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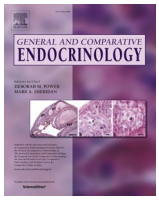

Research paper

Sex steroid profiles align with phenotype in sex-reversed female lizards

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ABSTRACT

Vertebrate sex determination is remarkably diverse. In species with genetic sex determination, reproductive physiology and sex-specific behaviour are influenced by sex chromosomes and sex steroid hormones. However, some species experience sex reversal where the influence of sex chromosomes on sexual development is modulated by mutations or environmental conditions, leading to animals that phenotypically mismatch with their genotype. In these species, sex-reversed ('discordant') individuals can theoretically exhibit traits that are similar to either their phenotypic or genotypic sex. In the central bearded dragon (*Pogona vitticeps*), high incubation temperatures produce sex-reversed females with male-type sex chromosomes (ZZf). ZZf display a mix of male-like and intermediate traits compared to males (ZZm) and concordant females (ZWf). Sex steroid profiles could drive these differences but are yet to be examined in *P. vitticeps*. We measured testosterone (T) and estradiol (E2) in ZWf, ZZf, and ZZm dragons at three timepoints across a breeding season. As sex steroids can potentially affect offspring if maternal hormones are transmitted to eggs, we also compared T and E2 levels in egg yolks from ZZf and ZWf females. Sex-reversed ZZf had lower T levels than males and similar T and E2 levels to ZWf across the breeding season. ZWf and ZZf laid eggs with similar levels of T and E2. We found a negative association between maternal and yolk E2 levels, but no association between maternal and yolk T. Our results show that sex steroid profiles in sex-reversed ZZ female *P. vitticeps* reflect phenotypic, not genotypic sex, suggesting circulating sex steroids do not drive trait differences between discordant and concordant females.

1. Introduction

Vertebrates exhibit remarkably diverse mechanisms for sex determination. Species with genetic sex determination inherit sex chromosomes that initiate either male or female development (Bull, 1980). Early in development, genes carried on sex chromosomes induce the development of testes or ovaries, which produce sex steroid hormones that reinforce sexual development (Capel, 2017; Arnold, 2019). Sex reversal occurs when genetic sex determination pathways are overridden, either through genetic mutations, exposure to endocrine disruptors, or specific environmental conditions (Stelkens and Wedekind, 2010; Weber and Capel, 2018). For example, in some ectothermic vertebrates, sex is influenced by the incubation temperature of developing eggs, which can reverse genetic sex determination (known as "temperature-induced sex reversal"; Quinn et al., 2007; Radder et al., 2007; Hill et al., 2022). This phenomenon differs from temperature-dependent sex determination (occurring in species without sex chromosomes) because individuals who experience temperature-induced sex reversal retain genes linked to their chromosomal sex (Stelkens and Wedekind, 2010; Whiteley et al., 2018). Thus, sex reversal in species with genetic sex determination results in a novel sex class of individuals with mismatched genetic and phenotypic sex, hereafter referred to as 'discordant' individuals (Holleley et al., 2015).

Across species, discordant individuals vary in the degree to which they resemble non-sex-reversed individuals (i.e., 'concordant' individuals). Discordant individuals may display traits that resemble their phenotypic sex (like-phenotype), genotypic sex (like-genotype) or that are intermediate to concordant individuals (Saunders et al., 2016; Dussenne et al., 2022; Wild et al., 2023; see Table 1.). For example, in the central bearded dragon (*Pogona vitticeps*), discordant females (genetic males reversed to the female phenotype) are more similar to males than concordant females in aspects of body size, boldness, and

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thermoregulatory behaviour (Li et al., 2016). Discordant females have metabolic rates that are intermediate to concordant females and males (Wild et al., 2023), and their reproductive output is less than that of concordant females (Wild et al., 2022; Pearson, 2023). In general, sex differences in morphological, physiological, and behavioural traits result from sex-specific differences in genes or gene expression as well as differences in hormone profiles (Arnold, 2019; 2020; Blencowe et al., 2022; Veyrunes et al., 2024). Similarly, in discordant individuals, like-genotype traits may arise from the influence of genes located on sex chromosomes, while traits that are like-phenotype may be more heavily regulated by non-genetic factors such as gonadally-produced sex steroids (Li et al., 2016; Wild et al., 2022; Wild et al., 2023; Heitzmann et al., 2023).

