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Corticosterone does not have a role in temperature sex reversal in the central bearded dragon (*Pogona vitticeps*)

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Abstract

Environmental sex determination (ESD) is common among ectothermic vertebrates. The stress axis and production of stress hormones (corticosteroids) regulates ESD in fish, but evidence of a similar influence in reptiles is sparse and conflicting. The central bearded dragon (*Pogona vitticeps*) has a system of sex determination involving the interplay between sex chromosomes (ZZ/ZW female heterogamety) and the thermal environment. High egg incubation temperatures induce sex reversal of the ZZ genotype, feminizing chromosomally male individuals. Here we show that corticosterone elevation is not associated with sex reversal in the central bearded dragon, either during embryonic development or adulthood. We also demonstrate experimentally that sex determination is not affected by corticosterone injection into the yolk. This strongly suggests that stress axis upregulation by high temperature during incubation does not cause sex reversal in *P. vitticeps*. Our work is in general agreement with other research in reptiles, which suggests that the stress axis does not mediate sex in reptiles with ESD. Alternative biological systems may be responsible for capturing environmental conditions during reptile development, such as cellular calcium and redox regulation or the action of temperature-sensitive splicing factors.

KEYWORDS
corticosteroids, sex determination, sex reversal, stress axis

1 | INTRODUCTION

In many lineages of fish, amphibians and reptiles, environmental conditions experienced during early development influence sex (Bachtrog et al., 2014), a developmental process known as environmental sex determination (ESD). Among vertebrates, modes of sex determination are most diverse in fish, including conventional genetic sex determination (GSD), pure ESD, GSD with environmental influence, or sex change post-maturity (Baroiller & D'Cotta, 2016; Todd et al., 2016). A wide range of environmental cues determine sex in fish, including temperature, pH, salinity, social status or light regime (Baroiller & D'Cotta, 2016). The predominant environmental cue in reptile ESD is temperature (temperature dependent sex determination [TSD]), although moisture and egg size can also affect sex (Dupoué et al., 2019; Radder et al., 2009; Wyneken & Lolavar, 2015). Temperature has the capacity to direct sexual outcomes in the absence of sex chromosomes, in combination with an underlying genetic predisposition or even in the presence of differentiated sex chromosomes (Sarre et al., 2004). The epigenetic effectors of ESD are becoming well characterized in reptiles (Deveson et al., 2017; Ge et al., 2018; Weber et al., 2020), but the biological sensory mechanism which receives and translates the environmental signal into a biological cue is not yet known (Georges & Holleley, 2018).

The diversity among vertebrates in the environmental cues that are influential in sex determination (Bachtrog et al., 2014) and the poor concordance between modes of sex determination and phylogeny, particularly in reptiles (Sarre et al., 2004), suggests that ESD

has evolved repeatedly and independently many times, with frequent transitions in sex determining modes. Whether the biological translation mechanism of ESD is common to all species, or whether different mechanisms have been recruited during each emergence of ESD is not yet known. In fish, the vertebrate stress axis (hypothalamic-pituitary-interrenal/HPI axis) plays a fundamental role in ESD in both gonochoristic (Adolfi et al., 2019; Castañeda Cortés et al., 2019; Hattori et al., 2009; Hattori et al., 2020; Mankiewicz et al., 2013) and sequentially hermaphroditic fish (Chen et al., 2020; Goikoetxea et al., 2017; Solomon-Lane et al., 2013). Typically, environmental influence leads to masculinization in fish (Ospina-Álvarez & Piferrer, 2008), and the molecular pathway by which cortisol initiates androgen production is well understood (Goikoetxea et al., 2017). The production of cortisol can generate an increase in the enzyme HSD11B2/3. This enzyme inactivates cortisol as part of the negative feedback system of the stress axis, but also catalyzes the conversion of testosterone to 11-ketotestosterone, a potent androgen (Chen et al., 2020). The molecular pathway of cortisol involvement in sex determination in fish is thus well-characterized.

A causative role for the stress axis and glucocorticoids in reptile ESD not been conclusively demonstrated, though it must be noted that far less experimental research has been conducted in reptiles. Only five studies explicitly test this hypothesis. There was no effect of corticosterone (CORT) application on sex (Iungman et al., 2015; Uller et al., 2009; Warner et al., 2009; Wibbels & Crews, 1992) and no association between embryonic corticosterone production and male or female-producing temperatures (Marcó et al., 2015). One study did conclude that corticosterone may have a feminizing effect in a TSD agamid lizard (*Amphibolurus muricatus*), though sex-specific mortality in response to corticosterone application could not be excluded (Warner et al., 2009). Here we apply comparable methods to a third reptile species with high temperature sex reversal and investigate the role of corticosterone in influencing sexual outcome.

