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ORIGINAL PAPER

Offspring sex in a TSD gecko correlates with an interaction between incubation temperature and yolk steroid hormones

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Abstract We incubated eggs of the Japanese gecko *Gekko japonicus* at three temperatures, and measured yolk testosterone (T) and 17β-estradiol (E2) levels at three time points in embryonic development (oviposition, 1/3 of incubation, and 2/3 of incubation), to examine whether maternal influence on offspring sex via yolk steroid hormone deposition is significant in the species. Eggs incubated at 24 °C and 32 °C produced mostly females, and eggs incubated at 28 °C almost a 50:50 sex ratio of hatchlings. Female-producing eggs were larger than male-producing eggs. Clutches in which eggs were incubated at the same temperature produced mostly same-sex siblings. Yolk T level at laying was negatively related to eggs mass, and yolk E2/T ratio was positively related to egg mass. Results of two-way ANOVA with incubation temperature and stage as the factors show that: yolk E2 level was higher at 32 °C than at 24 °C; yolk T level was higher, whereas yolk E2/T ratio was smaller, at 28 °C than at 24 °C; yolk E2 and T levels were higher at 2/3 than at 1/3 of incubation. Our data in *G. japonucus* show that: (1) maternal influence on offspring sex via yolk steroid hormone deposition is significant; (2) incubation temperature affects the dynamics of developmental changes in yolk steroid hormones; (3) influences of yolk steroid hormones on offspring sex are secondary relative to incubation temperature effects; and (4) offspring sex correlates with an interaction between incubation temperature and yolk steroid hormones.

Keywords Sex determination · Incubation temperature · Yolk steroid hormones · Maternal effects · Egg size · *Gekko japonicus*

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Introduction

Sex determination is a developmental process after which the undifferentiated gonads develop into testes or ovaries (Matsumoto and Crews 2012). There are two major types of sex determination in vertebrates: genotypic sex determination (GSD), in which offspring sex is determined at the time of fertilization by genetic factors and environmental sex determination (ESD), where environmental factors that act after fertilization at a crucial embryonic stage determine offspring sex (Valenzuela and Lance 2004). Temperature-dependent sex determination (TSD), whereby the sex of embryos depends on the temperature at which they develop, is one form of ESD (Crews 1994). TSD is widespread among reptiles including all crocodilians studied to date, tuataras, many turtles and some lizards (Valenzuela and Lance 2004). The discovery that in TSD species temperature is not the only factor influencing sex determination indicates that other factors, especially maternal influences via yolk steroid hormone deposition, can influence the end result of the sexual differentiation process, although the influence (direction and/or magnitude) of a given steroid hormone may be species-specific (Janes et al. 2007; Radder 2007; Warner et al. 2008, 2009). For example, eggs with elevated levels of corticosterone are more likely to produce daughters in the Jacky dragon *Amphibolurus muricatus* and sons in the three-lined skink *Bassiana duperreyi* (Warner et al. 2009).

There has been some evidence that maternally derived yolk steroid hormones can influence offspring sex in several species of turtles and the American alligator *Alligator mississippiensis* with TSD (Conley et al. 1997; Janzen et al. 1998; Bowden et al. 2000; Elf 2003, 2004). Maternally derived yolk steroid hormones can also influence offspring sex even in species with GSD. For example, maternally derived yolk testosterone (T) might influence offspring sex by differentially influencing male- and female-inducing sperm in the green anole *Anolis carolinensis* (Lovern and Wade 2003a). In *B. duperreyi*, a species that exhibits multifactorial sex determination (Radder 2007), a negative correlation between yolk dihydrotestosterone (DHT) level and egg size provides an explanation for the result that large eggs are more likely to produce daughters and small eggs to produce sons (Radder et al. 2009).

Some previous studies have relied upon experimental manipulations, applying exogenous steroids directly to eggs or implanting hormone capsules to elevate steroid levels in mothers and eggs, to explore correlations between yolk steroid hormones and offspring sex in reptiles (Gutzke and Chymiy 1988; Elf 2003, 2004; Radder 2007; Radder and Shine 2007; Warner et al. 2009). Though not reflecting the natural situations, these studies generally show that yolk steroid hormones can influence offspring sex in both TSD and GSD species. In reptiles yolk steroid hormones that influence offspring sex are mostly, if not completely, maternally derived, because embryonically derived steroids are too low to play a key role before the sex of embryos is determined (Elf 2003, 2004; Radder 2007; Radder et al. 2007). The role of maternally derived yolk steroid hormones in sex determination was sometimes undetectable because the experimental protocols or statistical methods used limited its discovery. In *B. duperreyi*, for example, a negative correlation between yolk DHT level and egg size was not detected in an earlier study where the raw data were analyzed without reducing variance heterogeneity via logarithmic transformation (Radder et al. 2007, 2009).

