

# Environmental sex determination in a reptile varies seasonally and with yolk hormones

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Most hypotheses that have been put forward in order to explain the persistence of environmental sex determination (ESD) in reptiles assume a relatively fixed association of sex with temperature-induced phenotype and no maternal influence on offspring sex. Here we demonstrate the association of maternally derived yolk hormone levels with the offspring sex ratio and describe two new aspects of temperature-dependent sex determination (TSD), i.e. seasonal variation in both thermal response and yolk steroid levels. Eggs from painted turtles (*Chrysemys picta*) were incubated at 28 °C. The hatchling sex ratio at 28 °C (i.e. the phenotypic reaction norm for sex at 28 °C) shifted seasonally from ca. 72% male to ca. 76% female. Yolk oestradiol (E<sub>2</sub>) increased seasonally while testosterone (T) decreased. The proportion of males in a clutch decreased as E<sub>2</sub> levels increased and the E<sub>2</sub>:T ratio increased. These new findings are discussed in relation to heritability and adaptive explanations for the persistence of ESD in reptiles. Maternally derived yolk hormones may provide a mechanism for the seasonal shift in the sex ratio which in turn may help explain the persistence of ESD in reptiles. They may also explain those clutches of other reptiles with TSD that fail to yield only males at maximally masculinizing conditions.

**Keywords:** environmental sex determination; phenotypic reaction norm; pivotal temperature; turtle; yolk steroids

## 1. INTRODUCTION

The particular form of environmental sex determination (ESD) in reptiles, where incubation temperature determines offspring sex, i.e. temperature-dependent sex determination (TSD) (Pieau 1996), remains an 'evolutionary enigma' in reptiles (Shine 1999) despite considerable study (Bull & Vogt 1979; Ewert *et al.* 1994; Lang & Andrews 1994; Viets *et al.* 1994; Shine 1999). Specifically, TSD is seen as an enigma because many reptiles which have TSD are relatively long-lived organisms with delayed reproduction. Thus, in contrast to the best-studied system in fishes where longer growing time translates into increased female fecundity within one year (Conover 1984), in reptiles it is difficult to infer that a few weeks difference in hatching time will in itself result in differential fitness at the onset of reproduction several years later. Further, although it has been speculated that TSD can explain the sexual size dimorphism seen in many turtles and crocodylians, many reptiles with genetic sex determination (GSD) also have dimorphic sexes (and are long lived) (Janzen & Paukstis 1991). Finally, there would appear to be the potential for unusually warm or cool weather producing such strongly biased sex ratios as to result ultimately in local extinction of populations, at least in short-lived species (Bull & Bulmer 1989).

Prior attempts to resolve the enigma of TSD have assumed a relatively fixed association of sex with temperature-induced phenotype. In order to examine this assumption, we looked for seasonal variation in the association of temperature and sex, i.e. for seasonal variation in the phenotypic reaction norm (Schlichting & Pigliucci 1998) for sex ratio with temperature.

The mechanism for TSD also remains unclear. However, several studies have shown that steroids can

enhance or override the effects of incubation temperature in reptiles with TSD (Wibbles *et al.* 1991; Crews *et al.* 1996). In these studies, the exogenous application of oestradiol (E<sub>2</sub>) resulted in the production of females at male-producing temperatures with less E<sub>2</sub> needed to override temperature as the incubation temperature is closer to a female-producing temperature (Crews 1996). This indicates that steroids can influence sex determination and that relatively low levels of steroids can influence sex determination at intermediate incubation temperatures. Recent studies have found detectable levels of E<sub>2</sub> and testosterone (T) in the yolks of recently laid eggs (Conley *et al.* 1997; Janzen *et al.* 1998). Both of these hormones are known to have multiple functions in primary sexual differentiation in vertebrates (Wolfe & Wolfe 1951; Jost 1960; Crews 1996). Except for a recent suggestion that maternal condition might affect egg size as well as selection of nest sites (Roosenburg 1996), prior studies of the adaptive basis for TSD in reptiles have assumed no maternal influence over offspring sex. We examined this assumption by looking for any effect of maternally derived yolk hormone levels on offspring sex and for any seasonal variation in these hormone levels.

