

## WINDOWS OF EMBRYONIC SEXUAL LABILITY IN TWO LIZARD SPECIES WITH ENVIRONMENTAL SEX DETERMINATION

RICHARD SHINE,<sup>1</sup> DANIEL A. WARNER, AND RAJKUMAR RADDER

*School of Biological Sciences A08, University of Sydney, NSW 2006 Australia*

**Abstract.** Temperature-dependent sex determination (TSD) occurs in all major reptile lineages, but the selective forces and physiological mechanisms that link sex to incubation temperature may differ among and within those groups. Different models for TSD evolution make different predictions about when offspring sex will respond to environmental cues. Although TSD has evolved in several lizard lineages, there is less detailed information on these taxa than in turtles and crocodylians with TSD. We incubated eggs of an agamid lizard (*Amphibolurus muricatus*) and a scincid lizard (*Bassiana duperreyi*), two species with TSD. Rather than manipulate incubation temperature to identify periods of sexual lability (as in most previous studies of this topic), we topically applied the aromatase inhibitor fadrozole to eggs at a variety of times through the incubation period. Fadrozole application sex-reversed the resultant hatchlings if applied from the time of oviposition until at least 60% of the way through incubation. In all of the TSD lizard species studied so far, offspring sex is determined either while the eggs are held inside the mother's body or soon after oviposition, providing substantial maternal control over incubation temperatures at this critical period. Hence, the hypothesis that TSD evolves because it enables offspring sex to be matched to conditions that are unpredictable at the time of laying is less likely to apply to squamates than to turtles, sphenodontians, and (especially) crocodyles, in which the period of sexual lability is delayed until long after oviposition.

**Key words:** *Agamidae*; *Amphibolurus muricatus*; *aromatase inhibitor*; *Bassiana duperreyi*; *embryonic development*; *fadrozole*; *incubation*; *jacky dragon*; *Scincidae*; *temperature-dependent sex determination*; *thermosensitive period*.

### INTRODUCTION

Any classification system involves potential pitfalls: for example, continua will tend to be treated as dichotomies. Research on sex-determining systems of reptiles provides a good example of such potential dangers. In many reptile species, an individual's sex is determined by its genetic constitution, often involving heteromorphic sex chromosomes (Bull 1980, 1983, Valenzuela and Lance 2004). However, some species within all major reptile lineages display an alternative mode: environmental sex determination. In the tuatara, some lizards, some turtles, and all crocodylians, the temperature at which eggs incubate affects the sex of the offspring that emerge from those eggs (Valenzuela and Lance 2004). Phylogenetic reconstructions indicate multiple transitions between alternative sex-determining systems within the Reptilia (Janzen and Krenz 2004), genetic and physiological analyses suggest that many different mechanisms might evolve to link sex to temperature (Sarre et al. 2004), and theoretical models suggest that a wide range of selective forces could favor such a linkage (Bull 1983, Shine 1999b). Hence, there is

no a priori reason to expect conservatism in major features of temperature-dependent sex determination (TSD) within these disparate reptilian lineages (Bull 2004, Valenzuela and Lance 2004, Janzen and Phillips 2006).

The present study addresses one of the most fundamental issues in environmental sex determination: at what stages of embryogenesis is the gonadal development of the developing offspring sensitive to external influences? Undoubtedly, there will be periods when no external influence can modify the sex of the embryo and other periods when sex is malleable. Careful experimental work has documented the timing of this window of sexual lability for several crocodylians (e.g., Lang and Andrews 1994), turtles (e.g., Bull 1985, Bull et al. 1985, Georges et al. 1994, 2005), and sphenodontians (Mitchell et al. 2006), but there has been only one previous study of this topic in a lizard (the leopard gecko *Eublepharis macularius*; Bull 1987). Recent work has revealed that TSD also occurs in a range of species within in at least two other familial lineages of lizards, the Agamidae and the Scincidae (Harlow 2004). In this study we have experimentally examined the timing of the sensitive period (the interval of embryogenesis during which sex can be modified by environmental cues) in one agamid and one scincid species, both of which exhibit TSD.

Manuscript received 8 December 2006; accepted 15 December 2006; final version received 15 December 2006.  
Corresponding Editor: R. A. Relyea.

<sup>1</sup> E-mail: rics@bio.usyd.edu.au

## METHODS

*Study species*

Jacky dragons (*Amphibolurus muricatus*) are medium-sized (to 340 mm total length) agamid lizards abundant throughout coastal areas of southeastern Australia (Cogger 2000, Warner and Shine 2005). Each adult female lays two or three clutches of eggs per summer season, with each clutch containing about 4–8 eggs (Harlow and Taylor 2000). Offspring sex is determined by incubation temperature, with males hatching primarily from thermally intermediate nests ( $\sim 29^{\circ}\text{C}$ ), whereas warmer and cooler nests generally produce females (Harlow and Taylor 2000, Harlow 2004, Warner and Shine 2005). Our other study species is a smaller (to 150 mm total length) montane scincid lizard, *Bassiana duperreyi*, from highland areas of southeastern Australia. Females produce a single clutch of about 3–9 eggs early in the summer (Shine 1999a). These lizards possess heteromorphic sex chromosomes (XX–XY system), but at thermal regimes typical of relatively cool (high-elevation) nests, this genetic mechanism is overridden by TSD such that incubation at  $<20^{\circ}\text{C}$  generates a male-biased sex ratio among the hatchlings (Shine et al. 2002). The only information on the timing of sexual lability in either of these species comes from a study that documented sex ratio shifts in *Bassiana* eggs exposed to cold conditions, even if that exposure occurred quite close to the time of hatching (Shine et al. 2002).

