



## Research

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# Ovotestes suggest cryptic genetic influence in a reptile model for temperature-dependent sex determination

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Sex determination and differentiation in reptiles is complex. Temperature-dependent sex determination (TSD), genetic sex determination (GSD) and the interaction of both environmental and genetic cues (sex reversal) can drive the development of sexual phenotypes. The jacky dragon (*Amphibolurus muricatus*) is an attractive model species for the study of gene–environment interactions because it displays a form of Type II TSD, where female-biased sex ratios are observed at extreme incubation temperatures and approximately 50 : 50 sex ratios occur at intermediate temperatures. This response to temperature has been proposed to occur due to underlying sex determining loci, the influence of which is overridden at extreme temperatures. Thus, sex reversal at extreme temperatures is predicted to produce the female-biased sex ratios observed in *A. muricatus*. The occurrence of ovotestes during development is a cellular marker of temperature sex reversal in a closely related species *Pogona vitticeps*. Here, we present the first developmental data for *A. muricatus*, and show that ovotestes occur at frequencies consistent with a mode of sex determination that is intermediate between GSD and TSD. This is the first evidence suggestive of underlying unidentified sex determining loci in a species that has long been used as a model for TSD.

## 1. Background

The determination and differentiation of a sexual phenotype is a major event in vertebrate development, shaping the form and behaviour of individuals, and influencing the ecological properties of species [1]. Among terrestrial vertebrates, the evolution of sexual development in squamates (lizards and snakes) is particularly labile, unlike the stable genetic sex determination (GSD) mechanism of mammals. Squamates are, therefore, increasingly viewed as important models for understanding the molecular and developmental basis for sexual development in vertebrates [1–3].

Temperature-dependent sex determination (TSD), whereby incubation temperature determines sex in the absence of sex chromosomes, is a sex determination mode occurring in at least 10% of squamate species [1,4]. It is also possible for squamates to have genotypic sex determination, and for temperature to have a sex determining influence in the presence of sex chromosomes [5,6]. In such cases, extreme temperatures can override the influence of sex chromosomes, causing a discordance between an individual's sex chromosome complement and its phenotypic sex (sex reversal) [7,8]. There are only two known naturally occurring examples of such sex reversal: the Australian central bearded dragon *Pogona vitticeps*, and the three-lined skink *Bassiana duperreyi* [6,9]. In these two

species, it is clear that genetic factors and temperature can interact, so blurring the dichotomy between GSD and TSD [10]. These two species are unlikely to represent the only instances of sex reversal in squamates, and its occurrence is likely more widespread than currently appreciated in reptiles, as well as other vertebrate groups [7,11].

Through its influence on sex determination, temperature also plays an important role in the differentiation of gonads and genitalia. In many female squamates, male genitalia often develop concurrently with differentiated ovaries, and the hemipenes do not regress until late in development, or post-hatching. This asynchrony between gonadal and genital phenotypes in female squamates is termed temporary pseudo-hermaphroditism (TPH) [12] and requires a combination of concurrent histology and hemipenal morphology data to establish. TPH arises possibly because male genitalia may be a developmental default for some squamates (likely those with ZZ/ZW systems), that is overridden by other cues causing genital feminization [12,13]. In *P. vitticeps*, temperature-induced sex reversal causes the development of ovotestes, a rare gonadal phenotype with characteristics of both testes and ovaries [12]. Ovotestes were observed at a highly specific developmental period (stage 9) exclusively at sex-reversing temperatures [12]. It was hypothesized that ovotestes developing during sex reversal occurs due to antagonism between opposing cues from environmental stimuli and sex chromosomes and, therefore, can be used as cellular marker of sex reversal [12]. In TSD species, ovotestes can also occur due to incubation at the pivotal temperature (produces 50:50 sex ratios), drug manipulations or developmental abnormalities, though they are ultimately a rarely observed phenotype, particularly under natural conditions [14–18]. Importantly, ovotestes are not observed at the more extreme incubation temperatures that produce a single sex in TSD species.

