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Offspring Sex Is Not Determined by Gestation Temperature in a Viviparous Lizard (*Eremias multiocellata*) from the Desert Steppe of Inner Mongolia

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Abstract Sex-determining systems show a striking diversity not only among species, but also among populations. In reptiles, sex-determination is a continuum, from temperature-dependent sex determination (TSD) to genetic sex determination (GSD). The multi-ocellated racerunner (*Eremias multiocellata*) is reported to be a cryptic ZZ/ZW chromosomal TSD species, with male-biased sex ratios at high temperatures in two Gansu populations. However, the generality of the sex-determining pattern in different populations of this species remains unclear. To investigate the mode of sex determination in a population of *E. multiocellata* from the desert steppe of Inner Mongolia, we first identified sex chromosomes via comparative genomic hybridization (CGH). We then conducted a thermal manipulation experiment to determine the effect of gestation temperature on offspring sex ratios. From the CGH studies we found that lizards from the Inner Mongolia population possessed ZZ/ZW sex chromosomes. However, our thermal manipulation experiment showed that gestation temperature did not affect the sex ratio of neonates in this population. In combination, these results rule out TSD in the Inner Mongolia population of *E. multiocellata*, and suggest that there is widespread geographic variation in the sex-determining system of this species.

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1. Introduction

Vertebrates exhibit two broad categories of sex-determining systems: genotypic sex determination (GSD) where specific genetic elements direct the development of the gonads, and environmental sex determination (ESD) where external cues, such as temperature (temperature-dependent sex determination, TSD), determine the sex of an individual. All mammals and birds exhibit GSD. A dominant sex gene on the Y chromosome region (*Sry*) initiates male development in mammals (Kashimada and Koopman, 2010; Koopman *et al*., 1990). In birds, the dosage effects of double sex and mab-3 related transcription factor 1 (*Dmrt*1) on the Z chromosome control sex determination (Ellegren, 2009; Smith *et al*., 2007; Smith *et al*., 2009). In contrast, sex determination systems are highly variable among ectotherms. Both ZZ/ZW and XX/XY sex-chromosome systems occur in all these taxa including fish, amphibians and reptiles, as well as TSD in certain reptiles and fish (Bachtrog *et al*., 2014; Capel, 2017; Lambert *et al*., 2019; Nemesházi *et al*., 2020; Ospina-Alvarez and Piferrer, 2008; Janzen and Krenz, 2004). For example, reptiles have multiple sex-determining mechanisms. In all snakes, and some lizards and turtles (Ezaz *et al*., 2006a; Ezaz *et al*., 2006b; Janzen and Krenz, 2004; Schwanz *et al*., 2013) sex determination involves GSD (with ZZ/ZW or XX/XY sex chromosomes), whereas in all crocodiles, the tuatara, many turtles and several lizard species (Rhen and Schroeder, 2010; Shine *et al*., 2007) nest temperature (in oviparious species) or gestation temperature (in

viviparous species) is responsible for determining gonadal sex of offspring.

Environmentally influenced sex determination has been found in some ectothermic vertebrates with sex chromosomes, in which genes and environment interact to determine the phenotype of the sex (Holleley *et al*., 2015). Temperature can override the GSD effect and result in sex reversal in some fishes (Baroiller and D'Cotta, 2016; Ospina-Alvarez and Piferrer, 2008), amphibians (Alho *et al*., 2010; Bachtrog *et al*., 2014; Flament, 2016; Lambert *et al*., 2019; Nemesházi *et al*., 2020) and reptiles (Holleley *et al*., 2015; Quinn *et al*., 2007; Radder *et al*., 2008; Sarre *et al*., 2004; Shine *et al*., 2002). These species have sex chromosomes but exhibit thermal sensitivity in the development of gonadogenesis, representing mixed sex-determination systems. For example, high incubation temperatures reverse genotypic males (ZZ) to phenotypic females in the Australian central bearded dragon lizard (*Pogona vitticeps*), which has GSD with female heterogamety (ZW) (Holleley *et al*., 2015; Quinn *et al*., 2007). Analogously, low temperatures override chromosomal sex to generate XX male offspring in a montane scincid lizard (*Bassiana duperreyi*) from south-eastern Australia, which has male heterogamety (XY) (Radder *et al*., 2008; Shine *et al*., 2002). Further work on the interactive effects of sex chromosomes and early developmental (embryonic or larval) temperatures on offspring sex ratios is needed to elucidate the sex-determining systems across a greater diversity of lineages in ectothermic vertebrates.

Interestingly, recent studies have demonstrated that sex-determining mechanisms may also differ among populations within a species. For example, the association between phenotypic sex and the underlying genetic factor (linkage group 2) was perfect in a northern Swedish population, but weak and variable among families in a southern one in the common frog (*Rana temporaria*) (Rodrigues *et al*., 2016). Three Australian lizards, the snow skink (*Niveoscincus ocellatus*) (XX/XY system), the bearded dragon (ZZ/ZW system) and another skink (*Bassiana duperreyi*) (XX/XY system), are GSD species possessing a mixed sex determination system with temperature-induced sex reversal in some populations (Castelli *et al*., 2020a; Cunningham *et al*., 2017; Dissanayake *et al*., 2021; Hill *et al*., 2018; Pen *et al*., 2010). In the snow skink, variation in the magnitude of between-year temperature fluctuations might lead to natural selection on sex determination across altitudes (Cunningham *et al*., 2017; Pen *et al*., 2010). Similarly, climatic effects on sex determination also occur in the Atlantic silverside, *Menidia menidia*, which exhibits an exceptionally high level of clinal variation in sex determination across its geographic range, with TSD in southern populations, GSD in northern populations, and mixed systems in between (Duffy *et al*., 2015). However, whether such intra-specific divergence in sex-determining systems exists in other lineages remains largely unknown.