Sex steroids have been hypothesised to be a key driver of like-genotype or intermediate traits in discordant animals (Li et al., 2016; Veyrunes et al., 2024). Although most sex steroids (androgens, estrogens, and progestogens) are produced by the gonads, androgens and estrogens can also be produced through non-gonadal pathways (Barakat et al., 2016; Vajaria et al., 2024). For example, androgens are primarily produced by the Leydig cells in the testes but can also be synthesised in the brain and adrenal glands (Elzenaty et al., 2022). Similarly, estrogens are primarily produced in the ovaries when ovarian theca cells convert androgens to estrogens via aromatase enzymes in neighbouring granulosa cells (Barakat et al., 2016; Xu et al., 2022). However, aromatase is present in many tissues and significant amounts of estrogens are synthesised locally from androgens produced in brain and adipose tissue (Santen et al., 2009; Brann et al., 2022). In discordant females, gene products associated with male sex chromosomes may alter phenotype-like hormone production by increasing the production of non-gonadal androgens or reducing aromatase activity (Cisternas et al., 2018). Both scenarios would increase circulating androgen levels, with the latter also decreasing estrogen levels, resulting in a more male-like hormone profile.

Across taxa, discordant females with male sex chromosomes are often more aggressive, bold, and grow faster (in species with male-biased sexual size dimorphism) making them more akin to their male genotypic counterparts (Ovidio et al., 2002; Li et al., 2016; Saunders et al., 2016; Heitzmann et al., 2023). Such traits are often linked to androgens such as testosterone (Cox et al., 2009; Golinski et al., 2014; Cox et al., 2015; Celec et al., 2015; Lischinsky and Lin, 2020; Veyrunes et al., 2024), suggesting that male-like traits in discordant females could result from elevated levels of testosterone compared to concordant females (Li et al., 2016). Previous research (Table 1) has so far found the sex steroid hormone profiles of discordant individuals to be generally

Table 1
Summary of comparative studies on the hormone profiles of discordant sex-reversed (SR) individuals. Tested hormones include testosterone (T), 11-ketotestosterone (11-KT), 17β-estradiol (E2), cortisol, and corticosterone (Cort). Note that 11-ketotestosterone, rather than testosterone, is the main endogenous androgen in fish, and there is also taxonomic variation in primary glucocorticoids.

Study	Species	SR Trigger	Discordant Phenotype(s)	Discordant Traits	Hormones Tested	Discordant Phenotype Hormones
Rougeot et al., 2004	*Perca fluvialis* **European Perch**	Hormonal masculinisation	XX Male	Abnormal gonadal development (Rougeot et al., 2002) but no effect on reproductive capabilities (Rougeot et al., 2004).	T, 11-KT, E2	All hormones **Like-Phenotype** but XXm show lower 11-KT at beginning of spawning than XYm.
Babiak et al., 2012	*Hippoglossus hippoglossus* **Atlantic Halibut**	Aromatase inhibitor masculinisation	XX Male	No known differences (Babiak et al., 2012).	11-KT	**Like-Phenotype**.
Gennotte et al., 2017	*Oreochromis niloticus* **Nile Tilapia**	Hormonal masculinisation and feminisation	XX Male, XY Female, YY Female	Increased aggressive behaviours in SR XYf and YYf over XXf, but no differences between SR XXm and XYm (Gennotte et al., 2017).	T, 11-KT, E2	All hormones **Like-Phenotype** except XXm higher in 11KT than all other sex-classes and E2 higher in YYf than all other sex-classes (**Novel**).
Hu et al., 2019	*Cairina moschata* **Muscovy Duck**	Incomplete aromatase inhibitor masculinisation	ZW Male	ZWf-like slower growth, and ovotestis rather than testis (Hu et al., 2019).	T, E2	T **Intermediate,** but E2 **Like-Phenotype**
Dussenne et al., 2020; Dussenne et al., 2022	*O. niloticus* **Nile Tilapia**	Masculinising high temperatures	XX Male	More aggressive behaviours but fail to become dominant. Produce different sounds during courtship but not associated with any difference in female preference (Dussenne et al., 2022).	T, 11-KT, E2, Cortisol	T did not differ between sex-classes. E2, 11KT and Cortisol **Like-Phenotype**.
Veyrunes et al., 2024	*Mus Minutoides* **African Pygmy Mouse**	Feminising X chromosome variant	X*Y Female	Alternate maternal strategies (Heitzmann et al., 2023), earlier reproduction and larger litters linked to increased ovulation rate, XYm-like lowered anxiety and increased aggressiveness (Saunders et al., 2016).	Cort, T, E2	E2 and T **Like-Phenotype** but lowered baseline Cort **Like-Genotype**.
Castelli et al., 2021b; this study	*Pogona vitticeps* **Central Bearded Dragon**	Feminising high temperatures	ZZ Female	Increased exploratory behaviour and boldness, longer tails (Li et al., 2016). Possible reduced reproductive success in the wild (Wild et al., 2022), intermediate metabolic rate between ZWf and ZZm (Wild et al., 2023). Fewer, larger eggs over a reproductive season with hatchlings of higher body condition compared to ZWf (Pearson, 2023).	Cort, T, E2	E2 and Cort did not differ between sex-classes. T **Like-Phenotype**.