The central bearded dragon (*Pogona vitticeps*) is an agamid with both a ZZ/ZW system of GSD (Ezaz et al., 2005) and thermosensitivity (Holleley et al., 2015; Quinn et al., 2007). Adult ZZ sex-reversed females display transcriptomic signatures of an upregulated stress response, including upregulation of the stress hormone signaling precursor proopiomelanocortin (*POMC*) and downregulation of the negative feedback regulator corticotropin releasing hormone binding protein (*CRHBP*) (Deveson et al., 2017). Behavioral repertoires of juvenile sex-reversed females differ from those of either concordant sex, also indicative of sustained stress axis upregulation (Li et al., 2016). This evidence suggests that stress axis activation and glucocorticoid production are key effectors of the sex-reversed phenotype in adulthood, and so may also be involved in the initiation of sex reversal in the embryo. We predicted that (a) corticosterone and other indicators of prolonged hormonal stress will be naturally elevated in adult sex reversed females, and (b) experimental injection of corticosterone at low temperatures will initiate sex reversal. Contrary to our predictions, we did not find evidence that the stress axis mediates or is a long-term consequence of sex reversal in *P. vitticeps*. We did observe that acute exposure to high incubation temperature elevates embryonic corticosterone levels, but not until after sex determination has occurred.

2 | MATERIALS AND METHODS

2.1 | Colony details

Captive bred dragons from a breeding colony at the University of Canberra were used in this study. All dragons were caged communally in terrariums with sand substrate, water available *ad libitum*, and on equal feeding regimens. All adults were of known chromosomal sex (using the sex test of Holleley et al., 2015), and were either concordant males (ZZm), concordant females (ZWf), or sex-reversed females (ZZf).

2.2 | Animal breeding and egg allocation

Breeding groups consisting of one male and 2–4 females were set up to generate eggs. Both sex-reversed females (ZZf) and concordant females (ZWf) were used in this study. Gravid females were allowed to lay naturally, and eggs were recovered within 24 h of oviposition. Fertile eggs which had visible vascular systems were weighed and placed in boxes with divisions filled with moist vermiculite (5 vermiculite: 4 water) and immediately incubated at a constant 28°C or 36°C until egg treatment. Eggs from each clutch were allocated systematically to temperature and treatment blind to the genotypic sex of the embryo.

2.3 | Egg incubation and injection

Eggs were injected with corticosterone (Sigma C2505) in sesame oil vehicle (Sigma S3547) or sesame oil alone three days after lay. Uninjected controls were not altered. Firstly, the egg was candled to confirm the position of the embryo at the top of the egg. Injections were performed using disposable BD Insulin Syringes (Product #324910), which have 5 µl divisions and a 6 mm 31-gauge needle. The needle was inserted to its full length at a position approximately halfway down the lateral (long) side of the egg, to position the needle in the approximate center of the egg, depositing the vehicle or corticosterone solutions into the yolk. Once the injection was complete, a small amount of cyanoacrylate superglue (Selleys® QuickFix Gel SupaGlue™) was placed over the injection site, to prevent infection and desiccation. Eggs were then returned to their incubator and incubated at a constant 28°C or 36°C until either embryonic sampling or hatching. Doses of corticosterone were either 10 µg CORT total in 5 µl sesame oil or 25 µg CORT total in 5 µl sesame oil. We estimated the endogenous levels of yolk corticosterone in *P. vitticeps* based on those of two Australian agamids, *Amphibolurus muricatus* and *Ctenophorus pictus*. Using *A. muricatus*, we assumed an average

corticosterone concentration of 7.8 pg mg^{-1} of yolk mass (Warner et al., 2007). Using *C. pictus* we assumed an average corticosterone concentration of 446.16 p/g of total egg mass (Hansson & Olsson, 2018; Uller et al., 2009). The average total egg mass of *P. vitticeps* is approximately 4.2 g (unpublished data), and so making the conservative assumption that the egg is 100% yolk at the time of lay then the total amount of yolk corticosterone per egg in *P. vitticeps* is likely to range from 0.0019 to 0.0328 µg, significantly lower than even the low dose (10 µg) applied in these experiments.

2.4 | Experiment 1: Tissue corticosterone levels during development

Eggs from 12 males and 12 females were used in this experiment. For each clutch, within 24 h of oviposition half of the eggs were transferred to constant temperature incubators set at 28°C and the other half at 36°C. A total of 150 eggs were used in this experiment, split into the control (no injection; $n = 24$ at 28°C, $n = 31$ at 36°C), vehicle control (5 µl sesame oil; $n = 24$ at 28°C, $n = 27$ at 36°C) or high CORT (25 µg CORT in 5 µl sesame oil; $n = 29$ at 28°C only) treatments. Both control and vehicle injections were conducted at both 28°C and 36°C, and CORT injections were only conducted in eggs at 28°C.

Embryos from both incubation temperatures and all treatments and controls were sampled at embryonic development Stages 7 and 12. Stage 7 embryos still possess bipotential gonads when incubated at constant temperature, and we thus consider sex to still be labile and responsive to temperature at this point (Whiteley et al., 2017, 2018). Stage 7 embryos were sampled at 20 days post lay (dpl) for embryos at 28°C and 10 dpl for embryos at 36°C. Stage 12 embryos incubated at constant temperature possess fully differentiated gonads (ovaries or testes) and we thus consider sex to be fixed at this point during development (Whiteley et al., 2017, 2018). Stage 12 embryos were sampled at 35 dpl at 28°C and 20 dpl at 36°C (see 2.6 Hormonal and metabolite sampling).

