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EVIDENCE FOR A GENOTYPE × ENVIRONMENT INTERACTION IN SEX-DETERMINING RESPONSE TO INCUBATION TEMPERATURE IN THE LEOPARD GECKO, *EUBLEPHARIS MACULARIUS*

DANIEL E. JANES[1,2,3] AND MARTA L. WAYNE[1]

[1]*Department of Zoology, University of Florida, P.O. Box 118525, 223 Bartram Hall, Gainesville, FL 32611, USA*

ABSTRACT: Among vertebrates, sex is determined by environmental or genotypic sex determination, such that sex is determined by incubation environment or by the genetic contribution of parents, respectively. The selective advantage of one mechanism over the other is unclear and our understanding of the evolution of environmental sex determination (ESD) is obscured by the lack of description of the mechanism's genetic architecture. In this study, a reptile with environmental sex determination, the leopard gecko, *Eublepharis macularius*, was tested for a genotype × environment interaction (G×E) with respect to sex-determining response to incubation temperature. Five sires were each mated to five unique dams. The eggs from each sire/dam combination were randomly assigned to one of three incubation temperatures and resultant hatchling sex ratios were measured. Temperature and temperature × dam (sire) interaction significantly affected treatment group sex. We report a statistically significant effect of genotype by environment interaction (G×E) on the offspring sex in *E. macularius*. G×E for ESD may provide an opportunity for evolution for different threshold conditions for sex determination in this ESD species.

Key words: *Eublepharis macularius*; Genotype by environment interaction; Leopard gecko; Temperature-dependent sex determination

THE GENETIC architecture of sex-determining mechanisms differs among closely related species of reptiles. Among reptiles, two major classes of mechanisms are recognized: genotypic sex determination, or GSD, in which sex is determined by parental genetic contributions and environmental sex determination, or ESD, in which sex is predominantly determined by incubation environment. Among reptiles, crocodilians and tuataras exhibit ESD, snakes exhibit GSD, and lizards and turtles exhibit either ESD or GSD (Sarre et al., 2004). The distribution of ESD and GSD among lizards and turtles is not arranged in a noticeable pattern (Janzen and Krenz, 2004). Although much has been published about possible advantages of ESD and GSD, little consensus can be found concerning the differences that would make one mechanism adaptive for one species and the other mechanism adaptive for a different species (Bull, 1983). The enigma is further compounded by closely related taxa that have different sex-determining mechanisms.

Although ESD and GSD are not typically recognized as ancestral or derived in lizards and turtles, one has likely evolved into the other several times (Janzen and Krenz, 2004). However, like all traits, evolution of ESD depends on genetic variation. Of course, an absence of obvious genetic variation in the present does not imply that there is not or has never been genetic variation; a beneficial set of alleles may have fixed, and selection against new mutations may be strong enough to render them undetectable without large, controlled studies (Houle et al., 1996).

ESD must involve, at minimum, sensing and responding to environmental stimuli. Although temperature is known to affect the activation of genes that encode steroidogenic enzymes (Crews, 2003), the mechanism for temperature sensing remains poorly understood. Thus, genetic variation could affect sensing and/or responding to thermal stimuli. Indeed, few researchers dispute a genetic underpinning to ESD, but the patterns of inheritance that would allow microevolution of ESD or the conversion of one sex-determining mechanism to another are poorly understood (Crews, 2003; Janzen, 1992).

Patterns of inheritance will be crucial to understanding the mechanism(s) and evolution of ESD. Although patterns of sex-determining

[2] PRESENT ADDRESS: Department of Organismic and Evolutionary Biology, Harvard University, 26 Oxford St., Cambridge, MA 02138, USA.

[3] CORRESPONDENCE: e-mail, djanes@oeb.harvard.edu

response to incubation temperature differ among ESD lineages, developmental pathways should be highly conserved if the lineages share common ancestry (Smith et al., 1999). Shared patterns of inheritance of sex-determining response to incubation temperature also should follow from common ancestry. If all ESD lizards, for example, share a common pattern of inheritance in their sex-determining mechanism, then the trait may be more conserved than has been hypothesized (Kraak and Pen, 2002). In this study, we seek to describe the genetic architecture of ESD in leopard geckos, *Eublepharis macularius*, to which future studies can compare patterns of inheritance from other ESD lizards.