like-phenotype rather than like-genotype. However, these studies mostly focus on teleost fish with male heterogamety (XX/XY) and female-to-male sex reversal (but see Veyrunes et al., 2024). Systems with female heterogamety (ZZ/ZW), where male-to-female reversals occur, have yet to receive the same level of attention. Improved taxonomic coverage of discordant hormone profiles may provide new insights into the underlying mechanisms behind trait similarities and/or differences between discordant and concordant individuals. Although many aspects of sex determination in vertebrates with genetic sex determination are relatively conserved, there is considerable divergence in sex determining genes and levels of sex chromosome specialisation (Stelkens and Wedekind, 2010; Weber and Capel, 2018). It follows that the mechanisms responsible for discordant traits may also vary across taxa, as unique genetic sex factors interact with developing gonads.

Beyond directly affecting phenotype, sex-steroid profiles in discordant females could have cascading effects on their offspring. This is especially relevant in oviparous species, which comprise most known vertebrate sex reversal systems (amphibians, fish and reptiles; Flament, 2016; Hill et al., 2022; Tenugu and Senthilkumaran, 2022) because maternal hormones can strongly influence early developmental processes (Groothuis et al., 2019; Mouton and Duckworth, 2021). For example, maternal hormones can indirectly affect offspring by regulating the reproductive investment of mothers (e.g., egg size, clutch size; Williams, 2012a; 2012b). Maternal hormones can also directly affect offspring through the hormones females allocate to their eggs (von Engelhardt and Groothuis, 2011a). Maternal hormones can be transmitted to eggs (Radder, 2007; Groothuis et al., 2019) and yolk androgens potentially impact a variety of offspring traits depending on the species, including sex, aggressive behaviour, body condition, and growth (Uller et al., 2007; Müller et al., 2009; Jenni-Eiermann et al., 2020; Valli et al., 2023). Thus, sex steroid profiles in discordant females could affect offspring traits (and potentially fitness) if circulating levels differ from concordant females and maternal hormones are transmitted to eggs.

We tested for differences in sex steroid profiles of discordant and concordant adult females in the central bearded dragon (*P. vitticeps*) across a breeding season. Female *P. vitticeps* are heterogametic, with ZW sex chromosomes; however, male embryos with ZZ sex chromosomes can develop as phenotypic females under hot incubation conditions (>32 °C; Quinn et al., 2007). Discordant females (ZZf) have functional ovaries but differ from concordant females (ZWf) in reproductive output, behaviour, physiology, and morphology (Li et al., 2016; Wild et al., 2022; Wild et al., 2023; Pearson, 2023). Variations in sex steroid profiles may drive phenotypic differences between concordant and discordant female bearded dragons (Li et al., 2016), yet this hypothesis remains untested. Here, we tested how endogenous concentrations of two primary sex steroids, testosterone and estradiol, fluctuate in ZZf, ZWf and males (ZZm) at three points during the 6–9-month breeding season. Additionally, we tested whether testosterone and estradiol from eggs of ZWf and ZZf are correlated with maternal hormonal profiles. Previous work on *P. vitticeps* has shown 'male-like' traits in discordant females (Li et al., 2016; Wild et al., 2023). Therefore, we predicted that ZWf would have higher levels of testosterone and lower levels of estradiol compared to ZZf. We predicted ZZ males would have higher levels of testosterone and lower levels of estradiol than both female types. Finally, we predicted that hormonal profiles in egg yolks would reflect maternal hormonal profiles.

