

## The Developmental Effect of Sex Ratio on a Sexually Dimorphic Scale Count in the Garter Snake *Thamnophis elegans*

NICOLA M. OSYPKA<sup>1,2</sup> AND STEVAN J. ARNOLD<sup>1,3,4</sup>

<sup>1</sup>Department of Ecology and Evolution, University of Chicago, Chicago, Illinois 60637, USA

**ABSTRACT.**—In some mammals the development of embryos is affected by the sex of adjacent fetuses through increased or decreased levels of sex steroids present in the uterus. The effects of masculinization and de-feminization can be observed in behavior as well as in morphology. In this study we examined whether similar effects occur in a viviparous reptile, the garter snake *Thamnophis elegans*. We tested two populations of *T. elegans* for the effect of prenatal sex ratio on the development of a sexually dimorphic tail scale count, in which males have significantly more subcaudal scales than females. The results show that females from one population have significantly fewer subcaudal scales in litters with a higher proportion of males. No such trend was observed in males. The other population shows a significant difference in regression slopes comparing females to males, but the slopes cannot be shown to differ from zero in either sex. Unlike in mammals where testosterone is the organizing hormone for sexual differentiation, the results in *T. elegans* suggest that estrogen, after having been aromatized from testosterone, may influence the development of the subcaudal scale count in female garter snakes.

The intrauterine position of fetuses plays a significant role in the expression of sex-characteristic behavior and morphology in mammals. The classic condition is that of twinned cattle fetuses (Keller and Tandler, 1916; Lillie, 1916). The union of the fetal membranes and placentae results in anastomosis of the embryonic circulations and a masculinization of the female in male-female twins. Such females are infertile and are called freemartins. They have testes and a male gonoduct system, but the secondary (external) sex characteristics are feminine. Most subsequent studies on the influences of intrauterine positions have been done in rodents (Gandelmann et al., 1977; vom Saal and Bronson, 1980; Meisel, 1981; Richmond and Sachs, 1984; Houtsmuller and Slob, 1990; Clark et al., 1991) and swine (Rhode Parfet et al., 1990). The basic principle of sex differentiation that holds across mammalian species is that testosterone is the organizing hormone for most morphological and behavioral characteristics, while the female is the "default" sex. Studies on rodents support the contiguity hypothesis (influence by neighboring fetuses). Female mice that are surrounded by two male embryos during gestation exhibit less lordosis, are more aggressive, have longer neonatal anogenital distances, show later vaginal opening and have higher fetal testoster-

one titers than their female litter mates who are surrounded by female embryos (Gandelmann et al., 1977; vom Saal and Bronson, 1980). Similarly, in gerbils, androgen levels were found to be lower in male fetuses that developed between two females than in those not developing adjacent to female sibs, independent of sex ratio or number of males in their respective litters (Clark et al., 1991). Clark et al. (1991) also showed that, when intrauterine location relative to the opposite sex is held constant, there are (a) significant positive correlations between both the number and proportion of male gerbil fetuses in a litter and fetal androgen levels of that litter, and (b) a significant correlation between circulating concentrations of androgens in mothers and their respective male fetuses. In contrast, female rats are prenatally defeminized (i.e., show decreased lordosis behavior) and masculinized (i.e., show increased mounting behavior) not by adjacent males but by caudal males in the same uterine horn (caudal male hypothesis, Houtsmuller and Slob, 1990). Houtsmuller and Slob (1990) suggested that the effect of male littermates is additive, since even females from all-female litters and females with no caudal males display high levels of testosterone-induced mounting behavior.

In reptiles the prenatal environment is also known to affect the development of morphology and behavior. In leopard geckos (*Eublepharis*), as in many other reptiles, the sex of the offspring is determined by the incubation temperature (temperature-dependent sex determination or TSD). Flores et al. (1994) found that female *Eublepharis* born in litters from temperatures that produce male biased sex ratios are more ag-

<sup>2</sup> Present Address: Department of Psychology, University of California, Berkeley, California 94720-1650, USA, E-mail: nosypka@aol.com.

