Vol 451|31 January 2008|doi:10.1038/nature06519

LETTERS

The adaptive significance of temperature-dependent sex determination in a reptile

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Understanding the mechanisms that determine an individual's sex remains a primary challenge for evolutionary biology. Chromosome-based systems (genotypic sex determination) that generate roughly equal numbers of sons and daughters accord with theory[1], but the adaptive significance of environmental sex determination (that is, when embryonic environmental conditions determine offspring sex, ESD) is a major unsolved problem[2,3]. Theoretical models predict that selection should favour ESD over genotypic sex determination when the developmental environment differentially influences male versus female fitness (that is, the Charnov–Bull model)[4], but empirical evidence for this hypothesis remains elusive in amniote vertebrates—the clade in which ESD is most prevalent[5]. Here we provide the first substantial empirical support for this model by showing that incubation temperatures influence reproductive success of males differently than that of females in a short-lived lizard (*Amphibolurus muricatus*, Agamidae) with temperature-dependent sex determination. We incubated eggs at a variety of temperatures, and de-confounded sex and incubation temperature by using hormonal manipulations to embryos. We then raised lizards in field enclosures and quantified their lifetime reproductive success. Incubation temperature affected reproductive success differently in males versus females in exactly the way predicted by theory: the fitness of each sex was maximized by the incubation temperature that produces that sex. Our results provide unequivocal empirical support for the Charnov–Bull model for the adaptive significance of temperature-dependent sex determination in amniote vertebrates.

Why is an individual's sex determined by environmental variables (environmental sex determination, ESD) in some species, but by chromosomal factors (genotypic sex determination; GSD) in others? GSD plausibly enhances parental fitness by generating equal investment into sons versus daughters[1], but the adaptive significance of ESD remains a major unresolved problem, particularly for amniote vertebrates[2,3]. The problem is not a lack of plausible hypotheses, but rather the difficulty of testing those ideas. Mathematical models predict that ESD will be favoured by selection when an environmental variable (for example, temperature or photoperiod) differentially affects the fitness of sons versus daughters[4,6]. For example, the most common form of ESD is temperature-dependent sex determination (TSD), whereby incubation temperature determines offspring sex in many reptiles[5] and some fish[7]. The widely accepted Charnov–Bull model[4] predicts that TSD enhances parental fitness by matching offspring sex to incubation conditions; that is, eggs should produce sons when developing under conditions that promote high fitness for males, whereas eggs that encounter female-favourable conditions develop as daughters (see Fig. 1 for more detailed predictions). Genes creating such a link should be favoured by selection as they would confer higher fitness than the alternative GSD system.

Although the hypothesis and predictions are straightforward, tests of adaptive models are logistically difficult[8–12]. This is because, first, to evaluate sex-specific effects of incubation temperature, both sexes need to be produced at all incubation temperatures—an obvious problem if temperature determines offspring sex; second, most species with TSD exhibit long lifespans (60+ years) and delayed sexual maturation, precluding measurements of lifetime fitness; and, third, incubation temperature may differentially affect male versus female fitness by means of multiple pathways[3], complicating empirical tests of the Charnov–Bull model.

