detect medium time*sex effects, Table 4).

Testosterone-treatment Effects

Two hundred and sixty-nine offspring (132 males, 137 females) belonging to 17 families were used to test for differences in behaviour between testosterone-treatment group and the pooled sham- and flutamide-treatment groups (N=113 and 156, respectively). Crystalline testosterone was still present in implants upon their removal. Radioimmunoassay of blood samples collected when snakes were 195 days old (after completion of the second set of behavioural tests and before implants were removed) revealed that implants had a marked effect on circulating testosterone levels. Testosterone levels averaged 87.3 pg/ml (95% confidence interval=65.5, 116.4) among 66 sham- and flutamide-treatment males compared with 7950.6 pg/ml (6420.2, 9845.8) in 47 testosterone-treatment males and 8636.0 pg/ml (6840.4, 10 903.1) in 49 testosterone-treatment females (means and confidence intervals backtransformed from natural logarithms). Testosterone levels in elevated-testosterone treatment animals approached those reported in young male garter snakes during a pulse of high testosterone that occurs shortly after birth (24 540–122 490 pg/ml, Table 1 in Crews et al. 1985). However, this pulse was not evident in another natricine snake (King et al. 2000). Testosterone levels in elevated-testosterone treatment animals were similar to those seen in adult male garter snakes (1400–72 000 pg/ml, Table 1 in Weil 1985). Radioimmunoassay of blood samples collected when snakes where 318 days and 436 days of age revealed hormone manipulations had only temporary effects on testosterone levels. At 318 days of age, testosterone levels averaged 78.1 pg/ml (95% confidence interval=62.0, 98.4) among 76 sham- and flutamide-treatment males compared with 82.1 pg/ml (59.8, 112.8) in 53 testosterone-treatment males. At 436 days of age, testosterone levels averaged 1037.4 pg/ml (95% confidence interval=691.1, 1557.3) among 69 sham- and flutamide-treatment males compared with 1192.1 pg/ml (730.8, 1944.8) in 50 testosterone-treatment males. Analysis of covariance, with family and treatment as factors and ln(snout–vent length) as a covariate, revealed no effect of treatment on ln(testosterone) at 318 days ($F_{1,108}$=0.128, P=0.722, η^2=0.001, observed power=0.064, minimum η^2=0.035) or 437 days ($F_{1,98}$=2.554, P=0.113, η^2=0.025, observed power=0.353, minimum η^2=0.035).

MANOVA over the full 428-day experiment revealed that for latency, within-subjects effects involving treatment (time*treatment, time*family*treatment, time*sex*treatment) were consistently nonsignificant (Table 5). For strikes, within-subjects effects involving treatment were also nonsignificant over the full 428-day experiment. However, the time*treatment interaction approached significance over the full experiment (Table 5, P=0.076) and was significant in analyses of strikes at 39–190 days (MANOVA: $F_{1,201}$=4.55, P=0.034) and at 39–284 days ($F_{2,172}$=3.50, P=0.032). Furthermore, reverse-Helmert contrasts indicated that a significant

Table 4. *P* values from analysis of variance and from repeated measures multivariate analysis of variance of behaviour in pretreatment garter snakes (ANOVA: 39 days) and in the pooled sham- and flutamide-treated garter snakes (MANOVA: 39–428 days)

