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Constraints on temperature-dependent sex determination in the leopard gecko (*Eublepharis macularius*): response to Kratochvil et al.

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Abstract Kratochvil et al. (Naturwissenschaften 95:209–215, 2008) reported recently that in the leopard gecko (*Eublepharis macularius*) of the family Eublepharidae with temperature-dependent sex determination (TSD), clutches in which eggs were incubated at the same temperature produce only same-sex siblings. Interpreting this result in light of studies of sex steroid hormone involvement in sex determination, they suggested that maternally derived yolk steroid hormones could constrain sex-determining mechanisms in TSD reptiles. We have worked extensively with this species and have routinely incubated clutches at constant temperatures. To test the consistency of high frequency same-sex clutches across different incubation temperatures, we examined our records of clutches at the University of Texas at Austin from 1992 to 2001. We observed that clutches in which eggs were incubated at the same incubation temperature produced mixed-sex clutches as well as same-sex clutches. Furthermore, cases in which eggs within a clutch were separated and incubated at different temperatures produced the expected number of mixed-sex clutches. These results suggest that maternal influences on sex determination are secondary relative to incubation temperature effects.

Keywords Temperature-dependent sex determination · Maternal effects · Leopard gecko · Steroid hormones

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Introduction

Sex steroid hormones, whether maternally derived or produced in situ during incubation, play a central role in gonad differentiation in reptiles with temperature-dependent sex determination (TSD) (Crews et al. 1991; Elf et al. 2002). The influence of maternally allocated hormones on sex determination may be species-specific; the sex ratio of the painted turtles (*Chrysemys picta*) is influenced by season and yolk hormones although similar correlations between yolk estrogens and within-clutch sex ratios are not seen in other turtles or lizards (Bowden et al. 2000; St. Juliana et al. 2004; Radder et al. 2007). Individual variation of clutch sex-ratio bias is found in the painted dragon (*Ctenophorus pictus*) though consistency within individuals may be genetic or hormonal (Uller et al. 2006). In the leopard gecko (*Eublepharis macularius*), temperature-sensitive steroidogenic enzymes and hormone profiles during incubation affect gonad and brain differentiation (Elf 2004; Sakata and Crews 2004; Rhen et al. 2006; Endo et al. 2008). Studies of TSD in this species reveal a type II pattern with incubation temperatures (IncT) between 31 and 33°C produce male-biased sex ratios while temperatures 30°C and lower as well as 34°C and higher produce female-biased sex ratios; incubation temperatures of 26–28°C produce only females although no IncT has been identified that produces only males (Fig. 1) (Bull 1987; Gutzke and
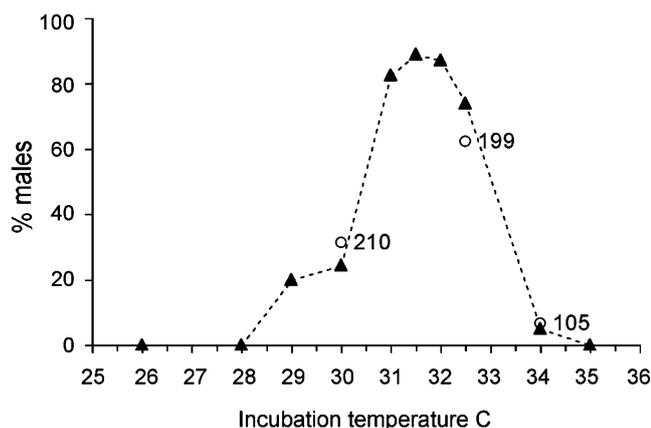
Fig. 1 Sex ratios (percent male hatchlings) across different incubation temperatures (IncTs) from Viets et al. (1993) (*filled triangles connected by dashed lines*) and from the University of Texas breeding colony from 1993 to 2001 (*open circles*). Presented are percentages of male hatchlings with different IncT with total number of hatchlings at the respective IncT. Clutch siblings were of different IncT that did not hatch or were not sexed

Crews 1988; Viets et al. 1993, 1994; Crews et al. 1998). There are two pivotal temperatures where the sex ratio of hatchlings is 1:1; the lower pivotal temperature (between 30 and 31°C) where sex ratio changes from female biased to male biased, and an upper pivotal temperature (between 33 and 34°C) where sex ratio changes from male biased to female biased (Mrosovksy and Pieau 1991; Viets et al. 1994). In the wild, these temperatures are within the range of soil temperatures (Anderson 1963). Sex determination occurs during the early to middle stages of embryonic development, after which the bipotential gonads become irreversibly ovaries or testes (Bull 1987).

