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Segregating variation for temperature-dependent sex determination in a lizard

T Rhen[1,2], A Schroeder[2], JT Sakata[1,3], V Huang[1] and D Crews[1]
[1]*Section of Integrative Biology, University of Texas at Austin, Austin, TX, USA;* [2]*Department of Biology, University of North Dakota, Grand Forks, ND, USA and* [3]*Department of Physiology, Keck Center for Integrative Neuroscience, University of California San Francisco, San Francisco, CA, USA*

Temperature-dependent sex determination (TSD) was first reported in 1966 in an African lizard. It has since been shown that TSD occurs in some fish, several lizards, tuataras, numerous turtles and all crocodilians. Extreme temperatures can also cause sex reversal in several amphibians and lizards with genotypic sex determination. Research in TSD species indicates that estrogen signaling is important for ovary development and that orthologs of mammalian genes have a function in gonad differentiation. Nevertheless, the mechanism that actually transduces temperature into a biological signal for ovary versus testis development is not known in any species. Classical genetics could be used to identify the loci underlying TSD, but only if there is segregating variation for TSD. Here, we use the 'animal model' to analyze inheritance of sexual phenotype in a 13-generation pedigree of captive leopard geckos, *Eublepharis macularius*, a TSD reptile. We directly show genetic variance and genotype-by-temperature interactions for sex determination. Additive genetic variation was significant at a temperature that produces a female-biased sex ratio (30 °C), but not at a temperature that produces a male-biased sex ratio (32.5 °C). Conversely, dominance variance was significant at the male-biased temperature (32.5 °C), but not at the female-biased temperature (30 °C). Non-genetic maternal effects on sex determination were negligible in comparison with additive genetic variance, dominance variance and the primary effect of temperature. These data show for the first time that there is segregating variation for TSD in a reptile and consequently that a quantitative trait locus analysis would be practicable for identifying the genes underlying TSD.
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Introduction

Sex determination, the developmental process of becoming female or male, results in extensive differences in gene expression profiles in animals (Jin *et al*., 2001; Ranz *et al*., 2003; Ellegren and Parsch, 2007). These differences translate into physiological, morphological and behavioral differences between the sexes that have profound effects on species at the ecological level (Reynolds, 1996; González-Solís *et al*., 2000; Coulson *et al*., 2001). Sex-determining mechanisms include genotypic sex determination with heteromorphic sex chromosomes, genotypic sex determination with homomorphic sex chromosomes, polygenic sex determination, haplo-diploid sex determination and environmental sex determination to name a few (Marín and Baker, 1998; Schütt and Nöthiger, 2000; Zarkower, 2001; Devlin and Nagahama, 2002; Beye *et al*., 2003; Haag and Doty, 2005; Pires-daSilva, 2007). The overall pace and pattern of evolutionary transitions among sex-determining mechanisms are quite variable. For example, there have not been any major changes in the mode of sex determination in birds or placental mammals since their origins. In contrast, a recent study by Pokorná and Kratochvíl (2009) indicates that environmental sex determination is the ancestral state in squamate reptiles and that genotypic sex determination and sex chromosomes have independently evolved several times within this group. This observation suggests that the mechanism underlying environmental sex determination might be homologous in all squamates. Whether this is true or not, the diversity observed within and among phyla suggests that sex-determining mechanisms are adaptive (Charnov, 1982; Bull, 1983; Uller *et al*., 2007). Charnov and Bull (1977) first proposed the hypothesis that temperature-dependent sex determination (TSD), a form of environmental sex determination, would be favored when temperature has a differential effect on male versus female fitness. Studies in three species, a fish, a turtle and a lizard, have shown that some temperatures produce better males, whereas other temperatures benefit females, thus supporting the notion that TSD is adaptive (Conover and Heins, 1987; Rhen and Lang, 1995; Warner and Shine, 2008a).

Although thermo-sensitive gonad development must be heritable for TSD to evolve, little is known about the gene(s) that transduces temperature into a biological signal for ovary versus testis development. Early research focused on the function of sex steroids in TSD. For instance, it has been shown that exogenous estrogens induce ovary development in embryos incubated at male-producing temperatures and that aromatase inhibitors, which block estrogen synthesis, interfere with

Correspondence: Dr T Rhen, Department of Biology, Box 9019, University of North Dakota, Starcher Hall, Cornell Street, Grand Forks, ND 58202, USA.
E-mail: turk.rhen@und.nodak.edu
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ovary development at female-producing temperatures (reviewed in Crews, 1996; Pieau and Dorizzi, 2004; Place and Lance, 2004; Ramsey and Crews, 2009). Thus, the leading model for TSD postulates that female-producing temperatures induce expression of the enzyme aromatase, which converts androgens into estrogens within the developing gonads. In turn, estrogens cause the bipotential gonads to develop into ovaries. Below some threshold level of estrogen exposure, the gonads develop into testes. Although this model emphasizes temperature-induced synthesis of estrogens by the embryo proper, some authors suggest that maternal steroids deposited in egg yolk may also influence sex determination (Conley *et al*., 1997; Janzen *et al*., 1998; Bowden *et al*., 2000; Elf *et al*., 2002; Elf, 2003; Radder, 2007; but see Huang *et al*., 2008; Kratochvíl *et al*., 2008). For instance, Bowden *et al*. (2000) reported a significant correlation between the initial estrogen content of eggs and hatchling sex ratios in the painted turtle: clutches of eggs with higher estrogen levels had more female-biased sex ratios than clutches of eggs with lower estrogen levels. Whether synthesized by the embryo or maternally derived in TSD species, estrogens induce ovary development in all non-mammalian vertebrates and have been shown to have a function in the maintenance of ovarian phenotype in mammals. In short, estrogen signaling is an evolutionarily conserved feature of ovary development (reviewed in Ditewig and Yao, 2005).