2.5 | Experiment 2: The effect of corticosterone manipulation on sex

A total of 168 eggs were used in this experiment. Eggs within each clutch were systematically allocated to control, vehicle control, low CORT and high CORT treatments at a ratio of 1:1:3:3. Eggs were allocated to control (no injection; $n = 27$), vehicle control (5 µl sesame; $n = 18$), low CORT (10 µg CORT in 5 µl sesame oil; $n = 61$), or high CORT (25 µg CORT in 5 µl sesame oil; $n = 62$). The high CORT treatment is approximately equivalent (by egg weight) to the dose applied to lizard eggs in (Warner et al., 2009).

In this experiment all eggs were brought to hatching. Hatchlings were removed from the incubator one day after hatching and placed into plastic tubs with newspaper substrate and cardboard hides. Hatchlings were provided with water *ad libitum* and were kept alive for 3 days before euthanasia and sampling. They were not fed during this time to prevent antagonistic competitive interactions between hatchlings. Before euthanasia via intraperitoneal injection of sodium pentobarbital, blood samples were taken to conduct plasma corticosterone measurements (see 2.6 Hormonal and metabolite sampling).

2.6 | Hormonal and metabolite sampling

Blood samples from adult dragons were taken between 10 a.m. and 4 p.m., allowing the dragons to behaviorally thermoregulate and reach an appropriate body temperature for blood sampling without the need for prior handling and positioning by researchers. Blood samples were taken from 20 sex-reversed females, 20 concordant females and 20 concordant males for corticosterone analysis. For DNA damage analysis, plasma samples of 14 sex-reversed females, 18 concordant females and 12 concordant males were used. It was assumed that as the dragons were bred in captivity and have become habituated to the presence of humans, the presence of the researcher in the terrarium room was likely not a major stressor, and so sampling time was considered to begin when the animal was first handled. Effort was made to keep handling time to a minimum.

Blood was drawn from the caudal vein, syringed into heparinised microcapillary tubes (Thomas Scientific 1202K16), sealed with capillary wax (Thomas Scientific 1202K13) and placed on ice until centrifugation in a microhematocrit centrifuge for 15 min. All samples were fractionated and frozen at −20°C within 8 h of collection. If the tail had been punctured three times, the caudal vein had not been found and the handling time reached 7 min, sampling was abandoned and the animal returned to its cage.

In experiment 1, Sampled embryos were euthanised via intracranial injections of sodium pentobarbital before being snap frozen in liquid nitrogen. Steroid fractions were extracted from subsamples of the embryo (see 2.7 corticosterone and DNA damage measurements).

In experiment 2, a total of 113 hatchlings were successfully blood sampled for comparisons of basal corticosterone levels between treatments. Before euthanasia, the tip of the tail was cut and blood from the tail drawn into a heparinised microcapillary tube (Thomas Scientific 1202K16). The tube was sealed with microcapillary wax (Thomas Scientific 1202K13) and spun down in a microhematocrit centrifuge for 15 min. Plasma was separated from the red blood cell pellet and frozen at −20°C for later analysis of corticosterone.

2.7 | Corticosterone and DNA damage measurements

Corticosterone and DNA damage levels were assessed using the Arbor Assays® DetectX Corticosterone EIA kit (K014) and DetectX DNA Damage EIA kit (K059) according to the manufacturer's instructions applied to blood plasma. Plasma samples were diluted to

an appropriate level to ensure that measurements fell within the standard curve generated during each plate run. Mostly, dilutions were performed to a factor of 12, but ranged from a 5 to 25-fold dilution factor, depending on the amount of plasma available and whether previous runs determined the sample to have exceedingly low or high amounts of corticosterone. A subset of samples were included on every plate to assess inter-assay variability. The endpoint absorbance values at 420 nm absorbance were read on a Bio-Rad Benchmark Plus Microplate Spectrophotometer and the raw values exported. Corticosterone and DNA Damage levels were calculated from the raw data using the MyAssays (www.myassays.com) calculator tools designed specifically for these two assays.

Steroid hormone extractions for tissue were conducted according to the Arbor Assays® protocol for tissue steroid extractions. In short, 5–15 mg of trunk tissue was pulverized using a handheld mortar and pestle in 1.5 ml acetonitrile (HPLC grade, 100%). The sample was centrifuged at 10,160*g* for 10 min at 4°C and the supernatant was transferred to a clean tube. 3 ml hexane (HPLC grade, 100%) was added to each sample and the tube was vortexed for 5 min, to solubilize the tissue-derived lipids in the hexane layer. The hexane was poured off and the acetonitrile transferred to a clean container before evaporation of the acetonitrile using a Christ SpeedVac system. One sample in each extraction batch was spiked with a known quantity of corticosterone to evaluate steroid recovery.