	ANOVA: 39 days						MANOVA: 39–428 days						Reverse-Helmert contrasts					
													190 versus 39 days		284 days versus previous times		428 days versus previous times	
Source	*df*	*F*	*P*	η^2	η^2_{min}	Power	*df*	*F*	*P*	η^2	η^2_{min}	Power	*F*	*P*	*F*	*P*	*F*	*P*
Latency to move																		
Between subjects																		
Family	25,325	14.54	<**0.001**	0.528	0.107	1.000	23,139	10.95	<**0.001**	0.644	0.211	1.000						
Sex	1,325	0.38	0.536	0.001	0.012	0.095	1,139	3.39	0.068	0.024	0.027	0.447						
Family*sex	25,325	0.70	0.860	0.051	0.107	0.612												
TrWithin subjects																		
Time							3,137	5.71	**0.001**	0.111	0.055	0.943	6.00	**0.016**	10.96	**0.001**	1.42	0.236
Time*family							69,417	2.62	<**0.001**	0.303	0.180	1.000	3.22	<**0.001**	3.28	<**0.001**	1.89	**0.013**
Time*sex							3,137	2.48	0.063	0.052	0.055	0.606	0.34	0.561	0.76	0.385	7.04	**0.009**
Number of strikes																		
Between subjects																		
Family	25,325	12.12	<**0.001**	0.483	0.107	1.000	23,139	6.60	<**0.001**	0.522	0.211	1.000						
Sex	1,325	9.13	**0.003**	0.027	0.012	0.854	1,139	16.03	<**0.001**	0.103	0.027	0.978						
Family*sex	25,325	0.77	0.780	0.056	0.107	0.671												
Within subjects																		
Time							3,137	81.50	<**0.001**	0.641	0.055	1.000	0.22	0.642	222.57	<**0.001**	15.43	<**0.001**
Time*family							69,417	3.32	<**0.001**	0.355	0.180	1.000	3.74	<**0.001**	3.30	<**0.001**	4.80	<**0.001**
Time*sex							3,137	0.25	0.860	0.005	0.055	0.097	0.04	0.839	0.08	0.780	0.51	0.478
Response distance																		
Between subjects																		
Family	25,325	6.73	<**0.001**	0.341	0.107	1.000	23,139	2.14	<**0.004**	0.261	0.211	0.992						
Sex	1,325	1.00	0.319	0.003	0.012	0.169	1,139	1.45	0.230	0.010	0.027	0.223						
Family*sex	25,325	1.03	0.425	0.073	0.107	0.833												
Within subjects																		
Time							3,137	30.14	<**0.001**	0.398	0.055	1.000	42.37	<**0.001**	49.42	<**0.001**	6.43	**0.012**
Time*family							69,417	2.18	<**0.001**	0.265	0.180	1.000	3.16	<**0.001**	2.69	<**0.001**	1.28	0.193
Time*sex							3,137	0.23	0.875	0.005	0.055	0.093	0.18	0.670	0.26	0.614	0.16	0.689

P values less than 0.05 are shown in bold.

