Temperature Sex Reversal Implies Sex Gene Dosage in a Reptile

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Sex is determined by genes on sex chromosomes in many vertebrates [genotypic sex determination (GSD)], but may also be determined by temperature during embryonic development [temperature-dependent sex determination (TSD)] (*1*). In reptiles, sex determination can involve GSD with XX and XY sex chromosomes (male heterogamety, as in mammals), GSD with ZZ and ZW sex chromosomes (female heterogamety, as in birds), or TSD (*1*, *2*). The distribution of TSD and GSD across reptiles suggests several independent evolutionary transitions in sex-determining mechanisms (*2*, *3*), but transitional forms have yet to be demonstrated. We show that high incubation temperatures reverse genotypic males (ZZ) to phenotypic females in the Australian central bearded dragon lizard (*Pogona vitticeps*), which, like birds, has GSD with female heterogamety (*4*). Temperature thus overrides gene(s) involved in male differentiation.

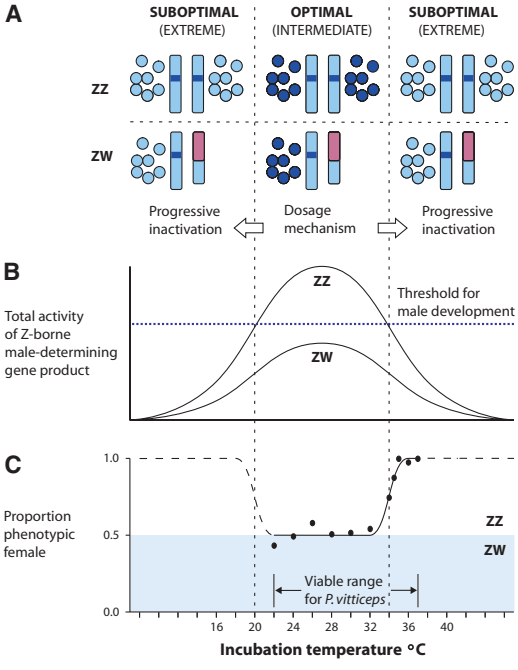

Fig. 1. Model for a ZZ dosage mechanism of sex determination in dragon lizards. (**A**) A Z-borne male-determining gene (dark blue line) that expresses a temperature-sensitive product (RNA or protein) is present in two copies in ZZ individuals but in only one copy in ZW individuals. At optimal (intermediate) incubation temperatures, the gene product is fully active (dark blue circles), but it is progressively inactivated at more extreme temperatures (light blue circles). (**B**) In ZW individuals, the total activity of the gene product is always half that of ZZ individuals. Activity exceeds a threshold level (dashed blue line) for male differentiation only within the optimal temperature range of ZZ individuals. At all other temperatures, female development proceeds. (**C**) Thus, the phenotypic sex ratio increases from 50% females at intermediate temperatures to 100% females at temperature extremes. Data points are for *P. vitticeps* (fig. S1).

We incubated eggs of *P. vitticeps* at constant temperatures between 20° and 37°C (*5*). No embryos survived to hatching at 20°C. Between 22° and 32°C, sex ratios did not differ significantly from 1:1, a response consistent with GSD (fig. S1A). However, between 34° and 37°C, there was an increasing female bias, suggesting that temperature was overriding genotypic sex in some males. Differential mortality cannot explain the deviation of the sex ratio from 1:1 at temperatures where survivorship allowed a test (at 34.5°, 35°, and 36°C), because the deviation remained significant even when all mortalities were conservatively scored as male (table S1).

We isolated a female-specific DNA marker for *P. vitticeps* by screening amplified fragment length polymorphisms (AFLPs) (*6*) to enable a test for sex reversal (*5*) (fig. S1B). To verify that the AFLP marker (designated Pv72W, GenBank accession no. ED982907) is specific to the W chromosome, we genotyped 15 juveniles and examined their metaphase chromosome spreads. There was 100% agreement between Pv72W genotype and karyotype as demonstrated by C banding (11 ZW and 4 ZZ).

Pv72W was used to identify genotypic sex of hatchlings from three clutches, each split between two incubation temperatures: a control treatment (28°C), which produced an unbiased phenotypic sex ratio, and a high-temperature treatment (34° or 36°C), which produced a strong female bias (table S2). Phenotypic sex was identified by hemipene eversion and examination of gonadal morphology. At 28°C, the phenotypic sex ratio was 1:1, compared with 2 males and 33 females at the high temperatures. All but one of the 30 lizards (97%) incubated at 28°C had concordant sex phenotype and genotype. However, only 18 of 35 animals (51%) from the high-temperature treatment were concordant. All discordant animals were genotypic males (ZZ) that developed as phenotypic females. Our data demonstrate that high incubation temperatures reverse sex (table S2). This finding extends a previous report of low-temperature sex reversal in a skink (*7*) by explicitly identifying discordance between genotype and phenotype and adds empirical support for the coexistence of TSD and GSD.