The multi-ocellated racerunner (*Eremias multiocellata*) is a small viviparous lizard (about 65 mm snout-vent length SVL) with an extensive distribution ranging from Mongolia, Kazakhstan, Kyrgyzstan to Xinjiang, Gansu, and Inner Mongolia of China, with the highest known population found at an altitude of 2900 m above sea level (asl) (Zhao, 1999). The chromosomal system of this species was identified by traditional Giemsa staining as $2n = 38$, with no obvious sex chromosomes. However, a recent study detected female-specific amplified signals in two Gansu populations by using comparative genomic hybridization (CGH), suggesting this species possesses a female-heterogametic sex chromosome system (ZZ/ZW) (Wang *et al*., 2015). Interestingly, gestation temperature has been shown to affect offspring sex ratio in two Gansu populations at elevations of 1400 m and 2900 m asl, with male-biased sex ratios at 35°C and female-biased sex ratios at 25°C (Tang *et al*., 2012; Zhang *et al*., 2010). In addition, compared to the low-elevation population from a relatively warmer climate, the high-elevation population of *E. multiocellata* exhibited a less skewed offspring sex ratio (Tang *et al*., 2012). In the Inner Mongolia population of *E. multiocellata* at a lower elevation of 1036 m asl, the edge of the species' altitudinal distribution range, extreme environments (i.e., low precipitation and extreme high temperatures) during reproductive season affect offspring sex (Wang *et al*., 2016). The effect of temperature on offspring sex varied between years with different precipitation levels (Wang *et al*., 2016), so a controlled experiment is needed to test if high temperature itself (without accompanying aridity) affects sex determination in the Inner Mongolia population of *E. multiocellata*. Therefore, here we used CGH to identify the sex chromosome system, and conducted temperature manipulation experiments to investigate the effects of materal thermal (gestation) environment on offspring sex.

2. Materials and Methods

2.1. Study site and species The *E. multiocellata* used in this study were collected from Shierliancheng countryside (40°12'17"N, 111°07'43"E; elevation 1036 m), Jungar Banner, in the desert steppe region of Inner Mongolia, China. Shierliancheng is characteristic of a cold semi-arid climate with an average annual temperature of 6–7°C and annual precipitation of 300–380 mm (http://www.nmic.gov.cn). This lacertid lizard is a viviparous species (adult SVL 54.0–67.3 mm, BM 4.7–8.2 g) that generally occupies arid or semi-arid regions. Female *E. multiocellata* have a gestation period of two months and produce a single litter of 2–5 offspring between July and August (Zhao, 1999). The selected body temperature and critical thermal maximum of *E. multiocellata* in Inner Mongolia population are

(35.2±0.2)°C and (45.1±0.1)°C, respectively (Li *et al*., 2017).

2.2. Identification of sex chromosomes Three male and three female adult *E. multiocellata* were used for sex chromosome identification, using the established protocol of CGH (Martinez *et al*., 2008). we prepared both female and male metaphase spreads of mitotic chromosomes from peripheral blood leukocytes following the published method of Ezaz *et al* (2005). Approximately 100 μL blood was used to set up 2 mL culture in RPMI1640 (Gibco) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 40 μg/mL phytohaemagglutinin M (PHA M). After incubated at 30°C in 5% CO_2 incubators for 72 h, we replaced new medium. Eight and six hours before harvesting, 35 mg/mL 5-bromo-2-deoxyuridine (BrdU; Sigma) and 75 ng/mL colcemid (Roche) were added to each culture. Metaphases were fixed in 3:1 methanol-acetic acid. Cell suspension was dropped onto wet cold slides prechilled at 4°C, and slides were dried on a hotplate at 60°C and saved at –80°C.

Total genomic DNA (gDNA) was extracted from whole blood following the phenol-chloroform method, and the DNA probe was then labeled with SpectrumGreen-dUTP and SpectrumRed-dUTP (Enzo Life Science, Farmingdale, NY). Metaphase chromosome slides dried for 2 h at 60°C and denatured for 2 min at 60°C in 70% formamide and 2×SSC, then dehydrated through a 70%, 80% and 100% ethanol series for 1 min each, and air-dried at room temperature until hybridization.