## 2. METHODS

### (a) Collection of turtles, incubation and determination of sex

We studied the painted turtle (*Chrysemys picta*) in south-central Indiana. Eggs were obtained from recent field nestings or by oxytocin injection (Ewert & Legler 1978) of recently collected gravid females.

For the sex ratio, clutches from each of five years (between 1992 and 1998) yielded a total of 425 hatchlings from a total of 91 clutches. Eggs were weighed, measured and marked as to clutch and individual. The eggs were randomized and placed into incubation boxes that contained 1:1 dry vermiculite to water

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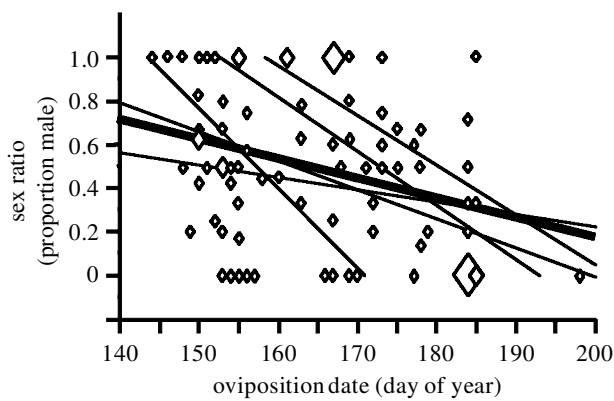


Figure 1. Clutch sex ratio (as % male) plotted against oviposition date (i.e. day of year). Diamond heights are proportional to the number of clutches with the same sex ratio and oviposition date. The bold line represents the regression for all years combined ( $r = 0.326$ ). The dashed lines represent the regressions for the five years individually, three of which were independently statistically significant.

by weight (ca.  $-170$  kPa); water was added periodically. The incubator temperature ( $28^{\circ}\text{C}$ ) was monitored frequently by placing an NBS traceable thermometer into one to three 500 ml bottles housed within the incubator. The boxes of eggs were rotated frequently within the incubators in order to reduce the influence of any small gradations in temperature. Sex was determined by macroscopic examination of gonads and Müllerian ducts (Schwarzkopf & Brooks 1985; Ewert & Nelson 1991; Janzen 1994).

#### (b) Steroid radioimmunoassays

We performed yolk extractions (Schwabl 1993) and competitive binding steroid radioimmunoassays for the 33 clutches collected in 1998 (Wingfield & Farner 1975) in order to determine yolk  $E_2$  and T levels at oviposition in a mean of 2.5 eggs (range of one to three) from each clutch. We estimated clutch-specific sex ratios from the hatchlings from the remaining eggs (mean = 4.2 hatchlings and range of three to eight).

Yolk samples were run in duplicate and hormone concentrations were compared to a standard curve that ranged from 500–1.95 pg. The recovery values ranged from 35–69% with a 50% average for  $E_2$  and from 37–74% with a 54% average for T. Due to the large number of samples, two assays were run. The intra-assay variations for  $E_2$  were 13.9 and 14.9% with an interassay variation of 5.9% and the intra-assay variations for T were 16.5 and 8.5% with an interassay variation of 16.8%.

#### (c) Statistical analyses

Initial associations were examined graphically using StatView 512. Data were analysed using programs from SPSS 6.1 for Macintosh for general linear models, linear regressions and stepwise linear regressions as specified in § 3.