More recent studies have used a molecular genetic approach to examine the mechanism underlying TSD. Researchers have cloned orthologs of sex-determining genes first identified in mammals and examined their expression patterns in TSD species (reviewed in Ramsey and Crews, 2009; Shoemaker and Crews, 2009; Rhen and Schroeder, 2010). Such investigations are important because they identify genes that appear to have a conserved function in sex determination in all vertebrates. For instance, Dmrt1 and Sox9 are expressed at a higher level in gonads from embryos incubated at male-producing temperatures (that is, presumptive testes) in several TSD species (Spotila *et al*., 1998; Smith *et al*., 1999; Western *et al*., 1999; Kettlewell *et al*., 2000; Valleley *et al*., 2001; Torres Maldonado *et al*., 2003; Murdock and Wibbels, 2003a, 2006; Rhen *et al*., 2007; Shoemaker *et al*., 2007a, b; Anand *et al*., 2008). Conversely, FoxL2, R-spondin 1, aromatase and estrogen receptors seem to be crucial for ovary development at female-producing temperatures (Desvages *et al*., 1993; Bergeron *et al*., 1998; Jeyasuria and Place, 1998; Gabriel *et al*., 2001; Place *et al*., 2001; Murdock and Wibbels, 2003b; Ramsey and Crews, 2007; Ramsey *et al*., 2007; Rhen *et al*., 2007; Valenzuela and Shikano, 2007; Shoemaker *et al*., 2007b; Smith *et al*., 2008). Expression profiling of candidate genes, however, has not led to the temperature-sensitive factor(s) that actually triggers ovary versus testis development in TSD species (Lance, 2009; Rhen and Schroeder, 2010).

Another way to identify the gene(s) underlying TSD is through a combination of classical and molecular genetics (that is, quantitative trait locus (QTL) analyses), an approach successfully adopted for other sex-determining systems (Spigler *et al*., 2008). This approach depends first and foremost on the presence of segregating variation for sex determination (Martin *et al*., 1997). Previous studies have examined among-family variation for sex ratio in several TSD reptiles (Bull *et al*., 1982; Janzen, 1992; Rhen and Lang, 1998; Dodd *et al*., 2006; Janes and Wayne, 2006; Warner *et al*., 2007, 2008). Those studies report broad-sense heritability (that is, family effects) and family-by-temperature interactions. However, their usefulness is limited because additive, dominance and non-genetic maternal effects are confounded in full-sib families and in families merely assumed to be full-sibs (paternity was neither controlled nor determined in most studies). Multiple paternity within families is common in reptiles in nature and has been directly shown in many of the species in the aforementioned studies (Galbraith *et al*., 1993; Davis *et al*., 2001; Pearse *et al*., 2001; Jensen *et al*., 2006; Uller and Olsson, 2008). Thus, uncertain genetic relationships among eggs collected from the wild confound heritability estimates. One study controlled paternity and used a half-sib/full-sib breeding design (Janes and Wayne, 2006), but missing data only allowed a full-sib analysis in which additive, dominance and maternal effects were confounded. These studies leave a fundamental question unanswered, is there genetic variation for TSD?

Here, we dissect and quantify genetic, maternal and environmental contributions to sex determination in the leopard gecko, *Eublepharis macularius*. We know the pedigree, incubation temperature and sex of most animals in our breeding colony, making the data ideally suited to analysis using the 'animal model' (Kruuk, 2004). Analysis of inheritance patterns for sexual phenotype in the 13-generation pedigree revealed segregating variation for TSD, including additive and dominance variance and significant genotype-by-temperature interactions. Demonstration of genetic variation for sex determination indicates that QTL analyses would be useful for identifying specific loci that control TSD in this species. We also tested for different kinds of maternal effects on sex determination. In particular, maternal identity, maternal body condition and clutch order (within breeding season) had no detectable influence on sex determination, but sex ratios varied significantly with breeding season.

Materials and methods

Leopard gecko colony

We bred and raised leopard geckos in environmental chambers at the University of Texas at Austin from 1986 to 2001. Leopard geckos are Eublepharid geckos indigenous to southeast Afghanistan, Pakistan and northwestern India. Although we do not know the geographic origin of colony founders (that is, where wild geckos were originally collected), we know that Dr James Bull started the colony at the University of Texas in 1985–1986 using leopard geckos received from Ernie Wagner of the Woodland Park Zoo, Seattle, WA, USA. The colony at the Woodland Park Zoo had been propagated for roughly 20 years before it was discontinued in 1985–1986. In addition, a few animals from Indiana University and the National Zoo were brought into the University of Texas colony as fresh breeding stock in the early to mid-1990s.

We have records for 2752 leopard geckos from the colony at the University of Texas. Animals were individually marked with a unique combination of toe

clips at hatch and raised to reproductive maturity in isolation (that is, one individual per container as described in Flores *et al*., 1994). Upon reaching sexual maturity, some males and females were placed into breeding cages to perpetuate the colony, whereas others were used in a variety of experiments (reviewed in Crews *et al*., 1998; Sakata and Crews, 2004). Each breeding cage contained a single male, one to four females, bricks and PVC pipe for environmental enrichment, a heat lamp for behavioral thermoregulation and a polypropylene box filled with moist sand as nesting substrate. Animals were fed mealworms dusted with vitamins and minerals twice a week, pinky mice once a week and had water *ad libitum*.