The jacky dragon (*Amphibolurus muricatus*), an Australian agamid lizard, is a model for studies on the evolution and adaptive significance of TSD [19–24]. In this species, female-biased sex ratios are obtained at high (30–32°C) and low (23–25°C) temperatures, whereas approximately 50:50 sex ratios are produced at intermediate temperatures (27–30°C) [25]. Though considered a classic TSD species, this sex ratio pattern has been hypothesized to occur by temperature overriding an underlying GSD system [26]. Under this hypothesis, sex chromosomes are the primary sex determining influence at intermediate temperatures and thus produce 50:50 sex ratios, while extreme temperatures induce sex reversal in half of the individuals (assuming half of the individuals are genetically male) [26]. Therefore, if ovotestes indeed indicate sex reversal [12], *A. muricatus* developing at temperatures outside of the pivotal range should develop otherwise rarely observed ovotestes at a frequency of approximately 50%.

In this study, we investigate Quinn *et al.*'s [26] hypothesis that *A. muricatus* has a cryptic GSD mechanism with thermal override by assessing the frequencies of ovotestes at extreme incubation temperatures. For this purpose, we provide the first simultaneous characterization of gonadal and genital development for *A. muricatus*. We also consolidate important baseline information on the development of this species, by assembling the first quantitatively rigorous confirmation of the thermal reaction norms of sex ratios in this species and also providing the first staging descriptions for this emerging model organism. Our data suggest that *A. muricatus* may indeed have an unidentified genetic influence on sex

determination that is overridden by extreme temperatures, highlighting the need for further study on the sex determination mode of this species.

## 2. Results

For the developmental data presented in this study, eggs from *A. muricatus* were incubated at 24, 28 and 34°C, temperatures that have been established to produce female-biased sex ratios at the extremes, and approximately even sex ratios at the intermediate temperature. Eggs were sampled throughout embryonic development (figure 1; electronic supplementary material, file S1) and staged according to the system developed for close relative, *P. vitticeps* [13].

### (a) Temperature reaction norms of sex ratios

Our combined dataset ( $n = 806$  individuals; electronic supplementary material, file S3) confirms that *A. muricatus* does exhibit Type II TSD (figure 2a). However, the proportion of female individuals is not 100% at extreme temperatures, as has been reported by incubation experiments with smaller sample sizes [19,21]. This is the most comprehensive profile of the temperature reaction norms for sex ratios in this species to date, and reveals that more variation in sex ratios exists than previously reported (electronic supplementary material, file S2). Pearson's  $\chi^2$  test showed that sex ratios differed significantly from 50:50 ratios at every temperature except for 27.5°C ( $p = 2.2 \times 10^{-16}$ , electronic supplementary material, file S3).