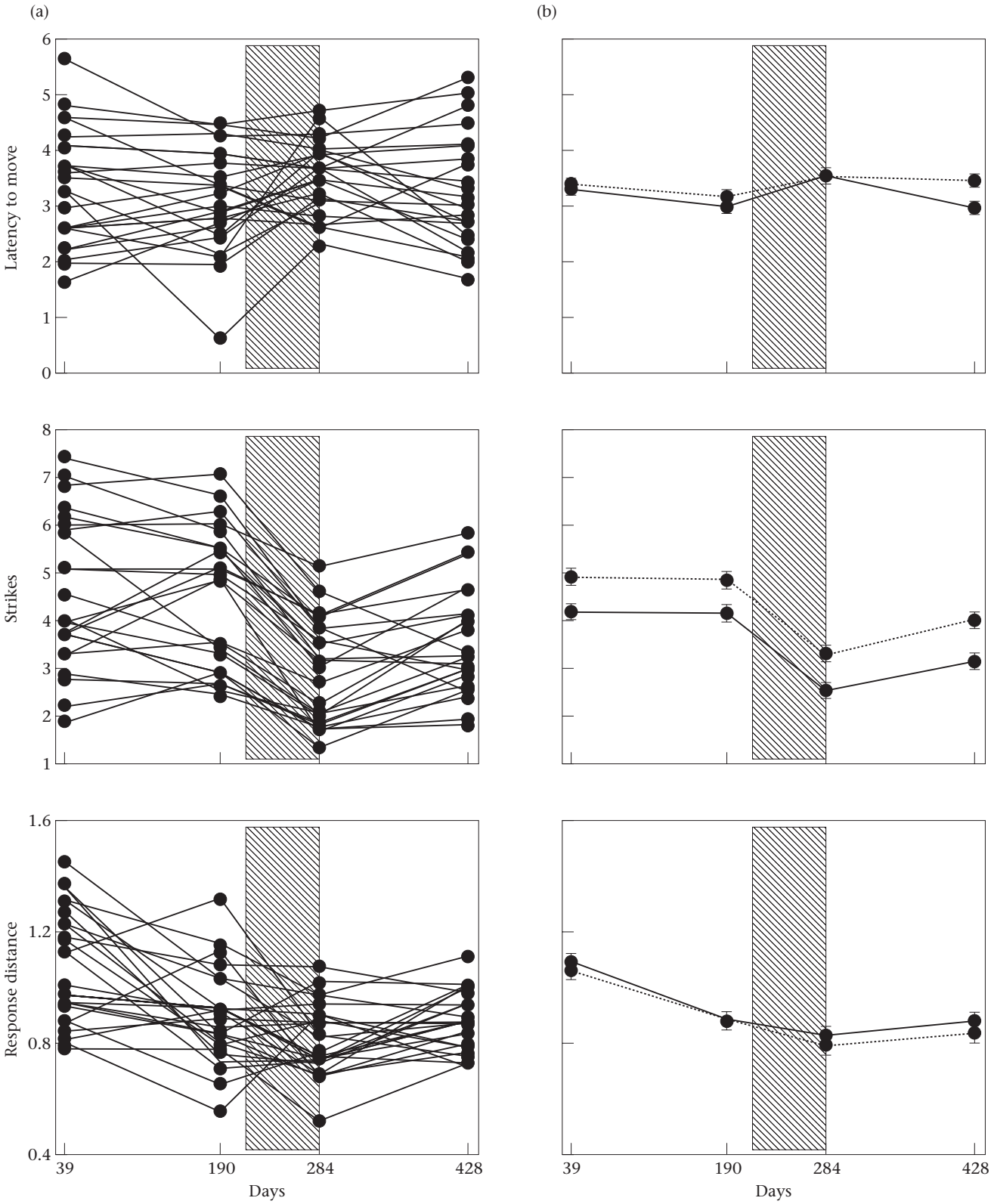

Figure 1. Profile plots showing variation in estimated marginal means for latency to move (ln transformed seconds), number of strikes (square-root transformed) and response distance (arcsine square-root transformed proportions) at 39 days, 190 days, 284 days and 428 days. (a) Variation among families, with families represented by separate lines. (b) Variation between male (—■—) and female (– –○– –) garter snakes (bars represent ±1 standard error of estimated mean). The between-subjects effect of family and the within-subjects time-by-family interaction were significant for all three behaviours; the between-subjects effect of sex was significant for strikes. Snakes underwent simulated hibernation between 213 and 283 days (▨).

Table 5. *P* values from repeated measures multivariate analysis of variance of behaviour in testosterone-treated versus pooled sham- and flutamide-treated garter snakes