2. Methods

2.1. Experimental animals and husbandry

We used dragons from a breeding colony at the University of Canberra, Australia. Dragons were housed in enclosures with breeding groups of four individuals. Single adult males (ZZm) were housed with three adult females all of the same genotypic sex (either 3 ZWf or 3 ZZf). Dragons were subjected to a 10:14 h light:dark cycle and housed at a constant ambient temperature of 22–18 °C, with temperatures lowered in preparation for brumation at the end of breeding. Enclosures included full spectrum UV bulbs and heat lamps that allowed for behavioural thermoregulation. Dragons were fed *ad libitum* quantities of live crickets (*Acheta domesticus*) dusted with calcium powder and a mixture of shredded vegetables 3 days per week. Females were hand fed approximately 10 g of meat mince mixed with Wombaroo Reptile Supplement (Wombaroo Food Products, Glen Osmond, South Australia) once per week to ensure weight maintenance during reproduction. All methods for husbandry and experimental protocols were approved by the University of Canberra Animal Ethics Committee (AEC 17-13, 2081).

Samples for this research were collected from September 2020–July 2021. During the reproductive season (September-January), females were briefly and gently palpated each week to check for developing eggs. When developing eggs reached approximately 2 cm, enclosures were monitored daily for nesting behaviours. Females were allowed to nest naturally in their cages in a sand substrate. Eggs were removed from the enclosure within 3 h of laying. During the sampling period, 6 of 19 ZW females and 11 of 22 ZZ females produced at least one clutch (hereafter: reproductive females). We analysed data for reproductive and non-reproductive females separately to account for potential hormonal changes associated with reproduction (see below).

2.2. Blood sample collection

Blood samples were collected from a total of 19 ZWf, 22 ZZf, and 15 ZZm dragons over three time points across the 2020–2021 breeding season. The first set of samples were collected in the early breeding season across four days (Sep 29th-Oct 2nd). The second set of samples were collected during the late breeding season (23-25th Nov). For the majority of dragons (n = 32; n = 11 ZZm, 10 ZZf, 11 ZWf), a final non-breeding season measurement was taken on the 6th July 2021, except for n = 17 dragons (n = 3 ZZm, 8 ZZf, 6 ZWf) that were sampled earlier (19th April 2021) due to logistical constraints.

Blood samples were collected between 9am and 12 pm within five minutes of removing a dragon from its enclosure. Blood (~500 μL) was collected from the caudal vein using non-heparinised syringes and haematocrit capillary tubes, transferred to microtubes, and allowed to clot at room temperature for 30 min. After clotting, samples were refrigerated at 4 °C for one hour before centrifuging at 3,000 rpm for 5 min to separate serum, which was transferred to a microtube and stored at −20 °C.

2.3. Serum hormone assays

Serum samples were assayed using Arbor Assay Enzyme Immunoassay (EIA) kits (testosterone: Cat. No. K032; 17β-estradiol: Cat. No. KB030). Serum samples were thawed at 4 °C prior to use in assays. We validated the use of Arbor Assay kits for *P. vitticeps* serum using analysis of covariance to confirm that optical density measurements of serially diluted samples were parallel to assay standards (testosterone: $F_{1,10}$ = 1.83, p = 0.95; estradiol: $F_{1,8}$ = 0.02, p = 0.90; Supplementary Fig. S1.1). We diluted serum samples 1:23.33 to measure estradiol (males and females) and testosterone (females only), and 1:25 to measure testosterone in males. All samples and standards were run in triplicate. Plates were read on a FLUOstar Omega microplate reader at 450 nm. Testosterone levels were calculated from a four parameter 7-point standard curve ranging from 40.96 to 10,000 pg/mL. Estradiol levels were calculated from a four parameter 6-point standard curve ranging from 3.75 to 120 pg/mL. External standards were assayed on each plate to determine inter-assay variation (testosterone: 500 pg/mL; estradiol: 15 pg/mL). We re-assayed n = 31 samples from females for which intra-assay variation was high (percent coefficient of variations (CV) > 15 %). We assayed these samples at a [1:8] dilution to minimize high intra-assay CVs associated with low hormone concentrations. Nine

testosterone samples from females had intra-assay CVs > 50 %. These data were retained in the dataset as 'not available', and accounted for as missing data in our statistical analyses (detailed below in section 2.5). Intra- and inter-plate variation was 15.5 % and 12.6 % for testosterone assays, and 3.1 % and 11.9 % for estradiol assays.