Within the family Eublepharidae, some species exhibit genotypic sex determination (GSD) while others exhibit TSD; in both sex determining systems, species have little intra-clutch variation of maternally derived yolk steroid hormones concentration (Kratochvil et al. 2006; Rhen et al. 2006). Maternal contributions to sex determination in the leopard gecko have only recently begun to be explored. Sex steroid hormones from the mother are deposited into the egg during follicular development, and the concentration of yolk steroids between eggs within a clutch have been found to be similar (Elf 2004; Rhen et al. 2006). The similarity of yolk steroid concentrations in eggs within a clutch therefore provide a good reason to study the leopard gecko in determining the extent of maternal influences in the context of temperature-dependent sex determination. Recently, Kratochvil et al. (2008) observed that unlike four GSD gecko species, in the leopard gecko siblings from the same clutch (two eggs sharing the same mother, father, and date of deposition) were always the same sex when both eggs were incubated at 30°C. The authors argued that the lack of mixed-sex clutches was attributed to the similar

concentrations of yolk steroid hormones deposited during follicular development; in other words, maternally derived steroids constrain sex differentiation in geckos with TSD but not in those with GSD.

We have monitored sex ratios in our breeding colony of leopard geckos at the University of Texas at Austin and reading Kratochvil et al. (2008) motivated us to assess whether mixed-sex clutches were also absent in our large dataset. Breeding records from 1992 to 2001 were examined to: (1) compare the observed frequency of same- and mixed-sex clutches when both eggs in a clutch were incubated at 30°C (the same temperature as that reported in Kratochvil et al. (2008)) and 32.5°C, (2) reexamine the sex ratios across incubation temperatures, and (3) assess the magnitude of maternal contributions to sex ratios of clutches where eggs were incubated at different incubation temperatures.

Materials and methods

Generating and hatching eggs Leopard geckos were bred in captivity at the University of Texas at Austin. Eggs were collected from moistened nest boxes within a day or two of oviposition. Clutches usually consisted of two eggs, though occasionally only of one. In same-IncT studies, both eggs within a clutch were incubated at either 30 or 32.5°C. In different-IncT studies, eggs within a clutch were split and incubated at different IncTs. Individual eggs were placed in plastic cups with moist vermiculite then covered with perforated plastic and maintained at a constant temperature within incubators (Precision Scientific, Chicago, IL, USA) monitored by calibrated mercury thermometers until hatching (Viets et al. 1993; Coomber et al. 1997). Across years, the moisture content ranged from 1:1 to 1.5:1 ratio of water: vermiculite. Moisture content during incubation has not been demonstrated to affect hatchling sex ratio in the laboratory under constant temperature (Viets et al. 1993); therefore, the data from 1:1 and 1:1.5 ratios of water: vermiculite were combined. The sex of the individual was determined by monitoring morphological development from hatching to sexual maturity (40–50 weeks). Individuals were assigned as males if they developed pre-anal pores and hemipenes bulges (Rhen et al. 2005).

Predicting frequencies of clutch siblings with different sexes We used a χ^2 test to examine whether the observed frequencies of same- vs. mixed-sex clutches differed from expected frequencies. We used the ratio of same- vs. mixed-sex clutches as a proxy for maternal effects because (1) females deposit sex steroids into the egg during follicular development, (2) steroid composition is comparable between eggs within a clutch, and (3) because sex steroid