							Reverse-Helmert contrasts					
							190 versus 39 days		284 days versus previous times		428 days versus previous times	
Source	*df*	*F*	*P*	η^2	η^2_{min}	Power	*F*	*P*	*F*	*P*	*F*	*P*
Latency to move												
Time	3,125	11.57	<**0.001**	0.218	0.060	**0.999**	11.46	**0.001**	22.61	<**0.001**	0.51	0.477
Time*family	36,381	4.19	<**0.001**	0.283	0.124	**1.000**	6.26	<**0.001**	4.59	<**0.001**	4.82	<**0.001**
Time*sex	3,125	0.96	0.417	0.022	0.060	0.256	0.48	0.490	0.02	0.892	2.29	0.132
Time*treatment	3,125	1.57	0.200	0.036	0.060	0.405	0.15	0.696	4.04	**0.047**	0.15	0.698
Time*family*sex	36,381	1.25	0.161	0.105	0.124	0.972	1.05	0.407	1.87	**0.045**	1.14	0.336
Time*family*treatment	36,381	0.78	0.820	0.068	0.124	0.797	0.74	0.709	0.67	0.774	0.95	0.499
Time*sex*treatment	3,125	1.46	0.229	0.034	0.060	0.379	2.87	0.093	0.97	0.326	0.52	0.472
Number of Strikes												
Time	3,125	86.09	<**0.001**	0.674	0.060	**1.000**	2.47	0.119	257.10	<**0.001**	2.36	0.127
Time*family	36,381	4.19	<**0.001**	0.484	0.124	**1.000**	3.77	<0.001	4.41	<**0.001**	4.85	<**0.001**
Time*sex	3,125	0.99	0.401	0.023	0.060	0.264	1.15	0.286	0.74	0.390	1.09	0.300
Time*treatment	3,125	2.35	0.076	0.053	0.060	0.578	5.89	**0.017**	0.65	0.424	0.53	0.469
Time*family*sex	36,381	0.97	0.528	0.084	0.124	0.900	1.04	0.414	1.08	0.379	0.91	0.542
Time*family*treatment	36,381	1.01	0.465	0.087	0.124	0.915	0.59	0.850	1.40	0.175	1.19	0.299
Time*sex*treatment	3,125	0.32	0.810	0.008	0.060	0.111	0.94	0.335	0.11	0.745	0.06	0.801
Response distance												
Time	3,125	43.81	<**0.001**	0.513	0.060	**1.000**	52.59	<**0.001**	70.48	<**0.001**	12.73	<**0.001**
Time*family	36,381	2.28	<**0.001**	0.177	0.124	**1.000**	2.62	**0.004**	2.73	**0.003**	1.95	**0.034**
Time*sex	3,125	0.21	0.891	0.005	0.060	0.088	0.06	0.802	0.45	0.506	0.10	0.752
Time*treatment	3,125	0.43	0.733	0.010	0.060	0.134	0.53	0.468	0.49	0.485	0.34	0.560
Time*family*sex	36,381	1.47	**0.043**	0.122	0.124	**0.991**	1.59	0.104	2.17	**0.017**	0.99	0.462
Time*family*treatment	36,381	1.49	**0.037**	0.124	0.124	**0.992**	1.88	**0.043**	1.15	0.325	1.48	0.141
Time*sex*treatment	3,125	0.36	0.783	0.009	0.060	0.119	0.80	0.372	0.01	0.914	0.34	0.560

Because the primary interest of this analysis is in treatment effects on changes in behaviour over time (i.e. the time*treatment interaction), only within-subjects sources of variation are shown. *P* values less than 0.05 are shown in bold.

treatment effect occurred while testosterone-containing implants were in place: the contrast between 190 days and 39 days was significant but other contrasts were nonsignificant (Table 5). The direction of the treatment effect paralleled the difference seen between males and females in that males (Fig. 1) and testosterone-treatment animals (Fig. 2) showed lower frequencies of strikes than did females and sham-treatment animals, respectively. For response distance, the time*family*treatment interaction was significant over the full 428 day experiment but other within-subjects effects involving treatment (time*treatment, time*sex*treatment) were nonsignificant (Table 5). Again, reverse-Helmert contrasts indicated that this treatment effect occurred only while testosterone-containing implants were in place: only the contrast between 190 days and 39 days was significant (Table 5). However, the time*family*treatment effect was nonsignificant in analyses of response distance measured at 39 and 190 days (MANOVA: $F_{16,201}$=1.52, P=0.095) and at 39, 190 and 284 days (MANOVA: $F_{32,346}$=1.37, P=0.091). Other within-subjects effects on response distance involving treatment (time*treatment, time*sex*treatment) were consistently nonsignificant. Power was sufficient to detect medium time*treatment and time*sex*treatment effects and medium to large time*family*treatement effects (Table 5).

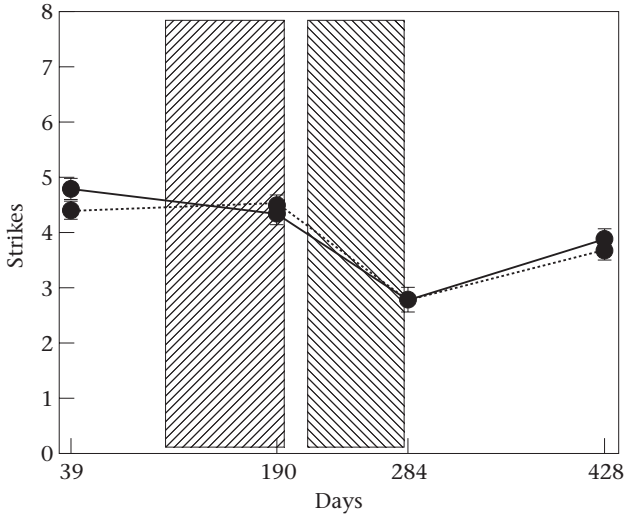

Figure 2. Profile plot showing variation in estimated marginal means for number of strikes (square-root transformed) between testosterone-treated (—■—) and pooled sham- and flutamide-treated garter snakes (– –●– –) (bars represent ±1 standard error of estimated mean). The within-subjects time-by-treatment interaction was significant over the 39–190-day interval. Implants were in place between 109 and 196 days (▨) and snakes underwent simulated hibernation between 213 and 283 days (▧).