Female leopard geckos can lay up to eight clutches of eggs during a single reproductive season and can reproduce for >8 years (~ 8 breeding seasons). The majority of eggs were collected from nest boxes within 1 day of oviposition. Each clutch usually has two eggs, though females occasionally produced one egg. The mother of a given clutch was almost always unambiguously identified because it takes ~ 35 days for females to produce one clutch of eggs and we monitored female reproductive status on a weekly basis: yolking follicles within ovaries and ovulated eggs within oviducts are visible through the abdominal wall as described in Rhen *et al*. (2000, 2006). In addition, females are gaunt after oviposition because the two eggs represent a significant fraction of gravid body mass. Finally, it was rare for more than one female in a cage to lay eggs on the same day. Thus, we know the maternity of $>96\%$ of the animals in the colony ($n=2648$). We do not know the maternity of a few animals ($n=104$) that were colony founders, fresh breeding stock from other institutions or colony members hatched from eggs that were laid by different females on the same day in the same breeding cage.

We are also certain of the paternity of most animals in the colony. Many breeding females (54%) were only mated to a single male during their lifetime. For females that had multiple mates, we are sure of the paternity of eggs produced when females were in residence with their first mate, but uncertain about paternity for subsequent mates. For this analysis, we assume that the resident male fertilized eggs produced by females in his cage (see below for caveats concerning potential errors in paternity assignment). All told, we were able to assign paternity (or putative paternity) for 92% of the animals ($n=2530$) in the colony. We do not know the paternity of animals ($n=222$) that were colony founders, fresh breeding stock from other institutions or colony members hatched from eggs that were laid by different females on the same day in the same breeding cage.

Following collection, each egg was placed in an individual plastic cup with a 1:1 or 1.5:1 ratio of water:vermiculite and covered with perforated plastic. Each cup containing a single egg was then placed in a different Precision brand incubator ($\pm 0.1\,^{\circ}$C) set to one of several constant temperatures (26, 29, 29.5, 30, 31, 31.5, 32.5, 34 or 35 °C) until the egg hatched (Viets *et al*., 1993; Crews *et al*., 1998). Temperature was also monitored by HOBO temperature loggers (Onset Computer Corporation, Pocassett, MA, USA) and by daily readings of in-incubator thermometers. Substrate water potential has no influence on sex ratios, so data from 1:1 and 1:1.5 ratios of water:vermiculite were combined (Viets *et al*., 1993). Sex determination occurs during the early to middle stages of embryonic development, after which the gonads irreversibly develop into ovaries or testes (Bull, 1987). Hatchlings were raised individually in polypropylene containers inside environmental chambers. The sex of each gecko was determined by monitoring morphological development from hatching to sexual maturity at 40–50 weeks of age. Individuals were classified as males if they developed androgen-dependent pre-anal pores and hemipenes, which are usually evident by 20 weeks, or as females if they did not develop these male-specific traits (Rhen *et al*., 2005). The use of secondary sexual characteristics is an accurate surrogate for direct examination of the gonads: we observed perfect correspondence between gonadal sex and the diagnosis of sex based on presence/absence of pre-anal pores and hemipenes in 73 males and 93 females that were gonadectomized at 1 year of age for studies of hormonal regulation of behavior (Rhen and Crews, 1999, 2000). Animals euthanized for other experiments or that died before secondary sexual characteristics developed were dissected to determine gonadal sex. In all, sexual phenotype was recorded for 2136 individuals from the colony (Table 1).

Genetic analysis of TSD using the 'animal model'

Our primary aim was to test whether there are genetic and/or maternal effects on sex determination in leopard geckos in addition to the major temperature effect previously reported (Bull, 1987; Gutzke and Crews, 1988; Viets *et al*., 1993; Janes and Wayne, 2006). The large, multigenerational pedigree from our breeding colony allows for such an analysis using the 'animal model' (reviewed in Kruuk, 2004; Wilson *et al*., 2010). The 'animal model' refers to the general linear mixed model when it is used for quantitative genetic analyses. This model uses all the available information about genetic relationships from pedigree records and has several advantages over full-sib or half-sib/full-sib breeding designs. Most importantly, we can directly test for additive genetic variance, dominance genetic variance and maternal effects. These causes of familial resemblance are confounded to various degrees with full-sib and half-sib/full-sib breeding designs (Falconer and Mackay, 1996). In addition, the 'animal model' produces parameter estimates with smaller standard errors than full-sib or half-sib/full-sib models (Kruuk, 2004). We can also test for interactions between random effects and

Table 1 Numbers of male and female leopard geckos produced at various incubation temperatures

Temperature (°C)	*Sexual phenotype*		*Sex ratio (% male)*
	Male	*Female*	
26.0	0	305	0.00
29.0	1	5	16.67
29.5	0	8	0.00
30.0	186	405	31.47
31.0	2	1	66.67
31.5	14	10	58.33
32.5	563	288	66.16
34.0	32	256	11.11
35.0	0	2	0.00

fixed effects even when certain combinations of predictor variables have not been sampled (that is offspring from a particular parent have not been incubated at all possible temperatures). Another benefit of the 'animal model' is that individuals with unknown phenotype, unknown maternity or unknown paternity can be included in the analysis. Finally, we can incorporate other factors/covariates in the model that may influence sex determination (that is breeding season, clutch order and maternal body condition).

We used ASReml 3 to fit the 'animal model' to our data on sexual phenotype in leopard geckos. Fixed effects in the model included the intercept, incubation temperature, breeding season and clutch order nested within breeding season. We used maternal body condition (mass/snout-vent length) immediately after oviposition as a covariate. Female snout-vent length does not change in adulthood, but mass can change with successive clutches within a breeding season and across breeding seasons (Rhen *et al*., 2006). Hence, maternal condition varies dramatically (coefficient of variation = 16.1%, $n = 1738$) and can serve as an index of maternal physiological state. Random effects in the model included additive genetic variation, genetic variation because of dominance, maternal identity as well as interactions between these factors and incubation temperature. Maternal identity tests for permanent maternal effects not because of aging (that is, breeding season) or physiological changes associated with clutch order or maternal body condition.