The evolution of sex-determining mechanisms likely depends on a genotype × environment interaction (G×E). In G×E, some genotypes are more sensitive to environmental differences than other genotypes or the order of merit of genotypes varies among different environments. In short, some genotypes are better fit to one environment than other environments (Falconer and Mackay, 1996). Through G×E, the evolution of ESD to GSD (and GSD to ESD) could be explained as a gradual change across generations in sensitivity to environmental variance. Within species or as a result of speciation, GSD could become ESD through increased thermal sensitivity of a species' sex-determining mechanism. Likewise, ESD could become GSD through decreased thermal sensitivity of a species' sex-determining mechanism.

Genetic variation for ESD has been investigated previously in several species of reptiles (Girondot et al., 1994; Rhen and Lang, 1998). The heritability of offspring sex ratio incubated under constant temperature has been reported in the temperature-dependent sex-determining (TSD) Ouachita map turtle, *Graptemys ouachitensis* (Bull et al., 1982*a*). Bull et al. (1982*a*) incubated *G. ouachitensis* eggs from different families at a single constant temperature, 29.2 C. Their threshold model for ESD yielded a heritability estimate of 0.82 for the sex-determining response to incubation temperature. They concluded that natural variation in nest temperatures reduced the realized heritability of the sex-determining character under natural conditions. Further, Janzen (1992) found strong genetic variation in the TSD response of the common snapping turtle, *Chelydra serpentina*, to incubation temperature. However, there was no statistically significant interaction of temperature and family. Therefore, Janzen (1992) concluded that variation in the sex-determining response to incubation temperature in the TSD species *C. serpentina* is not due to G×E but rather to factors directly affecting sex. Genetic variation for sex-determining response to incubation temperature can explain differences in hatchling sex ratios among families or populations but does not explain consistent hatchling sex ratios among ESD families or populations that face varying incubation temperatures. Variations in estimated hatchling sex ratios of ESD painted turtles, *Chrysemys picta*, balanced out at 1:1 over 49 years (Janzen, 1994). Such consistency could be explained by G×E. It should be noted that finer scale estimation of yearly sex ratios in ESD sea turtles showed significant yearly variation in hatchling sex ratios (Godfrey et al., 1996).

In this study, we estimated G×E for the sex-determining mechanism of *E. macularius*. G×E was estimated using a half-sibling breeding design (Lynch and Walsh, 1998), randomizing eggs across three different incubation temperatures, which were chosen to represent the range of thermal tolerance for the embryos of this species (Viets et al., 1993).

Materials and Methods

Experimental Design

On 5 January 2002, a group of five male and 25 female adult leopard geckos, *Eublepharis macularius*, were selected from a colony of more than 50,000 individuals at The Gourmet Rodent, a commercial reptile breeding facility in Archer, Florida, USA. The five males were selected based on their previously demonstrated fertility and the variation of color patterns on their dorsa. Dorsal coloration is an indicator of geographic origin in *E. macularius* (Borner, 1974, 1976). Thus, the five males were selected to avoid comparisons of closely related sires, as the variety of color patterns suggested that the animals probably represented a variety of patrilineal lines. Twenty-five virgin females were selected on the basis of body size (larger females are more likely to be fecund). At the facility, females are reared separately from

TABLE 1.—Sample sizes of offspring groups of leopard geckos from individual dams. Each letter represents a different sire. Each sire was mated to five dams. The offspring of each sire/dam were divided randomly among three temperature treatments. Sample sizes differed because of differential reproduction among the sire/dam pairs.

Temperature (C)	Sire										
	A	B	C	D	E	F	G	H	I	J	Total
26	12	3	3	4	11	5	7	6	4	9	64
30	8	8	8	6	4	8	8	6	10	12	78
32.5	7	6	6	9	5	11	7	7	6	11	75
Total	27	17	17	19	20	24	22	19	20	32	217

males. The reproductive status of each female is monitored by the staff at the facility. For this reason, identifying adult virgin females was a simple task. Each male was mated to five females. We employed a standard half-sibling design to estimate genetic variation for sex (Lynch and Walsh, 1998). Half-sibling experiments eliminate common maternal effects. The experiment was replicated the following year (2003) with a new group of similarly selected male (5) and female (25) adult *E. macularius* from a separately maintained breeding colony at the Gourmet Rodent. Thus, the sires and dams used in the first year's experiment were neither the sires nor dams used in the second year's replication, nor their immediate relatives. All subsequent procedures were identical for animals from both years. There was no statistically significant effect of year or its interactions in any of our analyses, so data from the 2 years were combined for presentation purposes. See Table 1 for experimental design and sample sizes. All work conducted in this study followed the guidelines of the Institutional Animal Care and Use Committee of the University of Florida (approval #Z010).