Dried hormone samples were kept frozen at −80°C for no more than three days until use in the Arbor Assays® DetectX Corticosterone Enzyme Immunoassay kit (K014). The extracted hormone fraction was dissolved in 10 µl of 100% ethanol, then with 40 µl of the diluted assay buffer. The sample was vortexed well and incubated at room temperature for 5 min three times, to ensure reconstitution of the hormone. The samples were again diluted with 350 µl diluted assay buffer and immediately run in duplicate in the assay. The manufacturer's protocol for the corticosterone EIA kit indicates that there is the potential for cross-reactivity mainly with 1-dehydrocorticosterone and desoxycorticosterone, metabolites of corticosterone with no glucocorticoid activity.

2.8 | Genotypic and phenotypic sexing

Clutches from both ZWf mothers and ZZf mothers were used in these experiments, so hatchling *P. vitticeps* were phenotypically sexed using the hemipene transillumination method while alive, and subsequently after euthanasia via manual eversion of the hemipenes. Sampled embryos were not phenotypically sexed, as all embryos possess hemipenes which are only regressed in females before hatching (Whiteley et al., 2017).

To confirm genotypic sex in both hatchlings and embryos, a blood sample was placed on an FTA Elute or Classic card and DNA was extracted using the Whatman Elute protocol or Qiagen DNeasy kit. A polymease chain reaction sex test was conducted to determine if the individual was ZZ or ZW following the method of Holleley et al. (2015) and primers characterized by Quinn et al. (2010).

A subset of gonads from each treatment and genotype (total of 15 individuals) from experiment 1 were used to confirm gonadal identity via histology. Five hatchlings of each genotype (ZZ and ZW) in each treatment were used (aside from control ZW, in which three hatchlings were used) to confirm that gonadal morphology matched with the presence or absence of hemipenes. The adrenal-kidney-gonadal complex was removed and fixed in 10% neutral buffered formalin for 24 h then transferred to 70% ethanol. Histology was performed using haematoxylin and eosin staining as described in Whiteley et al. (2018).

2.9 | Statistical analysis

All statistical analyses were conducted in RStudio (R version 4.0.2). Data were analyzed for normality, homogeneity of variance by analysis of residuals. Outliers were identified and data analysis performed both with and without outliers included to determine their impact on the result. Where data were not normally distributed (tissue corticosterone measurements from Experiment 1), a log10 transformation was applied and the data analyzed using a factorial analysis of variance (ANOVA) which accounted for specimen genotype (ZZ/ZW) and treatment.

3 | RESULTS

3.1 | Experiment 1: Tissue corticosterone throughout development

The purpose of this experiment was to examine, by way of a factorial design, the impact of treatments involving combinations of corticosterone injections, stage, and temperature (treatment) on embryonic tissue corticosterone (response), after accounting for differences between the sexes (Figure 1). Assumptions of homoscedasticity and normality were accommodated by a log 10 transformation. There was no significant interaction between treatment and sex as the main factors ($F = 0.883$; df = 5,79; $p = .497$), so it was appropriate to consider the significance of the main effects. There was no significant difference between the two sexes ($F = 0.232$; df = 1,79; $p = .631$). There was a significant effect of the treatment on embryonic corticosterone levels ($F = 4.805$; df = 5,79; $p < .001$). Neither corticosterone treatment nor incubation temperature affected embryo mortality in this experiment ($\chi^2 = 4.147$, df = 4, $p = .387$).

The data were re-analyzed as a single-factor ANOVA (on treatment) followed by Tukey multiple comparisons. Control and vehicle injection treatments did not differ significantly and were pooled. The 36°C control stage 12 embryos had significantly higher corticosterone levels than either of the 28°C control Stage 7 embryos (Tukey honest significance test [HSD] = 0.388, df = 85, $p < .001$). and the