*Experimental methods*

We captured jacky dragons in coastal areas near Sydney and maintained them in groups within large outdoor enclosures; gravid females nested in these enclosures and their eggs were transferred to the laboratory for incubation (for details, see Warner and Shine 2005). Female *Bassiana duperreyi* were collected in the Brindabella Range 50 km west of Canberra in the week or two prior to oviposition, and were held in the laboratory until oviposition (for details, see Shine et al. 2002, Shine 2004). In each case, regular checks meant that eggs were discovered  $<24$  hours after oviposition and were immediately transferred to individual glass jars for incubation. Vermiculite at  $-200$  kPa was used as the substrate, and plastic food wrap over the top of each incubation jar allowed oxygen exchange but prevented desiccation.

To quantify the time of onset and duration of the thermosensitive period (TSP) in TSD reptile species, most previous studies have relied upon thermal translocation experiments (e.g., Bull 1987, Lang and Andrews 1994; but see Gutzke and Chymiy 1988). That is, eggs are incubated at a mean temperature that will produce some known sex ratio, then moved for specified intervals to other thermal conditions expected to generate a different sex ratio, then returned to their original incubator for the rest of incubation. This method allows the investigator to discern the periods during which

temperature can influence offspring sex. We used a different method, involving direct application of an aromatase inhibitor (fadrozole) that blocks the biochemical conversion of testosterone to estradiol and, hence, induces embryonic development as a male rather than a female in species with sexual lability (Gutzke and Chymiy 1988, Crews 1994, Crews et al. 1996). We adopted this approach for two reasons. First, switching temperatures also modifies developmental rates, making it more difficult to quantify the percentage of the development period (or of the incubation period) when sex is capable of responding to external factors. Second, thermal translocation experiments have revealed dosage effects whereby the percentage of embryos affected by a given switch depends not only upon temperature but also upon duration of exposure (Pieau and Dorizzi 1981, Yntema and Mrosovsky 1982, Wibbels et al. 1991, Lang and Andrews 1994). Thus, thermal cues may fail to elicit sex reversal even if the embryo is potentially able to respond, because the stimulus for change was insufficiently strong. Allied to this issue is the possibility of lethal or sublethal effects from extreme temperatures (or prolonged exposure at relatively high temperatures), making it difficult to identify thermal “windows” that provide a sufficiently intense stimulus to detect any possibility for sex change while avoiding embryo death.

Use of fadrozole application instead of thermal windows avoids these problems, but also has potential disadvantages. First, fadrozole may be able to reverse embryo sex even at times in development when “normal” thermal cues would be unable to do so (i.e., the level of thermal stimulus required might be so high as to be lethal). Thus, the period of sexual lability (as revealed by our work) may not equate exactly to the thermosensitive period as defined in earlier studies, although presumably there will be close correspondence between the two. Second, fadrozole may continue to influence embryogenesis for some time after it is applied, whereas thermal effects presumably cease as soon as the egg equilibrates thermally with a change in ambient temperature. Fortunately, these problems seem minor, with the timing of sensitive periods proving to be very similar between hormonal manipulation and thermal cues (Gutzke and Chymiy 1988, Wibbels et al. 1991, Pieau et al. 1994, Chardard et al. 1995, Chardard and Dournon 1999). Research on humans suggests that fadrozole has a short effective life (10.5 hours in the study of Perez and Borja [1992]) and aromatase concentrations in developing reptile and avian embryos vary dynamically over brief periods, with reciprocal regulation of aromatase and anti-Mullerian hormone levels (Smith et al. 1995, Nishikimi et al. 2000). Thus, long-term effects of fadrozole application are likely to be a source of minor imprecision only. For example, Gutzke and Chymiy’s (1988) study on turtles showed that even a massive dose of estradiol (more than 20 times the minimum amount needed for sex determination) had no carryover effects, in that sex-sensitive periods

identified by this massive dose were similar to those identified by thermal-switching studies.

We incubated jacky dragon eggs at 23°C, a temperature expected to produce 100% female offspring (Harlow and Taylor 2000). Hence, any effect of fadrozole (in increasing the percentage of sons) should be evident even with relatively small sample sizes (we used  $\geq 6$  eggs per treatment group, for a total of 71 eggs). In *Bassiana duperreyi*, in contrast, most incubation temperatures produce  $\sim 50\%$  males, and there is no thermal range that results in overproduction of daughters (Shine et al. 2002). Thus, we incubated eggs of this species at 23°C (null expectation is 50% sons) and used larger sample sizes so that we could detect deviations from this expected value ( $N = 10$  per treatment group, for a total of 100 eggs). To quantify the timing of sexual lability, we applied 30  $\mu\text{g}$  of fadrozole per egg (Ciba-Geigy CGS016949A, kindly provided by Novartis Pharmaceuticals AG, Basel, Switzerland) dissolved in 5  $\mu\text{L}$  of ethanol and applied as drops from a pipette. The fadrozole was applied to each egg only once, at one of five stages in development corresponding to approximately 0%, 20%, 40%, 60%, or 80% of the total (post-laying) incubation period for each species. For jacky dragon eggs with a mean incubation of 113 days at 23°C, we applied fadrozole at day 0 (immediately post-oviposition) or at day 20, 40, 60, or 80. For *B. duperreyi* eggs with a mean incubation period of 56 days at 23°C, we applied fadrozole at day 0, 10, 20, 30, or 40. We used an equal number of control eggs on each of these days for both species; controls were given ethanol only. The eggs were then returned to their incubators and checked daily for hatching. Hatchlings were removed as soon as they emerged from the egg, and we determined their sexes by manually everting hemipenes of males (for details, see Harlow 1996, Shine et al. 2002). The validity of this sex-identifying method was checked by histological examination of gonads from 30 hatchlings at 70–84 days after hatching, comprising 20 *B. duperreyi* and 10 *A. muricatus*, with approximately equal numbers of each sex and from control vs. fadrozole groups.

