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## Transgenerational sex determination: the embryonic environment experienced by a male affects offspring sex ratio

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Conditions experienced during embryonic development can have lasting effects, even carrying across generations. Most evidence for transgenerational effects comes from studies of female mammals, with much less known about egg-laying organisms or paternally-mediated effects. Here we show that offspring sex can be affected by the incubation temperature its father experiences years earlier. We incubated eggs of an Australian lizard with temperature-dependent sex determination under three thermal regimes; some eggs were given an aromatase inhibitor to produce sons at temperatures that usually produce only daughters. Offspring were raised to maturity and freely interbred within field enclosures. After incubating eggs of the subsequent generation and assigning parentage, we found that the developmental temperature experienced by a male significantly influences the sex of his future progeny. This transgenerational effect on sex ratio may reflect an epigenetic influence on paternally-inherited DNA. Clearly, sex determination in reptiles is far more complex than is currently envisaged.

Environmental conditions experienced during development can have long-lasting effects that can persist across generations<sup>1–5</sup>. These transgenerational effects occur when the environment experienced by parents affects phenotypes in subsequent generations without modification of the DNA sequence. Such effects can influence evolutionary dynamics<sup>6</sup>, and may be adaptive in fluctuating environments<sup>7</sup>. Most studies of parental effects focus on the environmental conditions that parents experience during the period that coincides with offspring development. For example, stress experienced by human mothers during pregnancy can have lasting effects on the physiology and behavior of their babies<sup>8</sup>. Conditions experienced during embryonic development also can contribute to longer-lasting transgenerational effects; the nutritional state of pregnant female rodents can influence phenotypes not only of their progeny, but also of their grandchildren<sup>4</sup>. Although most research has focused on maternally-mediated transgenerational effects, these effects can also be transmitted via the father<sup>5,9</sup>.

The thermal environment plays a critical role in shaping embryonic development in ectothermic organisms. In reptiles, for example, the temperatures that embryos experience influence a diverse suite of fitness-relevant phenotypic traits (e.g., offspring body size, growth rate, behavior, and sex)<sup>10</sup>. To assess whether or not such effects persist across generations, we studied an Australian lizard (the jacky dragon, *Amphibolurus muricatus*) that exhibits temperature-dependent sex determination (TSD), whereby cool (23–26°C) and warm (30–33°C) incubation temperatures produce exclusively daughters and intermediate incubation temperatures (27–30°C) produce both sexes. In this species, egg incubation temperature also has strong effects on body size and reproductive success when offspring reach adulthood<sup>11,12</sup>, but transgenerational impacts are unknown.

## Results

We incubated eggs from wild-caught *A. muricatus* under one of three temperatures (23, 27, 33°C), to produce daughters at the extreme incubation temperatures and both sexes at the intermediate temperature<sup>11,12</sup>. In addition, by applying an aromatase inhibitor to approximately half the eggs in each temperature treatment, we were able to produce male offspring even at female-producing temperatures; the remaining eggs served as controls. This aromatase manipulation produced nearly all male offspring (> 90%) at all three incubation temperatures<sup>11</sup>. After receiving a unique toeclip (preserved for future parentage analysis) for identification, all offspring were housed in outdoor enclosures for 3.5 years, the approximate life span for *A. muricatus*. After performing parentage analyses



**Table 1 | Effect of paternal and maternal incubation temperature on offspring phenotypes.** Generalized linear mixed models used parental incubation temperature and season as nominal independent variables. The random effect corresponds to the paternal and maternal identity effects

Phenotype)	Paternal effect		Maternal effect	
	Incubation temperature	Random effect	Incubation temperature	Random effect
Sex ratio (percent sons)*	$F_{2,41.87} = 3.31, P = \mathbf{0.046}$	$\chi^2 = 331.1, P < \mathbf{0.001}$	$F_{2,16.8} = 0.56, P = 0.580$	$\chi^2 = 341.7, P < \mathbf{0.001}$
Snout-vent length (mm)	$F_{2,184} = 1.186, P = 0.309$	$\chi^2 = 100.2, P < \mathbf{0.001}$	$F_{2,186} = 0.24, P = 0.789$	$\chi^2 = 113.9, P < \mathbf{0.001}$
Body mass (g)	$F_{2,184} = 0.30, P = 0.738$	$\chi^2 = 227.0, P < \mathbf{0.001}$	$F_{2,186} = 0.68, P = 0.507$	$\chi^2 = 235.2, P < \mathbf{0.001}$
Locomotor performance (m/s over 25 cm)	$F_{2,154} = 0.26, P = 0.772$	$\chi^2 = 0.0, P = 0.999$	$F_{2,153} = 0.16, P = 0.852$	$\chi^2 = 0.0, P = 0.999$

\*Statistical results using parental incubation temperature as a continuous variable; Paternal incubation effect:  $F_{1,39.1} = 6.66, P = \mathbf{0.014}$ ; maternal incubation effect:  $F_{1,14.8} = 0.07, P = 0.796$ .

of the second-generation offspring, we assessed the effects of developmental temperature experienced by parents on the phenotypes of their offspring (size, locomotor performance, sex) produced several years later.