Table 6. Correlations among measures of the same behaviour over time and among different behaviours

	Latency to move				Number of strikes				Response distance			
	39 days	190 days	284 days	428 days	39 days	190 days	284 days	428 days	39 days	190 days	284 days	428 days
Latency to move												
39 days		**0.385**	**0.299**	**0.263**	0.009	−0.013	0.079	−0.039	0.053	−0.024	−0.081	−0.129
190 days			**0.291**	**0.365**	0.018	0.073	0.033	0.006	0.099	0.073	−0.002	−0.190
284 days				**0.197**	−0.064	0.108	−0.018	0.075	0.065	0.000	0.003	−0.031
428 days					−0.011	−0.038	0.060	−0.244	0.092	−0.087	−0.034	−0.219
Number of strikes												
39 days						**0.568**	**0.466**	**0.457**	−0.054	0.034	0.000	−0.007
190 days							**0.590**	**0.590**	−0.126	**−0.251**	0.071	0.015
284 days								**0.485**	−0.054	**−0.265**	0.075	0.059
428 days									−0.069	−0.075	0.048	0.126
Response distance												
39 days										0.099	0.098	−0.021
190 days											**0.286**	**0.257**
284 days												0.165
428 days												

Entries represent Pearson product-moment correlations among residuals from MANOVA analysis of behaviour over the full 428-day experiment. N=167. Statistically significant correlations (following adjustment for multiple tests by use of α=0.05/48 for correlations among different behaviours and α=0.05/6 for correlations among measures of the same behaviour over time) are shown in bold. Correlations obtained from analyses of behaviour at 39 days, 39–190 days and 39–284 days were similar in magnitude.

Correlations among Behaviours

Pearson correlations among residuals of the three behavioural scores at 39 days were nonsignificant (latency versus strikes, $r_{375} = -0.064$; latency versus response distance, $r_{375} = -0.045$; strikes versus response distance, r_{375}=0.090). Significant positive correlations were typically present between measures of the same behaviour over time (Table 6). These correlations were strongest for strikes (range 0.457–0.590), intermediate for latency to move (0.197–0.385), and weakest (and sometimes nonsignificant) for response distance (-0.021–0.286). Small but significant negative correlations were present between strikes at 190 days and response distance at 190 days and between strikes at 284 days and response distance at 190 days (Table 6). Use of residuals removes the effects of sex, family membership and treatment on these correlations.

DISCUSSION

Perhaps the clearest result of this investigation is the pervasive effect family membership had on garter snake behaviour. Such family effects have been found consistently in studies of the behaviour of natricine snakes (garter snakes and their allies) (reviewed by Brodie & Garland 1993; Burghardt & Schwartz 1999; see also Burghardt et al. 2000). However, this study goes further in documenting the presence of significant time*family interactions, indicating that ontogenetic trajectories also vary among families (Fig. 1). Patterns of variation within and between families have been used previously to estimate the heritability of defensive behaviour in natricine snakes (Brodie & Garland 1993; Burghardt & Schwartz 1999; Burghardt et al. 2000). Applying a full sibling analysis (Brodie & Garland 1993) to behaviours measured at 39 days of age yields heritability estimates of 0.65 (approximate 95% confidence interval: 0.37, 0.95) for latency, 0.56 (0.30, 0.86) for strikes, and 0.33 (0.13, 0.56) for response distance (heritability was computed as $2 \times \sigma^2_{family}/(\sigma^2_{family}+\sigma^2_{error})$ with σ^2_{family} and σ^2_{error} obtained from a two-factor ANOVA with family and sex included as factors; confidence intervals were computed as in Becker 1992, following modification for the inclusion of sex as a factor). These estimates assume that litters consist of full siblings and that maternal effects are negligible. Evidence is mounting that in some natricines, including *T. sirtalis*, multiple paternity within litters is commonplace (Barry et al. 1992; Garner 1998; Gibson & Falls 1975; McCracken et al. 1999; Prosser 1999; Schwartz et al. 1989). By itself, multiple paternity should lead to underestimates of h^2 using full sibling analysis. King et al. (2001) have taken advantage of the occurrence of multiple paternity within litters to explore the possibility that maternal effects also contribute to between-family variation. Based on an analysis of four litters each sired by two males (eight sireships total), it appears that maternal effects may markedly inflate estimates of h^2 in natricines obtained using full sibling analysis. The nature of these maternal effects remains unexplored and may include both maternal environmental effects (effects of the common uterine environment shared by littermates) and maternal genetic effects (effects of maternal genotype on offspring phenotype).