## RESULTS

Hatching success was relatively high in both species (for *Amphibolurus muricatus*, 53 of 71 eggs; for *Bassiana duperreyi*, 96 of 100 eggs), with no effect of fadrozole application or the timing of application on hatching success in either *A. muricatus* (logistic regression, likelihood ratio tests: for treatment,  $\chi^2 = 0.12$ ,  $df = 1$ ,  $P = 0.73$ ; for day of treatment,  $\chi^2 = 1.17$ ,  $df = 4$ ,  $P = 0.88$ ) or *B. duperreyi* (for treatment,  $\chi^2 = 1.09$ ,  $df = 1$ ,  $P = 0.30$ ; for day of treatment,  $\chi^2 = 1.77$ ,  $df = 4$ ,  $P = 0.78$ ). Our system of classifying sex based on external morphology (presence or absence of hemipenes) unambiguously identified 18 hatchling *A. muricatus* as males (17 fadrozole, 1 control) and 25 as females (8 fadrozole, 17 control). A further 10 *A. muricatus* hatchlings were provisionally identified as probable males ( $n = 4$ , of

which 3 were from the fadrozole treatment) or probable females ( $n = 6$ , of which 5 were from the control treatment). The percentages of “ambiguous” individuals with respect to sex thus were similar in the control and fadrozole-treated groups (for *A. muricatus*, 4 of 29 controls vs. 6 of 24 fadrozole-treated;  $\chi^2 = 0.48$ ,  $df = 1$ ,  $P = 0.49$ ). All hatchling *B. duperreyi* were sexed unambiguously based on hemipene presence or absence. The “ambiguous” *A. muricatus* are included in our analyses because histological examination suggests that our initial assessments of sex were correct; excluding them does not affect any of our major results.

The histology of hatchling gonads confirmed the initial sex assignment in every case, with gonads of fadrozole-treated males identical to those of males from the control treatments in all aspects that we examined (Fig. 1). As expected, most *A. muricatus* from control treatments hatched as females, whereas control *B. duperreyi* displayed a 50:50 sex ratio (Fig. 2). In both species, most fadrozole-treated eggs produced male hatchlings. The difference in sex ratio between control and fadrozole-treated groups was statistically significant in both species (for *A. muricatus*,  $\chi^2 = 17.51$ ,  $df = 1$ ,  $P < 0.0001$ ; for *B. duperreyi*,  $\chi^2 = 16.38$ ,  $df = 1$ ,  $P < 0.0001$ ). In both of these taxa, fadrozole application shifted sex ratios from the earliest application (on the day of oviposition) through to at least 60% of the way through incubation (Fig. 2). However, fadrozole application 80% of the way through incubation did not modify the expected sex ratio away from that of the control group (Fig. 2).

## DISCUSSION

Our experiments clearly show that fadrozole can effectively reverse the sex of offspring in both of the lizard species that we studied. This is not surprising in the case of the “classic” TSD species *Amphibolurus muricatus*, which lacks sex chromosomes, but is more interesting in the skink *Bassiana duperreyi*. This species exhibits strongly heteromorphic chromosomes (XX in females, XY in males), yet that system can be overridden such that XX individuals develop into sons rather than daughters as a result of fadrozole administration (present study) as well as by low incubation temperatures and differential allocation of maternal yolk (Shine et al. 2002; R. Radder and R. Shine, unpublished data). The gonadal anatomy of fadrozole-induced males appears to be identical to that of “control” males, as concluded in previous studies that have examined the masculinizing effects of fadrozole in other lizards, as well as in turtles and crocodylians (Crews and Bergeron 1994, Wibbels and Crews 1994, Wennstrom and Crews 1995).

In combination with previous studies, our work demonstrates a significant diversity in the timing of sexual lability within reptile species with TSD (Fig. 3). Direct comparisons between major groups of reptiles are complicated by lineage-specific differences in the degree