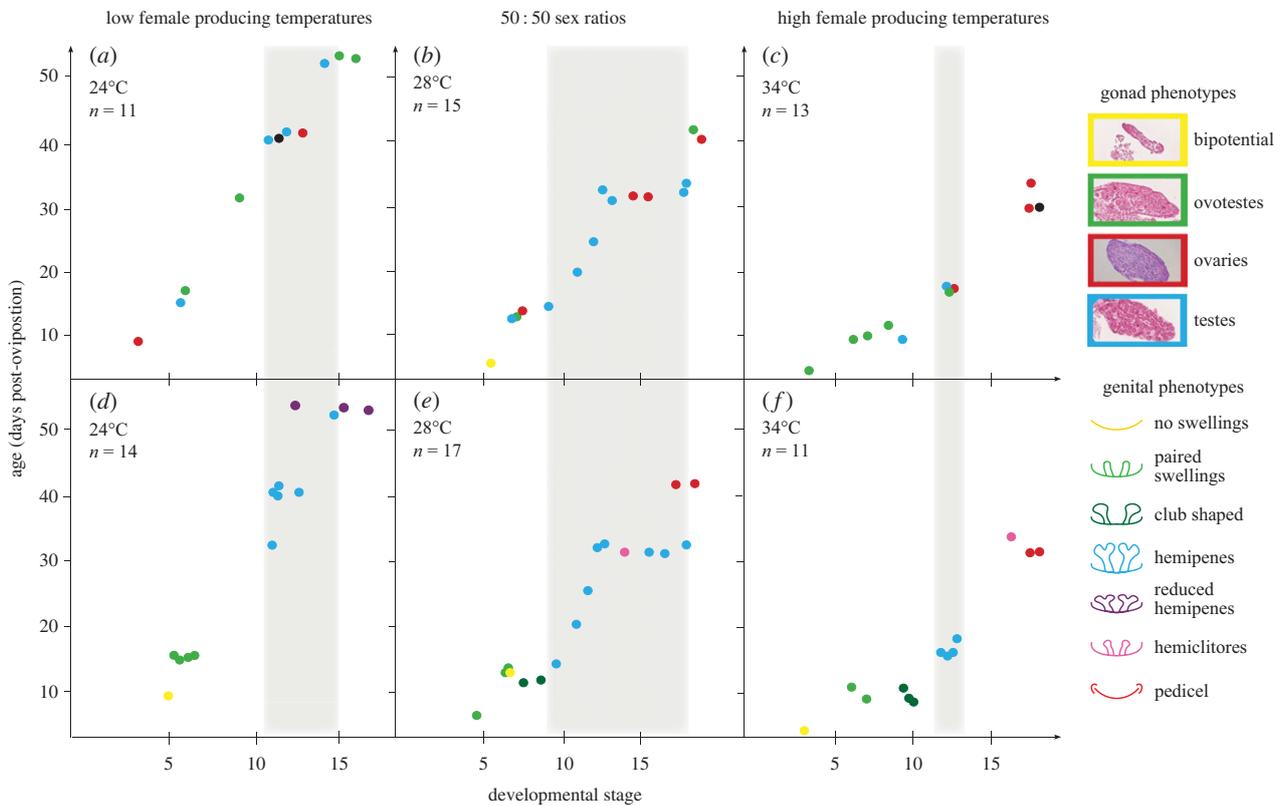
### (b) Frequency of ovotestes

Consistent with our hypothesis, assuming a GSD system with a thermal override, the proportion of ovotestes is highest at extreme temperatures and occurs at frequencies approaching 50% (table 1 and figure 2b). Of the samples with characterized gonadal phenotypes (the gonads of some samples were unable to be characterized, electronic supplementary material, file S1), ovotestes were observed more frequently at 24°C ( $n = 4$  of 11, 36%) and 34°C ( $n = 5$  of 11, 45%) compared to the moderate incubation temperature at 28°C ( $n = 2$  of 14, 14%; figure 2b). In total, across all samples with a characterized gonadal phenotype in all incubation temperatures, 31% had ovotestes ( $n = 11$  of 36) (table 1; electronic supplementary material, file S1).

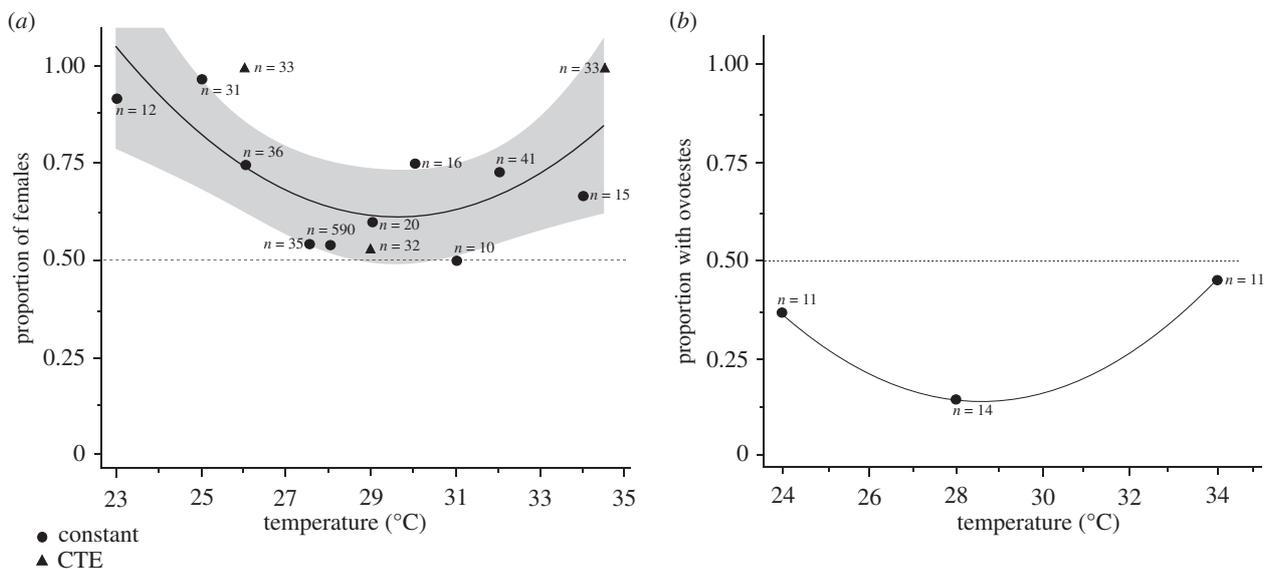
There was considerable morphological variation observed in the ovotestes. Some samples exhibited rudimentary seminiferous tubules and a cortex layer, while others exhibited well-defined tubules and a cortex layer (figure 3a). Unlike what is seen in *P. vitticeps*, where ovotestes were observed during a narrow developmental range (stage 9–9.5) [12], ovotestes were observed at disparate developmental stages in *A. muricatus*, spanning stages 3–16 (a range equivalent to approx. 72% of embryonic development) (figure 1; electronic supplementary material, file S1). Given the wide range of developmental stages at which ovotestes were observed, they were concurrent with every genital phenotype observed during development (figures 1 and 3b; electronic supplementary material, file S1).

