TEMPERATURE-DEPENDENT SEX DETERMINATION IN THE FRILLNECK LIZARD, *CHLAMYDOSAURUS KINGII* (AGAMIDAE)

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ABSTRACT: Frillneck lizards (*Chlamydosaurus kingii*) are large (0.75 m total length) agamid lizards from tropical Australian savannas. We incubated 56 eggs from six clutches of *C. kingii* at four thermal regimes in the laboratory: constant temperatures of 26, 29 and 32 C, and a diurnally cycling regime (33 ± 5 C) designed to mimic extreme natural nest temperatures. Incubation temperatures determined hatchling sex; all hatchlings from the hottest and coldest thermal regimes were female, with males produced only at intermediate temperatures. Thermal regimes also determined incubation periods and significantly affected hatchling sizes. The sexes differed in size (mass, snout–vent length, tail length) at hatching, but the influence of incubation temperature on hatchling morphology was similar in the two sexes. Several attributes of *C. kingii* suggest that it offers an ideal opportunity to investigate the adaptive significance of temperature-dependent sex determination in reptiles.

Key words: Agamidae; Temperature-dependent sex determination; Incubation; Morphology; Lizard

LIVING reptiles exhibit spectacular diversity in many aspects of their reproductive biology, but few phenomena are as puzzling as their modes of sex determination. Many reptilian taxa exhibit genotypic sex determination—with or without highly differentiated sex chromosomes—whereby an individual's sex is determined at fertilization. In others, however, sex is determined by the incubation environment that an individual experiences as an embryo. Temperature-dependent sex determination (TSD) has now been described as the norm in crocodilians (Lang and Andrews, 1994), as a common occurrence in turtles (Ewert and Nelson, 1991), and an occasional occurrence in lizards (Viets et al., 1994). Phylogenetic analyses indicate that TSD has evolved, or been lost, several times during the evolutionary history of reptiles (Janzen and Paukstis, 1991*a,b*). However, the adaptive significance of reptilian TSD (if any) remains unclear, with several competing hypotheses (Burke, 1993; Charnov and Bull, 1977; Roosenburg, 1996). Part of the problem lies with too few independent evolutionary events for robust testing using the comparative method (e.g., Harvey and Pagel, 1991) and part with the fact that most TSD species are long-lived, and hence poorly suited for experimental studies on the influence of incubation temperatures on survivorship and lifetime reproductive success (Bull and Charnov, 1989). We need to know more about the distribution of TSD and alternative sex-determining systems in reptiles, with the hope of discovering additional examples of this phenomenon, especially in relatively short-lived species that might prove more amenable to experimental studies. Our work provides an example of just such a system.

MATERIALS AND METHODS

The frillneck dragon (*Chlamydosaurus kingii*) is a large [28.5 cm snout–vent length (SVL), 75 cm total length] agamid lizard widespread over northern Australia. It is best known for its spectacular threat display, in which the large (to 25 cm diameter) "frill" is erected by the hyoid musculature (e.g., Greer, 1989). The clutch size in this species is between four and 23 eggs, with means of 15 and nine recorded in two different studies (Bedford et al., 1993; Shine and Lambeck, 1989). In the field, at least some females will multiple-clutch within a breeding season (Bedford et al., 1993) and longevity has been estimated as being >6 yr (Griffiths, 1994). The large size, spectacular appearance,

and high abundance of this species have stimulated a series of ecological and physiological studies over recent years (Bedford et al., 1993; Christian and Bedford, 1995; Christian et al., 1995, 1996; Griffiths and Christian 1996a,b; Shine, 1990; Shine and Lambeck, 1989) making this species one of the best known Australian lizards. However, the mode of sex determination of *C. kingii* has remained unknown.

We obtained six clutches or part-clutches of frillneck lizard eggs from Darwin, in Australia's Northern Territory. Four of these clutches were removed from nesting burrows shortly after the female lizard had been observed laying them, one clutch was removed from a lizard that was euthanased after being seriously injured by a car, and one clutch was obtained by injecting a gravid wild-caught lizard with synthetic oxytocin (Bedford et al., 1993). Eggs were packaged in damp vermiculite and airfreighted to The University of Sydney, where they were transferred to incubators within 2–5 days of laying. Each egg was weighed on arrival then individually buried ⅔ deep in a numbered 250 ml jar of moist vermiculite (−150 kPa water potential or 129% water by dry mass of vermiculite). The jar was sealed with plastic sandwich wrap, secured with a rubber band. Eggs from each clutch were divided between two incubation treatments.