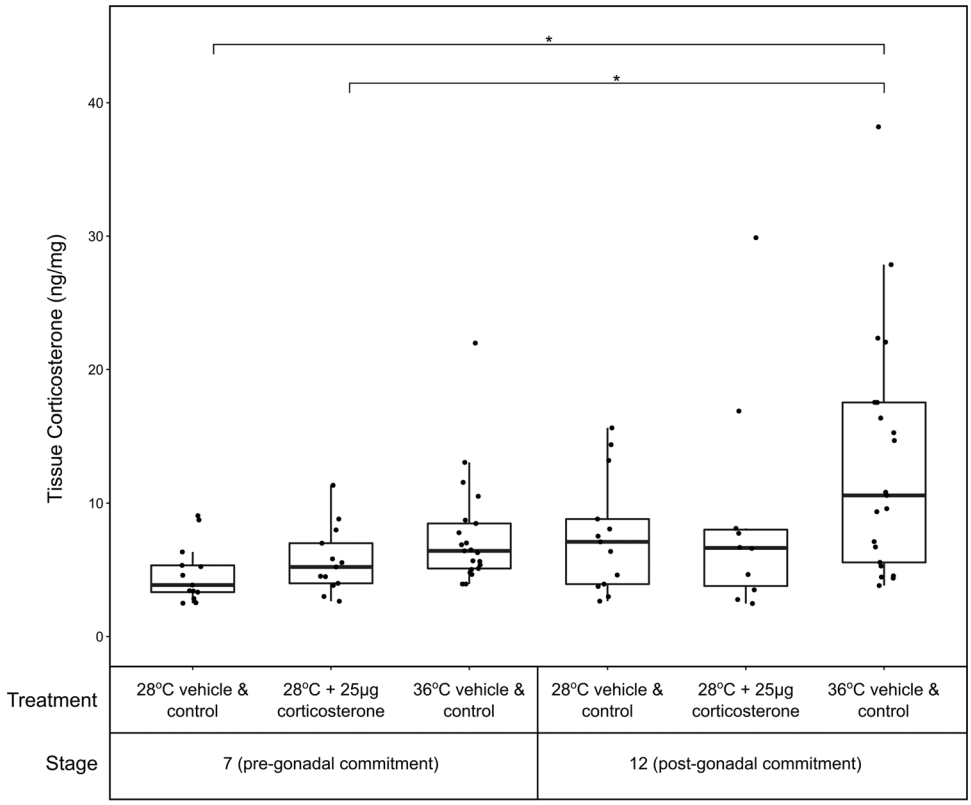

FIGURE 1 Embryonic tissue corticosterone levels are elevated in response to high temperature (36°C) incubation after gonadal commitment. Analysis was conducted on $\log_{10}$ transformed values. A factorial analysis of variance (ANOVA) with Tukey's pairwise comparisons determined that 36°C incubated stage 12 embryos had higher tissue corticosterone levels than both 28°C incubated control (Tukey honest significance test [HSD] = 0.388, df = 85, $p < .001$). and 28°C corticosterone-treated (Tukey HSD = 0.302, df = 85, $p = .009$) embryos. This indicates that the embryonic stress axis can be responsive to incubation temperature, but only after the thermosensitive period, strongly suggesting that the vertebrate stress axis does not initiate sex reversal in *Pogona vitticeps*. Each treatment group contains both ZZ and ZW embryos

28°C CORT Stage 7 embryos (Tukey HSD = 0.302, df = 85, $p = .009$) (Figure 1). There were no other significant differences between groups (Figure 1).

3.2 | Experiment 2: Effects of corticosterone on sex

The purpose of this experiment was to examine, by way of a factorial design, the impact of corticosterone injection concentration and genotype (treatments) on hatchling (3 days post-hatch) plasma corticosterone (response). Of the hatchlings which were produced ($n = 158$), sufficient plasma was recovered to allow corticosterone measurements from 21 control hatchlings, 8 vehicle injected hatchlings, 43 low CORT hatchlings and 40 high CORT hatchlings. Corticosterone treatment did not affect mortality in this experiment ($\chi^2 = 1.244$; df = 3; $p = .743$). There was a significant treatment and sex interaction ($F = 5.196$; df = 2, 104; $p = .007$). Opposite to what we would predict, the ZW control group displayed significantly higher corticosterone levels than the group experimentally treated with 25 µg corticosterone injection in ZW individuals (Tukey HSD = 46.63; df = 106; $p = .026$) (Figure 2). There were no other significant differences between treatment classes or genotype (Figure 2).

No sex-reversed individuals were identified in either the low CORT or high CORT treatment groups (Figure 3). All ZZ individuals were phenotypically male and all ZW individuals were phenotypically female. Histological examination of gonads confirmed typical ovary structure in ZW females and typical testes structure in ZZ males, regardless of treatment group.

3.3 | Corticosterone measurements in adults

The purpose of this experiment was to examine, by way of a factorial ANOVA, the differences in plasma corticosterone levels and DNA damage (an indicator of chronic stress) in adult specimens of the three sex classes, ZZm (male), ZZf (reversed to female), and ZWf (female). Mean basal levels of plasma corticosterone between the three sex classes did not differ significantly ($F = 2.737$; df = 2,57; $p = .073$; Figure 4). Exclusion of the two outliers did not alter the outcome ($F = 2.849$; df = 2,55; $p = .067$). There were no significant differences between levels of oxidized guanine in adult ZW females,