<sup>3</sup> Department of Zoology, Oregon State University, Corvallis, Oregon 97331-2914, USA, E-mail: arnolds@bcc.orst.edu

<sup>4</sup> Corresponding Author: Stevan J. Arnold.

gressive and less attractive to males, and have higher androgen levels, than females incubated at temperatures that produce female biased sex ratios. Morphological differences can also be seen in females that were incubated at male-biased temperatures; these develop pubic glands with secretory pores similar to those found in males, rather than the small glands and closed pores seen in females at female-biased temperatures (Crews, 1994). In the garter snake (*Thamnophis sirtalis*) males have a shorter jaw length (smaller head) and snout-vent length than females (Shine and Crews, 1988). If newborn males are castrated, they develop a larger head than females, unless they are treated with testosterone within their first two years of life. The snout-to-vent length of castrated males does not differ significantly from normal ones. However, jaw length differs significantly between castrates and normals, and testosterone seems to be the factor that inhibits head growth in the normal male.

Effects of adjacent fetuses on development have never been investigated in reptiles. However, masculinizing or de-feminizing effects such as those seen in mammalian species might be expected in viviparous reptiles. We tested for the effect of prenatal sex ratio on the development of a sexually dimorphic scale count in a viviparous snake, *Thamnophis elegans*. In this species, the sex of the offspring is not determined by temperature but by sex chromosomes. The number of subcaudal scales corresponds to the number of tail vertebrae.

In *T. elegans*, as in all garter snake species, males have significantly more tail vertebrae than females (Arnold, 1988). Although testosterone shortens jaw length in male *T. sirtalis* (Shine and Crews, 1988), it may enhance tail length by increasing the number of tail vertebrae. In a litter with a proportionally larger number of males, females might develop in an environment with more circulating androgens than in a litter with a 1:1 sex ratio. Consequently, females might be less feminized, as evidenced by a larger than average subcaudal scale count. Similarly, male fetuses developing in a predominantly female litter might be feminized and show a smaller than average subcaudal scale count.

We examined the possibility of intrauterine effects by testing for a statistical association between the sex ratio of litters and the average subcaudal scale counts of male and female neonates. We view this analysis as a first step in testing for intrauterine effects exerted by neighboring embryos in reptiles and a precursor to the more laborious procedure of establishing the sex of neighboring embryos.

#### MATERIALS AND METHODS

Subcaudal scale counts and sex were determined for neonates born to 268 females, which were collected at two localities in northern California, called coastal and inland (see Arnold, 1988 for a description of the localities). The coastal (107 litters) and the inland populations (161 litters) yielded 936 and 1545 offspring, respectively, for a total of 2481 neonates. The sex of the neonate was determined by eversion of hemipenes. The sex ratio used in statistical analyses was based on all littermates for which sex could be determined, including those few neonates whose subcaudal scales could not be counted due to broken tails or deformities (3.4% for the coastal, 6.4% for the inland population). The subcaudal scale counts of mothers was used in some statistical analyses, and in those cases mothers with incomplete tails were excluded from the sample.

Statistical analysis was conducted using Minitab, version 10 (Minitab Inc). In regression analyses, the mother's scale count was included as a covariate and each litter was weighted using the total number of offspring in the litter (i.e., including those without subcaudal counts). The mothers' counts were used as a covariate because the subcaudal count is moderately heritable (Arnold, 1988). By using the mother's count as a covariate, we hoped to control for genetic differences among litters in subcaudal count and thereby improve the chance of detecting the nongenetic effects of hormones.

For the purpose of statistical analysis, sex ratio [number of males / (number of males + number of females)] was transformed by taking the arcsine of the square root of sex ratio. This transformation often yields normal distributions for variables that are proportions (Sokal and Rohlf, 1981).

#### RESULTS

The inland population showed a unimodal distribution that deviated significantly from normality (Shapiro-Wilk  $W = 0.9389$ ,  $P < 0.0001$ , Fig. 1a). The sex ratio of the coastal population showed a unimodal, normal distribution (Shapiro-Wilk  $W = 0.9782$ ,  $P = 0.415$ , Fig. 1b). The modes in both populations are close to a 50:50 sex ratio. Note that on the transformed scale in Fig. 1, a value of 0.785 corresponds to a 50:50 sex ratio.