Dependent variables such as gonadal sex (a binary trait) can be analyzed in several ways. Three widely used approaches include the logistic model, the threshold model and a model in which sex is treated as a continuous variable with females assigned a phenotypic value of 0 and males a value of 1 (Chatterjee and Price, 1978; Mercer and Hill, 1984; Cox and Snell, 1989; Falconer and Mackay, 1996; Rhen and Lang, 1998; Krackow and Tkadlec, 2001; Vandeputte *et al*., 2007; Visscher *et al*., 2008). Each model has different assumptions and different strengths and weaknesses. We, therefore, use all three models to assess the robustness of our results.

The logistic model is most appropriate for analysis of binary-dependent variables such as gonadal sex (Chatterjee and Price, 1978; Cox and Snell, 1989; Krackow and Tkadlec, 2001). This model uses the log of ratios of probabilities as the dependent variable (that is $\ln [p_m/p_f]$, where p_m is the proportion male and p_f is the proportion female). Note that an individual's sex is the phenotype of interest in our analysis and that sex ratio is a nominally emergent property of populations (Grantham, 2007). In other words, genetic and environmental factors acting at the level of the individual determine population sex ratios. Therefore, we use the general linear mixed model as described above to draw inferences about genetic and environmental mechanisms underlying sex determination in the individual. We used the link function LOGIT BIN to run the logistic 'animal model' in ASReml 3. The significance of fixed effects was tested using conditional Wald F-statistics as described by Gilmour *et al*. (2006). To test the significance of random effects, we dropped each effect from the model and calculated the change in deviance between the reduced and the full model. The change in deviance is a likelihood ratio ($LR\chi^2$), which was compared with the χ^2 distribution with the appropriate degrees of freedom. Although the logistic model provides a robust framework for analysis of binary-dependent variables, we cannot use it to quantitatively estimate the heritability of sexual phenotype or to estimate the magnitude of maternal or dominance effects on sex determination. In contrast, these parameters can be estimated using the threshold model and a model in which sex is treated as a continuous variable.

The threshold model assumes that sex is inherited in the same way as continuously varying characters with a polygenic and environmental basis (Falconer and Mackay, 1996; Visscher *et al*., 2008). In brief, genetic and environmental factors contribute to an individual's 'liability' to develop as one sex or the other. It is assumed that the underlying trait, liability, is normally distributed and that individuals with liabilities above a certain threshold develop as one sex, whereas individuals with liabilities below the threshold develop as the other sex. Previous studies have indicated that estrogens of embryonic or maternal origin could contribute to liability, as could genes that have a conserved function in sex determination. Novel genes may also influence liability to develop ovaries versus testes. We assume that the temperature effect on sex determination is due to a major effect of developmental temperature on liability, so heritability estimates must be environment specific. Heritability of liability was estimated using population sex ratio at a given temperature (for example incidence of males at 30 °C) and the sex ratio in specific types of relatives incubated at the same temperature (for example 30 °C offspring from 30 °C males for parent–offspring regressions or paternal half-sibs incubated at 30 °C for half-sib analysis). We used a formula that corrects for unequal variances between the entire population and families with at least one 'affected' individual (Equation (18.3) in Falconer and Mackay, 1996). We calculated the standard errors for heritability of liability according to Falconer (1965) and Falconer and Mackay (1996).

The last model treats sex as a continuous trait by assigning a phenotypic value of 0 to females and a value of 1 to males (Falconer and Mackay, 1996; Visscher *et al*., 2008). This is akin to using 'a very coarsely graduated instrument for measuring liability; an instrument, in fact, with only one graduation mark' (Falconer and Mackay, 1996). We used ASReml 3 to fit the 'animal model' and calculate variance components and estimate the relative contribution of additive variance, dominance variance and maternal identity to the total phenotypic variance within incubation temperatures. Narrow-sense heritability (h^2), dominance variation (d^2) and maternal effects (m^2) were calculated as the ratio of the corresponding variance component to the total phenotypic variance within each temperature. Assumptions of the threshold model apply to the continuous model (that is liability is multi-factorial and normally distributed), with the added stipulation that measurement error increases as the population sex ratio increases or decreases from 0.5. As a result, this model typically underestimates heritability (Falconer and Mackay, 1996; Visscher *et al*., 2008). We used Equation (18.4) in Falconer and Mackay (1996) to transform heritability on the 0–1 scale to the liability scale, which allows a direct comparison with heritability estimates from the threshold model.

Caveats

The rate of unknown or erroneous maternity in this data set is low (<4%). The rate of unknown paternity is also relatively low (~8%). However, paternity may have been misassigned for some eggs from females mated to more than one male (that is for second or third mates). We assume that the resident male fertilized eggs produced by females in his cage. This assumption is reasonable if female leopard geckos do not store sperm from previous mates or if there is last male precedence in sperm competition. Even if there were errors in paternity assignment, they would have little effect on estimates of maternal effects (Charmantier and Réale, 2005; Morrissey *et al*., 2007). The main effect of such errors would be underestimation of additive genetic variation and heritability (Charmantier and Réale, 2005; Morrissey *et al*., 2007). Thus, our tests of significance for additive variation and heritability are likely to be conservative.