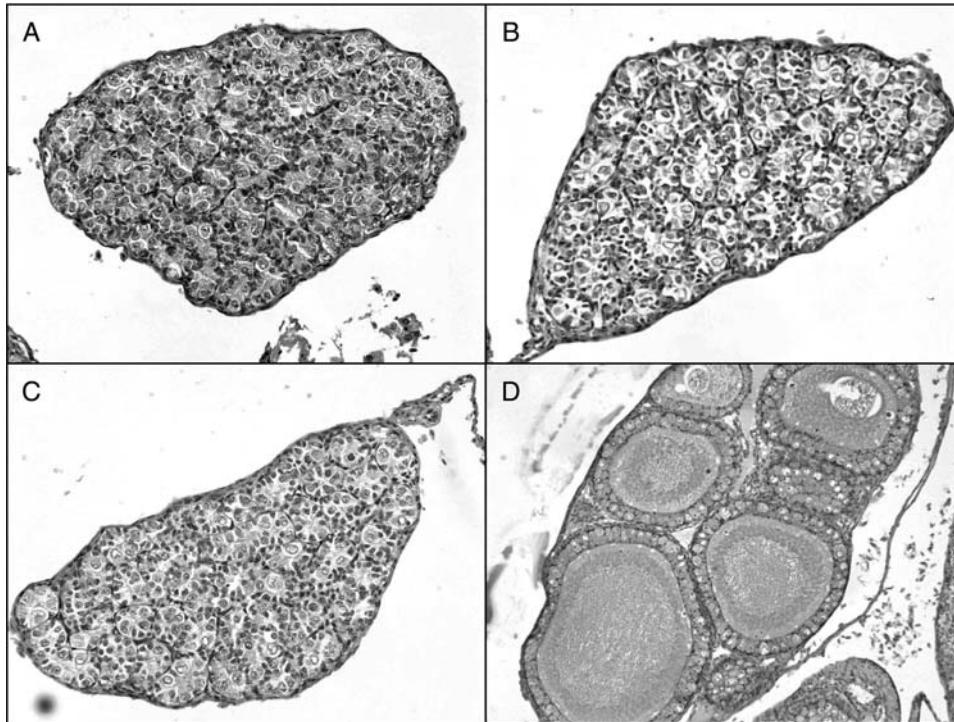


FIG. 1. Cross sections from gonads of 10-week-old hatchlings of two lizard species, showing sex differences and comparing gonads of control males and females to those of fadrozole-treated (sex-reversed) males. (A) Testis of *Amphibolurus muricatus* from an egg that had been treated with fadrozole on day 40 of incubation. (B) Testis of *Bassiana duperreyi* from an egg that had been treated with fadrozole on day 20 of incubation. (C) Testis of *B. duperreyi* from an egg that had been treated with ethanol (control) on day 20 of incubation. (D) Ovary of *B. duperreyi* that had been treated with ethanol (control) on day 20 of incubation.

to which developing embryos are retained in utero prior to oviposition. Turtles, sphenodontians, and crocodylians (and a few squamates) lay their eggs with relatively undeveloped embryos, whereas most squamates retain their developing eggs in utero for about 30% of the total period of embryogenesis (Shine 1983, Blackburn 1989). The exact percentage of development completed prior to oviposition differs significantly among squamate species and even among clutches within a single population, but probably ranges from about 25% to 40% in many squamate taxa (De Marco 1993, Andrews and Mathies 2000). Accordingly, the chronology of the period of sexual lability in TSD lizards differs considerably depending upon whether it is expressed in terms of (1) the total developmental period or (2) the incubation (post-ovipositional) period only. The left and right panels of Fig. 3 depict that difference graphically.

For both types of comparisons, our data on agamids and skinks accord well with Bull's (1987) estimates on leopard geckos (Fig. 3). In both cases the "sensitive" period comprises approximately the first half of the post-ovipositional incubation period. The primary difference between the results of Bull's (1987) study and our own involves sexual lability immediately post-oviposition; Bull's geckos did not respond to thermal cues for the first 7–14 days after the eggs were laid. This difference cannot be attributed to differences in devel-

opmental stages of the embryos; all three taxa of lizards oviposit with embryos at similar developmental stages (stage 30 in *Eublepharis* and *Amphibolurus* vs. stages 28–31 in *Bassiana*; Shine 1983 [as *Leiopisma trilineatum*], Bull 1987, Harlow 2004). However, fadrozole may have retained some activity for a few days after we applied it, reducing the temporal discrepancy between the sex-sensitive periods documented in the two studies. The ability of fadrozole to switch offspring sex late into development for *Bassiana* accords well with results of a previous temperature-switching experiment in this species (Shine et al. 2002).

What of other reptilian lineages with TSD? In the tuatara, sex is determined in the period between 25% and 55% of embryogenesis (Nelson et al. 2004, Mitchell et al. 2006); turtles are broadly similar (Ewert et al. 2004). The embryonic stages at which sex is labile in turtles and sphenodontians thus may be similar to those in lizards, although they occur much later when expressed as a percentage of the post-laying incubation period (because of the prolonged uterine retention of lizard eggs). Thus, offspring sex in TSD turtle and tuatara species is determined in the middle one-third of the post-laying incubation period compared to the first one-half or two-thirds of that period in lizards (Fig. 3, right-hand panels). Crocodylians appear to differ from the other reptile lineages in this respect. They lay eggs

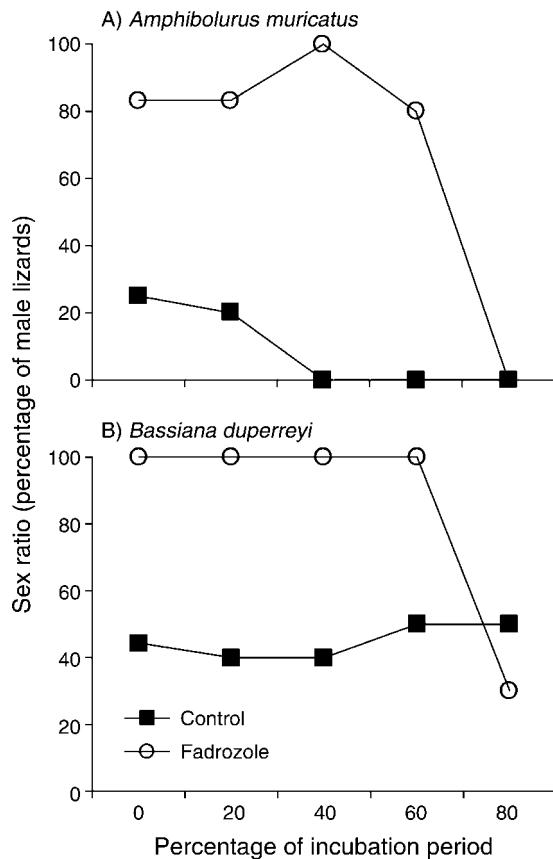


FIG. 2. Sex ratios (percentage male) of two lizard species as a function of the percentage of time through incubation when we applied the aromatase inhibitor fadrozole to the eggs. (A) Control eggs were primarily female in the agamid *A. muricatus*, and (B) about 50:50 male:female in the scincid *B. duperreyi*. Fadrozole treatment applied from 0% to at least 60% of the way through incubation generated mostly males in both species. Sample sizes were  $\geq 6$  eggs per treatment group for *A. muricatus*, and 10 eggs per treatment group for *B. duperreyi*.

with relatively undeveloped embryos, and sexual identity is determined in the third quarter of the incubation period, regardless of whether this is scored from ovulation or from oviposition (Lang and Andrews 1994; see Fig. 3).