On average males had 10–14% more subcaudal scales than females. The male mean in the coastal population was 80.7, whereas the female mean was 71.1 ( $t = 48.43$ ,  $P < 0.0005$ ). In the inland population the means were 90.7 and 80.6 for males and females, respectively ( $t = 54.41$ ,  $P < 0.0005$ ). Thus, sexual dimorphism for subcaudal scales is similar in the two populations.

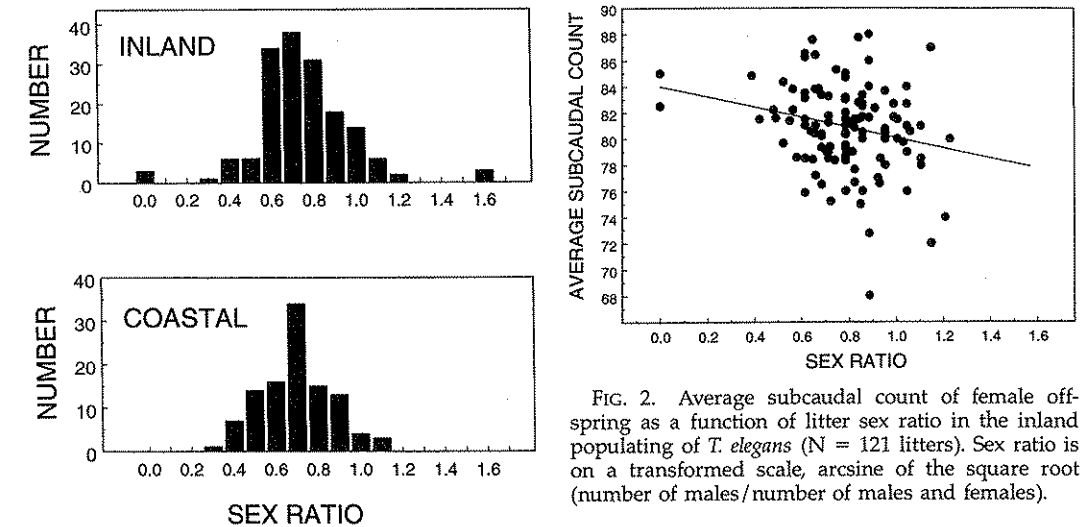


FIG. 1. Distribution of litter sex ratios in inland ( $N = 161$  litters) and coastal ( $N = 107$  litters) populations of *T. elegans*. Sex ratio is on a transformed scale, arcsine of the square root (number of males / number of males and females).

The partial regression coefficients relating subcaudal scale counts to sex ratio showed the same trend in both populations and in both sexes; the higher the proportion of males in the litter, the lower the number of scale counts in males and females. The only statistically significant regression with sex ratio, however, was obtained for females of the inland population (Fig. 2). We tested for a difference in regression slopes for males and females within each population by calculating the difference between the subcaudal means of females and males in each litter and regressing that difference on sex ratio. The regression slope for the difference variable on sex ratio was not different from zero in either population ( $P > 0.05$ ). Thus, there is no indication that regression slope relating female subcaudal count to sex ratio is greater than the corresponding male slope in either population.

#### DISCUSSION

Our populations adhere to the general rule that snake populations show 50:50 sex ratios (Shine and Bull, 1977; Gutzke et al., 1985). Past studies of snake sex ratios have been based on samples of less than 50 litters. Larger samples for our two *T. elegans* populations allow us to examine the distribution of sex ratios within those populations. That examination revealed unimodal distributions with modes near 50:50, which suggests that a single process of genetic sex determination prevails in these populations. In contrast, environmental sex determination or facultative parthenogenesis would have pro-

FIG. 2. Average subcaudal count of female offspring as a function of litter sex ratio in the inland population of *T. elegans* ( $N = 121$  litters). Sex ratio is on a transformed scale, arcsine of the square root (number of males / number of males and females).

duced bimodal distributions. Although facultative parthenogenesis has been proposed for one captive-reared *T. elegans* (Schuett et al., 1997), no signature of this reproductive mode could be detected in our populations.