### (c) Timing of gonad differentiation and sex ratios

The gonadal morphologies observed in *A. muricatus* are similar to those previously described for other reptile species (figure 3b–d). The gonad initially forms as a long ridge of undifferentiated tissue along the mesonephros, before



**Figure 1.** Development of gonad (a–c) and genital (d–f) phenotypes in *A. muricatus* at three different incubation temperatures (24, 28 and 34°C). Data for gonad and genital phenotypes are matched between individuals (electronic supplementary material, file S1). (Online version in colour.)



**Figure 2.** Temperature reaction norms for sex ratios (a) and proportion of ovotestes (b) for *A. muricatus*. The reaction norms were calculated by fitting a polynomial logistic regression model to the dataset, which was obtained by compiling incubation data from this study and pre-existing datasets (electronic supplementary material, file S2). Grey shading indicated 95% confidence intervals. The proportion of ovotestes was obtained from developmental data produced for this study, and fitted with a polynomial regression model (electronic supplementary material, file S1). Triangle, CTE (constant temperature equivalent) for fluctuating incubation regimes; circle, constant temperature incubations (electronic supplementary material, file S4). (Online version in colour.)

differentiating into ovaries or testes. Ovaries exhibit a distinct cortex and degenerating medulla (figure 3d). In testes, the cortex degenerates and the medulla proliferates with seminiferous tubules (figure 3c). However, there are key differences in the timing of gonadal differentiation between individuals. Differentiated ovaries were observed as early as stage 4 at 24°C, which is considerably earlier than has been observed

in other reptile species (table 2). By contrast, bipotential gonads were observed in a stage 5 specimen at 28°C (figure 1).

Of the specimens that had differentiated gonads (not including ovotestes), 67% had testes at 24°C and 40% had testes at 34°C. At 28°C, which produces 50:50 sex ratios, we observed a male bias (75% of samples with differentiated gonads had testes; electronic supplementary material, file S1).

**Table 1.** Embryos with ovotestes characterized in this study, including incubation temperature, developmental stage (based on staging system for close relative, *P. vitticeps* [13]) and corresponding genital phenotype. Ovotestes were observed at all incubation temperatures (though only two were observed at 28°C), and occurred alongside all possible genital phenotypes. They were also observed across a wide range of developmental stages. These data are also represented in figure 1, and electronic supplementary material, file S1. Age is days post-oviposition (dpo).

egg ID	incubation temperature	age (dpo)	stage	gonadal phenotype	genital phenotype
9144:01:03	24	16	6	ovotestes	paired swellings
81826:01:01	24	32	10	ovotestes	bilobed hemipenes
9171:01:06	24	53	16	ovotestes	reduced hemipenes
9131:01:04	24	53	16	ovotestes	reduced hemipenes
9165:01:01	28	13	7	ovotestes	club shaped
9130:01:01	28	41	18	ovotestes	pedicel
82431:01:03	34	3	3	ovotestes	developing cloaca
9130:01:07	34	10	6	ovotestes	paired swellings
9130:01:04	34	10	6	ovotestes	paired swellings
9165:01:02	34	10	9	ovotestes	club shaped
9138:01:01	34	17	12	ovotestes	bilobed hemipenes

#### (d) Genital development

Gross genital development follows the same processes as has been previously described for *P. vitticeps* [13]. The cloacal area forms early in development, followed by the growth of paired swellings. These swellings continue to grow and eventually become bilobed hemipenes (figure 3*b*). In females, hemipenes eventually regress to hemiclitoris, then a pedicel. In presumptive female specimens (those with differentiated ovaries), hemipenes occurred at all three incubation temperatures (figure 1).