In the present study, variation between families may have been inflated by year and site effects: gravid females were collected in 3 years and from five sites (Table 1). Efforts were made to minimize year effects by using uniform rearing and testing conditions. Furthermore, site effects are likely to be small because sites were separated

by less than 27 km and molecular genetic analyses suggest that gene flow among them is common (Bittner 1999; Lawson & King 1996). Examination of family means revealed no consistent year or site effects on the results presented here. However, small but significant differences in behaviour have been observed in wild-caught adult garter snakes from these sites (R. B. King & T. D. Bittner, unpublished data).

A second clear result of this investigation is the presence of sex differences in strikes but not in latency or response distance. For all three behaviours, my analyses were of sufficient power to detect even small differences between males and females using Cohen's (1988) effect size criteria (Table 4). Thus, the absence of sex effects on latency and response distance appears to be biologically meaningful and not simply a result of type II error. The effect of sex on strikes differs from the family effect described above in that it is smaller in magnitude and does not include an ontogenetic component: ontogenetic changes in strikes among males parallel those among females (Fig. 1). Sex differences in behaviour have been demonstrated in laboratory studies of some neonatal natricines but not others. Male Butler's garter snakes, *T. butleri*, and southern water snakes, *Nerodia fasciata*, strike more frequently than do females (Scudder & Burghardt 1983; Herzog & Burghardt 1986). In contrast, among the common garter snakes tested here, females struck more frequently than males. Sex differences are apparently lacking in other natricine snakes (e.g. *T. melanogaster*, *T. ordinoides, T. radix*, *N. cyclopion*, *N. rhombifera*, *N. sipedon*; Scudder & Burghardt 1983; Herzog & Burghardt 1986; E. D. Brodie III, unpublished data; A. Queral-Regil & R. B. King, unpublished data; R. B. King & D. Anderson, unpublished data). However, detection of sex differences may require moderately large sample sizes. In contrast to the results presented here, an earlier study of 90 common garter snakes born to seven females failed to reveal any difference between males and females (Herzog & Burghardt 1986). Sex differences in diet, foraging behaviour, spatial patterns and movement have been observed in snakes in the wild (Gregory et al. 1987; Shine 1991; Reinert 1993). Whether the sex difference in strikes documented here and in the studies cited above is related to behavioural differences in nature is unknown.

A third result of this investigation is an apparent effect of testosterone manipulation on strikes. Although this effect was relatively small, the difference between treatment groups parallels the difference seen between males and females. Males (the sex with intrinsically higher testosterone levels) struck less frequently than did females and snakes that received implants containing testosterone showed a decrease in strike frequency relative to sham-manipulated animals. Sexually monomorphic behaviours (latency, response distance) were generally unaffected by hormone manipulation although, admittedly, small treatment effects may have gone undetected because my analyses were only of sufficient power to detect medium effects using Cohen's (1988) effect size criteria (Table 5). The effect of treatment on strikes was only evident while implants were in place. Following implant removal, differences in behaviour and in testosterone level between treatment groups were nonsignificant. This suggests that testosterone has an activational effect on strikes. In contrast, testosterone has an organizational effect on garter snake courtship behaviour: elevating testosterone levels early in life elicits courtship behaviour later in life, well after effects on circulating hormone levels have passed (Crews 1985).