The probe mixture containing 20 mg glycogen, 500 ng female probe, and 500 ng male probewere co-precipitated with 5 mg of boiled *E. macquarii* female DNA. Following an overnight co-precipitation at –20°C, the probe mix was centrifuged at 15 000 g at 4°C for 15 min. The supernatant was discarded and the probe DNA pellet was resuspended in 40 μL of 37°C pre-warmed hybridization buffer and resuspended for at least 30 min at 37°C. The hybridization mixture was denatured at 70°C for 8 min, immediately placed on ice for 2 min, and 16 μL of the probe mixture was placed as a single drop per slide. For the purposes of this experiment we assumed that *E. multiocellata* have a female ZZ/ZW chromosome system, and therefore, non-labeled male gDNA was used to compete with DNA probes; in the reciprocal experiment, female gDNA was the competitor. After a humid hybridization chamber at 37°C for 3 days, slides were washed at 55°C in 0.4×SSC, 0.3% Tween-20 for 2 min, followed by a second wash in 2×SSC, 0.1% Tween-20 for 1 min at room temperature. Slides were immersed into a DAPI solution with a concentration of 1 μg/mL for 30 s. This procedure resulted in the respective hybridization of the competing DNA probes, with male and female metaphase spreads. Finally, we conducted fluorescene microscopy (Zeiss) to detect if there was any sex-specific signal in the spreads.

2.3. Thermal treatments A total of 60 gravid female and 18 adult male *E. multiocellata* were captured and brought back to the laboratory in Beijing in early May 2017. This timing of capture allowed us to start treating females prior to the temperature-sensitive period (TSP) of offspring sex determination, because the TSP is in the middle third of embryonic development in reptiles (Wibbels *et al*., 1991). After recording snout-vent length (SVL, ±0.01 mm) and body mass (BM, ±0.001 g), lizards were evenly assigned into three temperature treatments: low (29°C), moderate (35°C), and high (38°C). Within each treatment, lizards were randomly distributed into three terraria (55.6 cm×42.5 cm×34.6 cm, length×width×height) that were placed in temperature-controlled incubators, with each terrarium containing 6–7 females and 2 males. Incubators were set at 23°C for the low (29°C) and moderate (35°C) temperature treatments, and at 29°C for the high temperature treatment (38°C). We then used electronic heating pads with temperature control devices (YWK-F, Shanghai Medical Instruments, China) attached to each terrarium to increase the ambient temperature of terraria to 29°C, 35°C, and 38°C for the low, moderate, and high temperature treatments respectively, from 08:00 to 18:00 h each day. Data loggers (iButton, DS1921; Maxim Integrated Products Ltd., USA) were used to monitor the thermal environment within each terrarium for the duration of the study. Additionally, a 12:12 h (light: dark) photoperiod was provided in each terrarium from 07:00 to 19:00 h, using full UV-spectrum lamps. Each terrarium contained a sand substrate (5 cm deep) and several shelter items. Food (mealworms and crickets dusted with extra vitamins and minerals) and water were provided *ad libitum*. We recorded body temperatures of active female lizards four times on May 25th, June 1st, June 8th and June 15th by inserting a probe of UT325 electronic thermal meter into cloacae.

2.4. Female reproduction and neonate traits Females in the experimental terraria were palpated for gravidity every three days. Individual females containing soft and oval eggs (i.e., late developmental-stage embryos) were then transferred into small boxes with the same temperature as their original thermal treatments to give birth. Then, we checked these boxes every day for neonates. Once found, neonates were measured (SVL; ±0.01 mm) and weighed (BM; ±0.001 g). We calculated gestation length as the days between female capture and the parturition.

We obtained a total of 104 neonates, of which 22, 40 and 42 neonates produced by females from the low, moderate and high temperature treatment, respectively. In addition, 10 out of 22 neonates produced by gravid females from the low temperature treatment were stillborn. We identified the sex of these 104 neonates by the presence/absence of hemipenes. A subset of those neonates (n = 34) did not survive after birth and were used to validate our morphological sex identification

technique, by inspecting the gonads. Intact gonads were dissected out, snap-frozen in liquid nitrogen and embedded in an optimal cutting temperature compound (OCT) at –20°C to –30°C. Frozen gonads were cut into 6 μm slices with the microtome portion of the cryostat. Each slice was then placed on a glass slide and stained with hematoxylin and eosin (H&E stain). Sex was identified under an optical microscope based on standard testis and ovarian morphology (Figure 1). The sex of all of neonates identified by gonadal inspection was consistent with our morphological sex-identification technique (everting hemipenes; Figure 1); subsequently, neonate sex was identified using presence/absence of hemipenes.

2.5. Statistical analysis We used linear models (LM; "stats" package) (R Core Team., 2019) to detect the differences of maternal SVL and BM, and the ambient temperatures inside terraria between thermal treatments. We used the generalized linear models (GLM; "stats" package) (R Core Team., 2019) to detect the effects of thermal treatments on female reproductive success using a binomial distribution with a logit link. Generalized least squares models (GLS; "nlme" package) (Pinheiro *et al*., 2017) were used to detect the effects of thermal treatments on gestation period and litter mass with maternal SVL as a covariate, because within-group variance differed between treatment groups, we allowed for treatment-dependent variances in the GLS model by the varIdent function. The Kruskal-Wallis test was used to detect the effects of thermal treatments on litter size (the total number of offspring born by a female including stillborn). We used linear mixed-effects models (LME "nlme" package) (Pinheiro *et al*., 2017) to test the effects of thermal treatments on neonate SVL and BM with maternal ID as a random factor. The same