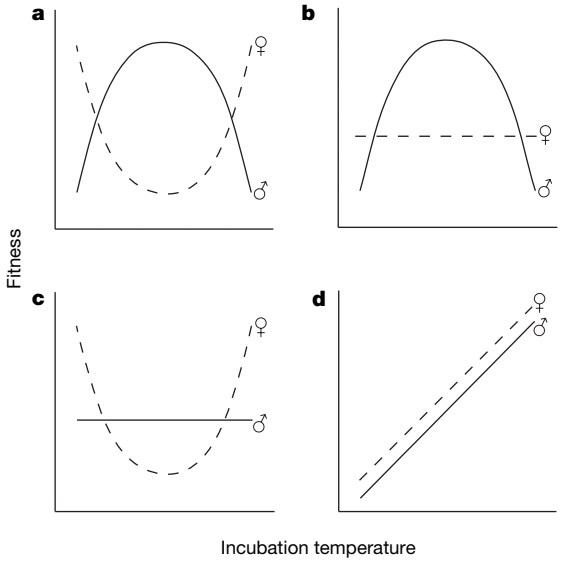

Figure 1 | Theoretical predictions of the Charnov–Bull model for the adaptive significance of TSD based on the TSD pattern in Jacky dragons (*A. muricatus*). In this species, females are produced at thermal extremes and males are produced at intermediate temperatures. The solid line represents fitness of sons and the dashed line represents fitness of daughters. The Charnov–Bull model predicts that TSD will enhance individual fitness if the fitness of sons is greatest for individuals that hatch from eggs incubated at temperatures that naturally produce males, and fitness of daughters is greatest for individuals from eggs incubated at temperatures that naturally produce females. These conditions might be satisfied if: **a**, male-producing temperatures are optimal for sons, and female-producing temperatures are optimal for daughters; **b**, female fitness is unaffected by incubation temperature, but fitness is optimized by intermediate incubation temperatures for males; or **c**, male fitness is unaffected by incubation temperature, but female fitness is optimized by cool and warm incubation temperatures. **d**, Many other scenarios should not favour TSD. For example, when incubation temperature affects fitness, but does so in similar directions for males versus females, TSD is not favoured by selection.

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We have overcome these obstacles in three ways: by hormonally manipulating eggs to decouple the confounded effects of sex and incubation temperature[13,14], by studying a short-lived species with TSD, and by using paternity analyses to evaluate the effects of incubation history on lifetime reproductive success for males as well as females. Our study organism, the Jacky dragon (*A. muricatus*), is a short-lived (probably 3–4 years) Australian agamid lizard with TSD[15]. Female offspring are produced from eggs incubated at low (23–26 °C) and high (30–33 °C) temperatures, and both sexes are produced at intermediate (27–30 °C) incubation temperatures[15]. We incubated eggs under each of these thermal regimes, and applied an aromatase inhibitor to half of the eggs early in development to override thermal effects on sex determination[16,17]. This manipulation blocked the conversion of testosterone to oestradiol during development, enabling us to produce male offspring at female-producing incubation temperatures. Thus, we were able to decouple the confounded effects of sex and incubation temperature. Importantly, this hormonal manipulation had no effect on morphology or survival of hatchling Jacky dragons[17], and gonadal histology showed that sex-reversed males do not differ from natural males[18]; similar non-effects of hormonal manipulations have been demonstrated in other reptiles[19,20]. Moreover, comparisons of males produced from eggs treated with an aromatase inhibitor versus naturally produced males (from 27 °C incubation) revealed no effect of aromatase inhibition on reproductive success ($F_{1,44} = 1.9$, $P = 0.17$). After eggs hatched, we followed the newly hatched individuals throughout their lives under semi-natural conditions by maintaining the lizards in large field enclosures for the next 3.5 years.

Incubation temperature had little direct effect on offspring phenotypes and survival, but had major effects through its covariation with the seasonal timing of hatching[17]. Warm incubation temperatures (that naturally produce daughters) accelerated embryonic development, allowing eggs to hatch early in the season. Consequently, incubation temperature had a strong positive effect (via its effect on the timing of hatching) on lizard body sizes before the onset of all three reproductive seasons monitored during this study (that is, when lizards were one, two and three years old; all P values <0.05). During the first reproductive season (2004), only two of the females produced viable offspring (two clutches comprising eight eggs in total); both females were from the warm (naturally female-producing) incubation treatment. The two males that sired their clutches were both from the intermediate (naturally male-producing) incubation treatment. These patterns fit the predictions of the Charnov–Bull model, albeit with small sample sizes.