Results

Analysis of inheritance of sexual phenotype using the logistic 'animal model'

As expected for a TSD species, this analysis revealed a strong incubation temperature effect on sex determination (Table 2). Controlling for temperature, breeding season had an effect on sex determination (Table 2). There was also significant additive genetic variation for sex determination across temperatures and a significant dominance-by-temperature interaction (Table 2). Clutch order (nested within breeding season), maternal body condition and other random effects in the model (that is dominance variance, maternal ID, additive-by-temperature interaction and maternal ID-by-temperature interaction) had no influence on sex determination (Table 2).

To further dissect genetic and non-genetic effects on sex determination, we analyzed the inheritance of sexual phenotype at 30 and 32.5 °C. Other temperatures were excluded because they displayed little phenotypic variation (that is very biased sex ratios) or had very small sample sizes (see Table 1). Temperature still had a strong effect on sex determination in the reduced model, as did breeding season, with a general increase in the proportion of males produced at 30 and 32.5 °C with successive seasons (Table 3; Figure 1). Furthermore, there was significant additive genetic variation for sex determination across temperatures and a significant dominance-by-temperature interaction (Table 3).

We also tested for genetic effects and non-genetic maternal effects within each of these temperatures. Additive genetic variation for sex determination was significant at 30 °C, but was not significant at 32.5 °C (Table 4). Conversely, dominance variation was not significant at 30 °C, but was significant at 32.5 °C (Table 4). Breeding season was significant, but maternal ID, clutch order and maternal condition were not significant at either temperature.

Analysis of heritability of sexual phenotype using the threshold model

Heritability of liability to develop ovaries versus testes was calculated at 30 and 32.5 °C. Estimates of narrow-

Table 2 Analysis of fixed and random effects on sex determination in the leopard gecko using the logistic model

Effect	*Num DF*	*Den DF*	*Con F*	*LR*χ^2	*DF*	P
Incubation temperature	8	189.6	24.57			<0.0001
Breeding season	8	1271.2	2.28			0.020
Clutch order (breeding season)	3	2057.0	0.01			0.999
Maternal condition	1	809.2	1.16			0.282
Additive variance				27.05	1	<0.0001
Dominance variance				0	1	1.0
Maternal ID				0	1	1.0
Additive × temperature				0	8	1.0
Dominance × temperature				18.21	8	0.0197
Maternal ID × temperature				0	8	1.0

Abbreviations: Con F, conditional Wald F statistic; Den DF, denominator degrees of freedom; DF, degrees of freedom; LRχ^2, likelihood ratio chi-square; Num DF, numerator degrees of freedom; P, probability of obtaining an F-value or LRχ^2-value greater than the observed value. Effect denotes source of variation.

Table 3 Analysis of fixed and random effects on sex determination in the leopard gecko using the logistic model and data from 30 to 32.5 °C

Effect	*Num DF*	*Den DF*	*Con F*	*LR*χ^2	*DF*	P
Incubation temperature	1	18.9	77.04			<0.0001
Breeding season	8	1,180.9	2.21			0.024
Clutch order (breeding season)	3	1,428	0.01			0.999
Maternal condition	1	912.8	0.54			0.463
Additive variance				12.65	1	0.0004
Dominance variance				0	1	1.0
Maternal ID				0	1	1.0
Additive × temperature				0	1	1.0
Dominance × temperature				20.98	1	<0.0001
Maternal ID × temperature				0	1	1.0

Abbreviations: Con F, conditional Wald F statistic; Den DF, denominator degrees of freedom; DF, degrees of freedom; LRχ^2, likelihood ratio chi-square; Num DF, numerator degrees of freedom; P, probability of obtaining an F-value or LRχ^2-value greater than the observed value. Effect denotes source of variation.

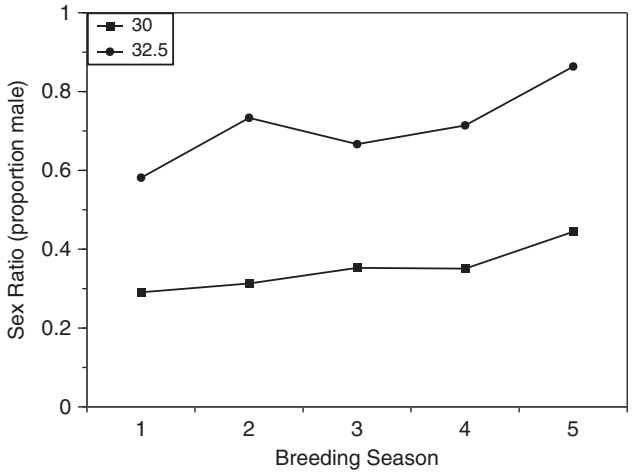

Figure 1 Sex ratio as a function of incubation temperature and breeding season in leopard geckos.

Table 4 Analysis of random effects on sex determination in the leopard gecko within incubation temperatures using the logistic model

Incubation temperature	*Random effect*	*LRχ^2*	*DF*	P
30 °C	Additive variance	25.0	1	<0.0001
	Dominance variance	2.26	1	0.133
	Maternal ID	0	1	1.0
32.5 °C	Additive variance	0	1	1.0
	Dominance variance	53.3	1	<0.0001
	Maternal ID	0	1	1.0

Abbreviations: DF, degrees of freedom; LRχ^2, likelihood ratio chi-square; P, probability of obtaining an F-value or LRχ^2-value greater than the observed value.
Random effect denotes source of variation.

sense heritability based on parent–offspring regression and paternal half-sibs were similar to each other within temperatures (Table 5). Although heritability estimates at 30 °C were not significantly greater than zero because of large standard errors, they were consistent with the logistic 'animal model' (see above) and the continuous 'animal model' (see below), which both revealed significant additive variance at 30 °C. The population-wide sex ratio was 31.47% male at this temperature ($n = 591$). However, males from 30 °C produced offspring with a more male-biased sex ratio of 39.13% male ($n = 46$) at this temperature. Likewise, paternal half-sib families with at least one male had a more male-biased sex ratio of 35.49% male ($n = 293$) at 30 °C.