These differences raise interesting issues about the mechanisms underlying the timing of sex determination as well as the selective forces that have favored the evolution of TSD in these disparate lineages. Based on available data, Bull (1987) concluded that there was a general similarity in the developmental stages at which sex determination occurs in all of the TSD reptiles studied to date. This remains true for squamates, sphenodontians, and turtles, but the careful work of Lang and Andrews (1994) showed that earlier reports about thermosensitive periods in crocodylians were in error. Crocodile sex is determined later than in the other reptilian groups, especially when expressed as a percentage of the post-laying incubation period (Fig. 3, right-

hand panels). In turn, this bears upon the validity of alternative explanations for the timing of sex-sensitive periods. Bull (1987) suggested that overall similarities among reptiles in periods of sexual lability might reflect conservative features of reptilian embryogenesis: that is, if gonadal differentiation occurs at about the same stages in all groups, then environmental sensitivity of sex determination may be restricted to such periods. The corrected estimates for thermosensitive periods in crocodylians compared to squamates, sphenodontians, and chelonians (Fig. 3) argue against his interpretation. "Delayed" gonadal differentiation in crocodylians is unlikely to explain this discrepancy, because morphological differentiation occurs earlier rather than later in the development of crocodylian embryos than of squamate embryos (Andrews 2004).

Another possible explanation for the timing of sexual lability is an adaptationist argument about the timing of fitness consequences. If incubation temperatures exert greater effects (and thus, potentially, differ most between the sexes in their effects on fitness) at some periods of embryonic development than at others, we might thus expect selection to move the window of sexual lability to coincide with the phase of incubation most important for offspring development (Bull 1987). Studies on squamates generally have concluded that conditions soon after laying are more important than those later in development, for developmental rates as well as phenotypic traits (e.g., Shine 1983, Andrews 2004, Lourdais et al. 2004). This generalization accords well with the observed timing of sex determination in TSD lizards, but a convincing test of the idea would require a broader range of temporal patterns in embryonic differentiation and sex determination. It would be instructive to know, for example, whether temperatures late in incubation have a disproportionately larger effect on offspring phenotypes in crocodylians than in squamates.

If mechanistic constraints cannot explain the timing of sex-labile periods in reptilian embryogenesis, could selective forces on that timing differ between groups? There is an important difference between the three main reptile lineages in the timing of sex determination relative to the timing of oviposition: sexual lability occurs almost immediately post-oviposition in oviparous squamates, in the middle one-third of development in turtles and the tuatara, and in the third quarter of development in crocodylians (Fig. 3, right-hand panels). One popular theoretical model for the adaptive significance of TSD places heavy emphasis on the idea that TSD enhances maternal fitness in circumstances where, at the time of oviposition, the mother cannot predict the fitness-relevant conditions that will be experienced by her developing offspring (Charnov and Bull 1977). This condition is more likely to be satisfied in crocodylians (where the thermosensitive period [TSP] occurs long after oviposition) than in squamates (where the TSP occurs immediately post-oviposition). Indeed, in com-