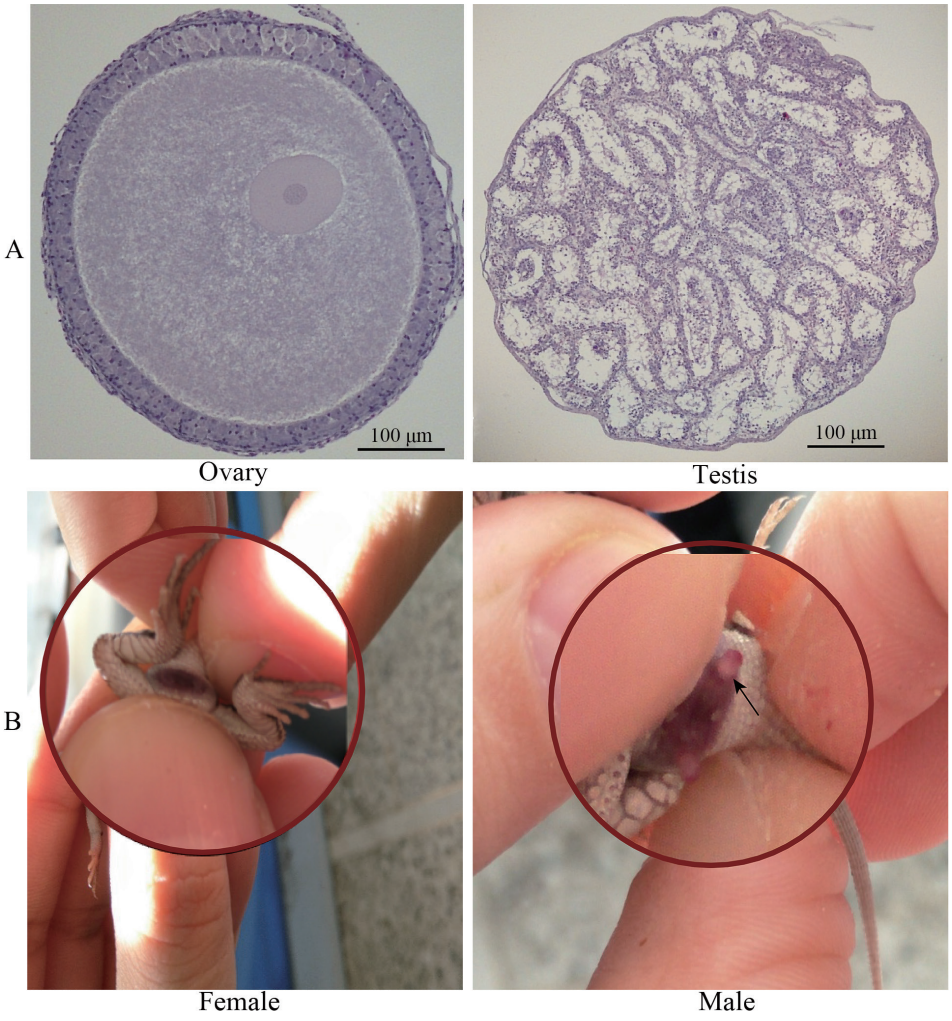

Figure 1 Methods of sex identification for *Eremias multicellata* used in this study. (A) Microscopy of frozen sections of gonads from *E. multicellata*. (B) Manual eversion of hemipenes. Sex of *E. multicellata* was determined by presence/absence of hemipenes. Arrow points to the hemipenes.

model LME was used to detect the effects of thermal treatments on body temperature of the *E. multiocellata*. In order to detect the effects of thermal treatments on neonate survival, total neonate (including both surviving and dead individuals) sex ratio, sex ratio of neonates that were dead after birth, and sex ratio of neonates that survived after birth, we used generalized linear mixed effect models (GLMMs; "lme4" package) (Bates *et al.*, 2015) with a binomial distribution and a logit link, and maternal ID as a random factor. We compared each model including thermal treatments with a model that only contained the random factor using the "anova" function to get an overall chi-squared and *P*-value for the thermal treatments effect. Post-hoc tests for the analysis of litter mass and gestation period were performed by emmeans function with "tukey" method ("emmeans" package) (Lenth R.V., 2018) and we used pairwise Mann-Whitney tests to carry out post-hoc tests for significant Kruskal-Wallis test and corrected the *P*-values by the "fdr" method ("stats" package) (R Core Team., 2019). We checked the GLMMs with binomial distribution for the dispersion parameter by "dispersion_glmer"function ("blmeco"package) (Korner-Nievergelt *et al.*, 2015), and the dispersion parameters of all binomial models are (close to) 1. We conducted all statistical analyses in R version 3.5.1 (R Core Team, 2019).

3. Results

3.1. Sex chromosomes of *E. multiocellata* We detected a significantly enhanced and specific hybridization signal in one of the microchromosomes in cells from all females, but not in males (Figure 2). This female-specific chromosome is therefore, by definition, a W chromosome, which means that *E. multiocellata* from the Inner Mongolia population possess a ZZ/ZW sex chromosome system.

3.2. Female body temperature and reproduction The ambient temperatures inside terraria were significantly different among the three temperature treatments ($F_{2, 616}$ = 126.59, P < 0.001; Figure 3A). Correspondingly, daily average body temperatures of gravid females from 08:00 to 18:00 were also significantly different among thermal treatments ($F_{2, 36}$ = 247.4, P < 0.001). Mean (±SE) body temperatures of gravid females were as follows: high temperature treatment (37.9±0.1°)C, moderate temperature treatment (34.6±0.1)°C, low temperature treatment (28.9±0.1)°C (Figure 3B).