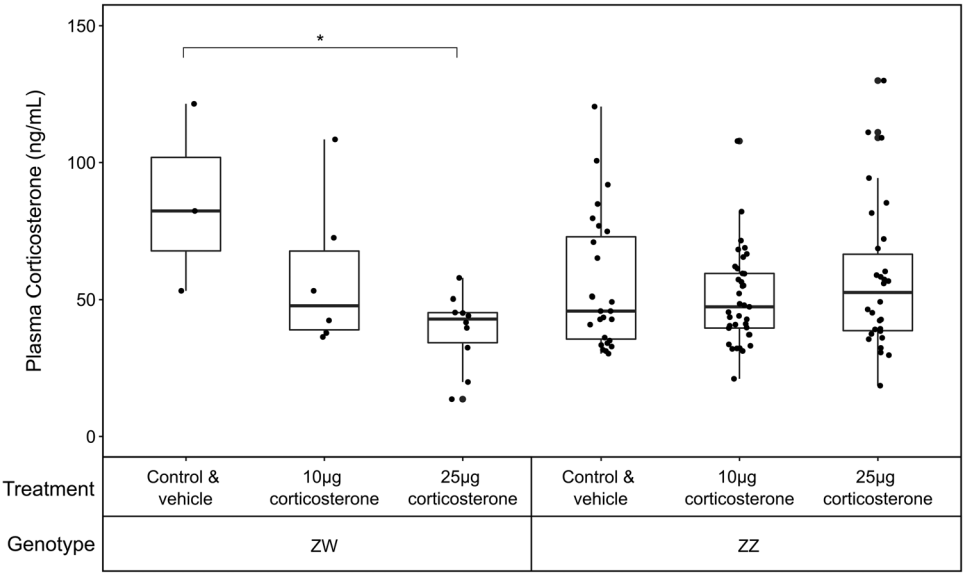

FIGURE 2 Hatchling basal plasma corticosterone (CORT) levels are not elevated by corticosterone yolk injections early in development. Genotypic female (ZW) and genotypic male (ZZ) eggs were treated with two doses of corticosterone (10 µg or 25 µg) during development. No hatchlings were sex-reversed as a result and all hatchlings had a physical sex concordant with their genotypic sex. Vehicle-treated (sesame oil injections) and uninjected control hatchlings were pooled for analysis. Control ZWf hatchlings had significantly higher basal plasma corticosterone levels than 25 µg CORT-treated ZWf hatchlings (Tukey HSD = 46.63, df = 107, $p = .026$), but there were no other significant differences between treatment groups, demonstrating that CORT injections early in development do not affect basal CORT levels. HSD, honest significance test

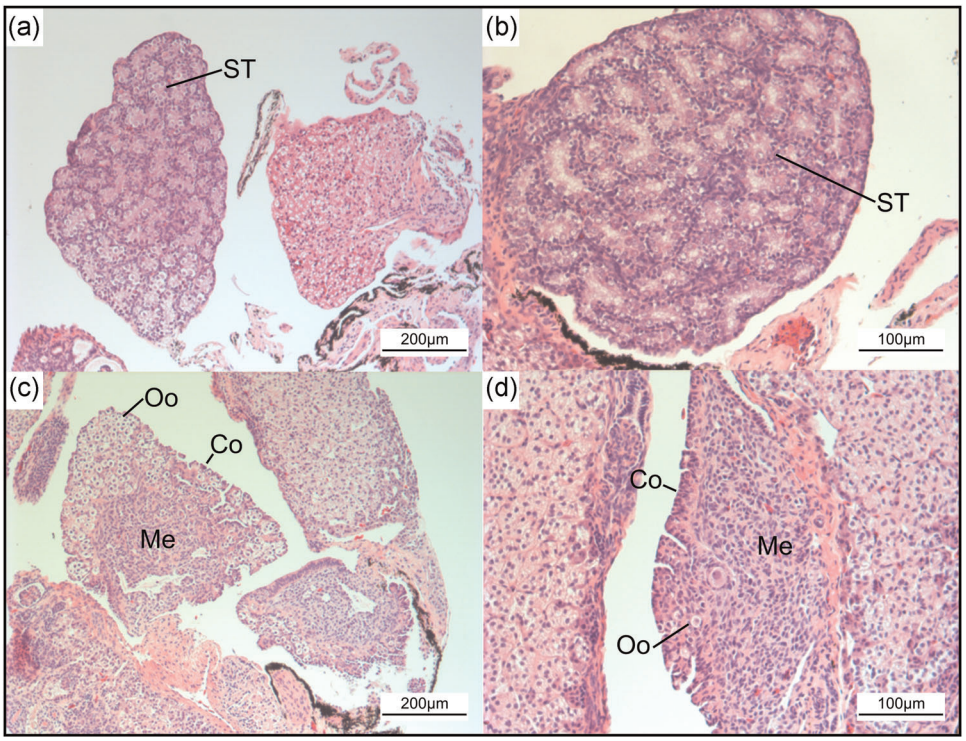

FIGURE 3 Gonadal phenotype matches genetic sex in corticosterone-treated *P. vitticeps* hatchlings incubated at a non sex-reversing temperature. Both control ZZ hatchlings (a) and ZZ hatchlings treated with corticosterone early in development (b) have normal testes with seminiferous tubules (ST). Both control ZW hatchlings (c) and ZW hatchlings treated with corticosterone early in development (D) have normal ovaries, with a clear medulla (Me) and cortex (Co) and developing oogonia (Oo) [Color figure can be viewed at wileyonlinelibrary.com]