We used a total of four incubation treatments: three at constant temperatures (26, 29, and 32 C, in refrigerated constant-temperature incubators) and one designed to mimic the strong diurnal thermal fluctuations typical of natural nests. In each case, incubation temperatures were monitored close to the eggs, in a sealed jar of vermiculite with a thermometer previously calibrated against a certified mercury thermometer. Temperatures were checked daily, at which time they were re-adjusted if they varied more than ±0.2 C from the set temperature.

To mimic natural nest temperatures, we used a Clayson programmable refrigerated incubator set to perform 10 temperature changes in each 24-h period. No data are available on the diurnal range of temperatures within natural nests of *C. kingii*, but

we know that eggs of this species are laid from late in the dry season (October) to late in the wet season (April: Bedford et al., 1993; Griffiths, 1994; Shine and Lambeck, 1989). The thermal regime within the nest will depend not only on air temperatures but also on the amount of solar radiation to which the nest site is exposed and the depth of the eggs below the soil surface. Bedford et al. (1993) described a single nest of *C. kingii* as being in an open, clear area with the eggs buried to a depth of 100–150 mm. One of the nests from which we obtained eggs for this study was partially shaded during the middle of the day, but it received full sun for most of the morning and afternoon, with an egg chamber between 50 and 90 mm deep. Another nest used in this study was laid at ground level under a 200-mm pile of newly cut grass clippings in an area of full sun. Finally, I. Morris (personal communication) has examined several nests of *C. kingii* in this area and found that all were about 120–130 mm in depth and mostly in full sun. From these observations, we surmise that frillneck lizard eggs are typically laid in shallow burrows in open, sunny areas. The temperature regime in our cycling-temperature incubator was based on thermal regimes that we recorded hourly at soil depths of between 50 and 100 mm in an unshaded, grassed area in Darwin over a period of 36 days during October–November 1996 (our unpublished data).

The time at which we recorded these soil temperatures, and the consequent thermal regime under which we incubated the eggs, corresponds to the late dry season. This is a time of hot days with clear skies, when daily solar radiation reaches its annual maximum (Bureau of Meteorology records). Thus, our chosen temperature regime was similar to the maximum temperatures that field nests would experience at the beginning of the breeding season, before the onset of the wet season in December–January with its soil-cooling monsoonal rains and associated heavy clouds.

Thermal variation within this cycling incubator followed a daily sinusoidal curve with a mean of 33 ± 5.0 C. Temperatures in the incubator were measured every 30

TABLE 1.—Effects of four incubation regimes on incubation period, sex ratio, mortality rate, and hatchling size in frillneck lizards, *Chlamydosaurus kingii*. SVL = snout–vent length. Mortality at full term includes dead, full-term embryos plus lizards that hatched but were neurologically damaged.

	Incubation temperature (C)			
	26	29	32	33 ± 5
No. of eggs	13	18	13	12
Incubation period				
Mean (days) ± SD	124.5 ± 9.2	87.5 ± 3.5	72.4 ± 4.3	72.0 ± 1.3
n	2	15	9	8
Sex ratio (males/females)	0/12	10/7	6/3	0/8
Mortality; eggs that died early in development (not sexed)	1	1	4	4
Mortality at full term (sexed)	8	4	2	1
Hatchling size				
Males:				
Mean SVL (mm) ± SD	—	51.44 ± 3.16	47.33 ± 1.21	—
Mean tail length ± SD	—	95.11 ± 8.60	83.33 ± 4.79	—
Mean mass (g) ± SD	—	4.56 ± 0.83	3.15 ± 0.43	—
Females:				
Mean SVL (mm) ± SD	49.59 ± 2.08	49.80 ± 3.27	46.83 ± 2.47	50.88 ± 1.33
Mean tail length ± SD	89.32 ± 5.67	84.60 ± 14.33	71.83 ± 5.06	91.13 ± 4.79
Mean mass (g) ± SD	3.87 ± 0.76	4.29 ± 1.03	3.22 ± 0.85	4.81 ± 0.53

min inside a jar of vermiculite by a calibrated temperature probe connected to a data logger. Subsequent analysis of the resulting data confirmed that this incubator maintained a mean temperature of 33.1 C (1 SD ± 3.62 C) over the incubation period. Using Georges' (1989) model for sinusoidal temperature variation (for incubation where the temperature does not go below the minimum for embryonic development), this cycle translates into a "constant temperature equivalent" of 34.6 C.