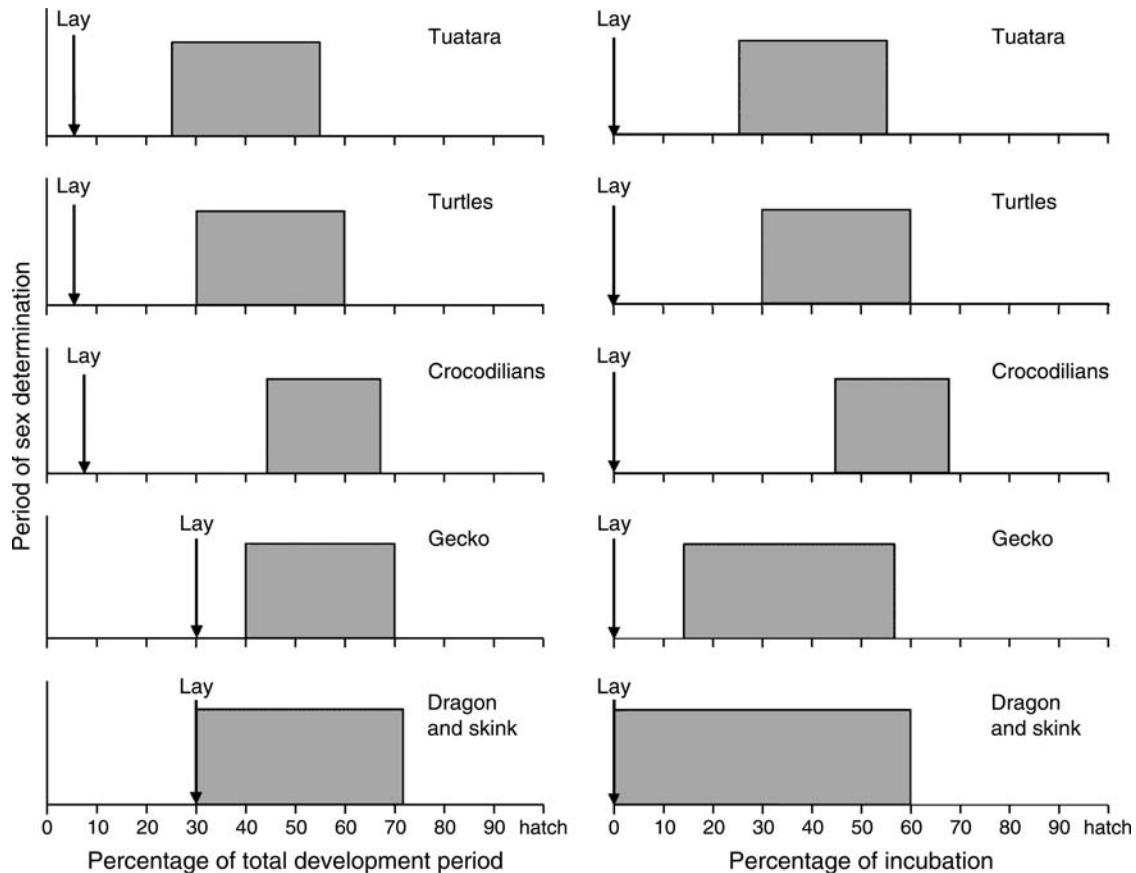


FIG. 3. A schematic comparison of the timing of sexual lability in reptile species with temperature-dependent sex determination (TSD). The arrow and word "Lay" indicate the time in development when the eggs are oviposited; the shaded region shows the duration of sexual lability (i.e., the period during which external cues can change offspring sex). Data on the tuatara come from Mitchell et al. (2006), on turtles from Bull (1987) and Ewert et al. (2004), on crocodilians from Lang and Andrews (1994), and on the leopard gecko *Eublepharis macularius* from Bull (1987). Data on the jacky dragon *Amphibolurus muricatus* and the skink *Bassiana duperreyi* come from the present study. The left-hand panels show the sex determination period expressed relative to the total duration of embryogenesis (fertilization through to hatching), whereas the right-hand panels show the same data expressed as a percentage of the post-oviposition incubation period (i.e., from laying until hatching). Differences among reptile lineages in the timing of sex determination are much greater in the latter comparison than in the former.

bination with recent demonstrations of TSD in viviparous squamates (Robert and Thompson 2001, Wapstra et al. 2004), our data suggest that reproducing female lizards in TSD species may have substantial control over incubation temperatures during the period of sex determination of their offspring. Thermal regimes prior to oviposition might influence offspring sex in oviparous species also; the sensitivity to fadrozole was present as soon as we could test for it post-oviposition, so perhaps was present even earlier. Thermoregulation by a gravid *Bassiana duperreyi* (i.e., pre-laying) can influence the phenotypic attributes of her offspring (Shine 2006), so that direct maternal control over offspring sex may be plausible in oviparous as well as viviparous squamates.

These considerations suggest that the original Charnov-Bull (1977) model for an adaptive significance to TSD may apply more strongly to turtles and (especially) crocodilians than to lizards. If a reproducing female lizard can predict (and control, through her own

thermoregulation and/or nest-site selection) the thermal regimes experienced by her offspring during the period of sex determination, ideas about unpredictability of developmental circumstances at that time seem unlikely to apply. This violates one of the two primary assumptions of the Charnov-Bull model. If reproducing females can select the incubation environments that will affect sex-specific fitness of their offspring, the models suggest that genetic sex determination (GSD) can match offspring sex to the incubation regime as well as can TSD. The recent discovery of TSD in viviparous lizards indicates that at least some TSD species have substantial control in this respect via maternal thermoregulation (Wapstra et al. 2004), although doubtless there are many circumstances in which weather extremes enforce thermal regimes different from those that would otherwise be selected by gravid females (Lourdais et al. 2004). Predictability may also be relatively high for egg-layers, at least in some types of environments. Statistical

analyses of a large data set on field nests of *B. duperreyi* revealed that incubation regimes were highly predictable at the time of laying, in terms of mean temperatures as well as variability (Shine 2002). Thus, the evolution of squamate TSD may not involve fitness advantages related to maternal ability to match offspring sex to unpredictable conditions. Instead, the adaptive significance of TSD in squamates may lie in other processes, such as the use of nest temperature as a predictor of seasonal timing of hatching (Harlow and Taylor 2000, Harlow 2004, Warner and Shine 2005) or direct sex-specific effects of low incubation temperatures on hatchling viability (Shine et al. 1995, Elphick and Shine 1999).

We conclude by returning to the theme of the *Introduction*: that the label “temperature-dependent sex determination” (TSD) may encompass a substantial diversity of phenomena (for a discussion of this point, see Janzen and Phillips 2006). TSD in some reptile lineages may have evolved as envisaged by the classic Charnov-Bull hypothesis: in such cases, we expect to see offspring sex determined long after oviposition, in circumstances that may be difficult or impossible for the mother to predict. In such taxa (as in many turtles, crocodiles, and the tuatara), TSD may have evolved because of fitness benefits related to adaptively matching offspring sex to that unpredictability. The great longevity of individuals in all of these groups may intensify selection for TSD (Bull and Bulmer 1989). In other lineages, however, an adaptive link between sex and incubation environment may be fashioned by different factors and, hence, sex may be determined during a period when the mother controls (to a greater or lesser degree) the conditions experienced by her offspring. This scenario may apply to viviparous lizards with TSD, or to any oviparous species where nest temperatures through the first half of embryogenesis are predictable at oviposition. Clearly, these situations represent end points of a continuum; it is easy to imagine circumstances in which predictability of incubation regimes at the time of oviposition is high even if the period of sex determination is delayed, and the reverse case in which predictability is low even though sex is determined shortly after the eggs are laid. Intermediate levels of predictability are equally easy to envisage, suggesting that we should be wary of classificatory schemes that oversimplify modes of reptilian sex determination.