The W chromosome is thus unnecessary for female differentiation, which suggests that the molecular mechanism directing sex determination is the dosage of a gene on the Z chromosome rather than the presence of a female-determining gene on the W. That is, male differentiation requires two copies of a Z-borne gene, the expression or activity of which is sufficient for male development only at optimal temperatures (Fig. 1). We have demonstrated sex reversal at high temperatures; low-temperature sex reversal may be obscured by mortality below 22°C.

Selection for a wider range of thermosensitivity in species such as *P. vitticeps* could result in the evolution of TSD from GSD. Reversal of the ZZ genotype to the female phenotype at extreme temperatures will bias the phenotypic sex ratio toward females and drive down the frequency of the W chromosome under frequency-dependent selection. This could account for the pattern observed in many TSD reptiles, where both low and high temperatures produce 100% females, yet intermediate temperatures produce predominantly (occasionally 100%) males (*1*, *2*).

References and Notes

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Supporting Online Material

www.sciencemag.org/cgi/content/full/316/5823/411/DC1
Materials and Methods
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Supporting Online Material

Materials and Methods

Egg incubation and phenotypic sexing. Eggs obtained from gravid lizards collected in New South Wales and Queensland, Australia, were randomly assigned across constant incubation temperature treatments from 20-34°C (at two degree intervals). Following indications of a temperature effect at 34°C, clutches of 20 eggs or more were split between a control temperature of 28°C (10 eggs), and either a low temperature (20, 22, or 24°C; remaining eggs) or a high temperature (34, 35, or 36°C; remaining eggs). These data were supplemented with (i) clutches incubated at a single constant temperature (28, 30, 34.5, or 37°C); (ii) clutches assigned to developmental staging experiments (constant 22, 24, 26, 28, 30, 32, 34, 35, or 36°C); and (iii) clutches split between constant 28°C and various temperature regimes fluctuating around a mean of 32°C. This paper presents only data from constant incubation temperatures (Fig. S1A). Eggs were individually incubated in moist vermiculite in plastic boxes at a high constant but unmeasured humidity, as described elsewhere (*S1*). Phenotypic sex was determined by everting hemipenes in male hatchlings and confirmed by examination of gonadal morphology after dissection. Sex of adult animals was determined using external morphological differences between the sexes; all adult females had at one time been gravid.

AFLP marker development. Genomic DNA was extracted from tail tissue of hatchlings or blood from the caudal vein of adults, using standard protocols (*S2*). AFLP analysis was performed either using the AFLP Analysis System I Kit (Invitrogen, Carlsbad, California), or according to the original protocol (*S3*). AFLP products were either separated by capillary electrophoresis and analyzed on a CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, California) or separated and analyzed on a Gel-Scan 2000 system (Corbett Life Science, Sydney, Australia). Initial AFLP screening for sex markers was performed on six male and six female hatchlings from a single clutch incubated at 28°C, a temperature that produces an unbiased sex ratio (Fig. S1A). Ninety-six combinations of selective AFLP primers were tested for amplification of sex linked markers. Eight candidate fragments were identified. These primer combinations were subsequently tested on an independent sample set of ten males and ten females (Table S2), increasing the total sample size to 16 of each sex. These 20 new individuals comprised four males and four females from a second 28°C clutch, plus six male and six female wild-caught adults. The primer combination *Eco*RI-AAG/*Mse*I-CTG amplified a 72bp fragment (Pv72W, accession no. ED982907) in 15 of 16 females, but in none of 16 males (Fig. S1B). Further testing of *Mse*I primers determined that the primer *Mse*I-CTGCT or -CTGCTG produced profiles with fewer bands, enabling unambiguous genotype scoring based on the presence or absence of Pv72W.

Testing for temperature-induced sex reversal by AFLP genotyping. Hatchlings and the mothers from three clutches split between high (34-36°C) and control (28°C) incubation temperature treatments were genotyped for sex based on the presence/absence of the Pv72W AFLP sex marker. Clutches were split between the different temperature treatments to control for maternal (clutch specific) effects upon sex ratio. Three criteria were used to select the three clutches for genotyping: (1) all ten eggs incubated at the control temperature survived to hatching; (2) ten or more eggs at the high temperature treatment survived to hatching (i.e. high survivorship); and (3) the phenotypic sex ratio in the control group was close to 1:1, suggesting that clutch-specific effects were unlikely to confound the test for a temperature effect. Two of the clutches selected had been incubated at 34°C for the high treatment experiment, and the third at 36°C (Table S2).