Maternal influence on offspring sex via yolk steroid hormone deposition has been hypothesized to be common, especially in species with TSD (Radder et al. 2009). Here, we describe a study testing whether such an influence is also significant in the Japanese gecko *Gekko japonicus*, a TSD gekkonid lizard (Tokunaga 1985). Female *G. japonicus* lay 1−3 clutches per season, 1−2 eggs per clutch, often between mid-May and early August; larger females generally produce more clutches and larger eggs than do smaller ones and, depending on the sampling year or season, and 7−16% of variation in egg size can be explained by maternal size (Ji et al. 1991; Xu and Ji 2001). *G. japonicus* is one of the most interesting gekkonid lizards with regard to sex-determining mechanism: the co-occurrence of heteromorphic sex chromosomes and TSD in specimens collected from a population in Japan (Yoshida and Itoh 1974; Tokunaga 1985) could have added evidence to the hypothesis that TSD and GSD are extremes of a continuum (Sarre et al. 2004; Shine et al. 2002; Gamble 2010). However, a different karyotype was reported for *G. japonicus* from eastern China, with no evidence of heteromorphic sex chromosomes (Chen et al. 1986). The differences in karyotype between the two sampling sites could indicate the presence of a cryptic species (Gamble 2010).

In the present study, we incubated eggs at 24 °C (low temperature females produced), 28 °C (male-producing temperature) and 32 °C (high temperature females produced) (Tokunaga 1985), and measured yolk T and 17β-estradiol (E2) levels at three time points in development, oviposition, 1/3 of incubation, and 2/3 of incubation. Specifically, we address three questions: (1) Do yolk T and/or E2 levels at laying influence offspring sex and vary with egg size, thus allowing us to evaluate the roles of maternally derived yolk steroids and maternal yolk allocation (and thus, egg size) in sex determination in *G. japonicus*? (2) Are yolk T and E2 levels at laying similar between eggs within a clutch, thus resulting in high frequencies of single-sex clutches across different incubation temperatures? (3) Are the dynamics of developmental changes in yolk T and E2 levels thermally dependent, thus providing evidence that incubation temperature can affect the hormonal environment of developing embryos? Answering the first two questions will allow us to assess the functional link between yolk steroid hormones and offspring sex, the extent to which maternal effects affect offspring sex, and the effect of an interaction between incubation temperature and maternally derived yolk steroid hormones on offspring sex. At the later stages of development, the embryo's gonads should be developed, and therefore the embryo itself would produce its own steroids (Elf 2003, 2004; Radder 2007; Radder et al. 2007). Thus, answering the third question will allow us to assess if offspring sex could be affected by temperature via an impact on yolk steroids at different stages of development.

Materials and methods

Egg collection and treatment

We collected females that were ready to lay eggs between May and July of 2009−2011 from various localities in Nanjing (32 °03′N, 118 °45′E), eastern China, and transported them to our laboratory at Nanjing Normal University. Females were housed individually in 200×150×150 mm (length × width × height) plastic mesh cages placed in a room where temperature varied naturally, often within the range of 24−28 °C. We lined the walls of each cage with paper, thereby allowing females to attach their eggs to the

paper which could be easily removed using wet towel. The time that females remained in the mesh cages was never more than 7 days and often less than 4 days. Eggs were collected, measured and weighed less than 6 h post-laying. We weighed eggs individually if they were not glued together, and weighed the entire clutch and then divided by two if they were glued together. Post-oviposition females were measured and weighed, and then released at their site of capture.

Of the 310 fertile eggs (171 clutches) collected, 279 (152 clutches) were incubated at three constant temperatures ranging from 24 °C to 32 °C, and the remaining 31 (19 clutches) were dissected at oviposition. Two eggs (occasionally one) from the same clutch were incubated individually in covered plastic jars (50 ml) that contained known amounts of vermiculite and water to produce approximately −220 kPa water potential (1 g dried vermiculite/1 g water; Ji and Braña 1999). These jars were assigned to three incubators (Sheldon MFG Inc., USA) inside which temperatures were controlled at 24 °C, 28 °C and 32 °C (±0.3), respectively. Of the 279 eggs incubated, 168 hatched, 35 failed to hatch, and 76 were dissected at either 1/3 (28 days since oviposition at 24 °C, 18 days at 28 °C, and 14 days at 32 °C; Ji et al. 1991; Xu and Ji 2001) of incubation, or at 2/3 of incubation.

Incubation length was recorded for each hatched egg. Hatchlings (N=168), numbered individually, often at 10-day intervals, using a non-toxic waterproof marker for future identification, were measured and weighed at hatching. Between 10 and 15 hatchlings were housed in one 400×400×200 mm mesh cage placed in the room mentioned above. A 40-W heating moonlight mounted in each cage allowed thermoregulation for 8 h daily during the night. Small mealworms (*Tenebrio molitor*), German cockroaches (*Blattella germanica*) and fruit flies (*Drosophila melanogaster*) were provided in excess and spread throughout the cage, such that hatchlings had free access to the food. Live hatchlings were sexed at 120 days of age, and re-sexed on 240 days of age; the presence of hemipenes allowed unequivocal identification of males. None of hatchlings was sexed wrongly, and our sex diagnoses in 120-day-old hatchlings were consistent with the results of sex determination on 240 days of age. None of hatchlings died in the first 45-day period. Bodies of the hatchlings (N=18) that died during the period of day 45−240 were dissected for gonadal identification, and those with testes allowed unequivocal identification of males.