hormones influence sex determination in TSD species (Elf 2004; Rhen et al. 2006); therefore, a maternal contribution would be manifested as an increase in the prevalence of same-sex siblings. We used a similar method to calculate the expected frequency of same- vs. mixed-sex clutches as Kratochvil et al. (2008). For the experiments in which we incubated both eggs in a clutch at the same IncT, we used the observed frequency of males and females at each IncT to calculate the expected frequencies of same- and mixed-sex clutches. For example, at the IncT of 30°C, the expected ratio was calculated as $(30M)^2 + (30F)^2 : 2 \times (30M) \times (30F)$, where 30M and 30F refer to the proportion of males and females, respectively, at 30°C, and 32.5M and 32.5F refer to the proportion of males and females, respectively, at 32.5°C. Instead of analyzing the frequency of two male siblings and of two female siblings separately as did Kratochvil et al. (2008), we simply asked whether the observed frequency of same- vs. mixed-sex clutches differed from expected. This was due to the hypothesis that maternal deposition of yolk steroids biases eggs in a clutch to be either both male or both female; there is no a priori prediction as to which scenario should dominate. Same-IncT clutches of 26 and 34°C were excluded from the analysis because the former produces only female offspring and the latter produces predominantly females (<5% males; Viets et al. 1993). We included data only from females who had a single clutch incubated at either 30 or 32.5°C to avoid pseudoreplication.

Another test of maternal contributions to sex determination is to examine whether the observed frequencies of same- vs. mixed-sex clutches differed from the expected frequencies when eggs within a clutch were separated and incubated at different IncTs. For example, if maternal deposition of yolk steroids strongly biases both embryos within a clutch to develop into females, then one should observe a higher incident of same-sex clutches even if eggs were separately incubated at 30 and 32.5°C; in other words, if maternal contributions are equally influential across various IncT, they should noticeably affect within-clutch sex ratios independently of IncT. For this analysis, we first computed the sex ratios at 30, 32.5, and 34°C from a group of hatchlings that was distinct the dataset used to analyze same-IncT and different-IncT clutches. Each of these hatchlings had a clutch sibling from a different IncT whose sex was unrecorded, so that no two individuals from the same clutch were used in the sex-ratio calculation. Eggs from the same clutch were separated and incubated in one of the following temperature pairs: 30 and 32.5°C, 30 and 34°C, or 32.5 and 34°C. Both 30 and 34°C are female-biased temperatures within a degree of the lower and upper pivotal temperatures, respectively (Viets et al. 1993, 1994). The expected ratio of same- vs. mixed-sex clutches for clutches separated and incubated at 30 and 32.5°C, for

example, was: $(30M) \times (32.5M) + (30F) \times (32.5F) : (30M) \times (32.5F) + (30F) \times (32.5M)$.

Results

Analysis of eggs incubated at the same incubation temperature We recorded the sexes of hatchlings from 32 clutches in which both eggs were incubated at 30°C and compared the frequency of same- vs. mixed-sex clutches against the expected frequencies. Of the 64 hatchlings, 20 developed into males and 44 developed into females (31.3% male). We observed both mixed- and same-sex clutches. Specifically, six mixed-sex and 26 same-sex clutches were observed. This observed frequency was significantly different from the predicted frequency of 13.75 mixed-sex and 18.25 same-sex clutches (χ^2=7.7, df=1, p=0.0056; Table 1). Of the 30 hatchlings from 15 clutches in which both eggs were incubated at 32.5°C, 18 developed into males and 12 developed into females (60% male). Again, we observed both mixed- and same-sex clutches. Specifically, we observed six mixed-sex and nine same-sex clutches, and this observed frequency did not differ significantly from the expected frequencies of 7.2 mixed-sex and 7.8 same-sex clutches (χ^2=0.4, df=1, p=0.54; Table 1).

Analysis of eggs incubated at different incubation temperatures Here, we analyzed whether the frequency of same- vs. mixed-sex clutches significantly differed from the expected frequencies of 128 clutches wherein clutches were split and the eggs placed in different IncT. The expected frequencies were calculated based on the observed sex ratios at 30, 32.5, and 34°C from a distinct set of hatchling data (514 hatchlings from 514 clutches); the sex ratios (percent male) at these IncTs were, respectively, 31.4%, 63.0%, and 7.0% (Fig. 1). We analyzed instances in which eggs within a clutch were separated and incubated at 30 and 32.5°C, 30 and 34°C, and 32.5 and 34°C. In each case, the observed frequencies of same- vs. mixed-sex clutches were not significantly different from the expected frequencies (Table 2). This analysis indicates that IncT effects on sex determination dominate potential maternal effects, and that high frequencies of same-sex clutches are not seen when eggs of one clutch are separated into different IncTs.