We moved the jars within and among shelves in the incubators twice each week to decrease any effects of thermal heterogeneity within each incubator. Day of hatching was recorded when the egg was pipped; hatchlings usually emerged 6–18 h after this time. Each hatching was brushed to remove adhering vermiculite, weighed, measured (SVL and total length), and then sexed by hemipene eversion (Harlow, 1996) under a low-power dissecting microscope. Any egg that collapsed at full term and did not hatch by the following day was opened and the dead embryo removed. Neurologically damaged hatchlings were euthanased, and together with dead full-term embryos, they were dissected to verify sex. Most other hatch-

lings were released at the sites where the eggs were collected.

As we anticipated that a constant temperature of 26 C would be too cool for successful completion of incubation in this tropical lizard, three eggs from this incubator were moved to the 29 C incubator on day 60, one on day 73, and another six on day 98 of incubation. For the morphological analyses, data for hatchlings from these shifted eggs are combined with those for the two hatchlings that were left to complete full incubation at 26 C.

RESULTS

Sex Ratio of Hatchlings

Table 1 shows the incubation periods, sex ratios, rates of mortality, and hatchling sizes from eggs allocated to each of the four treatments. All lizards from eggs that were incubated at 26 C for >60 days were female, a significant departure from the null expectation of a 50/50 sex ratio ($\chi^2 = 12.0$, $P = 0.0005$, df = 1). Likewise, the eight hatchlings from the hot cycling incubator were also female ($\chi^2 = 8.0$, $P = 0.005$, df = 1), although we cannot eliminate the possibility of sex-specific mortality for the eggs that died at this temperature

during incubation. In contrast, eggs incubated at intermediate temperatures (29 and 32 C) produced approximately equal numbers of male and female offspring (Table 1). Overall, temperature significantly affected hatchling sex ratios across the four incubation treatments ($\chi^2 = 19.0$, $P = 0.0003$, df = 3).

Incubation Periods

Temperature also influenced incubation periods (one-factor ANOVA, $F_{2,30} = 137.4$, $P < 0.0001$). Post-hoc (Fisher's PLSD) tests showed that all three constant-temperature incubation treatments produced different incubation periods ($P < 0.05$ in each case) but that there was no significant difference in mean incubation periods between eggs in the constant 32 C incubation and the 33 ± 5.0 C cycling treatment.

Viability of Hatchlings

Many of the lizards from 26 C incubation appeared to be neurologically damaged at hatching; these lizards had poor motor coordination, could not run normally, and were not capable of feeding. These effects did not improve after several days. An analysis of hatchling mortality across all treatments, including lizards that either died full-term or had obvious neurological damage, showed no significant differences among incubation temperatures ($\chi^2 = 5.31$, $P = 0.15$, df = 3). Although a total of 10 fertile eggs died during incubation, there were no significant differences among the incubation treatments in rates of early egg mortality ($\chi^2 = 6.21$, $P = 0.10$, df = 3) or rates of egg mortality for individual females ($\chi^2 = 4.26$, $P = 0.51$, df = 5). Five of the eight lizards from the hot cycling incubation had externalized yolk sacs attached at hatching, a condition not seen at any other incubation temperature.