Yolk steroid hormone analyses

Yolk steroid hormones, testosterone and 17β-estradiol, were determined for each dissected egg. On the appropriate sampling day, eggshells were cut open and the embryo and yolk were gently teased apart. The embryo was identified for developmental stage according to the criteria of Dufaure and Hubert (1961); the yolk was transferred into a 2-ml plastic Eppendorf tube and stored at −80 °C until analysis.

Concentrations of yolk steroid hormones were determined by chemiluminescence immunoassay via CLIA kits (T product no. CLA4660, and E2 product no. CLA4664; DRG International Inc). Before the CLIA assay, yolk T and E2 were extracted and chromatographically separated (Schwabl 1993; Lovern and Wade 2001). Yolk samples were thawed, homogenized, and 6–30 mg was mixed with 1.0 ml dH_2O for extraction. All samples were equilibrated overnight at 4 °C, extracted twice with 3 ml petroleum ether and diethyl ether (30:70 vol/vol), dried with nitrogen gas, and reconstituted with 90 % ethanol. The extracted samples were stored at −20 °C overnight, and then centrifuged at 2,000 rpm at 0 °C for 5 min to precipitate neutral lipids and any proteins that were extracted by the ether. The supernatant was dried and reconstituted in 500 μl of 10 % ethyl acetate in isooctane. To remove additional neutral lipids and to isolate T and E2, all samples were transferred to diatomaceous earth microcolumns for chromatographic separation. Purified T and E2 fractions were dried under nitrogen gas, resuspended in PBS, and then placed overnight at 4 °C. Samples were assayed in duplicate, averaged, and adjusted for individual recovery and initial sample mass. The interassay coefficient of variation was 10.9 % for T and 7.7 % for E2, and the intraassay coefficient of variation was 9.9 % for T and 5.0 % for E2. Yolk steroid levels are expressed as pg/mg.

Statistical analyses

We used G-test to examine whether hatchability and the sex ratio of hatchlings differed among the three temperature treatments, and whether eggs larger than the average size were more likely to produce female hatchlings. We used linear regression analysis to examine whether an examined variable (hatchling mass, E2 level, T level and E2/T ratio) was related to egg size. We used paired-sample t-test to examine whether yolk T and E2 levels measured at laying differed between eggs within a clutch. We used two-way ANOVA (for egg mass and incubation length) and two-way ANCOVA (for hatchling mass, to correct for egg size and to test for homogeneity of slopes) to examine whether these variables differed among the three temperature treatments and between the sexes. We also used two-way ANOVA to examined whether yolk T and E2 levels and E2/T ratio differed among the three temperature treatments and between the 1/3 and 2/3 of incubation. Tukey's post hoc test was performed on the traits that differed among the three temperature treatments. Data on hatchlings of the same sex were blocked by the clutch to avoid pseudo-replication. Ratio data were arcsine transformed, data on T and E2 levels were $\log_e$ transformed, and other data did not require

transformation to meet the assumptions for parametric tests. All statistical analyses were performed with the Statistica software (version 6.0 for PC, Tulsa, OK, USA). Throughout this paper, values are presented as mean±SE, and the significance level is set at α=0.05.

Results

Thermal dependence of egg incubation

Hatching successes, 86 % (48/56) at 24 °C, 83 % (60/72) at 28 °C, and 80 % (60/75) at 32 °C, did not differ among the three temperature treatments (*G*-test; G=0.76, df=2, P=0.683). Eggs incubated at the three temperatures did not differ from each other in mean mass, and female-producing eggs on average were larger than male-producing eggs; incubation length differed among the three temperature treatments but not between the sexes, with the mean length shortened by 31 days from 24 °C to 28 °C, and by 10 days from 28 °C to 32 °C; hatchling mass did not differ among the three temperature treatments, nor between the sexes after accounting for egg mass; the interaction between incubation temperature and sex was not a significant source of variation in egg mass, incubation length, or hatchling mass (Table 1). The mean embryonic stage was 27 (range=26–28) at oviposition, 34 (range=32–35) at 1/3 of incubation, and 37 (range=36–38) at 2/3 of incubation.

Sex ratio of hatchlings

Eggs incubated at 24 °C produced mostly females (88 %; G=30.37, df=1, P<0.0001), so did those incubated at 32 °C (85 %; G=32.45, df=1, P<0.0001); eggs incubated at 28 °C produced a almost 50:50 sex ratio of hatchlings (53 % females versus 47 % males; G=0.27, df=1, P=0.605) (Fig. 1a). Of the 63 two-egg clutches in which both eggs were incubated at the same temperature, 56 or 89 % (18 or 95 % at 24 °C, 17 or 77 % at 28 °C, and 21 or 95 % at 32 °C) produced same-sex siblings, and seven or 11 % (one or 5 % at 24 °C, five or 23 % at 28 °C, and one or 5 % at 32 °C) produced mixed-sex siblings. Eggs larger than the average size (mass) were more likely to produce female hatchlings than those smaller than the average size (92 % versus 83 % females at 24 °C, 73 % versus 33 % females at 28 °C, and 87 % versus 83 % females at 32 °C; Fig. 1b), but the difference was significant only in the 28 °C treatment (G=9.93, df=1, P<0.002).