Discussion

Based on the observation in the leopard gecko (*E. macularius*) that siblings were always either both male or both female (i.e., same-sex clutches) when both eggs within a clutch were incubated at 30°C, Kratochvil et al. (2008)

Table 1 Frequencies and sex ratios of same-clutch eggs at same incubation temperature (IncT)

Incubation temperature (°C)	Number of clutches	Number of hatchlings	Sex ratio (male:female)	Possible sibling sex outcomes	Observed sibling sex frequency	Expected sibling sex frequency	χ^2	p
Kratochvil et al. 2008 Same 30°C	26	52	20:32	Two ♂ or two ♀: one ♀ and one ♂	26 0	13.69 12.31	23.4	<0.00001
Univ. Texas Same clutch Same 30°C	32	64	20:44	Two ♂ or two ♀: one ♀ and one ♂	26 6	18.25 13.75	7.7	0.0056
Univ. Texas Same clutch Same 32.5°C	15	30	18:12	Two ♂ or two ♀: one ♀ and one ♂	9 6	7.8 7.2	0.4	0.54

Data from Kratochvil et al. (2008) of same-clutch, same-incubation temperature (30°C) siblings presented as reference on first row. Each clutch has two eggs sharing the same deposition date, father, and mother. χ^2 analysis for sibling same-sex vs. mixed-sex clutches ($df=1$, two ♂ or two ♀: one ♀ and one ♂). Data of same-clutch, same-IncT hatchlings were obtained from the University of Texas breeding colony from 1995 to 2001 and 1993–1999 for 30 and 32.5°C, respectively

hypothesized that maternal contributions (via sex steroid hormone deposition into the egg) significantly constrain sex-determining mechanisms. Here, we analyzed a larger dataset of offspring sex ratios from our colony of leopard geckos and assessed maternal contributions to sex ratios using two approaches. Maternal contributions were estimated by comparing the frequencies of same- vs. mixed-sex clutches for clutches in which both eggs were incubated at 30 or 32.5°C as well as for clutches in which eggs were separately incubated at 30, 32.5, or 34°C; therefore, we provide a more extensive test of maternal contributions to sex determination in this species.

If maternal contributions were significant, the frequency of same-sex clutches should be greater than expected for each experimental manipulation. First and foremost, in contrast to the data presented in Kratochvil et al. (2008), our analysis revealed the occurrence of mixed-sex clutches (~20%) when both eggs within a clutch were incubated at 30°C (Table 1). We also observed mixed-sex clutches (40%) when both eggs in the clutch were incubated at 32.5°C (Table 1). Second, the observed frequency of same-

vs. mixed-sex clutches was significantly different from expected at 30°C but not at 32.5°C (Table 1); this suggests a maternal contribution, albeit a weaker one than that observed in Kratochvil et al. (2008). This notion of relatively weak maternal contributions to sex determination was supported by the fact that the observed frequencies of same- vs. mixed-sex clutches were not different from expected when eggs within a clutch were separately incubated among 30, 32.5, and 34°C (Table 2). Taken together, these data strongly support the model proposed by Sakata and Crews (2004) that incubation temperature sets the threshold by which sex is determined via temperature-sensitive mechanisms that interact with circulating hormones or generate new hormones. The incubation temperature-as-threshold model is supported by temperature-correlated differential decreases in yolk steroid concentrations in leopard geckos as well as turtles (Elf et al. 2002). The model is also open to the possibility of temperature-sensitive gonad differentiation in the absence of yolk hormones or other steroidogenic tissue (Jeyasuria and Place 1998; Pieau and Dorizzi 2004; Ramsey and Crews 2007;

Table 2 Frequencies and sex ratios of same-clutch eggs separated and incubated at a different incubation temperature (IncT)

Incubation temperature pairs (°C)		Number of clutches	Number of hatchlings	Possible sibling sex outcomes	Observed sibling sex frequency	Expected sibling sex frequency	χ^2	p
30°C	32.5°C	56	112	Two ♂ or two ♀: one ♀ and one ♂	32 24	25.3 30.7	3.244	0.071
30°C	34°C	35	70	Two ♂ or two ♀: one ♀ and one ♂	23 12	23.1 12.9	0.001	0.971
32.5°C	34°C	37	74	Two ♂ or two ♀: one ♀ and one ♂	14 23	14.4 22.6	0.015	0.902

Each clutch has two eggs sharing the same deposition date, father, and mother. χ^2 analysis for same-sex vs. mixed-sex clutches ($df=1$; two ♂ or two ♀: one ♀ and one ♂). Data of same-clutch, different-IncT hatchlings were obtained from the University of Texas breeding colony from 1992 to 2001

Endo et al. 2008). Likewise, reptiles with GSD have sex ratios altered by certain temperatures, or even exogenous hormones; one mechanism of sex determination is not always exclusively found within a species (Shine et al. 2002; Quinn et al. 2007; Radder et al. 2008).