#### ACKNOWLEDGMENTS

We thank Melanie Elphick and Jai Thomas for assistance in the laboratory, and the NSW National Parks and Wildlife Service, the ACT Parks Service, and the University of Sydney Animal Ethics Committee for permits. The work was supported financially by the Australian Research Council.

#### LITERATURE CITED

- Andrews, R. M. 2004. Patterns of embryonic development. Pages 75–102 in D. C. Deeming, editor. *Reptilian incubation. Environment, evolution and behaviour*. Nottingham University Press, Nottingham, UK.
- Andrews, R. M., and T. Mathies. 2000. Natural history of reptilian development: constraints on the evolution of viviparity. *BioScience* 50:227–238.
- Blackburn, D. G. 1989. A saltatory model for the evolutionary origin of viviparity and placentotrophy in reptiles. *American Zoologist* 29:A133.
- Bull, J. J. 1980. Sex determination in reptiles. *Quarterly Review of Biology* 55:4–21.
- Bull, J. J. 1983. *Evolution of sex determining mechanisms*. Benjamin/Cummings Publishing, Menlo Park, California, USA.
- Bull, J. J. 1985. Sex ratio and nest temperature in turtles: comparing field and laboratory data. *Ecology* 66:1115–1122.
- Bull, J. J. 1987. Temperature-sensitive periods of sex determination in a lizard: similarities with turtles and crocodiles. *Journal of Experimental Zoology* 241:143–148.
- Bull, J. J. 2004. Perspective on sex determination: past and future. Pages 5–10 in N. Valenzuela and V. A. Lance, editors. *Temperature-dependent sex determination in vertebrates*. Smithsonian Books, Washington, D.C., USA.
- Bull, J. J., and M. G. Bulmer. 1989. Longevity enhances selection of environmental sex determination. *Heredity* 63: 315–320.
- Bull, J. J., J. M. Legler, and R. C. Vogt. 1985. Non-temperature dependent sex determination in two suborders of turtles. *Copeia* 1985:784–786.
- Chardard, D., G. Desvages, C. Pieau, and C. Dournon. 1995. Aromatase activity in larval gonads of *Pleurodeles waltl* (Urodele Amphibia) during normal sex differentiation and during sex reversal by thermal treatment effect. *General and Comparative Endocrinology* 99:100–107.
- Chardard, D., and C. Dournon. 1999. Sex reversal by aromatase inhibitor treatment in the newt *Pleurodeles waltl*. *Journal of Experimental Zoology* 283:43–50.
- Charnov, E. L., and J. Bull. 1977. When is sex environmentally determined? *Nature* 266:828–830.
- Cogger, H. G. 2000. *Reptiles and amphibians of Australia*. Sixth edition. Reed New Holland, Sydney, Australia.
- Crews, D. 1994. Temperature, steroids and sex determination. *Journal of Endocrinology* 142:1–8.
- Crews, D., and J. M. Bergeron. 1994. Role of reductase and aromatase in sex determination in the red-eared slider (*Trachemys scripta*), a turtle with temperature-dependent sex determination. *Journal of Endocrinology* 143:279–289.
- Crews, D., A. R. Cantu, and J. M. Bergeron. 1996. Temperature and non-aromatizable androgens: a common pathway in male sex determination in a turtle with temperature-dependent sex determination? *Journal of Endocrinology* 149:457–463.
- De Marco, V. 1993. Estimating egg retention times in sceloporine lizards. *Journal of Herpetology* 27:453–458.
- Elphick, M. J., and R. Shine. 1999. Sex differences in optimal incubation temperatures in a scincid lizard species. *Oecologia* 118:431–437.
- Ewert, M. A., C. R. Etchberger, and C. E. Nelson. 2004. Turtle sex-determining modes and TSD patterns, and some TSD pattern correlates. Pages 21–32 in N. Valenzuela and V. A. Lance, editors. *Temperature-dependent sex determination in vertebrates*. Smithsonian Books, Washington, D.C., USA.
- Georges, A., K. Beggs, J. E. Young, and J. S. Doody. 2005. Modeling development of reptile embryos under fluctuating temperature regimes. *Physiological and Biochemical Zoology* 78:18–30.
- Georges, A., C. J. Limpus, and R. Stoutjesdijk. 1994. Hatchling sex in the marine turtle *Caretta caretta* is determined by proportion of development at a temperature, not daily duration of exposure. *Journal of Experimental Zoology* 270:432–444.
- Gutzke, W. H. N., and D. B. Chymiy. 1988. Sensitive periods during embryology for hormonally induced sex determina-