### 3. RESULTS

#### (a) Overall sex ratio

The yearly sex ratios were between 0.46 and 0.54 ( $n = 8$ –33 clutches) with a range across clutches of 0.0–1.0 each year except 1993 ( $n = 7$ , mean = 0.64 and range = 0.33–1.0). The overall sex ratio produced for *C. picta* was 49% male (i.e. ca. 1:1 ratio expected at the

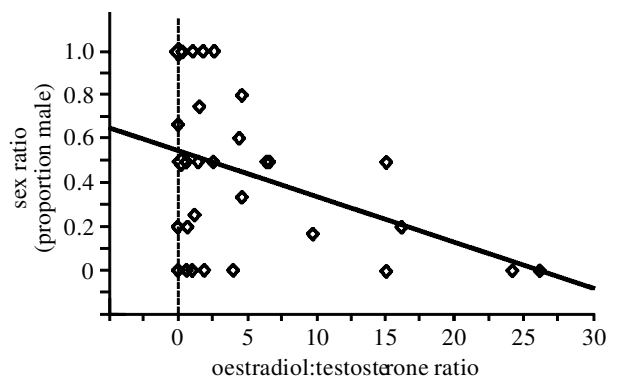


Figure 2. Clutch sex ratio plotted against the ratio of  $E_2$ :T ( $r = 0.402$ ). Diamond heights are proportional to the number of clutches with the same sex and hormone ratios.

‘pivotal’ temperature) (Mrosovsky & Picau 1991) in this study. This approximation of the pivotal temperature maximized our ability for detecting seasonal variation in offspring sex and (in 1998) any effects of yolk hormones on the sex ratio.

#### (b) Estimates of within- and between-clutch variation

The yolk hormone levels varied significantly between clutches for  $E_2$ , T and the  $E_2$ :T ratio (ANOVA,  $E_2$ ,  $F_{30,52} = 12.95$  and  $p = 0.0001$ ; T,  $F_{30,53} = 2.03$  and  $p = 0.012$ ; and  $E_2$ :T,  $F_{32,52} = 6.002$  and  $p = 0.0001$ ). Similar variation between clutches has been found for other reptiles with TSD (Conley *et al.* 1997; Janzen *et al.* 1998). We expected hormone levels within a clutch to be strongly correlated among eggs (Conley *et al.* 1997; Janzen *et al.* 1998) because all ovarian follicles contributing to a clutch undergo vitellogenesis simultaneously (Callard *et al.* 1978). Indeed, the correlation between clutch mates was positive and very high for  $E_2$  (mean  $r = 0.872$  and  $p = 0.0001$ ) and positive but not significantly so for T (mean  $r = 0.335$  and  $p = 0.129$ ).

#### (c) Seasonal variation in sex ratio

The average sex ratio at  $28^{\circ}\text{C}$  shifted seasonally from strongly male biased (ca. 72% male) for ovipositions prior to day 151 (day of year) to strongly female biased (ca. 24% male) subsequent to day 180 ( $F_{1,80} = 9.521$  and  $p = 0.003$ ) (figure 1). A general linear model indicated no between-year heterogeneity ( $F_{4,82} = 0.68$  and  $p = 0.607$ ). This seasonal shift is equivalent to a lowering of the pivotal temperature by ca.  $0.6^{\circ}\text{C}$  across the nesting season. This estimate is based on an observed 40% shift in the sex ratio over  $0.5^{\circ}\text{C}$  near the pivotal temperature in another population of *C. picta* (Rhen & Lang 1998). For comparison, the geographical variation in pivotal temperature in *C. picta* is only  $1.5^{\circ}\text{C}$  (Ewert *et al.* 1994).

#### (d) Correlation of yolk steroids, sex ratio and seasonal variation

Endogenous yolk hormone levels were directly correlated with the sex ratio (figure 2). In simple linear regressions,  $E_2$  alone was significantly correlated with the sex ratio ( $F_{1,31} = 4.49$  and  $p = 0.042$ ), but T was not ( $F_{1,32} = 1.37$  and  $p = 0.251$ ). In a stepwise linear regression that included  $E_2$ , T and the ratio of  $E_2$ :T, the ratio was the best