Manipulation of testosterone level had a similar effect on males and females (time*sex*treatment effects were consistently nonsignificant). However, it is unknown whether this effect was a direct result of testosterone manipulation or an effect mediated through the conversion of testosterone to dihydrotestosterone (DHT) or oestrogen. Significantly, cells that concentrate testosterone and oestrogen are equally common in many areas of the brains of male and female garter snakes, including areas such as the amygdala, which is associated with limbic responses like defensive behaviour (Halpern et al. 1982). Further experiments that directly manipulate DHT or oestrogen levels or that manipulate testosterone levels in the presence of inhibitors such as 5-α-reductase (the enzyme responsible for conversion of testosterone to DHT) or aromatase (the enzyme responsible for conversion of testosterone to oestrogen) would be useful (Moore & Lindzey 1992).

A fourth result of this investigation is the presence of a time*family*treatment interaction effect on response distance but not on latency to move and strikes: families responded differently to hormone manipulation for response distance. This result suggests that variation in response distance among families may be mediated through receptor- or postreceptor-level phenomena. In contrast, families responded similarly (or not at all) to hormone manipulation for latency to move and strikes although admittedly, my analyses were only of sufficient power to detect large effects using Cohen's (1988) effect size criteria (Table 5). Taken at face value, the lack of time*family*treatment interaction effects on latency and strikes suggests that variation between families in these behaviours is not mediated through receptor- or postreceptor-level phenomena. Instead, given that among males, families differ in circulating testosterone levels (R. B. King, J. H. Cline & C. J. Hubbard, unpublished data; see also King et al. 2001), it is possible that genetic or maternally induced variation in some garter snake behaviour (e.g. strikes) is mediated through processes that influence circulating hormone levels (e.g. responsiveness to environmental stimuli, responsiveness to releasing hormones, rate of secretion, androgen binding proteins in the plasma, hormone half-life).

Detecting an effect of individual variation in circulating testosterone level on behaviour using the data gathered in this study is complicated by the fact that testosterone level changes over time, precluding its use as a covariate in the repeated measures analyses used here to test for family, sex and treatment effects. In addition, testosterone was not assayed in all individuals (e.g. pilot assays indicated only marginally detectable levels in sham- and flutamide-treatment females, and insufficient blood volume was obtained from some individuals). To avoid these problems, I used analysis of covariance

(ANOVA) to test for a possible effect of testosterone level, sex and family on behaviour among 91 testosterone-treatment animals from 15 families at 190 days of age (while implants were in place). I also used ANCOVA to test for an effect of testosterone level and family on behaviour using data on 73 males from 10 families at 284 and 428 days (after implants had been removed and testosterone had returned to baseline). Covariation between testosterone level and strikes was consistently nonsignificant despite the fact that strikes was the one behaviour that most clearly showed a testosterone treatment effect. Covariation between testosterone level and behaviour did achieve statistical significance for response distance among testosterone-treatment animals at 190 days of age (P=0.049) and for latency to move among males at 284 days (P=0.041) but not at other times. Thus, evidence that individual variation in testosterone levels has detectable effects on behaviour is equivocal at best. However, the results of thus study suggest that future experiments designed specifically to test for such effects might be warranted.

Garter snakes grew markedly over the course of this investigation, from a mean of 1.6 g at birth to a mean of 19.7 g at 438 days of age. Furthermore, females exceed males in body size as adults, a difference thought to be mediated by an inhibitory effect of testosterone on growth of males (Crews et al. 1985). Thus, it is possible that the differences in garter snake behaviour among families, sexes and treatments reported here are attributable to differences in body size. Several lines of evidence suggest that this is not the case (a more detailed analysis of family, sex and testosterone effects on garter snake morphology will be presented elsewhere). (1) Neither mass nor snout–vent length (SVL) covaried significantly with behaviour at 39, 190 or 284 days of age. SVL did covary significantly with latency to move at 428 days of age (ANOVA with sex, family and treatment as factors, P=0.016). (2) Size residuals and behaviour residuals generated from mutivariate analyses that included latency, strikes, response distance and mass or SVL as dependent variables, and sex, family and treatment as factors were uncorrelated at 39, 190 and 284 days of age. Residual SVL was positively correlated with residual latency at 428 days (Pearson's correlation: r_{162}=0.22, P<0.05) indicating that by this age, larger snakes were slower to move than were smaller snakes. (3) Differences in body size between males and females and between treatment groups, although statistically significant, were small. At 438 days, males exceeded females in SVL by just 1.4% (358 versus 353 mm) and females exceeded males in mass by 2.6% (20.1 versus 19.6 g). At 195 days (immediately following implant removal), testosterone-treated animals exceeded sham- and flutamide-treated animals in SVL by less than 1% (276 versus 278 mm) and sham- and flutamide-treated animals exceeded testosterone-treated animals in mass by 3% (9.8 versus 9.5 g).