At 24°C, hemipenes had not regressed completely by the latest stage assessed (stage 16). However, these specimens also possessed ovotestes suggesting that the lack of total hemipenis regression may have occurred because of insufficient hormone signalling from the gonad (assuming development was ultimately on a female trajectory). One stage 12 specimen exhibited TPH, which is characterized with the concurrent appearance of differentiated ovaries and bilobed hemipenes [12]. At 28°C, hemipenes are observed between stages 9 and 17, with one stage 16 specimen exhibiting TPH. Two stage 18 specimens exhibited a pedicel, with one having ovaries and the other ovotestes, suggesting that hemipenis regression can still occur without fully differentiated gonads. At 34°C, one stage 13 specimen exhibited TPH. Hemipenis regression was observed in three specimens, with two developing a pedicel at stage 18 (figure 1; electronic supplementary material, file S1).

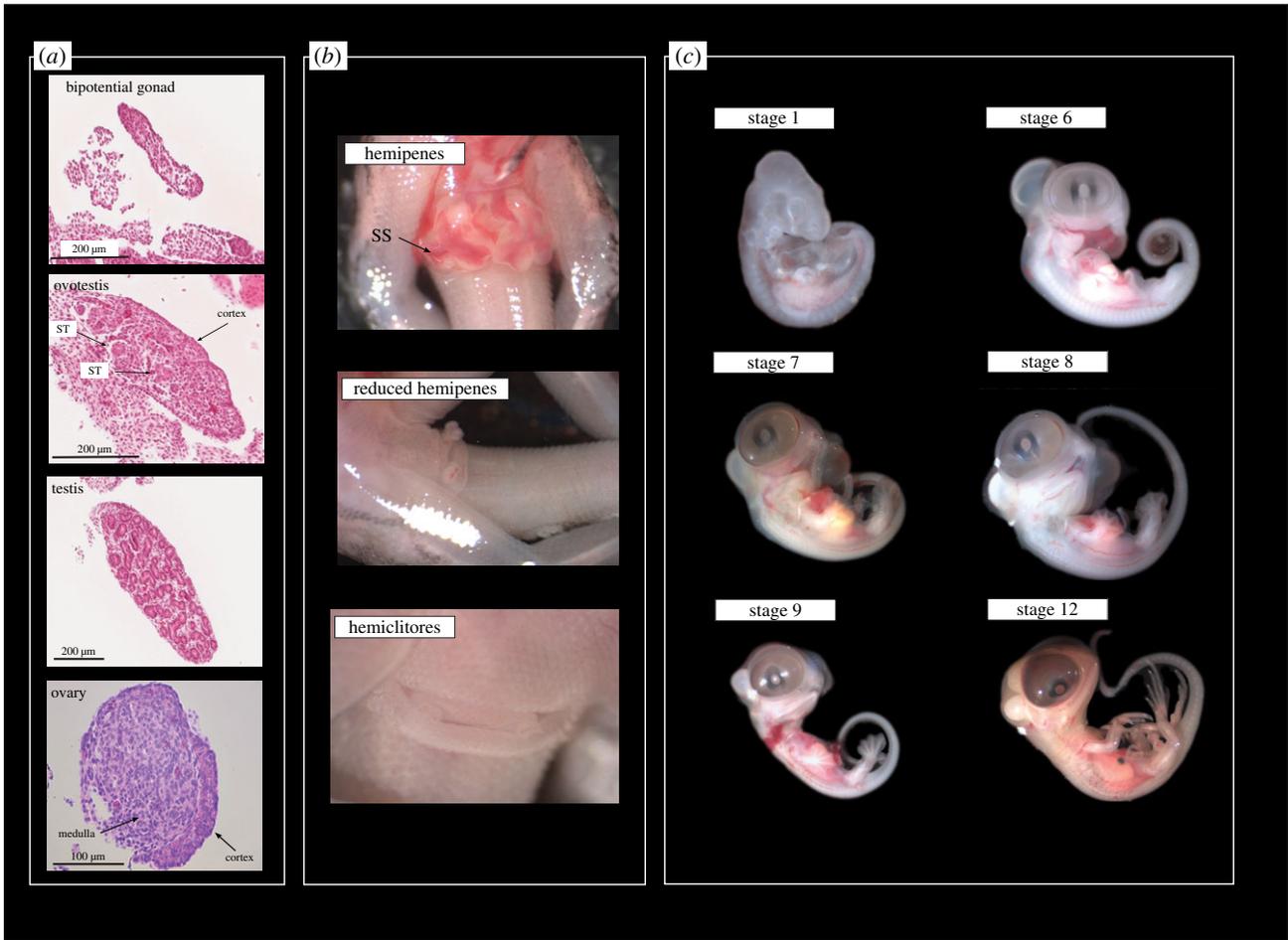
While the gross genital morphologies are similar between *A. muricatus* and *P. vitticeps*, the timing of development differs. In *P. vitticeps*, bilobed hemipenes have developed in both sexes by approximately stage 11. In females, hemipenis regression leading to hemiclitoris occurs by approximately stage 16.5 [13]. In *A. muricatus*, hemipenes develop earlier and persist for longer during development; the first specimens observed with hemipenes were at stage 9 and the oldest possessed hemipenes at stage 17. In *P. vitticeps*, the TPH phase in females persists from approximately stages 8 to 15. In *A. muricatus*, the timing of the TPH phase is less well established due to having fewer samples; however, we estimate the TPH phase in *A. muricatus* as occurring between approximately stages 9 and 17, so although it might begin slightly later it probably