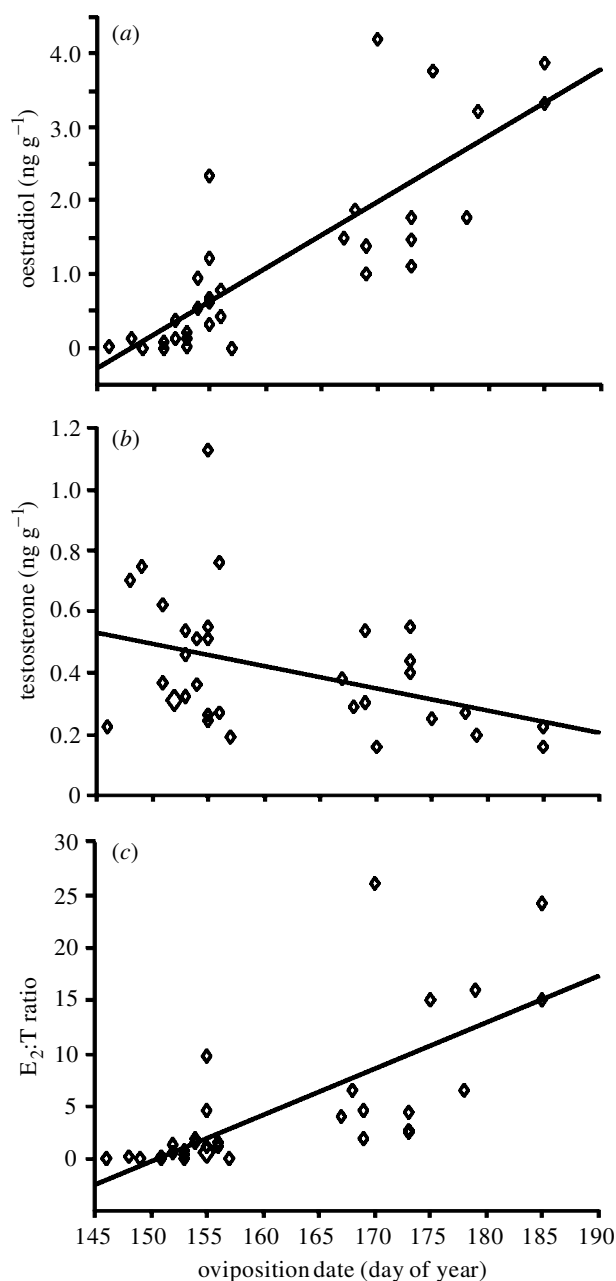


Figure 3. Endogenous hormone levels from recently laid eggs plotted against oviposition date for (a) oestradiol ( $r = 0.824$ ), (b) testosterone ( $r = 0.390$ ) and (c) the ratio of  $E_2$ :T ( $r = 0.718$ ).

predictor of the clutch sex ratio ( $F_{1,31} = 5.92$  and  $p = 0.021$ ) (neither  $E_2$  nor T significantly improved the model). As the ratio (or  $E_2$  alone) increased, the clutch sex ratio shifted from male biased to female biased (figure 2).

Seasonally, mean clutch values increased for  $E_2$  ( $F_{1,32} = 51.04$  and  $p = 0.0001$ ) (figure 3a), decreased for T ( $F_{1,32} = 6.28$  and  $p = 0.018$ ) (figure 3b) and, consequently, increased for the  $E_2$ :T ratio ( $F_{1,31} = 29.57$  and  $p = 0.0001$ ) (figure 3c). All of these shifts were statistically significant and coincided temporally with the seasonal shift observed for the clutch sex ratio (figure 1).

#### (e) Examination of potential confounding factors

Egg mass, hatchling mass, incubation period and year are potentially confounding factors but, in a stepwise

linear regression, oviposition date was the best predictor of the clutch sex ratio ( $F_{1,62} = 4.058$  and  $p = 0.048$ ) and these other four factors did not significantly improve the model. Similarly, a seasonal shift in incubator temperature could produce a corresponding shift in the sex ratio. The incubation temperature was monitored (to  $0.1^\circ\text{C}$ ) frequently and no seasonal shift in incubator temperature was observed. Moreover, had such an increase occurred, it would have decreased the incubation period, but the relationship between incubation period and oviposition date was flat (slope =  $-0.010$ ,  $r = 0.089$  and  $p = 0.80$ ). Further, although the incubation period increased significantly with egg mass (slope =  $0.244$ ,  $r = 0.365$  and  $p = 0.001$ ), egg mass was independent of oviposition date (slope =  $-0.003$ ,  $r = 0.045$  and  $p = 0.991$ ) and, thus, could not have masked a change in temperature. In addition, there was no significant association of egg mass with  $E_2$ , T or  $E_2$ :T.