Yolk steroid hormones

Of the 31 eggs dissected at oviposition, 24 were from two-egg clutches. We found in these clutches that yolk E2 (paired-sample *t*-test; t=0.61, df=11, P=0.551) and T (t=0.91, df=11, P=0.380) levels did not differ between eggs within a clutch. Yolk E2 level was independent of egg mass ($F_{1,\ 17}$=0.69, P=0.419), yolk T level was negatively related to eggs mass ($F_{1,\ 17}$=6.13, P=0.024; Fig. 2), and yolk E2/T ratio was positively related to egg mass ($F_{1,\ 17}$=9.16, P=0.008).

Incubation temperature affected yolk E2 level, T level and E2/T ratio, and eggs dissected at 1/3 of incubation differed from those dissected at 2/3 of incubation in yolk E2 and T levels but not in E2/T ratio (Fig. 3, Table 2). None of these three variables was affected by the interaction between incubation temperature and developmental stage (Table 2). The mean yolk E2 level was higher at 32 °C than at 24 °C, the mean yolk T level was higher at 28 °C than at 24 °C, the mean yolk E2/T ratio was greater at 24 °C than at 28 °C, and the mean yolk E2 and T levels both were higher at 2/3 than at 1/3 of incubation (Table 2).

Discussion

Consistent with the results (93 % females produced at 24 °C, and 76 % females produced at 32 °C) reported previously for *G. japonicus* (Tokunaga 1985), eggs incubated at 24 °C (88 %) and 32 °C (85 %) both produced mostly female hatchlings (Fig. 1a). Eggs incubated at 28 °C produced mostly male hatchlings (75 %) in the previous study (Tokunaga 1985), but a almost 50:50 sex ratio of hatchlings (53 % females versus 47 % males) in the present one (Fig. 1a). Does this difference contradict the previous conclusion that *G. japonicus* is a TSD species with a female–male–female (FMF) pattern (Tokunaga 1985)? Our answer to this question is no because: (1) smaller-than-average eggs did produce mostly male hatchlings at 28 °C (67 %), and mostly female hatchlings at 24 °C (83 %) and 32 °C (83 %); and (2) even though larger-than-average eggs produced female-biased hatchlings at all three temperatures, they were more likely to produce male hatchlings at 28 °C (27 %) than at 24 °C (8 %) or 32 °C (13 %) (Fig. 1b). These observations indicate that: (1) *G. japonicus* does exhibit a FMF pattern of TSD, but the ease of its discovery depends on egg size; (2) as in *B. duperreyi* (Radder et al. 2009), large eggs are more likely to produce female hatchlings and small eggs to produce male hatchlings in *G. japonicus*; and (3) offspring sex is more likely affected by egg size at 28 °C than at 24 °C or 32 °C in the gecko.

Consistent with the result reported for *B. duperreyi* (Radder et al. 2007), yolk E2 level at laying was not related to egg size. This result, together with the observation that yolk E2 levels did not differ between eggs within a clutch, suggest that the effect (if any) of maternally allocated yolk E2 levels on offspring sex is minimal or probably indirect. Yolk DHT level at laying, which was negatively related to egg size in *B. duperreyi* (Radder et al. 2009), was not determined in this study. Nonetheless, we did find that yolk

Table 1 Descriptive statistics, expressed as mean±SE (range), for egg mass, incubation length and hatchling mass of both sexes at three incubation temperatures, and results of two-way ANOVA (for egg mass and incubation length) or ANCOVA (for hatchling mass with egg mass as the covariate) with incubation temperature and sex as the factors

		N	Egg mass (g)	Incubation length (days)	Hatchling mass (g)
24 °C	F	25	0.73±0.02 (0.47–0.91)	82.2±0.3 (80.1–86.8)	0.48±0.02 (0.28–0.63)
	M	5	0.66±0.05 (0.51–0.79)	83.7±0.9 (81.3–86.3)	0.42±0.03 (0.35–0.50)
28 °C	F	25	0.69±0.02 (0.43–0.86)	51.1±0.3 (48.1–54.2)	0.46±0.02 (0.24–0.59)
	M	24	0.63±0.02 (0.48–0.81)	51.5±0.5 (48.4–54.3)	0.41±0.02 (0.19–0.58)
32 °C	F	35	0.68±0.01 (0.48–0.84)	41.6±0.2 (40.0–43.7)	0.46±0.01 (0.23–0.59)
	M	7	0.63±0.04 (0.54–0.76)	41.7±0.4 (40.0–43.1)	0.43±0.03 (0.34–0.57)
Temperature			$F_{2,\ 115}$=0.69, P=0.504	$F_{2,\ 115}$=3200.16, P<0.0001 24>28>32	$F_{2,\ 114}$=1.08, P=0.344
Sex			$F_{1,\ 115}$=9.62, P<0.003 F>M	$F_{1,\ 115}$=3.39, P=0.068	$F_{1,\ 114}$=0.02, P=0.878
Interaction			$F_{2,\ 115}$=0.28, P=0.757	$F_{2,\ 115}$=1.55, P=0.216	$F_{2,\ 114}$=0.16, P=0.850

Data on hatchlings of the same sex were blocked by the clutch. Tukey's post hoc comparison was performed on the trait that differed among the three temperature treatments