Gestation period of gravid females was significantly affected by thermal treatment, decreasing as female gestation temperature increased (Table 1 and Figure 4). Female reproductive success (the proportion of females producing live offspring) was 65.0% (13/20) for females in the high temperature group, 80.0% (16/20) in the moderate temperature group, and 50.0% (10/20) in the low temperature treatment (Figure 4). This difference in reproductive success among temperature treatments was not statistically significant (Table 1). Thermal treatment significantly affected litter mass and litter size. Females from the high temperature treatment had greater litter mass and larger litter size than those from the low temperature treatment (Table 1 and Figure 4).

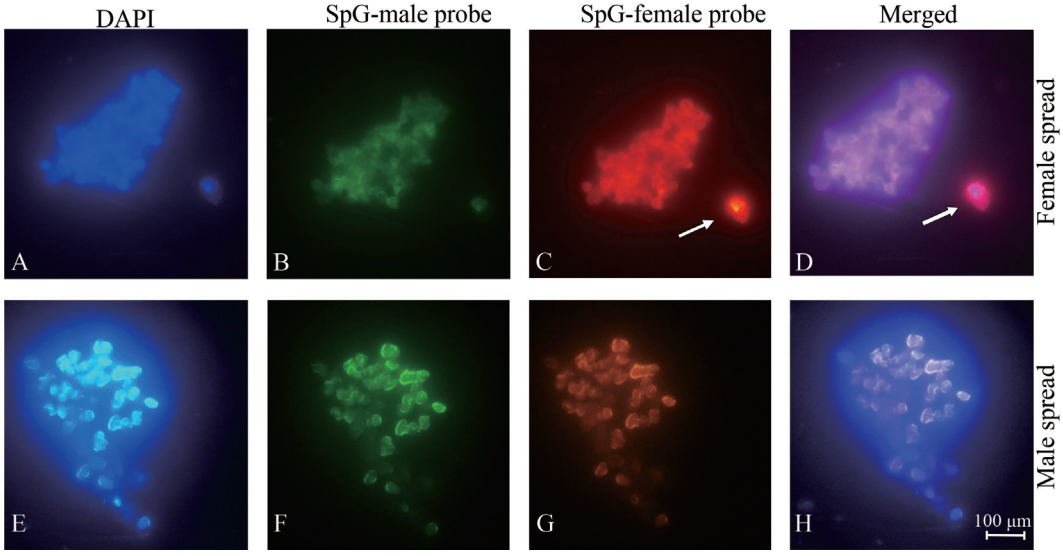

Figure 2 Results of comparative genomic hybridization (CGH) in female (upper) and male (lower) metaphases from *Eremias multiocellata* in our study. (A, E) DAPI stained metaphase chromosome spread; (B, F) Metaphase hybridized with SpectrumGreen-dUTP labeled male whole genomic DNA; (C, G) Metaphase hybridized with SpectrumRed-dUTP labeled female whole genomic DNA; (D, H) Merged images of metaphase hybridized with male and female whole genomic DNA. Scale bar indicates 100 μm. Arrow indicates W chromosome.

3.3. Neonate phenotypes and sex ratio Neither neonate SVL nor BM differed among thermal treatments (Table 1 and Figure 5). The proportion of neonates that did not survive after birth was higher in the high (78.6%) and low (75.0%) temperature treatment than the moderate (45.0%) temperature treatments (Figure 5).

Most notably, female gestation temperature did not affect neonate sex ratio (Table 1). The male ratio of neonates (54.5%, 52.5% and 52.4% for the low, moderate and high temperature treatments, respectively, which was very close to 50:50 in all three groups, did not differ between the three temperature treatments (Figure 5 and Table 1).

4. Discussion

Our study revealed that *E. multiocellata* from the Inner Mongolia population have a female ZZ/ZW chromosome system, and that female gestation temperature does not affect offspring sex ratio; this is inconsistent with the TSD pattern found in the two Gansu populations of *E. multiocellata* (Tang *et al*., 2012; Zhang *et al*., 2010). This between-population discrepancy in sex-determining system adds another example of intraspecific variation in TSD and GSD patterns in reptiles, in addition to the phenomenon first reported in a viviparous Australian montane lizard, the snow skink, *Niveoscincus ocellatus* (Cunningham *et al*., 2017; Pen *et al*., 2010). Sex-determining systems are quite variable in reptiles, involving environmental and genetic sex determination, and only some reptiles have been identified to possess sex chromosome differentiation (Bachtrog *et al*., 2014; Georges *et al*., 2010; Martínez-Juárez and Moreno-Mendoza, 2019). Traditional Giemsa staining methods did not elucidate sex chromosomes in *E. multiocellata* because the traditional way identifies chromosome pairs based on morphological differences (Wang *et al*., 2015). Instead, we used CGH, which distinguishes sex chromosomes through the non-homologous region, to reveal that *E. multiocellata* from the Inner Mongolia population had ZZ/ZW sex chromosomes (Figure 2). The sex chromosomes of some reptiles are extremely cryptic, and can only be detected via such high-resolution cytogenetic techniques (Badenhorst *et al*., 2013; Ezaz *et al*., 2005; Ezaz *et al*., 2006b; Kawai *et al*., 2007; Martinez *et al*., 2008). The Gansu populations of *E. multiocellata* also have ZZ/ZW sex chromosomes, but the sex was affected by temperature (Wang *et al*., 2015). These results suggest that the *E. multiocellata* populations we have studied all have sex chromosomes, but that the genetic effects on offspring sex can be overridden by extreme temperatures in the Gansu populations (Tang *et al*., 2012; Zhang *et al*., 2010).