#### 4. DISCUSSION

Various studies clearly support a causal linkage between hormones and TSD in reptiles. For example, administration of  $E_2$  to reptiles with ESD during the middle third of incubation shifts sex ratios towards more females (Crews *et al.* 1991, 1996; Lance 1997) and application of aromatase inhibitors (which block the conversion of androgens to oestrogens) shifts ratios towards more males (Wibbles *et al.* 1991; Lance 1997). Recent work (Sheehan *et al.* 1999) has demonstrated that even very small amounts of exogenously applied  $E_2$  ( $0.4\text{ ng}$  per egg) can produce appreciable increases ( $14.4$  and  $18.5\%$  in two experiments) in the proportion of females in *Trachemys scripta*.

Our data strongly suggest a seasonal shift in the sex ratio caused by seasonal variation in female deposition of yolk steroids. Further, our checks on confounding factors excluded the most reasonable alternatives to the hypothesis that the seasonal shift in the sex ratio is driven by seasonal shifts in maternally derived hormone levels. Sheehan *et al.* (1999) estimated endogenous  $E_2$  levels in *T. scripta* to be  $0.8 \pm 0.28\text{ ng}$  per egg for early season eggs collected in 1997 and  $1.7 \pm 0.9\text{ ng}$  per egg for late season eggs collected in 1996, suggesting that a seasonal shift may also occur in *T. scripta*.

The only previous study of hatchling sex ratios and endogenous yolk hormone levels in turtles with ESD failed to find a correlation of yolk T with the sex ratio (Janzen *et al.* 1998). This parallels our finding of no significant association of the sex ratio with T by itself. Most prior work supports a stronger role in sex determination for oestradiol or for the ratio of oestrogens to androgens (Bogart 1987; Crews *et al.* 1996; Conley *et al.* 1997; Crain *et al.* 1998).

Seasonal changes in the phenotypic reaction norm, i.e. in the propensity of eggs to become males (or females), will affect two important models for an adaptive advantage for ESD in reptiles (Charnov & Bull 1977; Shine 1999). First, the association of sex to any other temperature-induced aspects of phenotype (Rhen & Lang 1995; Shine 1999) is altered directly. Second, the association of sex to temperature-determined time of hatching (Conover 1984; Shine 1999) will also be altered, although not necessarily in a simple way.

The altered association of sex with other temperature-induced phenotypic characteristics and with time of hatching follows directly from the observed shift in the phenotypic reaction norm for sex. The concurrent seasonal shift in the maternally derived  $E_2:T$  ratio suggests a mechanism for explaining both of these effects as well as the seasonal shift in the reaction norm itself. It also strengthens the suggestion (Conley *et al.* 1997) that part of the within-clutch concordance (or 'heritability') in sex in reptiles with ESD (e.g. Bull *et al.* 1982; Bulmer & Bull 1982; Janzen 1992; Rhen & Lang 1998) reflects clutch-specific hormone levels. We found very little variation in hormone levels among eggs from the same clutch.

Hormone levels could also affect yet another potentially adaptive explanation for the persistence of ESD in some species, a match of sex to egg size (Roosenburg 1996). Specifically, hormone levels provide a mechanism for achieving such a match, a new alternative to nest-site selection (Roosenburg 1996). One study did find an association of egg mass and the sex ratio in a turtle with ESD (Ewert *et al.* 1994), but we found no such association in *C. picta*.

In species like *C. picta* in which individual females often lay two clutches per nesting season, our finding of a seasonal pattern raises the possibility that first and second clutches from the same female laid within one year will respond differently to the same thermal regime, i.e. more male hatchlings from an early clutch and more female hatchlings from a later clutch at equivalent temperatures. Such seasonal shifts would tend to bring the annual production of individual females closer to 1:1 but variation in ambient temperature or in nest-site selection could moderate or amplify this tendency. Sex ratios closer to 1:1 would make the persistence of ESD (versus GSD) less enigmatic.