N the number of clutches, *F* females, *M* males

T level at laying was negatively related to egg size (Fig. 2). T can be converted to DHT by the action of 5α-reductase (Crews 1994). In *A. carolinensis*, eggs that give rise to males contain higher yolk T concentrations than eggs producing females (Lovern and Wade 2001). Thus, taken together, we tend to conclude that the physiological basis by which yolk steroid allocation modifies offspring sex is similar between *B. duperreyi* and *G. japonicus*. E2/T ratio was positively related to egg size, but this was mathematically a byproduct of a negative correlation between yolk T level and egg size. The role of the E2/T ratio in offspring sex determination still remains unclear, and data generated in previous studies have not yet allowed us to draw any general conclusion in both TSD and GSD species (Radder 2007). Bowden et al. (2000) demonstrated in the painted turtle *Chrysemys picta* that the proportion of males in a clutch

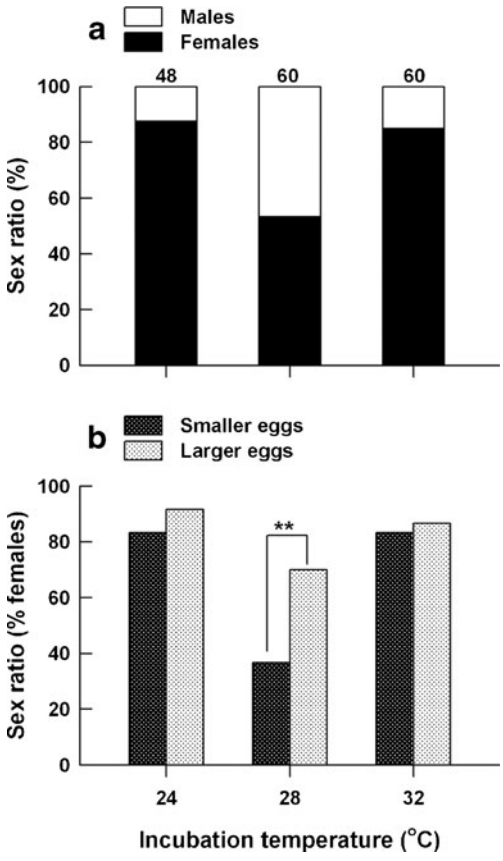

Fig. 1 Influence of incubation temperature on the sex ratio of *G. japonicus* hatchlings. **a** The overall sex ratios. Numbers in the plot indicate sample size for each temperature treatment. **b** The sex ratios of hatchlings from smaller-than-average and larger-than-average eggs. **P<0.01

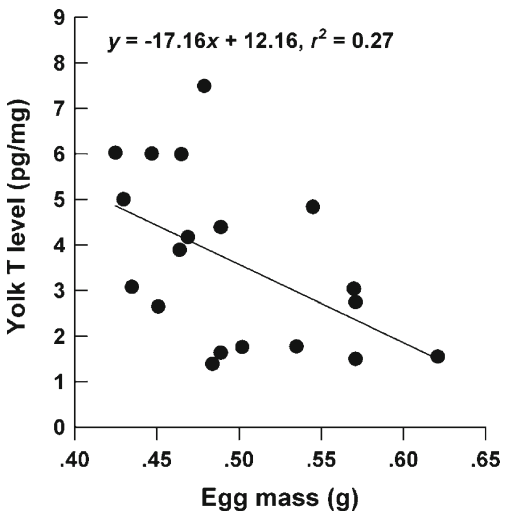

Fig. 2 Yolk T level in relation to egg mass in *G. japonicus*. Hormone measurements were taken at laying. Regression equation and coefficient are given in the figure