2.4. Yolk sample collection and hormone assays

Three to four eggs from the clutches of eight females (n = 4 ZZf and 4 ZWf) were collected within three hours of deposition. Embryos were excised from eggs with dissection scissors and egg yolks were isolated in falcon tubes and stored at −20 °C. Eggs were generally collected from each female's second clutch of the season, except for two of the ZZf clutches one of which was a first for the season, and the other a third clutch. Yolks were thawed and blended with a tissue homogeniser (LK Lab, HGD-10). We weighed a subset of each yolk (~120—240 mg) to the nearest ± 0.001 mg using a Sartorius microbalance and added 1.5 mL of doubly deionized water to create a yolk solution of known concentration.

We extracted steroids from yolk solutions using solid phase extraction (SPE) with silica bonded vacuum columns (United 8 Chem. Cat. No. CEC18156; methods from Crino et al., 2024). We washed columns twice with 5 mL of doubly deionized water prior to running 1 mL of yolk solution through the columns. Then, we washed columns with 5 mL of 40 % methanol to remove lipids and then soaked columns in 100 % methanol for two minutes before eluting samples with vacuum filtration. We dried sample extractions under nitrogen gas at 35 °C (Organomation Classic MICROVAP) and stored desiccated samples at −20 °C overnight.

We tested assay parallelism for yolk samples using two serially diluted pooled yolk samples ranging from [1:1] to [1:16] (range of yolk concentration of 7.5 – 240 mg/mL). For estradiol, the optical density measurements for the pooled sample were not parallel to the standard curve when all dilutions were included ($F_{1,10}$ = 8.80, p = 0.02; Supplementary Fig. S1.2). However, optical density measurements for the pooled sample were parallel to the standard curve for dilutions from [1:1] – [1:8] ($F_{1,9}$ = 0.0042, p = 0.95). Similarly, for testosterone, the serial dilution curve was not parallel to the standard curve when all dilutions were included ($F_{1,10}$ = 26.54, p < 0.001; Supplementary Fig. S1.2) but was parallel for dilutions from 1:1 – 1:4 ($F_{1,9}$ = 2.74, p = 0.15). To ensure yolk concentration did not affect hormone measures, we reconstituted all samples at a [1:1] resulting in a yolk solution concentration from 95 – 165 mg/mL (see below).

To measure extraction efficiency, we stripped hormones from a pooled yolk solution using a 1 % Dextran coated charcoal solution (Sigma, Cat. No. C6241). We spiked aliquots with testosterone (200 pg/mL) or estradiol (9.0 pg/mL) and used SPE to extract hormones from spiked and control aliquots (methods as above). We assayed a spiked and control samples on each EIA plate and compared the percentage of hormone recovered compared to the control sample to determine extraction efficiency. The average extraction efficiency was 55.45 % (std dev = 0.04) and 28.34 % (std dev = 0.01) for testosterone and estradiol respectively.

Extracted yolk samples were reconstituted in 1 mL of assay buffer for both testosterone and estradiol assays. Yolk samples were assayed across three plates each for testosterone and estradiol, using methods identical to those for serum samples. Intra-assay variation was 5.8 % and 2.1 % for testosterone and estradiol (respectively). Inter-assay variation for testosterone was 37.1 % due to the inter-assay standard on one plate which was ~ 1.67 times higher than expected (inter-assay CV 6.3 % for remaining two plates). Similarly, the inter-assay standard for one estradiol plate was ~ 1.49 times higher than expected, resulting in an initial inter-assay CV of 25.9 % (inter-assay CV 1.8 % for remaining two plates). Both plates with high inter-assay standards were assayed on the same day. To account for these high measurements, we adjusted all yolk hormone values by the percent difference between the observed and expected values of the inter-assay standard (Table S2.1). We then corrected hormone values by the concentration of the yolk hormone solution to obtain final hormone values for statistical analyses.