- tion in turtles. *General and Comparative Endocrinology* 71: 265–267.
- Harlow, P. S. 1996. A harmless technique for sexing hatchling lizards. *Herpetological Review* 27:71–72.
- Harlow, P. S. 2004. Temperature-dependent sex determination in lizards. Pages 42–52 in N. Valenzuela and V. A. Lance, editors. *Temperature-dependent sex determination in vertebrates*. Smithsonian Books, Washington, D.C., USA.
- Harlow, P. S., and J. E. Taylor. 2000. Reproductive ecology of the jacky dragon (*Amphibolurus muricatus*): an agamid lizard with temperature-dependent sex determination. *Austral Ecology* 25:640–652.
- Janzen, F. J., and J. G. Krenz. 2004. Phylogenetics: which was first, TSD or GSD? Pages 121–130 in N. Valenzuela and V. A. Lance, editors. *Temperature-dependent sex determination in vertebrates*. Smithsonian Books, Washington, D.C., USA.
- Janzen, F. J., and P. C. Phillips. 2006. Exploring the evolution of environmental sex determination, especially in reptiles. *Journal of Evolutionary Biology* 19:1775–1784.
- Lang, J. W., and H. V. Andrews. 1994. Temperature-dependent sex determination in crocodylians. *Journal of Experimental Zoology* 270:28–44.
- Lourdais, O., R. Shine, X. Bonnet, M. Guillon, and G. Naulleau. 2004. Climate affects offspring phenotypes in a viviparous snake. *Oikos* 104:551–560.
- Mitchell, N. J., N. J. Nelson, A. Cree, S. Pledger, S. N. Keall, and C. H. Daugherty. 2006. Support for a rare pattern of temperature-dependent sex determination in archaic reptiles: evidence from two species of tuatara (*Sphenodon*). *Frontiers in Zoology* 3:9.
- Nelson, N. J., M. B. Thompson, S. Pledger, S. N. Keall, and C. H. Daugherty. 2004. Do TSD, sex ratios, and nest characteristics influence the vulnerability of tuatara to global warming? *International Congress Series* 1275:250–257.
- Nishikimi, H., N. Kansaku, N. Saito, M. Usami, Y. Ohno, and K. Shimada. 2000. Sex differentiation and mRNA expression of P450c17, P450arom and AMH in gonads of the chicken. *Molecular Reproduction and Development* 55:20–30.
- Perez, N., and J. Borja. 1992. Aromatase inhibitors: clinical pharmacology and therapeutic implications in breast cancer. *Journal of International Medical Research* 20:303–312.
- Pieau, C., and M. Dorizzi. 1981. Determination of temperature sensitive stages for sexual differentiation of the gonads in embryos of the turtle, *Emys obicularis*. *Journal of Morphology* 170:373–382.
- Pieau, C., M. Girondot, N. Richard-Mercier, G. Desvages, M. Dorizzi, and P. Zaborski. 1994. Temperature sensitivity of sexual differentiation of gonads in the European pond turtle: hormonal involvement. *Journal of Experimental Zoology* 270:86–94.
- Robert, K. A., and M. B. Thompson. 2001. Viviparous lizard selects sex of embryos. *Nature* 412:698–699.
- Sarre, S. D., A. Georges, and A. Quinn. 2004. The ends of a continuum: genetic and temperature-dependent sex determination in reptiles. *Bioessays* 26:639–645.
- Shine, R. 1983. Reptilian reproductive modes: the oviparity–viviparity continuum. *Herpetologica* 39:1–8.
- Shine, R. 1999a. Egg-laying reptiles in cold climates: determinants and consequences of nest temperatures in montane lizards. *Journal of Evolutionary Biology* 12:918–926.
- Shine, R. 1999b. Why is sex determined by nest temperature in many reptiles? *Trends in Ecology and Evolution* 14:186–189.
- Shine, R. 2002. An empirical test of the “predictability” hypothesis for the evolution of viviparity in reptiles. *Journal of Evolutionary Biology* 15:553–560.
- Shine, R. 2004. Seasonal shifts in nest temperature can modify the phenotypes of hatchling lizards, regardless of overall mean incubation temperature. *Functional Ecology* 18:43–49.
- Shine, R. 2006. Is increased maternal basking an adaptation or a pre-adaptation to viviparity in lizards? *Journal of Experimental Zoology* 305A:524–535.
- Shine, R., M. Elphick, and S. Donnellan. 2002. Co-occurrence of multiple, supposedly incompatible modes of sex determination in a lizard population. *Ecology Letters* 5:486–489.
- Shine, R., M. J. Elphick, and P. S. Harlow. 1995. Sisters like it hot. *Nature* 378:451–452.
- Smith, C. A., P. F. Elf, J. W. Lang, and J. M. P. Joss. 1995. Aromatase enzyme activity during gonadal sex differentiation in alligator embryos. *Differentiation* 58:281–290.
- Valenzuela, N., and V. A. Lance. 2004. *Temperature-dependent sex determination in vertebrates*. Smithsonian Books, Washington, D.C., USA.
- Wapstra, E., M. Olsson, R. Shine, A. Edwards, R. Swain, and J. M. P. Joss. 2004. Maternal basking behavior determines offspring sex in a viviparous reptile. *Proceedings of the Royal Society of London Series B* 271:S230–S232.
- Warner, D. A., and R. Shine. 2005. The adaptive significance of temperature-dependent sex determination: experimental tests with a short-lived lizard. *Evolution* 59:2209–2221.
- Wennstrom, K. L., and D. Crews. 1995. Making males from females: the effects of aromatase inhibitors on a parthenogenetic species of whiptail lizard. *General and Comparative Endocrinology* 99:316–322.
- Wibbels, T., J. J. Bull, and D. Crews. 1991. Chronology and morphology of temperature-dependent sex determination. *Journal of Experimental Zoology* 260:371–381.
- Wibbels, T., and D. Crews. 1994. Putative aromatase inhibitor induces male sex determination in a female unisexual lizard and in a turtle with temperature-dependent sex determination. *Journal of Endocrinology* 141:295–299.
- Yntema, C. L., and N. Mrosovsky. 1982. Critical periods and pivotal temperatures for sexual differentiation in loggerhead sea turtles. *Canadian Journal of Zoology* 60:1012–1016.