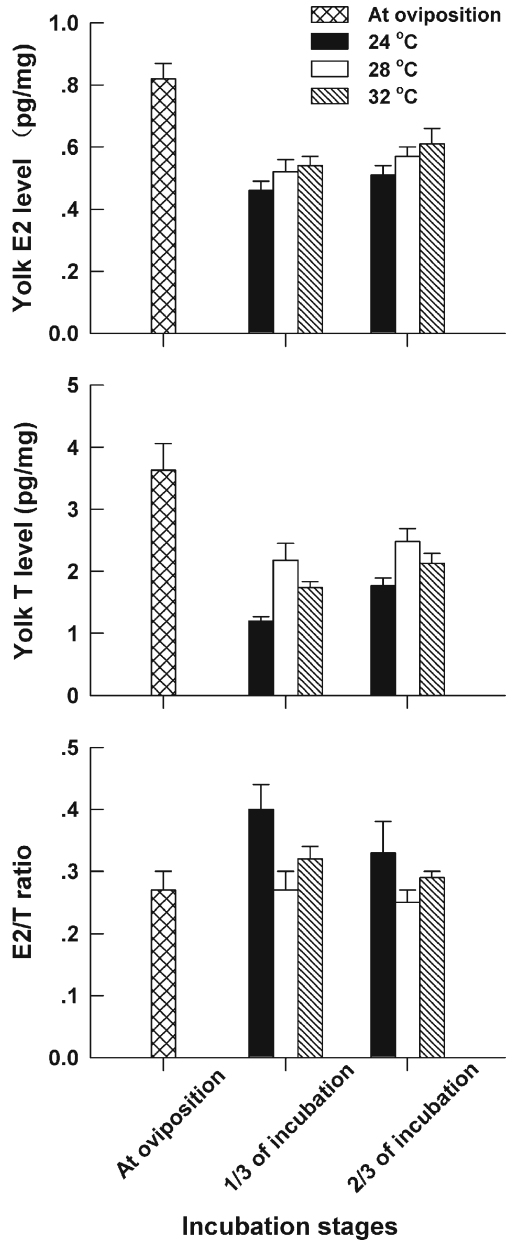

Fig. 3 Mean values (+SE) for E2, T and E2/T ratio in yolks of *G. japonicus* eggs dissected at oviposition, 1/3 of incubation and 2/3 of incubation. The mean embryonic stage was 27 at oviposition, 34 at 1/3 of incubation, and 37 at 2/3 of incubation. For lizards, hatching occurs at the embryonic stage of 40 (Dufaure and Hubert 1961)

decreased as the E2/T ratio increased. This is the only study that demonstrates the importance of the E2/T ratio in influencing offspring sex in TSD reptiles ever studied to date.

We found in *G. japonicus* that maternally derived T and E2 levels did not differ between eggs within a clutch. This may reflect physiological constraints on differential yolk steroid allocation among simultaneously developing follicles in oviparous reptiles where all follicles for a given clutch are exposed to the same maternal hormonal environment (Bowden et al. 2002a; Janzen et al. 2002; Lovern and Wade 2003b; Rhen et al. 2006). The lack of differences in yolk steroid hormones between eggs within a clutch provided an explanation for the result that clutches in which eggs were incubated at the same temperature (30 °C) always produced same-sex siblings in one study of the leopard gecko *Eublepharis macularius* (Kratochvíl et al. 2008). However, in another study of *E. macularius* clutches in which eggs were incubated at the same temperature produced same-sex as well as mixed-sex siblings, with clutches more likely to produce same-sex siblings at 30 °C (81 %) than at 32.5 °C (60 %) (Huang et al. 2008). Huang et al. (2008) interpreted the presence of mixed-sex clutches across different incubation temperatures to reflect the effect of gene × environment interactions, and concluded that in *E. macularius* incubation temperature sets the threshold to which yolk steroid hormones influence sex determination. In the present study, although the frequency of same-sex clutches was high across the three incubation temperatures, clutches were more likely to produce same-sex siblings at the two female-producing temperatures (95 % at 24 °C, and 95 % at 32 °C) than at 28 °C (77 %), the male-producing temperature. This result indicates that in *G. japonicus*, as in *E. macularius* (Huang et al. 2008), the effect of maternal influences via yolk steroid hormone deposition into the egg on offspring sex depends on incubation temperature. Our pattern that maternal influences on offspring sex are more likely to occur at intermediate temperatures is similar to that reported for *A. muricatus* where eggs are more likely to produce female offspring at extreme temperatures, and mixed-sex offspring at intermediate temperatures (Wapstra and Warner 2010). Most female *A. muricatus* are found to deposit eggs in nests that are near the intermediate mixed-

Table 2 Results of two-way ANOVA on E2, T levels and E2/T ratio in yolks of *G. japonicus* eggs with incubation temperature and stage as the factors

	Incubation temperature	Incubation stage	Interaction
Yolk E2 level	$F_{2,\ 70}$=3.67, P=0.031; 24(=28)<32(=28)	$F_{1,\ 70}$=4.94, P=0.029; 1/3<2/3	$F_{2,\ 70}$=0.05, P=0.952
Yolk T level	$F_{2,\ 70}$=16.68, P<0.0001; 24(=32)<28(=32)	$F_{1,\ 70}$=14.25, P<0.0004; 1/3<2/3	$F_{2,\ 70}$=0.94, P=0.395
E2/T ratio	$F_{2,\ 70}$=6.17, P<0.004; 24(=32)>28(=32)	$F_{1,\ 70}$=2.91, P=0.092	$F_{2,\ 70}$=0.58, P=0.561

24, 28 and 32: eggs incubated at 24, 28 and 32 °C; 1/3 and 2/3: eggs dissected at 1/3 and 2/3 of incubation. Tukey's post hoc comparison was performed on the traits that differed among the three temperature treatments

sex producing temperature, which would allow maternal effects on sex ratio to occur under TSD (Wapstra and Warner 2010).