2.5. Statistical analysis

Hormone concentrations were log-transformed before use in statistical analysis. Data were analysed using linear mixed-effects models in R version 4.3.2 (R Core Team, 2023), using the packages *brms* (Bürkner, 2017) and *lme4* (Bates et al., 2015). Mean and 95 % CI are reported for all data and α was set to 0.05 for evaluating statistical significance.

Throughout the experiment, reproductive females laid 1–4 clutches asynchronously. There was no significant difference between the proportion of females who became reproductive in each genotype (X^2 = 1.374, df = 1, p = 0.241). However, the mean first clutch date of ZZ females was significantly later than that of ZW females (t = -3.109, df = 10.011, **p = 0.011**) suggesting that reproductive ZZ and ZW females were at different reproductive stages during the three sampling dates. To account for potential differences in reproductive stages, we used inferred reproductive state (days since most recent clutch) as a predictor for the reproductive female model, whereas sampling date was in the model for non-reproductive females (see below).

Hormone levels in males and non-reproductive females were compared over the three sampling periods with a multiresponse Bayesian mixed-effects model, accounting for individual identity and ELISA plate identity as random effects. This analysis jointly models log testosterone and log estradiol as response variables which allowed for the estimation of the correlation between these two hormones within and among individuals, after accounting for the effect of predictor variables (O'Dea et al., 2022). The model also included an interaction term between genetic sex and sampling period to test for differences in how hormone concentrations changed over time for each sex class for each response variable (testosterone/estradiol).

Not all individuals had samples available for all three timepoints because of missing data resulting from uncollected, lost, or degraded samples. In addition, some testosterone concentrations in ZZ and ZW females which were missing due to a) high %CV in a sample, and b) limited sample volume. In the non-reproductive model, seven missing samples (2 ZZf, 5 ZWf) had no testosterone plate ID, as the sample was only run for estradiol. A further three missing samples (1 ZZf, 2 ZWf) were due to high %CV (>50 %). While such missing data should not result in any bias as it was largely random, it can result in a loss in statistical power (Noble and Nakagawa, 2021). As such, we accounted for missing testosterone data in our models. Prior to running the non-reproductive model, missing testosterone concentrations and/or testosterone plate IDs were imputed 50 times using the package *mice* (m = 50; van Buuren et al., 2024), with imputed values based on all variables in the dataset. These 50 imputed datasets were then run through the non-reproductive model, and the posterior distributions pooled for final model results. All figures show only complete data, but all statistical models account for uncertainty in missing data.

Hormone levels in reproductive ZZ and ZW females were compared using a similar model to that used for non-reproductive females and males, but instead of sample period, the second predictor variable was a proxy for reproductive state (days since most recent clutch; Fig. 3). To infer reproductive state, we assumed that females at similar distances from their most recent reproductive event (laying of a clutch) were of a similar reproductive stage. We then calculated the distance between a female's three sampling dates and her most recent clutch. The result was an index, either negative (before a clutch) or positive (after last clutch) related to each reproductive female's hormone samples (max. n = 3). The model for reproductive females was simplified, and did not include the interaction term between genotype and days since most recent clutch. This improved power to test for differences in hormone levels between sex class given modest and unbalanced sample sizes (n = 6 ZWf and n = 11 ZZf). To test for changes in reproductive female hormones over time, we selected three index points that correlated to the average

reproductive index for each of our original three sampling periods: 20 days before most recent clutch (Sep-Oct), 40 days after most recent clutch (Nov), and 190 days after most recent clutch (April/Jul). Mean log hormone concentration was then contrasted both over these three reproductive timepoints, and between the two female types. Again, the multivariate model allowed for calculation of residual correlation between testosterone estradiol within a sample (Fig. 2). In this reproductive model, six missing female testosterone samples were all due to high %CVs (>50 %). As such we used data augmentation during model fitting (i.e., including the term *mi()*) to impute these missing data based on all other parameters (genotype, sample period, individual ID, and plate number).