Estimates of narrow-sense heritability based on parent–offspring regression and paternal half-sibs were both close to zero at 32.5 °C (Table 5). These results were also consistent with the logistic 'animal model' (see above) and the continuous 'animal model' (see below), which did not detect additive variation at 32.5 °C. The population-wide sex ratio at 32.5 °C was 33.84% female ($n = 851$). Females from 32.5 °C produced offspring with a slightly higher sex ratio of 35.71% female ($n = 154$). Paternal half-sib families with at least one female had a sex ratio of 33.06% female ($n = 487$), which was essentially the same as the population-wide sex ratio.

Table 5 Estimates of heritability of liability to develop ovaries versus testes in leopard geckos incubated at 30 and 32.5 °C

Parameter	*Incubation temperature*	
	30 °C	*32.5 °C*
h^2 (Parent–offspring)	0.37 ± 0.33	0.09 ± 0.19
h^2 (Paternal half-sibs)	0.40 ± 0.27	0.00 ± 0.22

The threshold model was used to calculate heritability and standard errors as described in Falconer and Mackay, (1996).

Table 6 Estimates of heritability (h^2), dominance variance (d^2) and maternal effects (m^2) on sexual phenotype in leopard geckos incubated at 30 and 32.5 °C.

Parameter	*Incubation temperature*	
	30 °C	*32.5 °C*
h^2	0.257 ± 0.115*	0.038 ± 0.061
d^2	0.040 ± 0.061	0.134 ± 0.052*
m^2	0.020 ± 0.049	0.061 ± 0.041

Sexual phenotype was treated as a continuous variable (female = 0 and male = 1) as described in Materials and methods. Parameters are shown with their standard errors. Parameters significantly greater than zero are indicated with an asterisk.

Analysis of inheritance of sexual phenotype using the continuous 'animal model'

This model treats sex as a continuous trait (female = 0 and male = 1), which allows estimation of variance components and their contribution to total phenotypic variance. Overall estimates of additive variance, dominance variance and maternal effects on sex determination across temperatures have little meaning because of the genotype-by-temperature interactions described above. We, therefore, used ASReml 3 to calculate variance components and estimate heritability (h^2), dominance variation (d^2) and maternal ID effects (m^2) within temperatures that displayed phenotypic variation and large sample sizes. Heritability was significantly greater than zero at 30 °C, but was not different from zero at 32.5 °C (Table 6). However, heritability on the 0–1 scale is known to underestimate heritability of liability. When Equation (18.4) from Falconer and Mackay (1996) was used to transform estimates from the 0–1 scale to the underlying continuous scale, heritability of liability was 0.439 ± 0.115 at 30 °C. Heritability of liability was 0.064 ± 0.061 at 32.5 °C, which was not different from zero. These estimates are similar to heritability estimates from the threshold model (Table 5). Dominance variance for sex determination was significantly greater than zero at 32.5 °C, but was not different from zero at 30 °C (Table 6). In contrast, maternal effects were not different from zero at either temperature (Table 6). These results are perfectly consistent with the results from the logistic 'animal model' and the threshold model.

Genotype-by-environment interactions

We found significant genotype-by-temperature interactions in the previous analyses. Two factors contributed to this interaction, which can be illustrated using a reduced data set from 12 breeding males that had numerous offspring at each temperature (mean = 13.4 offspring/temperature; range = 7–27 offspring/temperature). The first

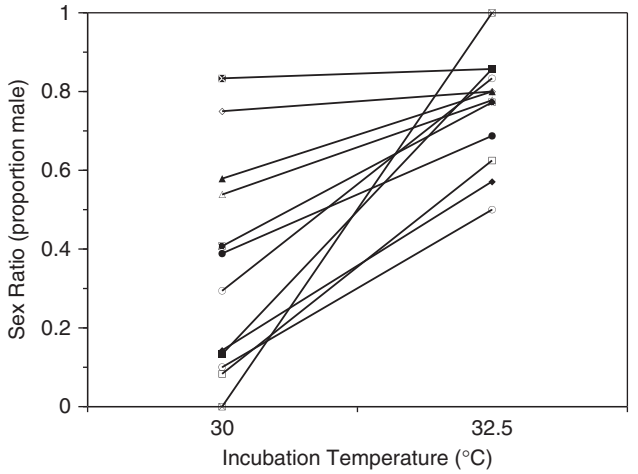

Figure 2 Sex ratio reaction norms as a function of incubation temperature and family identity in leopard geckos. Each line connects the sex ratio for an individual half-sib family (that is offspring sired by a single male) that was divided between two incubation temperatures as described in the text.

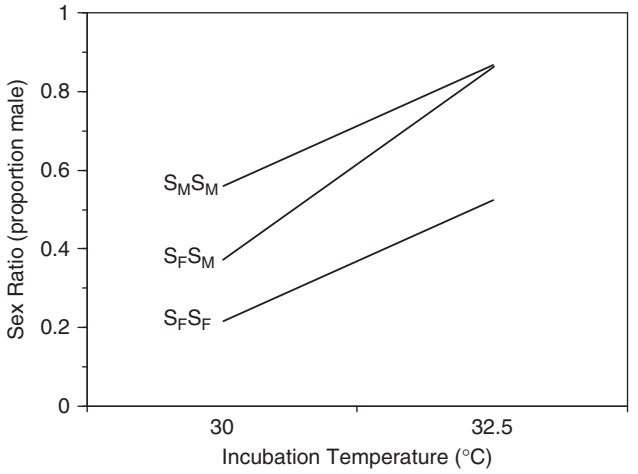

Figure 3 Hypothetical model for temperature-dependent allelic effects on sex ratio at 30 and 32.5 °C. Although the S_M and S_F alleles have additive effects on sex ratio at 30 °C, the S_M allele displays dominance at 32.5 °C. This model could explain the observed pattern of genotype–environment interactions and environment-specific heritability/dominance.