Maternally derived yolk steroid hormones have been measured in some 20 species of TSD and GSD reptiles (Radder 2007). However, data on the dynamics of developmental changes in yolk steroid hormones have been available for only four species, of which three [the snapping turtle *Chelydra serpentina* (Elf et al. 2002a,b; Elf 2003), the red-eared slider turtle *Trachemys scripta* (Bowden et al. 2002b; Paitz and Bowden 2009), and *A. mississippiensis* (Conley et al. 1997; Hamlin et al. 2010)] exhibit TSD, and one [*A. carolinensis* (Lovern and Wade 2001, 2003b)] exhibits GSD. The highest concentrations of yolk steroid hormones (T, E2 and/or progesterone) have been measured at oviposition in the three TSD species (Conley et al. 1997; Bowden et al. 2002b; Elf et al. 2002a,b; Elf 2003, 2004; Paitz and Bowden 2009; Hamlin et al. 2010), but not in *A. carolinensis* (Lovern and Wade 2001, 2003b). A marked decrease in yolk steroid concentrations throughout development, especially following the period of gonadal differentiation [stages 16−22 in turtles (Matsumoto and Crews 2012), and 21−23 in the American alligator (Conley et al. 1997)], is a common occurrence and has been shown to occur in all the three TSD species (Conley et al. 1997; Bowden et al. 2002b; Elf et al. 2002b; Elf 2003; Paitz and Bowden 2009; Hamlin et al. 2010). In *A. carolinensis*, yolk T concentrations are higher in the latter half of incubation than at oviposition, because embryos at the latter stages of development produce T that, due to its lipophilic nature, passes into the yolk (Lovern and Wade 2001, 2003b). Like the three TSD species, *G. japonicus* demonstrated a marked decline in yolk steroid concentrations (both T and E2) from the day of oviposition to the period (stages 32−37 in lizards; Bull 1987) of sex determination (Table 2). Unlike the three TSD species, *G. japonicus* demonstrated a slight but significant increase in yolk steroid concentrations at the latter stages of incubation, presumably because steroids produced in situ passed into the yolk. It seems likely that the dynamics of developmental changes in yolk steroid hormones differ between TSD and GSD species. However, as only one GSD species has been studied, any general conclusion cannot be made at this time. Future work could usefully investigate other lineages of reptiles with both TSD and GSD species, preferably in a phylogenetic context, to determine whether the differences observed do exist between species with different modes of sex determination.

In the present study, we documented differences in yolk E2 level, T level and E2/T ratio among the three temperature treatments. This allows us to conclude that incubation temperature affect the dynamics of developmental changes in yolk steroid hormones and thus the hormonal environment of developing embryos in *G. japonicus*. Such an effect has been also detected in *C. serpentina* (Elf et al. 2002b; Elf 2003, 2004), but not in *A. mississippiensis* (Conley et al. 1997) and *T. scripta* (Bowden et al. 2002b). Yolk E2 level was significantly lower at 24 °C than at 32 °C (Fig. 3, Table 2), but eggs incubated at the two temperatures produced nearly the same proportion of female hatchlings (G=0.14, df=1, P=0.708; Fig. 1a); yolk T level and E2/T ratio both did not differ significantly between eggs incubated at 28 °C and 32 °C (Table 2), but eggs produced a much lower proportion of female hatchlings at 28 °C than at 32 °C (G=14.63, df=1, P<0.001; Fig. 1a). These results provide an inference that, although yolk steroid hormones play a central role in offspring sex determination in reptiles with TSD, their influences are secondary relative to incubation temperature effects. Our study strongly demonstrates that offspring sex in *G. japonicus* depends on an interaction between incubation temperature and yolk steroid hormones.

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References

Bowden RM, Ewert MA, Freedberg S, Nelson CE (2002a) Maternally derived yolk hormones vary in follicles of the painted turtle, *Chrysemys picta*. J Exp Zool 293:67–72

Bowden RM, Ewert MA, Nelson CE (2000) Environmental sex determination in a reptile varies seasonally and with yolk hormones. Proc R Soc Lond B 267:1745–1749

Bowden RM, Ewert MA, Nelson CE (2002b) Hormone levels in yolk decline throughout development in the red-eared slider turtle (*Trachemys scripta elegans*). Gen Comp Endocrinol 129:171–177

Bull JJ (1987) Temperature-sensitive periods of sex determination in a lizard: similarities with turtles and crocodilians. J Exp Zool 241:143–148

Chen JC, Peng XB, Yu DW (1986) Studies on the karyotypes of three species of the genus *Gekko*. Acta Herpetol Sin 5:24–29

Conley AJ, Elf PK, Corbin CJ, Dubowsky S, Fivizzani AJ, Lang JW (1997) Yolk steroids decline during sexual differentiation in the alligator. Gen Comp Endocrinol 107:191–200

Crews D (1994) Temperature, steroids and sex determination. J Endocrinol 142:1–8

Dufaure JP, Hubert J (1961) Table de développement du lézard vivipare: *Lacerta* (*Zootoca*) *vivipara* Jacquin. Arch Anat Microsc Morph Exp 50:309–328

Elf PK (2003) Yolk steroid hormones and sex determination in reptiles with TSD. Gen Comp Endocrinol 132:349–355

Elf PK (2004) Yolk steroid hormones and their possible role in TSD species. In: Valenzuela N, Lance VA (eds) Temperature-