We used a linear mixed model to test for differences in yolk hormone concentrations in relation to maternal genotype (Fig. 4a–b). We used yolk hormone levels as the dependent variable, maternal genotype as a fixed effect, and maternal identity as a random effect to account for data from multiple eggs (3–4) within a clutch. High between mother variation, relative to the total variation, indicated that variation in egg yolk hormones could be explained by mother identity. To determine the variation explained by maternal identity, we calculated bootstrapped repeatability estimates of the model's random effect using the *rptR* package (Stoffel et al., 2017; Fig. 4c–d). We used linear regression models to look at the relationship between average maternal circulating hormone levels, and the average hormone levels of her clutch. Maternal T or E2 concentration was used as the predictor variable, and clutch T or E2 concentration as the dependent variable.

3. Results

3.1. Comparison of ZZ males and non-reproductive ZZ and ZW females

Discordant females (ZZf) that did not reproduce over the breeding season had hormone profiles like those of concordant ZW females (like-phenotype; Fig. 1). Mean testosterone levels for both ZZ and ZW females were lower than those of ZZ males across all three sampling points. However, regardless of genotype, there was overlap between females with relatively high testosterone and males with relatively low testosterone early in the breeding season (ZZf: 38.3–4845.0 pg/ml, ZWf: 142.5–3165.0 pg/ml, ZZm: 2425.0–90517.5 pg/ml; Fig. 1a). Mean testosterone levels decreased from the early to late breeding season in both ZZ and ZW females (first and second samples; ZWf: model estimate = 1.669, CI = [0.699, 2.672], **p < 0.001**; ZZf: model estimate = 1.188,