factor was variation in temperature sensitivity among genotypes: offspring from some sires were very sensitive to temperature, whereas others were less sensitive (note crossing reaction norms in Figure 2). The second factor was decreased sex ratio variation among paternal half-sib families at 32.5 °C compared with 30 °C: variation among these sires was significant at 30 °C ($LR\chi^2 = 51.5$, $df = 11$, $P < 0.0001$), but was not significant at 32.5 °C ($LR\chi^2 = 13.1$, $df = 11$, $P = 0.287$) (see Figure 2).

Discussion

Genetic, maternal and thermal effects on sex determination have never been fully dissociated or quantified in a TSD reptile (Valenzuela, 2004). Here, we present several important findings concerning TSD. For the first time, we directly showed genetic variation for sex determination in a TSD reptile. We also detected significant genotype-by-temperature interactions. In depth analysis of the inheritance of sexual phenotype within and between temperatures revealed a change in the nature of genetic variance across temperatures. There was additive genetic variation for sex determination at a temperature (30 °C) that produces a female-biased sex ratio, but not at a temperature (32.5 °C) that produces a male-biased sex ratio. Yet, there was still genetic variance for sex determination at the male-biased temperature: that is sex-determining genes that display dominance. Finally, our findings refute the hypothesis that maternally derived factors such as yolk steroids have a major impact on sex determination in this species. Maternal identity, maternal body condition and clutch order (nested within breeding season) had no detectable influence on sex determination. However, sex ratios did vary with breeding season, by way of an increase in the proportion of male offspring produced at 30 and at 32.5 °C as females aged. Consistency among the logistic, threshold and continuous models strengthens our inference that there is segregating variation for sex determination. Indeed, estimates of heritability of sexual phenotype at 30 °C ranged from 0.37 to 0.44. These estimates are likely to be conservative because paternity may have been misassigned for some individuals (that is a few animals may have been treated as first degree relatives when they were not in fact related). Dominance variance accounted for 13% of the phenotypic variance on the 0–1 scale at 32.5 °C, or 22% of the variance when transformed to the underlying liability scale. These findings indicate that a combined classical and molecular genetic approach (that is a QTL analysis) would be practical for identifying the locus or loci underlying TSD. These findings are also important because genetic variation for sex determination is the raw material for adaptive evolution of sex-determining mechanisms (Bull, 1983; Conover and Van Voorhees, 1990; Janzen and Paukstis, 1991; West and Sheldon, 2002).

It has long been recognized that genotype-by-temperature interactions are essential for the evolution of TSD (Conover and Kynard, 1981; Bull, 1983; Conover and Van Voorhees, 1990; Janzen, 1992; Rhen and Lang, 1998; Janes and Wayne, 2006). Yet, our results provide the first unequivocal evidence of genetic variation in thermal sensitivity in a TSD reptile. Genotype-by-temperature interactions in the leopard gecko could result from temperature-dependent allelic effects. In other words, alleles that have additive effects on sex ratio at 30 °C might display dominance at 32.5 °C. This proposition is based on a decline in additive variance and a parallel increase in dominance variance for sex ratio moving from 30 to 32.5 °C. This corresponds to the allelic sensitivity model for phenotypic plasticity (Via *et al*., 1995; Lacaze *et al*., 2009). Co-localization of QTLs for sex determination at 30 and 32.5 °C would be consistent with this hypothesis, whereas unique QTLs at 30 and 32.5 °C would refute the hypothesis.

We present a genetic model in Figure 3 showing how a temperature-dependent change in allelic effects might explain the observed pattern of genotype-by-temperature interactions. We envision a sex-determining locus S with alternative alleles. The first allele (S_F) favors development of females, whereas the second allele (S_M)

favors development of males regardless of incubation temperature. Holding all other genetic and environmental factors constant, the difference in sex ratio between S_FS_F and S_MS_M homozygotes is the same at 30 and 32.5 °C. In contrast, allelic effects in S_FS_M heterozygotes depend upon incubation temperature. At 30 °C, the S_F and S_M alleles have additive effects such that S_FS_M heterozygotes have a sex ratio intermediate between homozygotes. Allelic interactions change at 32.5 °C such that the S_M allele now displays dominance over the S_F allele and S_FS_M heterozygotes have the same sex ratio as S_MS_M homozygotes. Environment-dependent changes in dominance between alleles have been reported in other experimental systems and have a strong theoretical basis (Wright, 1977; Bourguet *et al*., 1996; Leips and Mackay, 2000; Corella *et al*., 2001).

This model of gene action is intriguing from an evolutionary perspective because it provides a mechanism for the maintenance of genetic variation for TSD. If temperature has a differential effect on male versus female fitness as theory predicts, and 30 °C produces better females and 32.5 °C produces better males, heterozygotes would have higher fitness than either homozygote for this hypothetical sex-determining gene (that is there is overdominance). We have, in fact, documented strong incubation temperature effects on phenotype in male and female leopard geckos that could produce this pattern of sex-specific selection (reviewed in Crews *et al*., 1998; Rhen and Crews, 1999; Rhen and Crews, 2000; Sakata and Crews, 2003).