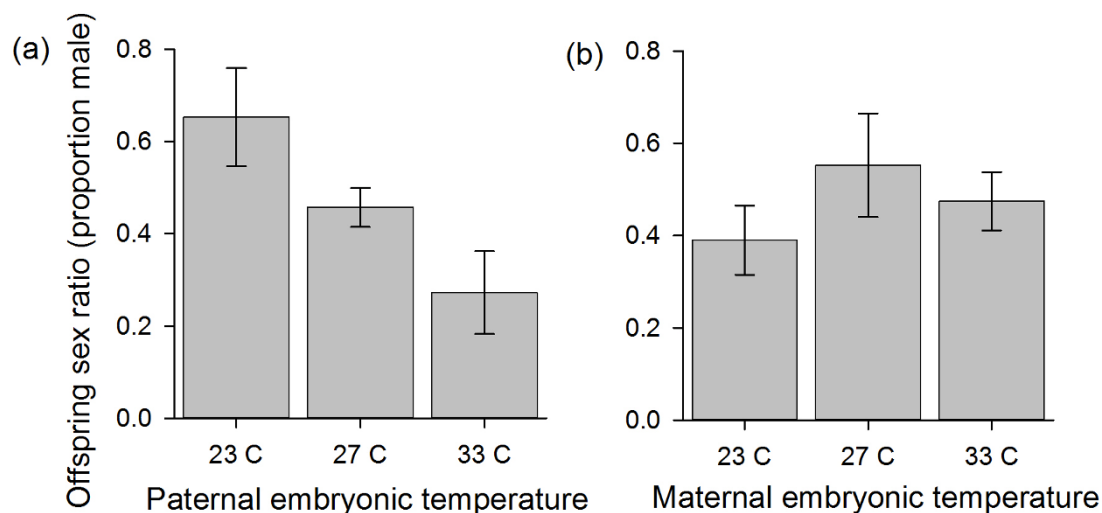
The embryonic temperature experienced by a father influenced the sex of his offspring, whereas maternal incubation temperature showed no such effect (Table 1; Fig. 1). Fathers that had hatched from cool-incubated eggs sired more sons than daughters, whereas fathers that had experienced warmer embryonic conditions produced more daughters than sons. Intermediate embryonic temperatures experienced by fathers resulted in a balanced sex ratio of their offspring. The body size of the parents did not affect the sex ratio of their offspring (father SVL:  $F_{1,48} = 1.29, P = 0.26$ ; father mass:  $F_{1,41.2} = 2.04, P = 0.16$ ; mother SVL:  $F_{1,37.7} = 0.19, P = 0.67$ ; mother mass:  $F_{1,33.3} < 0.53, P = 0.47$ ). The temperature experienced by parents when they were embryos had no significant impact on the morphology or performance of their offspring, but interactions between maternal and paternal identity affected morphology (but not locomotor performance: Table 1).

## Discussion

Plausibly, the transgenerational effect of incubation temperature on offspring sex ratio may reflect thermally-induced epigenetic changes in pathways of sexual differentiation. In sea bass, incubation temperature causes masculinization in females by methylating the gonadal aromatase promoter (*cyp19a*)<sup>13</sup>. If similar patterns occur within *A. muricatus*, males produced at extreme incubation temperatures (due to our application of aromatase inhibitors to eggs) can be viewed as

highly masculinized females with well-developed testes<sup>14</sup> that behave like “normal” males<sup>15</sup>, and can sire offspring<sup>12</sup>. Because we used an aromatase inhibitor to produce males at high and low temperatures, the transgenerational effects that we detected may have been due to either temperature or aromatase inhibition. For example, if aromatase inhibition interferes with downstream pathways in the sex-determining cascade, males from ‘female temperatures’ may still carry female-specific epigenetic marks on upstream genes, which may influence gene expression in the next generation (analogous to the transgenerational effects of vinclozolin in mice<sup>5,16</sup>). However, any straightforward effect of aromatase inhibition (rather than incubation temperature) on offspring sex ratios should have produced a similar sex ratio in both upper and lower temperature treatments, rather than a negative relationship with paternal incubation temperature. Thus, our data suggest a causal effect of incubation temperature *per se*.

Because male offspring are normally produced only at intermediate incubation temperatures, transgenerational effects via fathers may not be realized under natural conditions. However, the thermal regimes that eggs experience in natural nests are complex, and offspring sex ratios are influenced by an interaction between mean nest temperature and the thermal variance experienced each day<sup>17</sup>. Moreover, many TSD species produce at least some male offspring at temperatures that predominately result in daughters<sup>18</sup>. Thus, although the artificial incubation conditions used in the current study do not normally generate males at extreme temperatures, the complexities of natural field conditions can result in mixed sex ratios even at relatively high or low mean temperatures (depending on



**Figure 1 | Transgenerational effect of egg incubation temperature on offspring sex ratios in the jacky dragon (*Amphibolurus muricatus*).** (a), for fathers. (b), for mothers. Error bars represent one standard error.



thermal variance). Therefore, these transgenerational effects plausibly occur in nature because males can be produced at temperatures that might predominately produce females<sup>17</sup>. Whether or not they confer any fitness benefits is unknown.