Breeding Conditions

Each female was housed alone in a cage containing a food bowl, a water bowl, and a nest box filled with moist vermiculite. Males were mated to females by moving them to a different female's container every day. Males were rotated among their five mates every 24 h and isolated for 48 h every 5 d. Between 5 January and 15 May 2002, nest boxes were checked daily. If new eggs were found during daily nest box inspections, they were placed in a plastic box filled with vermiculite and transported to the University of Florida. In the laboratory, the eggs were placed in containers that consisted of six 188-ml plastic cups banded together in rings. Each cup contained 6 g of perlite and 17.5 ml of tap water and was sealed with a tight-fitting lid punctured with one gas-exchange hole. The eggs were placed randomly in containers. Each container held six eggs. Each egg was placed individually within one of the six cups in a container. The egg containers were placed randomly with respect to sire and dam in one of three environmental chambers maintained at either 26, 30, or 32.5 C for the duration of the experiment. Eggs of *E. macularius* are known to hatch in distinguishable and predictable sex ratios from these temperatures (26:0% males; 30: 24.4% males; 32.5: 74% males; Viets et al., 1993). No reported temperature produces 100% male offspring in this species. The sire, dam, egg container, and egg cup were recorded for each egg. Chamber temperatures were recorded every minute throughout the experiment with Hobo® temperature loggers (Onset Computer Corporation; Bourne, MA); temperature varied by a maximum of 0.5 C. This variation did not hinder our ability to produce offspring sex ratios similar to those previously reported (Viets et al., 1993; D. E. Janes, unpublished data). Every day, the environmental chambers were opened and the egg containers were removed. Each cup was opened momentarily in order to release metabolic gas waste and to check for hatchlings. Eggs that grew mold were discarded upon discovery. Position of egg containers within environmental chambers was randomized daily.

Histology and Microscopy

Upon hatching, geckos were euthanized by exposure to halothane (Fluothane: 2-bromo-2-chloro-1,1,1-trifluoroethane). Geckos were fixed in Bouin's fixative and preserved in 75% ethanol. The reproductive organs were removed from each gecko and prepared for analysis by light microscopy. The reproductive organs are opaque, white, cylindrical structures on either side of the posterior end of the dorsal artery. After removal, they were stored in 75% ethanol, dehydrated by increasing

concentrations of ethanol, cleared in two changes of Citrosolv, and infiltrated with paraffin (Fisher 55; Fisher Biotech, Orangeburg, NY) under increasing pressure (12, 15, 21, 23.5 lb/in^2). The resulting paraffin blocks were sectioned at 8 μm and stained with a modified trichrome of Harris (Humason, 1997). Two researchers independently analyzed sections of reproductive tissue from each gecko. If seminiferous tubules were identified within the tissue sections, the gecko was scored as male. If oogonia were identified along the edges (germinal ridge) of the tissue section, the gecko was scored as female. Seminiferous tubules were identified as clusters of simple, circular structures with a narrow lumen and oogonia were identified as small circular structures surrounded by larger concentric circles (the follicle; Berman, 2003). These structures are clear and distinct at 200× magnification. Histological examination is the most efficacious technique for sexing animals that lack heteromorphic sex chromosomes, as it avoids potential macroscopic misidentification of male and female reproductive organs. Errors in tissue preparation resulted in missing data, an issue dealt with subsequently with sensitivity analyses and bootstraps.

Statistical Analyses

The dependent variable, sex (1: female; 2: male), was analyzed using ANOVA (Falconer and Mackay, 1996). Terms in the original ANOVA included main effects of temperature (fixed) and sire (random), as well as their interaction (random). Dams were nested within sires (random) and their interaction with temperature (random) was included in the model. Genetic variation for sex ratio would be represented by the sire and dam terms; G×E would be represented by the terms sire × temp and temp × dam(sire). Egg containers were nested within temp × dam (sire[random: Falconer and Mackay, 1996]). Effects of temperature, sire, dam, and temp × sire (dam) interaction on proportion of male progeny per treatment group were analyzed using SAS version 6.10 for the MacIntosh. ANOVAs were performed using PROC GLM.

Clutch sizes and number of clutches per dam were variable. Some eggs did not hatch (63/281); further, unambiguous gonad identity could not be assigned to a number of those eggs that did hatch (55/218). The missing data had the result of making the sire term sufficiently unbalanced that an F statistic could not be calculated due to negative denominator means squares. Accordingly, this term and the temp × sire term were excluded from further analysis, effectively placing them in the error.

We performed a sensitivity analysis wherein missing data were replaced with all males or all females to test whether data were missing at random with respect to temperature (i.e., if individuals without definitive sex assignment were more likely to be one sex than the other; Rubin, 1976). We also analyzed a subset of data as a full-sibling design. These subsets were selected from sire/dam pairings that produced sufficient numbers of offspring at each temperature to permit analysis.