Although variation in body size does not appear to explain the family, sex or treatment effects on behaviour reported here, increasing body size may have contributed to temporal changes in behaviour. This is especially true for response distance, which decreased consistently over the course of this investigation (Fig. 1, Table 4). One interpretation is that larger (older) snakes are less vulnerable to predators and thus are less responsive to an approaching threat. Temporal changes in latency to move and strikes may be associated with emergence from simulated hibernation. Snakes were slower to move and struck less frequency at 284 days (1–2 days after emergence) than on earlier test dates (Fig. 1, Table 4). Changes in behaviour associated with recovery of physiological functions following emergence from hibernation have been reported in natural populations of garter snakes as well (Shine et al. 2000).

North American natricine snakes have become a model system in evolutionary quantitative genetics (Brodie & Garland 1993; King & Lawson 1995; Arnold & Phillips 1999). Much previous work with these snakes has focused on estimating heritability, genetic correlation and selection on quantitative characters in an effort to assess potential for evolutionary change. These studies have been groundbreaking in demonstrating how genetic correlations among traits may constrain evolutionary change (Arnold 1988), how combinations of traits (e.g. behaviour and morphology) can be the target of correlational selection (Brodie 1989), and how gene flow can slow adaptive evolution (King & Lawson 1995). In contrast, the work reported here and other recent studies of natricines have focused on how proximate mechanisms (e.g. hormonal pathways, maternal effects, experience) mediate the expression of quantitative traits (Burghardt et al. 2000; King et al. 2001). This emphasis on proximate mechanisms is complimentary to an evolutionary quantitative genetic approach. One interest in evolutionary quantitative genetics is the relative constancy of genetic correlations among traits (the genetic variance–covariance matrix, **G**) (Arnold & Phillips 1999). A constant **G** simplifies prediction of long-term evolutionary change but implies that such change is constrained by the specific form of **G**. Tests for constancy have typically involved comparisons of **G** among populations or closely related taxa (e.g. Arnold & Phillips 1999). Alternatively, insight into the constancy of **G** can come from investigations of proximate mechanisms. Studies such as this one can reveal the degree to which suites of traits are influenced by common pathways (e.g. a single hormonal control mechanism) and the ease with which expression of such traits might become uncoupled. The observation that number of strikes is influenced by testosterone levels, whereas latency to move and response distance are not, suggests that these behaviours may evolve relatively independently.

More generally, this study demonstrates the utility of combining quantitative genetic and endocrinological approaches to the study of behaviour. Evidence is accumulating for heritable variation in circulating levels of a range of hormones and hormone-binding proteins (e.g. Jaquish et al. 0000; Meikle et al. 1986, 1988a, b; Zarazaga et al. 1998). Furthermore, a number of studies provide evidence for correlations between hormonal variation and variation in other phenotypic traits, including behaviours related to activity, avoidance and aggression (e.g. Glowa et al. 1992; Compaan et al. 1993; Gupta &

Brush 1998). These studies lend credence to the proposal that genetic variation in phenotype may be mediated through hormonal pathways. However, studies of gene–hormone–behaviour interactions outside of humans and domesticated animals remain rare. A notable exception involves the evolutionary endocrinology of crickets, in which juvenile hormone esterase, wing polymorphism and migratory traits appear to be functionally interrelated (Fairbairn & Roff 1999; Zera 1999; Zera & Huang 1999). The widespread occurrence of geographical variation in behaviour (Foster & Endler 1999) as well as behavioural differences among species and higher taxonomic levels suggests that studies of gene–hormone–behaviour interactions might also benefit from geographical and phylogenetic perspectives.

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