A similar buffering of extreme sex ratios, with a consequent reduced stringency of Fisherian limitations, has been suggested (Ewert *et al.* 1994) for species with ESD in which it appears impossible to induce 100% males in some clutches at any constant temperature (Ewert & Nelson 1991; Viets *et al.* 1994). Indeed, in the one species examined intensively (the alligator snapping turtle *Macrolemys temminckii*), 100% males could not be induced even by using a series of multiple temperature shifts specifically designed to maximize the proportion of males (Ewert *et al.* 1994). These clutches that appear to resist complete masculinization may guarantee that at least some females are produced in cool habitats or years that would otherwise yield exclusively males (Ewert *et al.* 1994). In such clutches, maternally derived  $E_2$  (or  $E_2:T$ ) may be partly or entirely overriding TSD. Such clutches provide a unique opportunity for examining the effects of maternally derived steroids on offspring sex, since some individuals from each clutch would clearly be produced at a non-optimal temperature.

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## REFERENCES

- Bogart, M. H. 1987 Sex determination: a hypothesis based on steroid ratios. *J. Theor. Biol.* **128**, 349–357.
- Bull, J. J. & Bulmer, M. G. 1989 Longevity enhances selection of environmental sex determination. *Heredity* **63**, 315–320.
- Bull, J. J. & Vogt, R. J. 1979 Temperature-dependent sex determination in turtles. *Science* **206**, 1186–1188.
- Bull, J. J., Vogt, R. J. & Bulmer, M. G. 1982 Heritability of sex ratio in turtles with environmental sex determination. *Evolution* **36**, 333–341.
- Bulmer, M. G. & Bull, J. J. 1982 Models of polygenic sex determination and sex ratio control. *Evolution* **36**, 13–26.
- Callard, I. P., Lance, V., Salhanick, A. R. & Barad, D. 1978 The annual ovarian cycle of *Chrysemys picta*: correlated changes in plasma steroids and parameters of vitellogenesis. *Gen. Comp. Endocrinol.* **35**, 245–257.
- Charnov, E. L. & Bull, J. J. 1977 When is sex environmentally determined? *Nature* **266**, 828–830.
- Conley, A. J., Elf, P., Corbin, C. J., Dubowsky, S., Fivizzani, A. & Lang, J. W. 1997 Yolk steroids decline during sexual differentiation in the alligator. *Gen. Comp. Endocrinol.* **107**, 191–200.
- Conover, D. O. 1984 Adaptive significance of temperature-dependent sex determination in a fish. *Am. Nat.* **123**, 297–313.
- Crain, D. A., Guillette Jr, L. J., Pickford, D. B., Percival, H. F. & Woodward, A. R. 1998 Sex-steroid and thyroid hormone concentrations in juvenile alligators (*Alligator mississippiensis*) from contaminated and reference lakes in Florida, USA. *Environ. Tox. Chem.* **17**, 446–452.
- Crews, D. 1996 Temperature-dependent sex determination: the interplay of steroid hormones and temperature. *Zool. Sci.* **13**, 1–13.
- Crews, D., Bull, J. J. & Wibbles, T. 1991 Estrogen and sex reversal in turtles: a dose-dependent phenomenon. *Gen. Comp. Endocrinol.* **81**, 357–364.
- Crews, D., Cantú, A. R., Rhen, T. & Vohra, R. 1996 The relative effectiveness of estrone, estradiol-17 $\beta$ , and estriol in sex reversal in the red-eared slider (*Trachemys scripta*), a turtle with temperature-dependent sex determination. *Gen. Comp. Endocrinol.* **102**, 317–326.
- Ewert, M. A. & Legler, J. M. 1978 Hormonal induction of oviposition in turtles. *Herpetologica* **34**, 314–318.
- Ewert, M. A. & Nelson, C. E. 1991 Sex determination in turtles: diverse patterns and some possible adaptive values. *Copeia* **1991**, 50–69.
- Ewert, M. A., Jackson, D. R. & Nelson, C. E. 1994 Patterns of temperature-dependent sex determination in turtles. *J. Exp. Zool.* **270**, 3–15.
- Janzen, F. J. 1992 Heritable variation for sex ratio under environmental sex determination in the common snapping turtle (*Chelydra serpentina*). *Genetics* **131**, 155–161.
- Janzen, F. J. 1994 Vegetational cover predicts the sex ratio of hatchling turtles in natural nests. *Ecology* **75**, 1593–1599.
- Janzen, F. J. & Paukstis, G. L. 1991 A preliminary test of the adaptive significance of environmental sex determination in reptiles. *Evolution* **45**, 435–441.
- Janzen, F. J., Wilson, M. E., Tucker, J. K. & Ford, S. P. 1998 Endogenous yolk steroid hormones in turtles with different sex-determining mechanisms. *Gen. Comp. Endocrinol.* **111**, 306–317.
- Jost, A. 1960 Hormonal influences in the sex development of bird and mammalian embryos. *Mem. Soc. Endocrinol.* **7**, 49–62.
- Lance, V. A. 1997 Sex determination in reptiles: an update. *Am. Zool.* **37**, 504–513.