The gestation temperatures we used in our experiment, which mimicked female body temperatures measured in the field during the reproductive season (Li *et al*., 2017), did not affect offspring sex ratio in the Inner Mongolia population of *E. multiocellata*. However, a field warming experiment on this population showed that females produced male-skewed offspring under extremely high temperatures and dry environment, but not in response to extremely high temperatures alone (Wang *et al*., 2016). The sex-ratio bias found under warm and dry conditions could have been due to water deprivation or maternal manipulation of offspring sex ratios in response to nutrient deficiency caused by the extreme conditions. For example, water deprivation during pregnancy could induce a male-biased secondary sex ratio in *Aspic viper* and *Zootoca vivipara* (Dupoué *et al*., 2019). In addition, in an agamid lizard (*Amphibolurus muricatus*) from Australia, females fed a poor-quality diet produced highly male-biased sex ratios (Warner and Shine, 2007). These instances indicate that female-to-male sex reversal may be triggered by stressors, perhaps due to the effects of glucocorticoid hormones and reactive oxygen species on sex determination (Castelli *et al*., 2020b). In addition, the ecological driving forces

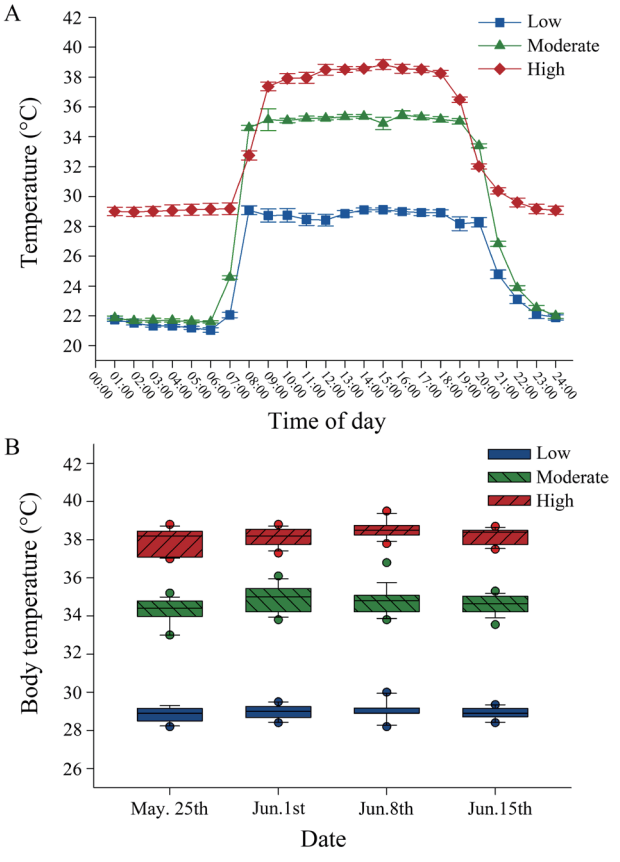

Figure 3 Experimental thermal treatments. (A) Ambient temperatures recorded in the terraria from the three temperature treatments, and (B) active body temperatures recorded during gestation in gravid female *Eremias multicellata*. Values are expressed as mean±SE.

Table 1 Differences between gestation temperature treatments in maternal and neonate traits in the multi-ocellated racerunner *Eremias multicellata* from Inner Mongolia.

Variable	Treatment			Statistical analysis
	29°C	35°C	38°C	
Maternal SVL (mm)	65.108±1.017	64.468±0.774	65.068±1.147	$F_{2,36} = 0.147$, $P = 0.864$
Maternal body mass (g)	5.615±0.179	5.576±0.201	5.890±0.332	$F_{2,36} = 0.472$, $P = 0.628$
Gestation period (day)	60.100±2.681	45.560±1.494	28.230±0.533	$F_{2,36} = 119.808$, $\mathbf{P < 0.001}$ 29°C^a>35°C^b>38°C^c a-b: $P = 0.001$; a-c: $P < 0.001$; b-c: $P < 0.001$
Reproductive success	50.0% (10/20)	80.0% (16/20)	65.0% (13/20)	$\chi^2 = 4.054$, $df = 2$, $P = 0.132$
Litter mass (g)	1.021±0.031	1.292±0.070	1.755±0.159	$F_{2,35} = 17.885$, $\mathbf{P < 0.001}$ 29°C^a<35°C^b<38°C^c a-b: $P = 0.004$; a-c: $P = 0.001$; b-c: $P = 0.032$
Litter size	2.200±0.133	2.500±0.158	3.230±0.281	$\chi^2 = 9.131$, df=2, $\mathbf{P = 0.010}$ 29°C^a <38°C^c a-b: $P = 0.217$; a-c: $P < 0.019$; b-c: $P = 0.056$
Neonate SVL (mm)	29.319±0.247	29.257±0.208	29.172±0.204	$F_{2,36} = 0.201$, $P = 0.819$
Neonate body mass (g)	0.498±0.011	0.517±0.013	0.543±0.012	$F_{2,36} = 1.589$, $P = 0.218$
Neonate survival ratio	75.0% (9/12)	45.0% (18/40)	78.6% (33/42)	$\chi^2 = 2.240$, $df = 2$, $P = 0.326$
Neonate sex ratio (♂%)	54.5%(12/22)	52.5% (21/40)	52.4% (22/42)	$\chi^2 = 0.029$, $df = 2$, P =0.986
Neonate sex ratio (Dead after birth, ♂%)	66.7% (2/3)	63.6% (14/22)	55.6% (5/9)	$\chi^2 = 0.267$, $df = 2$, $P = 0.875$
Neonate sex ratio (Survived after birth, ♂%)	55.6% (5/9)	38.9% (7/18)	51.5% (17/33)	$\chi^2 = 0.971$, $df = 2$, $P = 0.615$