Body Size of Hatchlings

We used two-factor ANOVA to evaluate the influence of sex and incubation temperature on the sizes (mass, SVL, and tail length) of the hatchling lizards. We also performed a multivariate ANOVA (MANOVA) combining information on all three dependent variables, to avoid spurious inflation of "significant" results through multiple non-independent tests. The interaction between sex and incubation treatment was not significant for any of these traits ($P > 0.59$ in all cases; from MANOVA: $F_{3,34} = 0.61$, $P = 0.62$), but the ANOVAs showed strong main effects of incubation treatment on all three variables (on SVL: $F_{3,36} = 4.72$, $P < 0.008$; on tail length: $F_{3,36} = 7.81$, $P < 0.001$; on hatchling mass: $F_{3,36} = 7.42$, $P < 0.001$; MANOVA: $F_{9,98} = 5.25$, $P < 0.001$). In contrast, sex affected tail length ($F_{3,36} = 10.81$, $P < 0.003$) but did not affect either SVL ($F_{3,36} = 1.03$, $P = 0.32$) or hatchling mass ($F_{3,36} = 0.09$, $P = 0.76$). Nonetheless, the MANOVA showed a highly significant sex effect on hatchling morphology ($F_{3,34} = 9.27$, $P < 0.0001$).

These data show strong incubation and sex effects on hatchling morphology, but an even stronger comparison between the sexes is possible if we take inter-clutch differences in egg size into account, and restrict attention to eggs incubated at the temperatures (29 or 32 C) that produced offspring of both sexes. Analysis of covariance (ANCOVA) with egg mass as the covariate (to remove confounding effects of inter-clutch differences in egg mass) showed that at both incubation temperatures, male hatchlings were heavier and had longer SVLs and tail lengths than did their sisters (hatchling mass: $F_{1,16} = 7.48$, $P < 0.02$; SVL: $F_{1,16} = 11.11$, $P < 0.005$; tail length: $F_{1,16} = 15.14$, $P < 0.001$; nonsignificant interaction terms deleted in each case).

DISCUSSION

Our laboratory incubation experiments show that incubation temperatures affect a series of biologically important traits in frillneck lizards. It is unsurprising to find that incubation temperatures influence developmental rates (and, thus, incubation periods), because such effects are probably ubiquitous in reptiles (Packard and Packard, 1988). However, our data also show that incubation temperatures influence the morphology (body size) and sex of the hatchling lizards. It is evident that sex is determined by incubation temperature

(Table 1) making *Chlamydosaurus kingii* one of only a small number of species for which such an effect has been described in detail (see Viets et al., 1994, for review). However, temperature-dependent sex determination (TSD) undoubtedly occurs in other lizard species as well, including other, but not all, agamid taxa (e.g., see Harlow, 1994, for *Physignathus lesueurii*; Langerwerf, 1983, for *Agama caucasia*). Indeed, the first report of TSD in any vertebrate species was Charnier's (1966) note on the African species *Agama agama*.

The pattern of cool and hot incubation temperatures producing all females, with males hatching from intermediate temperatures, is typical of other TSD lizard species where the full range of incubation temperatures has been examined (Harlow and Shine, unpublished data; Tokunaga, 1985; Viets et al., 1993). There may be no constant incubation temperature at which 100% of hatchlings are male; this is the case also in other TSD lizards for which good data are available (Viets et al., 1994, for *Eublepharis macularius* and *Hemithecamyx caudicinctus*; Harlow and Shine, unpublished data, for *Physignathus lesueurii*).

Although our coolest and hottest incubation treatments were chosen to be within the estimated all-female producing temperature regimes for a tropical TSD species, both these extreme temperatures produced less than optimal hatchlings. Cool-incubated lizards were significantly more likely to have serious neurological problems, and hatchlings from the hot cycling treatment were incompletely developed at hatching (i.e., had externalised yolk sacs). The unexpectedly long incubation period of eggs at the highest temperature treatment may also reflect thermal stress. The developmental rates of squamate eggs increase linearly with increasing temperature over the middle range of constant incubation temperatures, but decrease markedly (i.e., incubation periods increase) when the eggs are incubated at near-lethal high temperatures (Muth, 1980; Viets et al., 1993). The predicted incubation period for the 33 ± 5.0 C incubator (based on the mean of 33 C) would

be 60 days (rather than the 72 days that we recorded) if this developmental rate was within the linear section of the regression.