The difference in absence and presence of mixed-sex clutches between Kratochvil et al. (2008) and our results is possibly due to clutch effects from incubation methods or gene×environment interaction. Both studies incubated eggs within a clutch at constant temperatures, but the method of incubating eggs was slightly different; Kratochvil et al. (2008) placed eggs within the same plastic container, whereas we incubated eggs individually in covered plastic containers. It is possible that eggs placed in the same enclosed container are exposed to the same concentrations of metabolic waste such as heat and carbon dioxide, which can subsequently homogenize the external nesting environment that may not necessarily clarify the role of maternal contributions. Heat would be less uniform within a clutch where eggs are incubated individually, potentially creating differences in temperature during incubation (Ewert and Nelson 2003). Carbon dioxide concentration, which alters the activity of pH-sensitive 5-alpha-reductase, can subsequently homogenize steroidogenic activity of eggs in the same container (Jeyasuria and Place 1998). Neither our study nor that of Kratochvil et al. (2008) measured temperature or carbon dioxide concentration next to each egg. Differences in the observed sex ratios at 30°C cannot account for the presence of mixed-sex clutches, as our observed sex ratio at 30°C was comparable to that reported in Kratochvil et al. (2008), and, moreover, our reported sex ratios across incubation temperatures followed the same type II pattern as observed by Viets et al. (1993). The presence of mixed-sex clutches at 30°C, albeit at a frequency lower than expected, could result from gene× environment interactions (Janes and Wayne 2006). One clutch was represented per mother, but some fathers had more than one clutch represented. As we have aimed to follow up on Kratochvil et al. (2008) in the context of maternal contribution in TSD reptiles, we must further assess gene×environment interaction with full and half siblings with the same father.

The magnitude of maternal influences via yolk steroid hormone deposition on sex-determining mechanisms is unclear. In the leopard gecko, the concentration of yolk estradiol is positively correlated to circulating estradiol levels in gravid females, indicating that individual differences in circulating estrogens during follicular development could lead to variation in the amount of estrogens deposited into the egg (Rhen et al. 2006). The influence of estrogens on female development in this species as well as other TSD species is well established, suggesting that maternal estrogen deposition into the egg could bias hatchlings to

develop into females (Bull et al. 1988; Rhen and Lang 1994; Tousignant and Crews 1994). We have previously documented that initial yolk steroid hormones do not differ significantly among eggs within a clutch due to synchronous follicular development and oviposition (Elf 2004; Rhen et al. 2006). Therefore, maternal contributions to gonadal differentiation should be comparable across eggs within a clutch. Despite similar initial yolk concentrations, we observed instances in which hatchlings from eggs incubated at the same incubation temperature developed into a male and female sibling (mixed-sex clutches; Table 1) and the frequency of same- vs. mixed-sex clutches was not significantly different from expected when clutches were split across IncTs (Table 2). Consequently, if maternally derived yolk steroids do influence sex determination in this TSD lizard, it is of secondary importance to the temperature of the incubating egg.

Our primary objective of the current study was to assess the extent to which maternal effects influence hatchling sex within a clutch observed by Kratochvil et al. (2008). Specifically, we set out to examine whether the frequency of same- vs. mixed-sex clutches, a proxy for maternal contributions to sex determination as used in the previous study, deviated from expected under a variety of experimental manipulations. Our objective was not to fully quantify maternal contributions to sexual differentiation; this would require a more extensive analytical approach akin to techniques used in quantitative genetic analyses. Unlike Kratochvil et al. (2008), we observed that in the same-incubation temperature studies, 20% and 40% were mixed-sex clutches in which both eggs were incubated at either 30 or 32.5°C, respectively. Furthermore, by testing maternal contributions when eggs within a clutch were incubated at different temperatures, we failed to find consistently significant maternal effects. Taken together, we documented much stronger support for the notion that incubation temperature sets the threshold to which yolk steroid hormones influence sex determination.

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