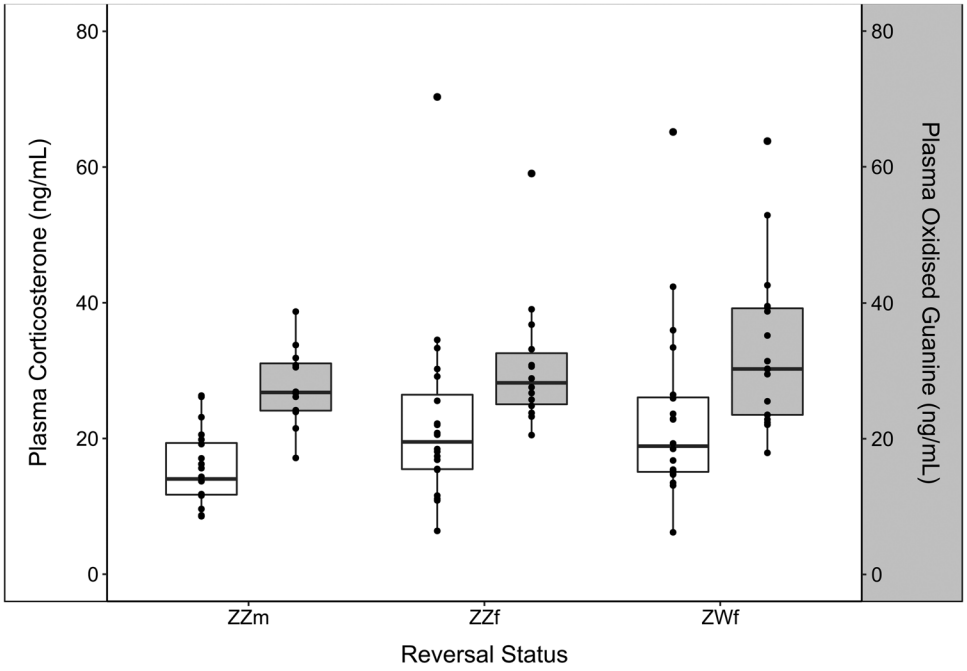

FIGURE 4 Adult basal plasma corticosterone (white) and oxidized guanine (gray) levels of concordant female (ZWf), sex-reversed female (ZZf) and concordant male (ZZm) bearded dragons (*Pogona vitticeps*). A one-way ANOVA detected no significant difference between mean corticosterone or oxidized guanine levels in any of the sexes, indicating that there is no difference in basal hormonal stress levels or DNA damage levels (as an indicator of long-term chronic stress) between the sexes. ANOVA, analysis of variance

ZZf sex reversed females and ZZm males ($F = 0.987$; df = 2,41; $p = .381$; Figure 4). Exclusion of the two outliers did not alter the outcome ($F = 0.819$; df = 2,39; $p = .448$).

4 | DISCUSSION

We set out to determine whether the vertebrate stress axis and corticosterone production are either a cause or consequence of sex reversal in *P. vitticeps*. We found no difference in the basal corticosterone levels of sex-reversed adult dragons or embryos at high temperature during a thermolabile period of development. Exposure to high incubation temperature does induce a temporary acute stress response to temperature *in ovo* during late embryonic development (Stage 12, after gonadal commitment). However, this corticosterone elevation does not occur during a period of development where sexual differentiation is labile, and it does not persist post-hatching or in adults. Our results are consistent with previous work in other reptile species that do not support a role for the vertebrate stress axis in reptile ESD (Iungman et al., 2015; Marcó et al., 2015; Uller et al., 2009; Warner et al., 2009; Wibbels & Crews, 1992).

We did not observe either acute (corticosterone) or chronic (DNA damage) stress axis upregulation in adult sex reversed females (Figure 4). Nor did we observe upregulation of corticosterone at the sexually labile period of development (Stage 7; Figure 1). Here we can confidently conclude that sex reversed females are not inherently more stressed than concordant males and females. This result is contrary to our predictions based on a previous RNA-seq study of sex reversal. This discrepancy could be for several reasons. The previously observed upregulation *POMC* (a pre-protein which signals for stress hormone production) in sex-reversed females could have been biased by random acute upregulation of the stress response during manual handling and/or low sample sizes (Deveson et al., 2017). However, similar *POMC* upregulation occurs in temperature treated fish with a sex reversal system (Yao et al., 2021), corroborating the Deveson et al. (2017) result. We assumed that the observed *POMC* upregulation in sex-reversed females (Deveson et al., 2017) is indicative of a stress response because one of its derived proteins (ACTH) is released into the circulatory system and signals glucocorticoid secretion from the adrenals. However, POMC has a range of other non-stress related biological functions. For example, the POMC pre-protein can be cleaved for roles in melanin production and steroidogenesis (Cawley et al., 2016; D'Agostino & Diano, 2010; Denver, 2009; Narayan, 2017). Thus, it is likely that *POMC* upregulation is a genuine consequence of sex reversal, but activation of the stress response via corticosterone is not.