At the onset of the second reproductive season (spring 2005), the minimum size at sexual maturity (72 mm snout-vent length[15]) had been attained by 70.6% and 69.0% of individuals from the intermediate and warm incubation treatments, respectively, but by only 37.2% of the individuals from the cool treatment ($\chi^2 = 11.1$, $P = 0.004$). Sixteen viable clutches (comprising 58 eggs in total) were produced during the second season, and our parentage analyses indicated that larger individuals (and hence, those that hatched early) were more likely to produce offspring (for both males and females, Fig. 2a; $F_{1,97} = 11.3$, $P = 0.001$). The same patterns (at least up to one year) occur under natural conditions in the field[21]. Reproductive success was strongly affected by the interaction between incubation temperature and sex (Fig. 3a, b; $F_{2,96} = 4.0$, $P = 0.021$) in a pattern consistent with the prediction in Fig. 1a. Similar to the patterns in the first year, males from eggs incubated at intermediate temperatures had the highest reproductive success, whereas more extreme (low and high) incubation temperatures enhanced female reproductive output.

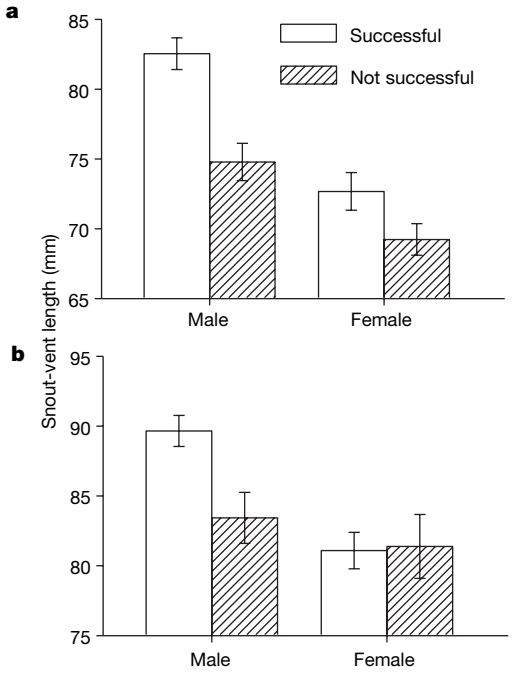

Figure 2 | Sex-specific body-size comparisons between individuals that successfully reproduced versus those that did not reproduce. Reproductive success was not evaluated statistically during the first reproductive season (2004–2005) because only two clutches were produced. **a**, Body-size comparisons in the second reproductive season (2005–2006). **b**, Body-size comparisons in the third reproductive season (2006–2007). Bars represent means ± 1 standard error.