Inland females appear to be feminized (fewer subcaudal scales) in litters in which males predominate. Mammalian studies show the opposite effect: females are masculinized by adjacent male fetuses. In mammals, testosterone is the organizing hormone during sexual differentiation, while the female is the "default" sex. Garter snakes may have a different system.

The female feminization result might be explained by conversion of testosterone to estrogen. In gravid garter snakes the amount of circulating testosterone may be higher during development if litters consist predominantly of males. Female neonates exposed to these higher levels of testosterone may aromatize it to estrogen and thus become feminized. The conversion of testosterone into 17 $\beta$ -estradiol (an estrogen) is catalyzed by a complex of enzymes known as the aromatase system, which is present only in the endoplasmic reticulum of ovaries and placenta (Abeles et al., 1992). Aromatization of excess testosterone is thought to occur in the egg-laying red-eared slider turtle (*Trachemys scripta*). The temperature at which *Trachemys* eggs are incubated determines the sex of the hatchlings, as in many other reptile species with temperature-dependent sex determination (TSD). Eggs incubated at all male-producing and male-biasing intermediate temperatures were injected with testosterone. This hormone treatment resulted in a significant increase in the number of female offspring (Wibbels et al., 1992; Wibbels and Crews, 1992, 1995; Crews and Bergeron, 1994; Crews et al., 1995). The authors suggest that the

effect is due to aromatization of testosterone to 17 $\beta$ -estradiol. At temperatures producing mixed sex ratios, single treatments of 17 $\beta$ -estradiol, norethindrone, tamoxifen, and testosterone all had feminizing effects (Wibbels and Crews, 1995). Administration of estrogen to gecko eggs early in development overrode the male-determining incubation temperature: all of the young developed ovaries (Bull et al., 1988; Crews et al., 1996). Administration of androgens at female-biased temperatures had no effect. These results further support the hypothesis that estrogen rather than testosterone may be the organizing hormone for sex differentiation in reptiles.

In one population there was a significant relationship between female subcaudal count and sex ratio, but in neither population was the relationship significantly stronger in females than in males. These results suggest that feminization of females in male biased litters, although statistically significant in one population, may be a statistical artifact. The alternative explanation is that sex ratio has a real effect on female subcaudal counts, but we succeeded in detecting significant regression in only one population. Only additional work in other populations of snakes can distinguish between these alternatives.

The hypothesis that female embryos are feminized by neighboring brothers could be further investigated by determining the sex of adjacent embryos, as has been done in mammalian studies. Experiments in which androgens are administered during prenatal development could establish whether female embryos are feminized by aromatizing testosterone. Such experiments could test the hypothesis that estrogen is the organizing hormone in garter snakes, as it might be in all reptiles.

*Acknowledgments.*—We are grateful to Jeanne Altmann, the Altmann lab group at the University of Chicago, William Gutzke, Lynn Houck, Martha McClintock, Robert Mason and an anonymous reviewer for helpful comments and suggestions. This work was supported by NSF Grant BSR-9119588.