High incubation temperature did result in elevated corticosterone levels, but only later in development after gonadal commitment. Specifically, Stage 12 embryos incubated at 36°C have significantly higher levels of corticosterone than Stage 7 embryos incubated at 28°C. They were not significantly higher, however, than Stage 12 28°C incubated embryos. The timing of this increase in corticosterone in response to temperature is important because it occurs after the bipotential gonad has transitioned to a committed gonad

(Whiteley et al., 2018). At constant incubation temperature, by developmental Stage 12 the embryo has exited the thermosensitive window where sex can be influenced by environmental stimuli. While nothing is known about the ontogeny of the stress axis in reptile embryos, in chickens the adrenals are capable of low-level autonomous corticosteroid production throughout development. Corticosteroid production does not come under cerebral control until two thirds of the way through development in the egg (Jenkins & Porter, 2004). Our result indicates a similar ontogeny of the stress axis in the beaded dragon. Proximal exposure to high temperature in the egg activates the stress axis late in development when the cerebral components of the stress axis (hypothalamus and pituitary) are integrated fully with the adrenal.

Direct injection of corticosterone (10 and 25 μg doses) did not induce sex reversal in the absence of high temperature exposure (Figure 3). Utilizing sex reversal to detect the effect of our treatment on sex determination is more powerful and more definitive than previous work which relied upon detecting differences in resulting sex ratios (Iungman et al., 2015; Marcó et al., 2015; Warner et al., 2009). Thus, here we are confident in the absence of an effect of our treatment on sex determination.

Experimental approaches that rely upon injecting compounds into the yolk of an egg make the assumption that this is an effective mode of delivery for the embryo. It must be considered that yolk hormone manipulation may not have been taken up by the embryo here and in other reptile experiments (Iungman et al., 2015; Uller et al., 2009; Warner et al., 2009; Wibbels & Crews, 1992), and the lack of a detectable increase in tissue corticosterone concentrations in treated embryos in this experiment (Figure 1) may suggest this. However, strong evidence from this literature supports the successful delivery of corticosterone to the embryo and the lack of an effect of corticosterone on sex. Using identical delivery methods, Wibbels and Crews (1992) demonstrated that a number of estrogenic compounds generated females at male-producing temperatures, but that neither corticosterone nor metyrapone (a corticosterone suppressing drug) affected sex. Despite the absence of an effect of corticosterone/dexamethasone treatment on sex, Iungman et al. (2015), Warner et al. (2009), and Uller et al. (2009) all detected phenotypic differences in hatchlings as a result of treatment.

Given the demonstrated phenotypic effects of corticosterone treatment in similar contexts, the lack of increase in tissue corticosterone could be explained by what we know of yolk hormone movement. The metabolism of corticosterone by the embryo is rapid (Carter et al., 2018), as is the conversion of the steroid from its lipid-soluble to water-soluble forms via reversible sulfonylation (Moore & Johnston, 2008; Paitz & Bowden, 2008). Our tissue corticosterone extractions and measurements targeted only lipophilic free corticosterone, and it is possible that this method obscured differences in levels of corticosterone metabolites. However, increasing corticosterone dosage resulted in decreasing plasma corticosterone concentrations in ZW hatchlings (Figure 2). This suggests that corticosterone was successfully delivered to the embryo and that gross administration of high concentrations may result in a negative feedback loop, suppressing endogenous corticosterone production. A similar manipulation study involving the application of estrogen to alligator eggs raised the possibility of such a mechanism having a suppressive effect on the steroid of interest (Milnes et al., 2002). Further experimentation and method improvement would remove this ambiguity.

We have demonstrated that corticosterone production is not elevated at high temperatures during the temperature sensitive period of sex determination, and that stress axis dysregulation is not an inherent consequence of sex reversal. This agrees with most other studies in reptiles in which no association has been found between corticosterone and sex (Iungman et al., 2015; Marcó et al., 2015; Uller et al., 2009; Warner et al., 2009; Wibbels & Crews, 1992). The theoretical basis of stress-induced sex reversal in fish, which has been borne out in many experimental papers, relies on the androgen-inducing effect of corticosterone production (Straková et al., 2020). In our model species high temperatures induce feminization, and so the paradigm for stress-induced sex reversal in fish cannot be applied directly to *P. vitticeps*. We suggest that the mediator of sex in ESD reptiles is unlikely to be corticosterone but reptilian environmental sex determination may involve cellular, rather than hormonal stress. For example, the reciprocal regulation of calcium levels and reactive oxygen species have the potential to encode and transduce environmental stimuli and influence sexual outcomes (Castelli et al., 2020). Recent experimental evidence supports this hypothesis, with calcium levels found to regulate the epigenetic regulators of sex determination in a TSD turtle (Weber et al., 2020). Due to the apparent lack of a responsive stress axis during the temperature sensitive period in *P. vitticeps*, a biochemical and gonad-autonomous sensor may be a better candidate for the biological transducer of environmental cues in ESD reptiles.

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DATA AVAILABILITY STATEMENT

All data used in this manuscript are provided as a supplementary file.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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SUPPORTING INFORMATION

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