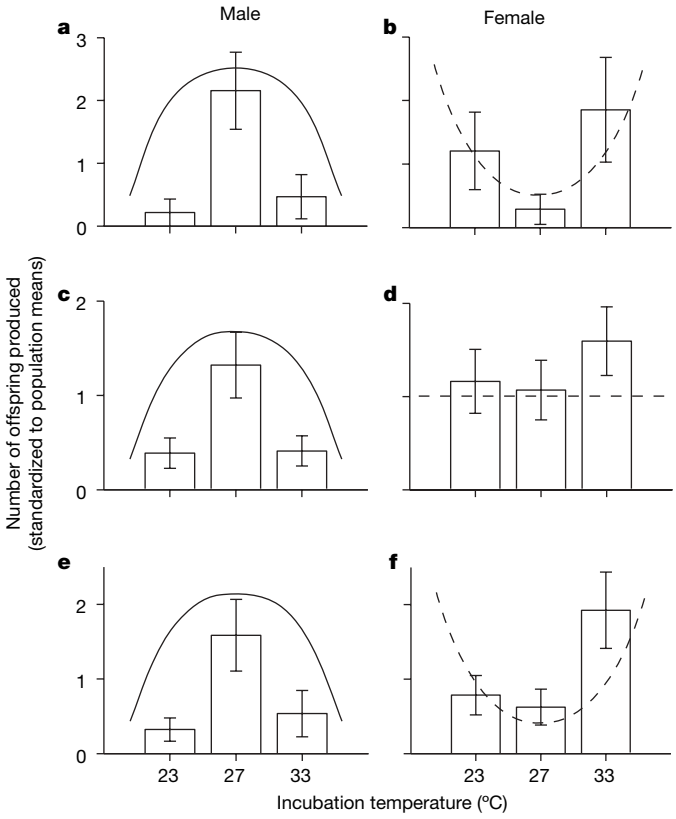

Figure 3 | Incubation temperature affected the fitness of sons differently from that of daughters. The left-hand panels show male reproductive success, and the right-hand panels show female reproductive success. Because only two clutches were produced in the first reproductive season, we did not include data from this season. **a**–**f**, The graphs show male (**a**) and female (**b**) reproductive success during the 2005–2006 season; male (**c**) and female (**d**) reproductive success during the 2006–2007 season; and male (**e**) and female (**f**) lifetime reproductive success, representing pooled reproductive data over all three seasons. Bars represent means ± 1 standard error. The curves on each graph represent the patterns predicted from Fig. 1 that best fit the results.

At the onset of the third reproductive season (spring 2006), all but three individuals (3.7%) had reached sexual maturity. Nonetheless, warmer-incubated (and thus, earlier-hatched) individuals still exhibited larger body sizes at this time (3 years after hatching; $F_{2,68} = 4.5$, $P = 0.015$). During this season, 50 clutches were produced (comprising 227 eggs). Larger body size again enhanced reproductive success for males, but not for females (Fig. 2b; interactive effect: $F_{1,71} = 4.3$, $P = 0.043$). Consequently, reproductive success followed a pattern broadly consistent with Charnov–Bull predictions, but different from that found in the second reproductive season (resembling Fig. 1b): male reproductive success was greatest for individuals produced at the intermediate incubation temperature, whereas female reproductive success was not significantly influenced by incubation treatment.

Lifetime reproductive success (pooled reproductive output for all three seasons, Fig. 3e, f) strongly conformed to the prediction in Fig. 1a (sex by temperature interaction: $F_{2,130} = 4.3$, $P = 0.016$). Males hatched from eggs incubated at naturally male-producing (intermediate) temperatures sired more offspring than did males from eggs incubated at naturally female-producing (extreme) temperatures. The reverse was true for females, with reproductive success greatest for females that hatched from eggs incubated at female-producing (low or high) temperatures. Thus, reproductive success of each sex was optimized by the incubation temperature that produces that sex in nature, as predicted by the differential-fitness (Charnov–Bull) model for the adaptive significance of TSD. These results provide the first unequivocal demonstration that incubation temperature differentially affects male versus female fitness in a way that will favour the evolution and maintenance of TSD in amniote vertebrates.

METHODS SUMMARY

Methodological details for the experimental design and early stages of the study are given elsewhere[17]. In spring 2003, we allocated eggs among three temperature regimes (23 °C, 27 °C or 33 °C) that mimicked natural nests[22]. Half the eggs in each treatment were given an aromatase inhibitor to produce male offspring at all temperatures[16]. After eggs hatched, all offspring were marked and raised in six replicate field enclosures for 3.5 years. Our releases ensured that each treatment and clutch were equally represented among replicate populations. When lizards became sexually mature, second-generation eggs were incubated, and tissue samples were taken from the resultant hatchlings. Samples were genotyped at nine microsatellite loci[23,24]. Paternity analyses were performed using CERVUS software (version 3.0.3)[25].

The effect of incubation temperature on body size at each season was evaluated with a mixed model analysis of variance (ANOVA) using temperature and sex as independent variables and enclosure as a random effect. Reproductive success was calculated as the number of offspring produced or sired by each individual that survived to the onset of each season; the number of offspring produced by each individual was standardized to the population mean for each enclosure. Two-way mixed model ANOVA was used to evaluate the interactive effect of sex and incubation temperature on reproductive success; enclosure was included as a random effect. Individuals that died throughout the study were not included in the within-season analyses, but were considered in the final analysis with pooled data across years (that is, lifetime reproductive success). The effect of body size on reproductive success was evaluated with mixed model ANOVA by comparing body sizes of individuals that reproduced versus those that did not. Before analysis, body size was standardized (as *z*-scores) for each enclosure, and enclosure was included as a random effect.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 1 October; accepted 26 November 2007.
Published online 20 January 2008.