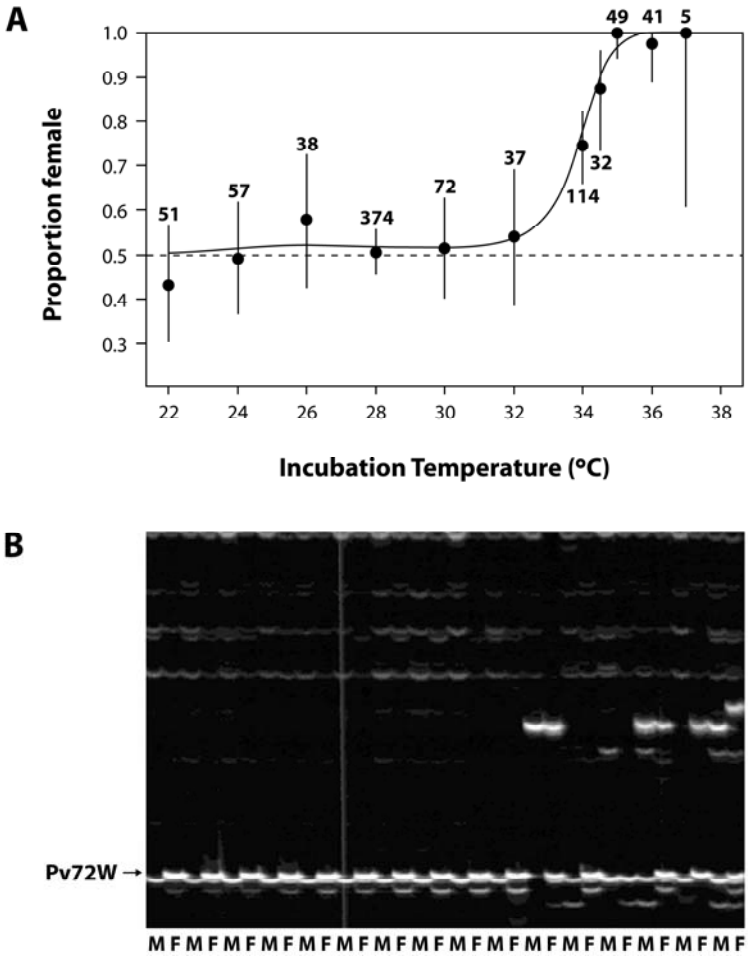

Figure S1

Panel A: Sex ratio of *Pogona vitticeps* as a function of constant incubation temperature. The fitted curve is a third order polynomial logistic regression of best fit, scaled to fall within 0.5 and 1.0, with a binomial error structure. Numbers denote sample sizes (males plus females). Vertical bars are 95% binomial confidence limits. Panel B: Partial AFLP profiles showing female-specific (W chromosome) 72bp marker (Pv72W). M, phenotypic male; F, phenotypic female.

Table S1

Differential mortality does not cause sex ratio bias. Mortalities were assigned sex to reduce observed bias resulting in significant differences at high temperatures ($\chi 2$ goodness of fit, with Yates' correction). Only clutches where more than 50% of embryos survived to hatching are included.

Incubation Temperature (°C)	Males	Males + Mortalities	Females	X^2	Significance
34	9	16	28	2.75	p=0.09
34.5	6	19	52	14.42	p<0.0001
35	0	8	49	28.07	p<0.0001
36	1	5	25	12.03	p<0.0005

Table S2

Sex phenotypes and sex genotypes for the 65 hatchlings from the three experimental clutches, the mothers of the three clutches, and the 32 animals tested during development of the Pv72W marker.

		Control temperature treatment (28°C) or wild-caught adults				**High temperature treatment (34°C or 36°C)**			
		Gonad phenotype	AFLP genotype	Sex reversed phenotype		Gonad phenotype	AFLP genotype	Sex reversed phenotype	
Marker discovery									
28°C hatchlings		6 females	6 ZW						
n = 12		6 males	6 ZZ						
Marker testing									
28°C hatchlings		4 females	4 ZW						
n = 8		4 males	4 ZZ						
Wild-caught adults		6 females	5 ZW						
n = 12			1 ZZ	1 of 20	5.0%				
		6 males	6 ZZ						
Sex reversal experiments									
All hatchlings									
Clutch 1	28°C / 36°C	6 females	5 ZW			13 females	5 ZW		
n = 24			1 ZZ	1 of 10	10.0%		8 ZZ	8 of 14	61.5%
		4 males	4 ZZ			1 male	1 ZZ		
Clutch 2	28°C / 34°C	5 females	5 ZW			10 females	7 ZW		
n = 21							3 ZZ	3 of 11	27.3%
		5 males	5 ZZ			1 male	1 ZZ		
Clutch 3	28C° / 34°C	4 females	4 ZW			10 females	4 ZW		
n = 20							6 ZZ	6 of 10	60.0%
		6 males	6 ZZ			0 males	0 ZZ		
Dams of clutches 1, 2 and 3									
Wild-caught adults	n = 3	3 females	3 ZW						
Overall									
All adults and clutches		34 females	32 ZW			33 females	16 ZW		
n = 100			2 ZZ	2 of 65	3.1%		17 ZZ	17 of 35	48.6%
		31 males	31 ZZ			2 males	2 ZZ		

Supporting References

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