- Lang, J. W. & Andrews, H. V. 1994 Temperature-dependent sex determination in crocodylians. *J. Exp. Zool.* **270**, 28–44.
- Mrosovsky, N. & Pieau, C. 1991 Transitional range of temperature, pivotal temperatures and thermosensitive stages for sex determination in reptiles. *Amphibia-Reptilia* **12**, 169–179.
- Pieau, C. 1996 Temperature variation and sex determination in reptiles. *BioEssays* **18**, 19–26.
- Rhen, T. & Lang, J. W. 1995 Phenotypic plasticity for growth in the common snapping turtle: effects of incubation temperature, clutch, and their interaction. *Am. Nat.* **146**, 726–747.
- Rhen, T. & Lang, J. W. 1998 Among-family variation for environmental sex determination in reptiles. *Evolution* **52**, 1514–1520.
- Roosenburg, W. M. 1996 Maternal condition and nest site choice: an alternative for the maintenance of environmental sex determination? *Am. Zool.* **36**, 157–168.
- Schlichting, C. D. & Pigliucci, M. 1998 *Phenotypic evolution: a reaction norm perspective*. Sunderland, MA: Sinauer Associates, Inc.
- Schwabl, H. 1993 Yolk is a source of maternal testosterone for developing birds. *Proc. Natl Acad. Sci. USA* **90**, 11446–11450.
- Schwarzkopf, L. & Brooks, R. J. 1985 Sex determination in northern painted turtles: effect of incubation at constant and fluctuating temperatures. *Can. J. Zool.* **63**, 2543–2547.
- Sheehan, D. M., Willingham, E., Gaylor, D., Bergeron, J. M. & Crews, D. 1999 No threshold dose for estradiol-induced sex reversal of turtle embryos: how little is too much? *Environ. Health Perspect.* **107**, 155–159.
- Shine, R. 1999 Why is sex determined by nest temperature in many reptiles? *Trends Ecol. Evol.* **14**, 186–189.
- Viets, B. E., Ewert, M. A., Talent, L. G. & Nelson, C. E. 1994 Sex-determining mechanisms in squamate reptiles. *J. Exp. Zool.* **270**, 45–56.
- Wibbles, T., Bull, J. J. & Crews, D. 1991 Synergism between temperature and estradiol: a common pathway in turtle sex determination? *J. Exp. Zool.* **260**, 130–134.
- Wingfield, J. C. & Farner, D. S. 1975 The determination of five steroids in avian plasma by radioimmunoassay and competitive protein-binding. *Steroids* **26**, 311–327.
- Wolfe, E. & Wolfe, E. 1951 The effects of castration on bird embryos. *J. Exp. Zool.* **116**, 59–97.