lasts slightly longer in *A. muricatus* compared to *P. vitticeps* (electronic supplementary material, figure S1).

### 3. Discussion

Our results confirm our prediction that approximately half of *A. muricatus* specimens incubated at extreme temperatures display ovotestes. This provides support for Quinn *et al.*'s [26] suggestion that *A. muricatus* possess a cryptic genetic component to sex determination while simultaneously exhibiting a thermal override. We expect that ovotestes in *A. muricatus* are occurring due to antagonism between genetic and thermal influences on sex, as proposed for *P. vitticeps* [12]. A GSD system with thermal override in *A. muricatus* would also explain why extreme incubation temperatures do not produce 100% females, because sex reversal generally occurs at slightly lower than absolute frequencies (approx. 96%) in *P. vitticeps* [6].

In *P. vitticeps*, ovotestes were observed exclusively in association with sex reversal, and occurred during a very limited developmental period [12]. In *A. muricatus*, ovotestes occurrence was far less stable. Ovotestes were observed at all three incubation temperatures (though at a low frequency at 28°C), and across a wide range of developmental stages. We also observed male-biased sex ratios (67% at 24°C, 75% at 28°C and 40% at 34°C). Understanding that sex determination modes can exist on a continuum between GSD and TSD can clarify such observations in *A. muricatus*. Even in TSD species, heritable genetic variation in thermal thresholds can influence sex ratios, particularly at the pivotal temperature [41]. These differences in thresholds can subsequently shift an individual embryo's propensity for developing as one sex or the other at a given temperature, which can create sex ratio biases, such as those we observed in *A. muricatus* [42–45]. We argue that such genetic variation in thermal thresholds likely exists alongside other genotypic determinants of sex in *A. muricatus*, so explaining the variation observed in both sex ratios and ovotestes frequency at different incubation temperatures [46]. This is akin to observations in close relative, *P. vitticeps*, where rates of sex reversal increase as temperature increases, though some



**Figure 3.** Embryonic development of gonads, genitalia and gross morphology of *A. muricatus*. (a) Gonadal phenotypes observed in *A. muricatus*: bipotential gonad, ovotestis showing rudimentary seminiferous tubules (ST) in the medulla, and a thickening cortex, differentiated testis with abundant, well-defined seminiferous tubules in the medulla and a completely degraded cortex, and differentiated ovary with developing cortex. (b) Genital phenotypes observed in *A. muricatus*: bilobed hemipenes with sulcus spermaticus (SS), reduced hemipenes with a bilobed appearance but sulcus spermaticus is no longer apparent, and female hemiditiores with no sulcus spermaticus, some protrusions remain at the genital terminus but are no longer bilobed. (c) Subset of morphological stages obtained staged according to the criteria development for *P. vitticeps* [13]. (Online version in colour.)

individuals do not reverse sex [6]. Maternal genotype also influences rates of sex reversal; offspring of sex-reversed mothers reverse at lower temperatures compared to offspring of concordant mothers [6]. We propose that differences observed between the two species may be due to the genetic determinant of sex being less fixed in *A. muricatus* compared with *P. vitticeps*, which possess differentiated sex microchromosomes [47]. A particularly intriguing scenario might be that *A. muricatus* represents an early stage of sex chromosome evolution, where a small number of sex-linked genes produce the unusual timing of ovotestes that we observed.