dependent sex determination in vertebrates. Smithsonian Institution Press, Washington DC, pp 111–118
Elf PK, Lang JW, Fivizzani AJ (2002a) Yolk hormone levels in the eggs of snapping turtles and painted turtles. Gen Comp Endocrinol 127:26–33
Elf PK, Lang JW, Fivizzani AJ (2002b) Dynamics of yolk steroid hormones during development in a reptile with temperature-dependent sex determination. Gen Comp Endocrinol 127:34–39
Gamble T (2010) A review of sex determining mechanisms in geckos (Gekkota: Squamata). Sex Dev 4:88–103
Gutzke WHN, Chymiy DB (1988) Sensitive periods during embryogeny for hormonally induced sex determination in turtles. Gen Comp Endocrinol 71:265–267
Hamlin HJ, Lowers RH, Albergotti LC, McCoy MW, Mutz J, Guillette LJ (2010) Environmental influence on yolk steroids in American alligators (*Alligator mississippiensis*). Biol Reprod 83:736–741
Huang V, Sakata JT, Rhen T, Coomber P, Simmonds S, Crews D (2008) Constraints on temperature-dependent sex determination in the leopard gecko (*Eublepharis macularius*): response to Kratochvíl et al. Naturwissenschaften 95:1137–1142
Janes DE, Bermudez D, Guillette LJ Jr, Wayne ML (2007) Estrogens induced male production at a female-producing temperature in a reptile (leopard gecko, *Eublepharis macularius*) with temperature-dependent sex determination. J Herpetol 41:9–15
Janzen FJ, Wilson ME, Tucker JK, Ford SP (1998) Endogenous yolk steroid hormones in turtles with different sex-determining mechanisms. Gen Comp Endocrinol 111:306–317
Janzen FJ, Wilson ME, Tucker JK, Ford SP (2002) Experimental manipulation of steroid concentrations in circulation and in egg yolks of turtles. J Exp Zool 293:58–66
Ji X, Braña F (1999) The influence of thermal and hydric environments on embryonic use of energy and nutrients, and hatchling traits, in the wall lizards (*Podarcis muralis*). Comp Biochem Physiol A 124:205–213
Ji X, Wang PC, Hong WX (1991) The reproductive ecology of the gecko *Gekko japonicus*. Acta Zool Sin 37:185–192
Kratochvíl L, Kubička L, Landová E (2008) Does the mechanism of sex determination constrain the potential for sex manipulation? A test in geckos with contrasting sex-determining systems. Naturwissenschaften 95:209–215
Lovern MB, Wade J (2001) Maternal plasma and egg yolk testosterone concentrations during embryonic development in green anoles (*Anolis carolinensis*). Gen Comp Endocrinol 124:226–235
Lovern MB, Wade J (2003a) Yolk testosterone varies with sex in eggs of the lizard, *Anolis carolinensis*. J Exp Zool A 295:206–210
Lovern MB, Wade J (2003b) Sex steroids in green anoles (*Anolis carolinensis*): uncoupled maternal plasma and yolking follicle concentrations, potential embryonic steroidogenesis, and evolutionary implications. Gen Comp Endocrinol 134:109–115
Matsumoto Y, Crews D (2012) Molecular mechanisms of temperature-dependent sex determination in the context of ecological developmental biology. Mol Cell Endocrinol 354:103–110
Paitz RT, Bowden RM (2009) Rapid decline in the concentrations of three yolk steroids during development: is it embryonic regulation? Gen Comp Endocrinol 161:246–251
Radder RS (2007) Maternally derived egg yolk steroid hormones and sex determination: review of a paradox in reptiles. J Biosci 32:1213–1220
Radder RS, Ali S, Shine R (2007) Offspring sex is not related to maternal allocation of yolk steroids in the lizard *Bassiana duperreyi* (Scincidae). Physiol Biochem Zool 80:220–227
Radder RS, Pike DA, Quinn AE, Shine R (2009) Offspring sex in a lizard depends on egg size. Curr Biol 19:1102–1105
Radder RS, Shine R (2007) Sex-based hatchling asynchrony in an oviparous lizard (*Bassiana duperreyi*, Scincidae). Austral Ecol 32:502–508
Rhen T, Crews D, Fivizzani A, Elf P (2006) Reproductive tradeoffs and yolk steroids in female leopard geckos, *Eublepharis macularius*. J Evol Biol 19:1819–1829
Sarre SD, Georges A, Quinn A (2004) The ends of a continuum: genetic and temperature-dependent sex determination in reptiles. Bioessays 26:639–645
Schwabl H (1993) Yolk is a source of maternal testosterone for developing birds. Proc Natl Acad Sci USA 90:11446–11450
Shine R, Elphick MJ, Donnellan S (2002) Co-occurrence of multiple, supposedly incompatible modes of sex determination in a lizard population. Ecol Lett 5:486–489
Tokunaga S (1985) Temperature-dependent sex determination in *Gekko japonicus* (Gekkonidae, Reptilia). Dev Growth Differ 27:117–120
Valenzuela N, Lance VA (2004) Temperature-dependent sex determination in vertebrates. Smithsonian Institution Press, Washington DC
Wapstra E, Warner DA (2010) Sex allocation and sex determination in squamate reptiles. Sex Dev 4:110–118
Warner DA, Lovern MB, Shine R (2008) Maternal influences on offspring phenotypes and sex ratios in a multi-clutching lizard with environmental sex determination. Biol J Linn Soc 95:256–266
Warner DA, Radder RS, Shine R (2009) Corticosterone exposure during embryonic development affects offspring growth and sex ratios in opposing directions in two lizard species with environmental sex determination. Physiol Biochem Zool 82:363–371
Xu XF, Ji X (2001) Female reproduction and influence of incubation temperature on duration of incubation and hatchling traits in the gecko, *Gekko japonicus*. Chin J Ecol 20(6):8–11
Yoshida M, Itoh M (1974) Karyotype of the gecko, *Gekko japonicus*. Chrom Inf Serv 17:29–31