Despite clear demonstration of segregating variation for sex determination, the 'animal model' cannot reveal the genetic architecture underlying TSD (that is number of loci, individual allelic effects, interactions among loci and so on…). To simplify our analysis, we assumed that additive and dominance variance and non-genetic maternal effects were the only factors that contributed to sex ratio variation. Heritability estimates based on parent–offspring regressions, paternal half-sib analyses and the 'animal model' were consistent with each other, which suggests that this assumption was reasonable. Nevertheless, epistatic interactions between two (or more) loci may be involved in sex determination. More complicated breeding designs with much larger sample sizes would be required to estimate variance because of epistasis (Falconer and Mackay, 1996). Our main conclusions, however, are robust: we detected significant genetic variation for TSD, but negligible maternal effects.

Our findings shed light on other studies of sex determination in the leopard gecko. Janes and Wayne (2006) reported significant family-by-temperature interactions in the leopard gecko. These researchers set up a controlled breeding study using 10 male geckos and 50 female geckos: each male was mated to five females in a nested design. Eggs were incubated at 26, 30 or 32.5 °C until hatching, and the sex of offspring was analyzed using ANOVA and the continuous model. This breeding design allows phenotypic variation to be partitioned among sires, among dams nested within sires and to progeny within a dam (Falconer and Mackay, 1996). Variation among sires is solely due to additive genetic variance, whereas additive variance, dominance variance and maternal effects all contribute to variance among dams (Falconer and Mackay, 1996). Janes and Wayne (2006) reported a significant dam-by-temperature interaction, but no main effect of dam. Our findings support their conclusion that this interaction was in fact a genotype-by-temperature interaction (see Discussion above).

In another leopard gecko study, Kratochvíl *et al*. (2008) reported an extreme level of concordance for sexual phenotype between clutch mates incubated at 30 °C: eggs in a clutch always developed into two males or two females, but not into a male and a female. The authors suggested that unisexual clutches were evidence of strong maternal effects on sex determination. Yet, those data alone cannot distinguish whether full siblings develop as the same sex because of maternal effects (for example yolk steroids) or because they are related (for example genetic effects). Another possible explanation for unisexual clutches is that clutch mates were incubated next to each other in the same container (Kratochvíl *et al*., 2008), which may allow exchange of steroids between eggs as hypothesized by Braña (2008). In contrast, we incubated eggs individually, which eliminates this potential 'common environment' effect. We found little evidence for most non-genetic maternal effects: maternal identity, maternal condition and clutch order did not influence sex determination. However, there was significant variation in sex ratio with successive breeding seasons. Variation across breeding seasons within females may be an 'age effect'. The mechanism underlying the increased production of sons as females aged is unclear at this time, but this pattern could be due to changes in maternal physiology and deposition of yolk steroids with age and could, therefore, be considered a 'maternal effect'.

Maternally derived yolk steroids have been proposed to be a primary cause of sex ratio variation among families in TSD species (Radder, 2007). However, there are currently no direct tests of this hypothesis and only six studies examine correlations between yolk steroids and offspring sex ratios (Janzen *et al*., 1998; Bowden *et al*., 2000; St Juliana *et al*., 2004; Radder *et al*., 2007; Warner *et al*., 2007, 2008). Of these studies, only one detected a significant correlation between yolk steroids and sex ratios (Bowden *et al*., 2000). One study showed a significant effect of maternal nutrition on offspring sex ratios, but did not observe a link with yolk steroids: female jacky dragons on a poor quality diet produce fewer but larger eggs that are more likely to become males (Warner *et al*., 2007). Measurements of maternal condition immediately after oviposition were available for many of the leopard gecko eggs in our study. Furthermore, we have shown that maternal condition varies with successive clutches within a breeding season and can change between breeding seasons (Rhen *et al*., 2006). Maternal condition is also related to egg size and to steroid levels in egg yolk in leopard geckos: females in poor condition lay smaller eggs and deposit more dihydrotestosterone in their eggs (Rhen *et al*., 2006). Nevertheless, there was no relationship between maternal body condition and sex ratios in our colony. Given the lack of empirical support, claims about a major impact of maternally derived yolk steroids on sex determination in TSD species seem premature.

At this point, it is important to distinguish different kinds of maternal effects. Our study provides a test for various physiological effects including maternal identity, maternal body condition, clutch order and breeding

season and experimentally eliminates behavioral effects. For instance, maternal selection of specific microenvironments can influence nest temperatures in the wild and in the laboratory, which could in turn affect offspring sex ratios (Bull *et al*., 1988; Bragg *et al*., 2000; Janzen and Morjan, 2001; Ewert *et al*., 2005; Kamel and Mrosovsky, 2005; Doody *et al*., 2006; Kamel and Mrosovsky, 2006; Warner and Shine, 2008b; McGaugh *et al*., 2010). However, the focus of this study was on the embryo's response to temperature and not maternal nest site selection.

In summary, we found that there was segregating variation for TSD in captive leopard geckos. This finding opens a new approach for identification of genes directly involved in transducing temperature into a biological signal for sex determination. For example, QTL analyses can be used to identify and estimate the number of loci that influence sex determination (Spigler *et al*., 2008). Such a study would also allow estimation of dominance deviations for particular loci at different temperatures to test the allelic sensitivity model versus other mechanisms of genotype-by-environment interaction (Via *et al*., 1995). In addition, our findings raise questions about patterns of genetic variation for TSD in wild leopard geckos. Are populations genetically differentiated as appears to be the case in some TSD species? Snapping turtle populations occupying different thermal niches display different TSD profiles (Ewert *et al*., 2005). Although it is tempting to speculate that among-family variation for sex ratio in other TSD reptiles is also due to genetic variation, this hypothesis can only be tested by carefully designed, prospective genetic studies.

Conflict of interest

The authors declare no conflict of interest.

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