Developmental temperatures influence offspring sex ratio in many types of organisms<sup>19</sup>, and sire phenotype influences progeny sex ratio in another lizard species<sup>20</sup>. However, our data on *A. muricatus* are the first to show that the developmental conditions experienced by fathers can impact the sex ratios of their future progeny. Our finding that this effect is mediated only through fathers is consistent with an epigenetic influence on paternally-inherited DNA. Our results highlight the complexity of sex determination in squamate reptiles, with roles for epigenetics as well as for genetics, nest environments, and other maternal effects – and with multiple mechanisms simultaneously at work within a single population<sup>21–23</sup>. Current classification schemes that attribute sex determination within a given species to some single type of mechanism may be in error: in many taxa of lizards, an individual's sex appears to be the outcome of multiple factors operating over a diversity of timescales<sup>24</sup>.

## Methods

**Experimental design.** We provide only a brief description of methodology because most details are described elsewhere<sup>11,12</sup>. In the 2003/2004 austral summer, eggs ( $N = 221$ , from 41 clutches) from wild-caught *A. muricatus* (collected from natural areas around Sydney, Australia) were incubated under one of three temperatures (23, 27, 33°C) with a diel thermal fluctuation of  $\pm 5^\circ\text{C}$  for all treatments. The intermediate temperature produces both sexes; the two extremes both generate daughter-only clutches. Approximately half the eggs in each temperature treatment were given an application of an aromatase inhibitor to produce male offspring even at female-producing temperatures; the remaining eggs served as controls. After eggs hatched, each individual was given a unique toeclip (preserved for future parentage analysis) for identification. All offspring were housed in outdoor enclosures for 3.5 years, the approximate life span for *A. muricatus*. When females became gravid (which occurred at ages of 1 to 3 years), they were put in separate enclosures until they laid eggs, and then returned to their original enclosure. Eggs were collected and incubated under a constant 28°C, a temperature that produces both sexes in *A. muricatus*<sup>18</sup>.

After second generation eggs hatched, all offspring were weighed, measured, and their sex identified<sup>25</sup>. Each lizard was individually marked by toe-clipping and we snipped a small piece of tail to use for DNA extraction for parentage analyses. DNA extraction followed published methodology<sup>15</sup> and individuals were genotyped at eight microsatellite loci<sup>26,27</sup>. Genotypes were scored with Genemapper software (Applied Biosystems) and CERVUS software was used for paternity analyses<sup>12,15</sup>. At 11 to 16 days of age, offspring were tested for locomotor performance in an electronically-timed racetrack<sup>28</sup>.

**Statistical analyses.** Our goal was to quantify how developmental temperatures experienced by parents (as embryos) influenced the phenotypes of their future offspring. To do this, we used two separate mixed models to assess the effects of maternal and paternal embryonic temperature. Dependent variables in mixed model Analyses of Variance included hatchling snout-vent length (SVL), mass, and locomotor performance. Analyses of hatchling size measurements used egg mass as a covariate, and those of locomotor performance used SVL as a covariate. Generalized linear mixed models with a logit link function and binomial error structure were used to assess the effects of parental developmental temperature on offspring sex ratio. The analyses of sex ratio were weighted by 'total offspring per clutch' to account for variation in clutch size (e.g., a clutch of two eggs was given less weight than a clutch size of eight when assessing variation in the percentage of males produced). All analyses included maternal identity, paternal identity and their interaction as random variables, and effects were assessed by comparing the difference in  $-2$  restricted log-likelihood values between models that included the random effect with those that did not include the random effect.

Because incubation temperature experienced by parents influenced progeny sex ratio and has previously been shown to affect body size in our study species<sup>11,12</sup>, an additional analysis was used to determine if maternal and paternal body size at the onset of the reproductive season influenced sex ratio. For this analysis, we used a generalized linear mixed model with paternal size and maternal size (for SVL and mass at the onset of the reproductive season) as independent variables and clutch sex ratio as a dependent variable; parental identities were random effects and the analysis was weighted by total offspring per clutch. For all analyses, reproductive data across three seasons were pooled and season was included as a fixed effect; results did not change when only data from the third season was used, suggesting that offspring sex ratios can be influenced by the incubation environment of the father at least three years later. This research was approved by the Animal Care and Ethics Committees of The University of Sydney and Macquarie University.

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## Author contributions

D.A.W. designed and conducted the experiment, genotyped all individuals, analysed the data, and wrote the first draft of the manuscript. T.U. contributed to the conceptual basis of the study, data interpretation and the preparation of the final manuscript. R.S. contributed the design of the experiment, provided conceptual expertise and helped prepare the final manuscript.

## Additional information

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