We also performed a bootstrap on our data to assess the significance of the ANOVA results. The term that was of the most interest to us was the dam*temperature term. Given that there was a main effect of temperature, we bootstrapped the data within each of the three temperatures 1000 times across dams, generating 1000 new datasets that did not disrupt the temperature effect but reassigned parentage (or missing data status) within each temperature to evaluate the dam*temperature interaction. Bootstrapped datasets were created using the R statistical software package. The bootstrapped datasets were then analyzed using the same model in PROC GLM as the original ANOVA, and the distribution of F statistics for the dam*temp term was recorded.

Results

Significant genotype × environment interaction (G×E) was found for sex in leopard geckos, *Eublepharis macularius*. The effect of temperature was significant on sex determination in *E. macularius*, such that the proportion of male progeny increased with temperature ($P < 0.0001$, Table 2). There was no evidence for genetic variation for sex determination per se, as the dam source of variance was not significant. However, there was evidence for G×E, as the temp × dam(sire) term was statistically significant ($P < 0.0261$, Table 2). G×E for sex is apparent at higher incubation temperatures (see Fig. 1), which is not surprising given that the sex ratios are closer

TABLE 2.—ANOVA for sex ratio in leopard geckos, entire dataset.

Source	df	EMS	*P*
Temp	2	2.728	0.0001
Dam(Sire)	41	0.199	0.7581
Temp*Dam(Sire)	50	0.247	0.0261
Container(T*S*D)	60	0.146	0.8414
Error	4	0.250	

to 1:1 at the higher temperatures, and thus our ability to perceive G×E will be greater at these temperatures.

Results of the sensitivity analysis were qualitatively consistent with the analysis using the entire dataset: the frequency of males increased with temperature, regardless of whether missing data were replaced with males, with females, or not replaced (Table 3). Further, for both sensitivity analyses, the results of ANOVA were consistent with the results from the entire dataset: temperature was significant ($P < 0.0004$ and $P < 0.0031$, missing data replaced with males or females respectively), and temperature × dam was the only other term significant or nearly so ($P < 0.0332$ and $P < 0.0930$, missing data replaced with males or females respectively).

We also selected one full sibling family from each set of half-sibling families. We selected only families that had 100% of data present (definitive assignment of sex to all progeny). Where a given sire had multiple dams with all data present, the dam with the largest number of progeny was chosen, so that the model was not overdetermined. Although this sample was very small such that no *P*-values were <0.05, results were again qualitatively consistent with other analyses (Tables 3, 4).

Finally, we evaluated the significance of the temperature × dam term by bootstrap (see Materials and Methods for details). Our results were further supported by the bootstrapping exercise. The *F* value for temperature × dam that we obtained from the actual data exceeded 993 of the *F* statistics from the bootstrapped data, generating a *P* value of 0.007.

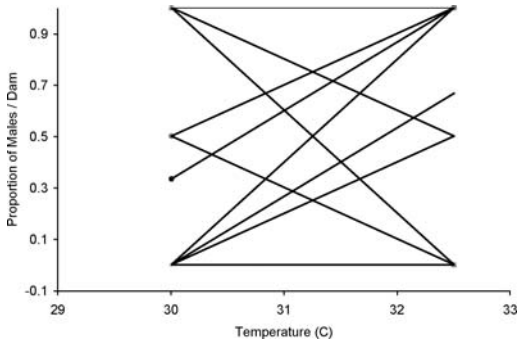

FIG. 1.—Offspring sex ratios of leopard geckos incubated at 30 or 32.5 C during one of two years of experimentation. Each line represents the offspring sex ratio of one of 21 dams used in this study. A subset of dams was selected for representation based on hatching success of their offspring at the two temperatures shown in the figure. Some lines overlap and are indistinguishable. Incubated at the same temperature, different dams produced different offspring sex ratios. The ranks of the genotypes of different dams changed depending on the environment, indicating a gene × environment interaction.

TABLE 3.—Means (± standard errors) for sex ratio of leopard geckos at different temperatures, for different analyses (please see text for description of analyses). The percent male progeny increases with temperature regardless of analysis (100% female would be represented by 1.00; 100% male would be represented by 2.00).