#### LITERATURE CITED

- ABELES, R. H., P. A. FREY, AND W. P. JENCKS. 1992. Biochemistry. Jones and Bartlett Publ., Boston, Massachusetts.
- ARNOLD, S. J. 1988. Quantitative genetics and selection in natural populations: microevolution of vertebral numbers in the garter snake *Thamnophis elegans*. In B. S. Weir, E. J. Eisen, M. M. Goodman, and G. Namkoong (eds.), Proceedings of the Second International Conference on Quantitative Genetics, pp. 619–638. Sinauer Assoc. Inc., Sunderland, Massachusetts.
- BULL, J. J., W. H. N. GUTZKE, AND D. CREWS. 1988. Sex reversal by estradiol in three reptilian orders. Gen. Comp. Endocrinol. 70:425–428.
- CLARK, M. M., D. CREWS, AND B. G. GALEF, JR. 1991. Concentrations of sex steroid hormones in pregnant and fetal mongolian gerbils. Physiol. Behav. 49:239–243.
- CREWS, D. 1994. Animal sexuality. Sci. Amer. 279:108–114.
- , AND J. M. BERGERON. 1994. Role of reductase and aromatase in sex determination in the red-eared slider (*Trachemys scripta*), a turtle with TSD. J. Endocrinol. 143:279–289.
- , A. R. CANTU, J. M. BERGERON, AND T. RHEN. 1995. The relative effectiveness of androstenedione, testosterone, and estrone, precursors to estradiol, in sex reversal in the red-eared slider turtle (*Trachemys scripta*), a turtle with TSD. Gen. Comp. Endocrinol. 100:119–127.
- , A. R. CANTU, AND J. M. BERGERON. 1996. Temperature and non-aromatizable androgens: A common pathway in male sex determination in a turtle with TSD? J. Endocrinol. 149:457–463.
- FLORES, D., A. TOUSIGNANT, AND D. CREWS. 1994. Incubation temperature affects the behavior of adult leopard geckos (*Eublepharis macularius*). Physiol. Behav. 55:1067–1072.
- GANDELMANN, R., F. S. VOM SAAL, AND J. M. REINISCH. 1977. Contiguity to male fetuses affects morphology and behavior of female rats. Nature 266:722–724.
- GUTZKE, W. H., G. L. PAUKSTIS, AND L. L. MCDANIEL. 1985. Skewed sex ratios for adult and hatchling bullsnakes, *Pituophis melanoleucus*, in Nebraska. Copeia 1985:649–652.
- HOUTSMULLER, E. J., AND A. K. SLOB. 1990. Masculinization and defeminization of female rats by males located caudally in the uterus. Physiol. Behav. 48:555–560.
- KELLER, K. AND J. TANDLER. 1916. Über das Verhalten der Eihäute bei der Zwilligsträchtigkeit des Rindes. Wiener Tierärztl. Wochenschrift 3:513–527.
- LILLIE, F. R. 1916. The theory of the free-martin. Science 43:611–613.
- MEISEL, R. L. 1981. Fetal female rats are masculinized by male littermates located caudally in the uterus. Science 213:239–242.
- RHODE PARFET, K. A., W. R. LAMBERSON, A. R. RIEKE, T. C. CANTLEY, V. K. GANJAM, F. S. VOM SAAL, AND B. N. DAY. 1990. Intrauterine position effects in male and female swine subsequent survivability, growth rate, morphology and semen characteristics. J. Anim. Science 68:179–185.
- RICHMOND, G., AND B. D. SACHS. 1984. Further evidence for masculinization of female rats by males located caudally in utero. Horm. Behav. 18:484–490.
- SCHUETT, G. W., P. J. FERNANDEZ, W. F. GERGITS, N. J. CASNA, D. CHISZAR, H. M. SMITH, J. B. MINTON, S. P. MACKESSAY, R. A. ODUM AND M. J. DEMLONG. 1997. Production of offspring in the absence of males: evidence for facultative parthenogenesis in bisexual snakes. Herpetol. Nat. Hist. 5:1–10.
- SHINE, R., AND J. J. BULL. 1977. Skewed sex ratios in snakes. Copeia 1977:228–234.
- , AND D. CREWS. 1988. Why male garter snakes

have small heads: the evolution and endocrine control of sexual dimorphism. Evolution 42:1105–1110.

- SOKAL, R. R., AND F. J. ROHLF. 1981. Biometry. Freeman, San Francisco, California.
- VOM SAAL, F. S., AND F. H. BRONSON. 1980. Sexual characteristics of adult female mice are correlated with their blood testosterone levels during prenatal development. Science 208:597–599.
- WIBBELS T., AND D. CREWS. 1992. Specificity of steroid

hormone-induced sex determination in a turtle. J. Endocrinol. 133:121–129.

- , AND ———. 1995. Steroid-induced sex determination at incubation temperatures producing mixed sex ratios in a turtle with TSD. Gen. Comp. Endocrinol. 100:53–60.
- , J. J. BULL, AND D. CREWS. 1992. steroid hormone-induced male sex determination in an amniotic vertebrate. J. Exp. Zool. 262:454–457.

Accepted: 1 October 1999.