The maternal body size (SVL and BM) were analyzed with linear models; the female reproductive success were analyzed with generalized linear models; the gestation period and the litter mass were analyzed with linear generalized least squares models; the litter size were analyzed with Kruskal-Wallis test; the neonate SVL and BM were analyzed by LME with mother ID as random factor; neonate survival and neonate sex ratio were analyzed with generalized linear mixed effect models.

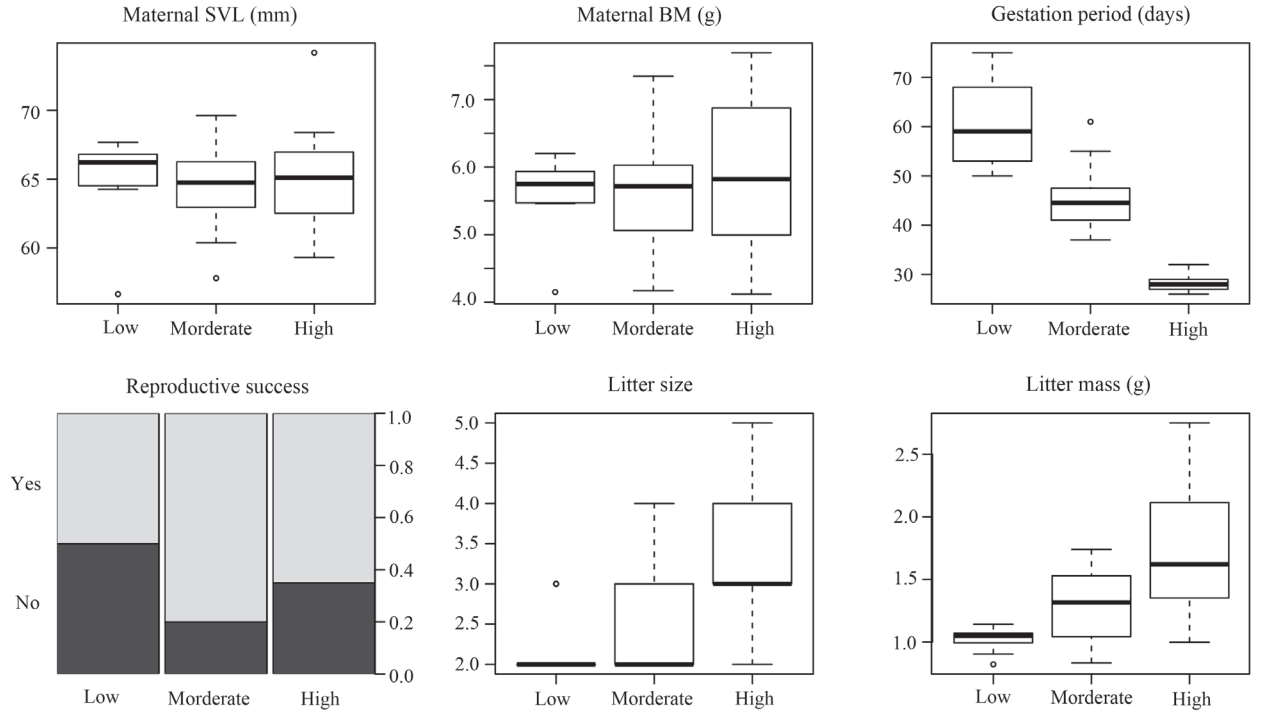

Figure 4 Differences between gestation temperature treatments in maternal snout-vent length (SVL), body mass (BM), gestation period length, reproductive success, litter size and litter mass in the multi-ocellated racerunner *Eremias multicellata* from Inner Mongolia.