Analysis	26 C	30 C	32.5 C
Entire dataset	1.065 ± 0.250	1.517 ± 0.504	1.537 ± 0.503
Sensitivity (male)	1.328 ± 0.473	1.641 ± 0.482	1.667 ± 0.475
Sensitivity (female)	1.047 ± 0.213	1.385 ± 0.490	1.387 ± 0.490
Full sibling subset	1.200 ± 0.422	1.556 ± 0.511	1.900 ± 0.316

DISCUSSION

We tested for a genotype × environment interaction (G×E) for sex in an environmentally sex-determined (ESD) reptile, leopard geckos, *Eublepharis macularius*, by estimating genetic variation in the sex of progeny in response to a range of incubation temperatures. If demonstrable G×E exists for sex in ESD species, then potential for microevolution

TABLE 4.—ANOVA for sex ratio in leopard geckos, full-siblings.

Source	df	EMS	*P*
Temp	2	1.053	0.0675
Genotype	7	0.160	0.7815
Temp*Genotype	9	0.288	0.1306
Container(T*G)	18	0.148	0.9712
Error	1	0.500	

exists (Falconer and Mackay, 1996). Some genotypes are more sensitive to environmental effects than other genotypes; individuals of high environmental sensitivity comprise ESD species while individuals of low or no environmental sensitivity comprise GSD species. By this logic, G×E could explain the difference between ESD and GSD species. G×E could bridge the two ends of the continuum of sex-determining mechanisms by explaining the pathway by which one becomes the other. In the case of ESD, G×E could allow the production of male and female offspring even if nest site temperatures fluctuate beyond either male- or female-producing temperatures, as appears to be occurring in nature (Leslie and Spotila, 2001). G×E could sufficiently decouple sex ratio from nest site temperature to allow Fisherian mechanisms to shape adult sex ratios as they are expected to do in GSD species (Fisher, 1930). According to Fisher, the sex that is most negatively affected by environmental stress will be produced less frequently and will garner more parental input. The cost paid by the parents for the rarer, more energetically expensive sex offspring should balance with the cost they pay for the more common, less energetically expensive sex offspring. Our results demonstrate that there is no genetic variation for sex determination per se, consistent with our understanding of *E. macularius* as an ESD species. However, we report G×E for sex in this sample.

We have demonstrated a statistically significant interaction between genotype and environment with respect to sex determination in the leopard gecko. It is worth noting that our study is small, and that there were significant missing data. Both these issues may increase our risk of Type I error. In addition, while ANOVA is robust to binary variables, scale effects can be important when testing interaction terms (Neter et al., 1990). In short, though we have been as rigorous as possible in the analysis of our data, further experiments are needed to test the generality of our result.

For microevolution to occur, genetic variation must exist for the threshold temperatures at which embryos become either male or female. We have provided evidence that suggests such G×E exists for sex determination in this species. Other intrinsic and extrinsic factors could also drive evolution of sex-determining mechanisms. For example, the evolution of ESD to GSD, GSD to ESD, or threshold temperatures for male or female determination may be further complicated by nest choice and construction. Bull et al. (1982*a*) suggested that sex-ratio evolution in ESD species is driven primarily by changes in maternal behavior varying nest construction or timing of oviposition. Genetic variation for behavioral placement or construction of nests may work in concert or in opposition to variation for sensivity of ESD, as has been considered in *E. macularius* as well as a viviparous skink and a turtle (Bull et al., 1988; Morjan, 2003; Robert and Thompson, 2001). Bull et al. (1982*a*,*b*) demonstrated a heritable effect of sex determination among families of map turtles but did not demonstrate local adaptation of sex-determining threshold temperatures between populations of map turtles from different latitudes and climates.

Microevolution of sex-determining mechanisms can explain reptilian phylogenetics, evolution of sex, and the likelihood of extinction as a result of global warming. If mean July temperature in the central United States rises 4 C in the next 100 yr as predicted (Manabe and Stouffer, 1993) and there is insufficient genetic variation for the sex-determining response, the painted turtle, *Chrysemys picta* will become extinct because of an inability to produce males (Janzen, 1994). Offspring sex ratios of temperature-dependent sex-determining (TSD) species are highly correlated with mean air temperature when most clutches are in the middle third of the incubation period: the thermosensitive period (Janzen, 1994). As a consequence of this correlation, TSD species are considered vulnerable to climate change (Girondot et al., 1998; Janzen, 1994; Leslie and Spotila, 2001). Other ESD and GSD reptiles should be tested in a similar manner in order to compare patterns of inheritance. If other ESD lizards also demonstrate G×E, then ESD may be more conserved than it appears (Janzen and Krenz, 2004). Shared patterns of inheritance between ESD and GSD species would also suggest genetic differences between ESD and GSD species are small which would explain the apparently facile conversion of one to the other. Despite the complexities and irregularities of

this experiment, our work suggests a broad and deep field of study that will elucidate the genetic architecture of sex determination across Reptilia.

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