The development of the European pond turtle, *Emys orbicularis*, offers support for an interaction between a genetic and thermal sex determination mechanism we propose for *A. muricatus*. *Emys orbicularis* was presumed to have TSD based on incubation experiments and hatchling sex ratios in the laboratory, until additional research revealed that temperature can override a weak genetic mechanism of sex determination identified by differential expression of H-Y antigens in gonadal tissues [48,49]. H-Y antigens are widely associated with XX/XY and ZZ/ZW systems in a variety of reptiles (reviewed in Dournon *et al.* [50]). The joint action of H-Y expression and thermal sensitivity in *E. orbicularis* thus implies that some genetic factors likely influence thermally

sensitive sex determination in many reptiles, and a similar process may be occurring in *A. muricatus* (reviewed in Sarre *et al.* [10]). However, it is important to note that the functional roles of H-Y antigens are not well elucidated, particularly how they may influence sex determination [7].

As with *A. muricatus*, the embryonic development of *E. orbicularis* is often characterized by the presence of ovotestes. In the turtle, they may persist post-hatching but ultimately resolve as testes [51]. It appears that ovotestes occur readily in this species due to a high sensitivity to small fluctuations in oestrogens, which can rapidly drive the development of an ovarian cortex but fails to fully repress the seminiferous tubule proliferation in the medulla [52,53]. It is possible that oestrogen sensitivity may also drive ovotestes development (and its lability) in *A. muricatus*, though it is unknown how oestrogen levels may be influenced by varied incubation temperatures in *A. muricatus*. Testosterone, or the balance between testosterone and oestrogen, may also influence ovotestes development; however, further study is required. It is currently unknown if ovotestes persist post-hatching in *A. muricatus*, or if they resolve by hatching, as has been reported for *P. vitticeps*. The timing in ovotestes occurrence greatly differs to that of *P. vitticeps*, and may be more similar to *E. orbicularis*; however, this remains to be investigated fully.

**Table 2.** Timing of gonadal differentiation in species with TSD in which gonadal development has been characterized. The stage and staging system used in the original publication is provided, which has been calibrated to the staging system used for *P. vitticeps* and *A. muricatus* to compare the timing of differentiation. Where only the thermosensitive period (TSP) is given, stages of the lower and upper bounds of the period or the average to the *P. vitticeps* staging system are provided.

species	gonad differentiation/TSP period	staging system	<i>P. vitticeps/A. muricatus</i> equivalent	original reference
<i>Alligator mississippiensis</i>	stage 23	[27]	stage 13	[28]
<i>Apalone spinifera</i>	stages 18–20	[29]	stage 9	[30]
<i>Calotes versicolor</i>	stage 34	[31]	stage 9	[32]
<i>Chelydra serpentina</i>	stages 14–16 (TSP period)	[29]	stage 14 = stage 5 stage 16 = stage 6	[33]
<i>Crocodylus palustris</i>	stages 21–25 (TSP period)	[27]	stage 13 (average)	[34]
<i>Emys orbicularis</i>	male differentiation at stage 17, female differentiation at stage 19	[29]	stage 17 = stage 8 stage 19 = stage 9	[35]
<i>Eublepharis macularius</i>	stages 33–37 (TSP period)	[36]	stage 33 = stage 6 stage 37 = stage 14	[37]
<i>Malayemys macrocephala</i>	stage 17	[29]	stage 8	[38]
<i>Pogona vitticeps</i>	stage 8	[13]	NA	[13]
<i>Trachemys scripta</i>	stages 14–20 (TSP period)	[39]	stage 14 = stage 5 stage 20 = stage 9	[40]

Understanding the genetic underpinnings of ovotestes development in reptiles would also be of great benefit. To date, no ovotestes in reptiles have been sequenced to reveal the gene expression profiles of this unusual phenotype. In many fish species, ovotestes occur comparatively often, and RNA sequencing has revealed novel insights into the genetic machinery responsible for ovotestis development, for example, in the rice field eel and black porgy [54–56]. In mammals, ovotestes are typically only associated with disorders of sexual development; however, the Iberian mole possess ovotestes and RNA sequencing has shown how they develop [57]. Interestingly, despite the wide phylogenetic divide between these groups, many of the same genes (e.g. aromatase and DMRT1) have been implicated, so it would be particularly intriguing for future research to assess this in reptiles.