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Acknowledgements We thank D. Allsop, J. Cuervo, W. Du, M. Elphick, H. Giragossyan, P. Harlow, T. Langkilde, M. Olsson, R. Peters, B. Phillips, S. Ruggeri, T. Schwartz, F. Seebacher, J. Thomas, M. Thompson, D. Van Dyk, M. Wall and Novartis Pharmaceuticals for assistance. Comments by F. Janzen and members of his laboratory improved this manuscript. Research funding was provided by Sigma Xi, the American Society of Ichthyologists and Herpetologists, the Norman Wettenhall Foundation, the Linnean Society of New South Wales, the Society for Integrative and Comparative Biology, the Chicago Herpetological Society, the Royal Zoological Society of New South Wales, Environmental Futures Network (to D.A.W.) and the Australian Research Council (to R.S.). Grants awarded to D.A.W. funded the development of the microsatellite markers and the genetic work associated with paternity analyses, and grants awarded to R.S. funded all other aspects of the study. This research was approved by the New South Wales National Parks Service, and the Animal Care and Ethics Committees of The University of Sydney and Macquarie University.

Author Contributions D.A.W. conducted the experiment, maintained the lizard populations, genotyped all individuals, analysed the data and wrote the first draft of the manuscript. Both authors contributed equally to the design of the experiment, discussion of the results and preparation of the final manuscript.

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METHODS

Experimental design and paternity assignment. Methodological details for the experimental design and early stages of the study are given elsewhere[17]. In spring 2003, we allocated eggs from each of 41 clutches ($n = 221$ eggs) to one of three temperature treatments (23 °C, 27 °C or 33 °C). Half the eggs in each treatment were exposed to an aromatase inhibitor to produce male offspring at all temperatures[16]. Incubators were programmed to fluctuate ±5 °C and temperature regimes were within the range found in natural nests[22]. After eggs hatched, all offspring were marked by toe-clipping (toe-clips were preserved for genotyping) and released into one of six replicate field enclosures (4×8 m)[17] such that each treatment and clutch were equally represented among replicate populations (30–32 hatchlings per enclosure). Over the next 3.5 years, the offspring were monitored and re-measured at least three times per year.

When females became gravid, they were removed from their enclosure, housed individually until they nested, and then returned to their original enclosure; thus, eggs and offspring were assigned to their maternal parent with 100% confidence. All second-generation eggs were incubated at a constant 28 °C, and toe-clips from the resultant hatchlings were used for tissue samples for paternity analysis. The pool of potential fathers of specific clutches consisted only of 11 to 17 males (within a given enclosure), providing high confidence in paternity assignment. Samples were genotyped at nine microsatellite loci[23,24]. Paternity analyses were performed using CERVUS software (version 3.0.3)[25].

Statistical analyses. The effect of incubation temperature on body size (snout-vent length; dependent variable) at each season was evaluated with a mixed model analysis of variance (ANOVA) using temperature and sex as independent variables and enclosure as a random effect. Reproductive success was calculated as the number of offspring produced or sired by each individual that survived to the onset of each season; the number of offspring produced by each individual was standardized to the population mean for each enclosure. Two-way mixed model ANOVA was used to evaluate the interactive effect of sex and incubation temperature (independent variables) on reproductive success (dependent variable); enclosure was included as a random effect. Individuals that died throughout the study were not included in the within-season analyses, but were considered (with a reproductive success of 0) in the final analysis with pooled data across the three years of study (that is, estimate of lifetime reproductive success).

In an additional analysis that used data for all lizards (rather than just survivors up to each year), we evaluated the effects of incubation temperature, sex and their interaction on reproductive success using a repeated measures MANOVA with reproductive success in each year as the repeated variable. This analysis accounted for survival across years (non-survivors in each year were scored as having zero reproductive success), and still revealed a significant incubation temperature by sex interaction (Wilks' Lambda, $F = 2.3$, $P = 0.036$). The effect of body size on reproductive success was evaluated with mixed model ANOVA by comparing body sizes of individuals that reproduced versus those that did not. Before analysis, body size was standardized (as z-scores) for each enclosure, and enclosure was included as a random effect.