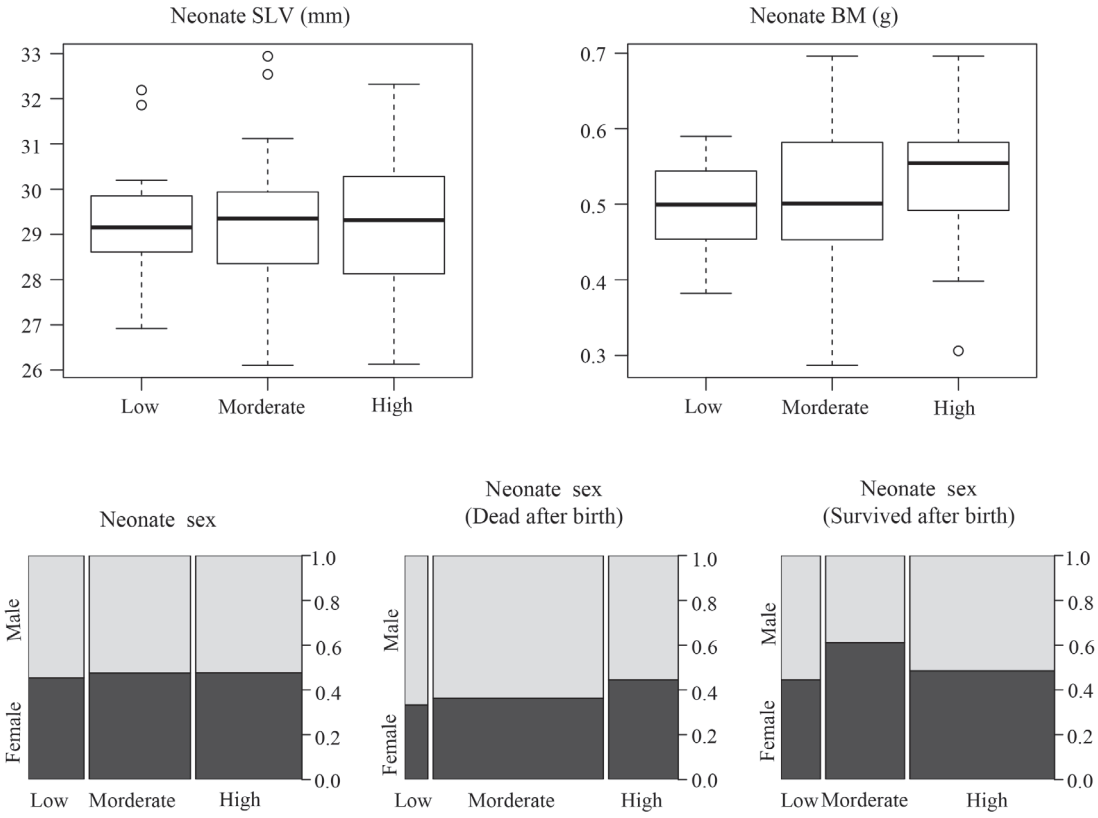

Figure 5 Neonate snout-vent length (SVL), body mass (BM), and sex ratio in different thermal treatments during maternal gestation of the multi-ocellated racerunner *Eremias multicellata* from Inner Mongolia.

of different sex determining systems among populations of *E. multiocellata* remain unrevealed, but could be related to among-population differences in climate conditions. Our study site at Inner Mongolian has lower ambient temperatures in terms of average, the lowest and highest temperatures and higher precipitation than two sites of Minqin and Tianzhu in Gansu Province (Table 1). The dry and warm condition in Minqin and Tianzhu might be a reason for the evolution of a mixed sex-determining system with temperature-induced sex reversal in these populations.

Currently there is a knowledge gap on intraspecific variation in sex-determining systems, concerning the evolutionary driver of different sex-determination patterns among populations. For example, climate or elevation did not explain within-species variation in sex reversal in the Australian dragon lizard (Cornejo-Páramo *et al.*, 2020a) or sex-chromosome differentiation in common frogs (Phillips *et al.*, 2020). The population divergence of sex-determining systems found in *N. ocellatus* was attributable to the increased rate of female maturation in the lowland population, and the higher magnitude of between-year temperature fluctuations in the highland population (Pen *et al.*, 2010). Therefore, climatic effects on lizard life history may induce divergent natural selection forces on sex determination across altitudinal clines e.g., a relatively long activity season favours an evolutionary shift from GSD to TSD in lowland populations of *N. ocellatus* (Cunningham *et al.*, 2017; Pen *et al.*, 2010). However, this explanation cannot be applied to the *E. multiocellata* system, because TSD occurs in high-altitude populations rather than low-altitude populations in this species. In addition, a meta-analysis on sex-determining systems of non-avian reptiles falsified the assumption that populations living in either highly variable or cold climatic conditions should evolve GSD to buffer the populations from extreme sex ratios (Cornejo-Páramo *et al.*, 2020b). The selective forces acting on the evolution of TSD-GSD systems remain a mystery and warrants further study in future.

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Appendix

File 1 Excel 1 presented the data of female gestation traits including maternal SVL, BM, Gestation period, litter size, litter mass and reproductive success in the multi-ocellated racerunner *Eremias multicellata* from Inner Mongolia with different temperature treatment. Excel 2 presented the data of neonate traits (SVL, BM, sex and survival) with different temperature treatment of their mother. The file can be downloaded from the website https://pan.baidu.com/s/1M8MuOyWLkyzBH_rp_S9sqw (access code: k9og).

File 2 Meteorological data of Tianzhu, Minqin and Eerduosi (Inner Mongolia) from http://data.cma.cn in recent 20 years. LowestT means the lowest temperature, highestT represents the highest temperature, and averageT means the average temperature. RH represents relative humidity. The file can be downloaded from the website https://pan.baidu.com/s/1AZ3OW91K-lij3LHjXsZ1BQ (access code: v9n5).