We present the first comprehensive thermal reaction norms for sex ratios in *A. muricatus* by combining our data with previously published sex ratio data. We show that contrary to previous reports, the only incubation temperature that did not exhibit significant deviation from 50:50 sex ratios was 27.5°C. This suggests that the intermediate temperature range of this species may be far narrower than previously reported [21,25,58], and that small sample sizes limit the accuracy of earlier reported sex ratios. This may also go some way to explaining the male-biased sex ratios we observed in our study. Further, our observation of two samples with ovotestes at 28°C can also be explained by this trend, as 50:50 sex ratios are not actually expected at this temperature.

Lastly, we also show *A. muricatus* is the fourth squamate discovered to exhibit TPH (male genitalia occurs alongside differentiated ovaries), another condition previously considered unusual. This supports suggestions that TPH may occur in female squamates and is associated with thermolabile sex determination (electronic supplementary material, figure S1; [12]). Histological studies on squamate gonads are rare, but we expect that further investigation of genital

and gonadal development will reveal TPH to be a common occurrence among squamates, particularly among those with retained hemipenes in female juveniles [36,59–61].

## 4. Conclusion

Our results indicate strong potential for extensive and unappreciated diversity in genetic, temperature and possibly other cues in the differentiation of sex in squamates. Our understanding of the interaction between genes and the environment in reptile sex determination remains poorly characterized, so this area provides many compelling avenues for future research. *Amphibolurus muricatus* emerges as a particularly important study species to identify the nature of genetic mechanisms influencing sex, such as evidence of cryptic sex chromosomes. It will also be imperative to identify loci that have sex-associated alleles in adults from intermediate temperatures. Definitive demonstration of the genetic mechanisms underlying sex, combined with identification of phenotype, will be required to confirm our suggestion that sex reversal occurs in this species. *Amphibolurus muricatus* would then represent the third squamate with sex reversal, and would be the first with sex reversal at both extremes of temperature. We hope that our suggestion of sex reversal in *A. muricatus* provides the impetus to examine the sex determination modes of TSD squamates more closely, and highlights novel approaches that can be taken to uncover previously unidentified complexities in reptile sexual development.

## 5. Material and methods

### (a) Egg incubations and sampling

During the 2018–2019 and 2019–2020 breeding seasons, eggs were obtained from both wild caught ( $n = 4$ ) and captive bred females ( $n = 4$ ) for the developmental data presented in this study. Females

were provided with nesting substrate and allowed to lay naturally. If the female retained eggs for a prolonged period of time, they were induced to lay with an intraperitoneal injection of 10–30 IU of oxytocin followed by a 10 IU dose of calcium carbonate solution. Eggs were weighed and randomly allocated to one of three incubation temperatures (24°C, 28°C, 34°C). These temperatures are within the range of those experienced in wild nests [24]. Eggs were placed individually in glass jars filled damp vermiculite (four parts vermiculite to five parts water by weight) and covered with Glad Wrap<sup>®</sup> known to allow the diffusion of oxygen. Eggs were subsequently randomly allocated to a target developmental stage (6, 12 and 15), the sampling day estimated based on incubation data from *P. vitticeps* and adjusted for differing incubation durations [13]. Six eggs were sampled at day of lay to establish stage at lay (two eggs from three clutches). This showed that eggs were consistently at stage 2 based on the staging system developed for *P. vitticeps* [13]. Every embryo was staged and photographed fresh, and the urogenital system (UGS) was dissected. In total, 44 embryos were obtained. While the sample size is small due to the low reproductive output of this species, these data can still provide valuable information on the embryonic development of *A. muricatus*. All procedures were carried out in accordance with animal ethics procedures from the University of Canberra (Project 270). Additional incubations were carried out at the University of Canberra and the University of New South Wales, and these data were used to generate the temperature reaction norms (electronic supplementary material, file S2). Constant temperature equivalent (CTE) [62] was calculated for fluctuating incubation data from [23]. Data used to calculate the CTE for these incubations are provided in electronic supplementary material, file S4.

## (b) Histology and phenotype characterization

All UGS samples for histology were prepared at the University of Queensland's School of Biomedical Science's Histology Facility.

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