In nature, the nests of *C. kingii* almost certainly display a high diurnal thermal variance, due primarily to solar radiation. In combination with other recent studies on reptilian development, our data suggest that embryogenesis (including sex determination) in this species may be sensitive to the variance as well as the mean of incubation temperatures (note that the sex ratios from our two hottest treatments were very different, despite the similarity in incubation periods: Table 1). Shallow nests (and individual eggs within a nest) laid in areas receiving solar radiation experience a larger daily temperature range than do deeper nests at the same location, despite their similarity in mean temperature (Jury et al., 1991). If high temperatures greatly accelerate embryogenesis, thermal variability may increase the overall rate of embryonic development. Thus, the "effective nest temperature" may be significantly higher in a shallow nest than in a deep nest with the same mean temperature but a smaller diel thermal range (Georges, 1989; Shine and Harlow, 1996). The potential for thermal variance to influence hatchling sex ratios has been elegantly shown in a laboratory study of the turtle *Caretta caretta* (Georges et al., 1994). In this study, 4% of eggs hatched as females at a constant temperature of 26 C while 100% females hatched at a cycling temperature of 26 ± 7.0 C. Recent work on scincid lizards has shown that thermal variance per se (independent of mean temperature) can influence not only incubation periods but also several aspects of the phenotypes (size, shape, locomotor performance) of the hatchling lizards (Shine and Harlow, 1996). Remarkably, such effects may differ between male and female hatchlings (Shine et al., 1997).

Our study provided no evidence of such "sex × incubation temperature" interactions in *C. kingii*—that is, incubation temperatures influenced morphology in a similar way in male and female hatchlings—but our results do indicate that incubation

regimes influenced hatchling morphology as well as sex. Plausibly, these phenotypic modifications might affect lifetime reproductive success differently in male and female lizards. All of the traits that we measured in hatchlings (mass, SVL, tail length) appear to be sexually dimorphic (at least at intermediate incubation temperatures), as well as being influenced by incubation temperature. These traits also show considerable sexual dimorphism in adults of *C. kingii* (Christian et al., 1995). In keeping with this dimorphism, available data suggest that body size influences reproductive success both in males (via male–male aggression) and females (via fecundity advantages of larger size: Shine and Lambeck, 1989). Thus, some linkage between incubation-induced phenotypic modifications and incubation-induced sex determination seems plausible for this taxon. Such a linkage is predicted by some (but not all) theoretical models for the adaptive significance of reptilian TSD (Bull and Charnov, 1989). Recent studies on North American snapping turtles showed that medium-temperature incubation produced mostly males that had a higher rate of survival and growth after hatching than did warmer-incubated females (Bobyn and Brooks, 1994; McKnight and Gutzke, 1993). Research on crocodilians has also indicated that incubation temperature can exert long-term effects on organismal growth (Hutton, 1987; Joanen et al., 1987).

Why do frillneck lizards display TSD? Several adaptive scenarios are plausible. For example, TSD might enhance maternal fitness in *C. kingii* by linking sex determination to time of hatching (because soil temperatures change predictably with the onset of wet season rains), or to incubation-induced modifications of the offspring phenotype that affect subsequent growth rates, body sizes, or behavior. Unfortunately, these possibilities remain entirely speculative. It is difficult to extrapolate a laboratory study such as ours to the field, particularly because we know relatively little about natural incubation environments or about the ways in which incubation effects might influence individual fitness in these lizards.

Our study suggests, nonetheless, that *C. kingii* might be a suitable model organism for studies of this question. On a purely logistical basis, it has several advantages over the crocodilians, turtles, and gekkonid lizards that have been the focus of most previous research in this field. First, *C. kingii* is a fast-growing, early-maturing taxon (our captive hatchlings reached maturity in <12 mo), unlike most crocodilians and turtles. Second, sex is easily determined at hatching, without the need to sacrifice the animal or to use laborious laparoscopic techniques. Thirdly, the species is abundant in an area that is logistically suitable for field studies. Fourthly, clutch sizes are much higher than in gekkonid lizards. Fifthly, the general biology of *C. kingii* is now relatively well known (Bedford et al., 1993; Christian and Bedford, 1995; Christian et al., 1996; Griffiths and Christian 1996a,b; Shine and Lambeck, 1989). Sixthly, the considerable phenotypic plasticity in response to incubation temperatures in our experimental work (Table 1) suggests that longterm effects of incubation conditions on organismal fitness may be particularly clear-cut in this taxon. Most of these advantages are shared by other Australian agamid lizards also, suggesting that this group may have considerable potential to clarify the intriguing phenomenon of temperature-dependent sex determination.

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