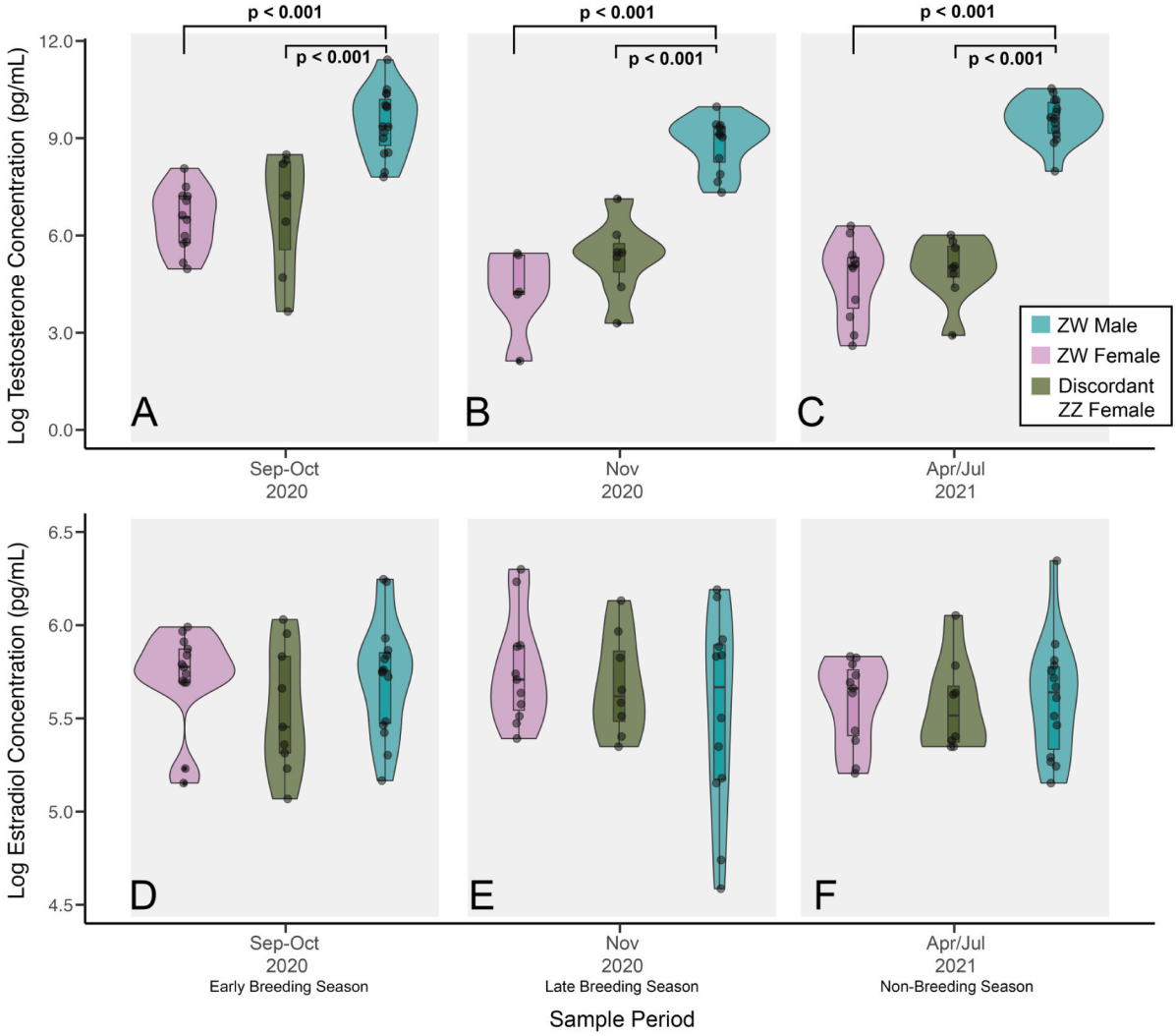

Fig. 1. Testosterone (A–C) and estradiol (D–F) concentrations from male and non-reproductive female *Pogona vitticeps* at three sampling periods across the 2020/2021 breeding season. Points show individual measurements, and violin plots the density distribution of points within a group. Boxplots within each violin plot show medians, lower and upper quartiles. Significant differences between the groups at each timepoint are highlighted with p-values from a Bayesian mixed-effects model accounting for certain missing samples and ELISA plate number. The three timepoints correlate to an early breeding season measurement, an end of breeding season measurement, and a non-breeding season measurement, respectively.

CI = [0.276, 2.102], **p = 0.01**). There was no difference between the mean testosterone levels of females in the late breeding season and non-breeding season (ZWf: model estimate = 0.084, CI = [-0.894, 1.022], p = 0.86; ZZf: model estimate = 0.590, CI = −0.330, 1.538], p = 0.22). Testosterone levels in males also decreased between the early and late breeding season (model estimate = 0.790, CI = [0.145, 1.448], **p = 0.02**). However, their testosterone levels during the non-breeding season were no different from either the early or late season timepoints (early: model estimate = -0.675, CI = [-1.371,0.048], p = 0.06; late: model estimate = -0.115, CI = [-0.794, 0.569], p = 0.74), suggesting that ZZ male testosterone levels decrease following reproduction, but gradually increase in the non-breeding season prior to reproduction.

Mean estradiol levels did not differ between ZWf and ZZf across the breeding season (ZZf: 158.7 – 459.7 pg/ml, ZWf: 172.7 – 543.7 pg/ml, p > 0.14). Similarly, there were no differences between mean estradiol levels in females and males across the breeding season (ZZm: 98.0 – 569.3 pg/ml, p > 0.10). Both ZWf and ZZf showed no change in estradiol across time (ZZf: p > 0.28, ZWf:, p > 0.20), but there was still a significant interaction between sex-class and time as estradiol levels in males decreased between the early and late breeding season (first and second timepoints; model estimate = 0.224, CI = [0.019, 0.417], **p = 0.03**). Tables showing the full model results are available in the Supplementary Material (Table S3.1 and S3.2).

Among individual samples for males and non-reproductive females, testosterone and estradiol measurements were not significantly correlated (within-sample residual correlation = 0.21 [-0.08, 0.48]; Fig. 2d–f).

3.2. Comparison of hormonal profiles of reproductive ZZ and ZW females

Reproductive discordant ZZ female dragons showed no difference from reproductive concordant ZW females in their concentrations of testosterone (model estimate = -0.375, CI = [-1.453, 0.695], p = 0.46; Fig. 3a) and estradiol (model estimate = 0.020, CI = [-0.240, 0.290], p = 0.89; Fig. 3b). As a group, reproductive females did not show changes in hormone concentrations at different stages of reproduction (between −20, 40 and 90 days since most recent clutch; Table S4.2; Fig. 3). Tables showing the full model results are available in the Supplementary Material (Table S4.1 and S4.2).

Unlike males and non-reproductive females, the testosterone and estradiol measurements in individual samples from reproductive females were significantly correlated (within-sample residual correlation = 0.53 [0.12, 0.79]; Fig. 2a–c).

3.3. Comparison of yolk hormones from ZZ and ZW female clutches

There were no differences in yolk hormone levels of eggs from ZW and ZZ females (Testosterone; p = 0.480, Estradiol; p = 0.640;

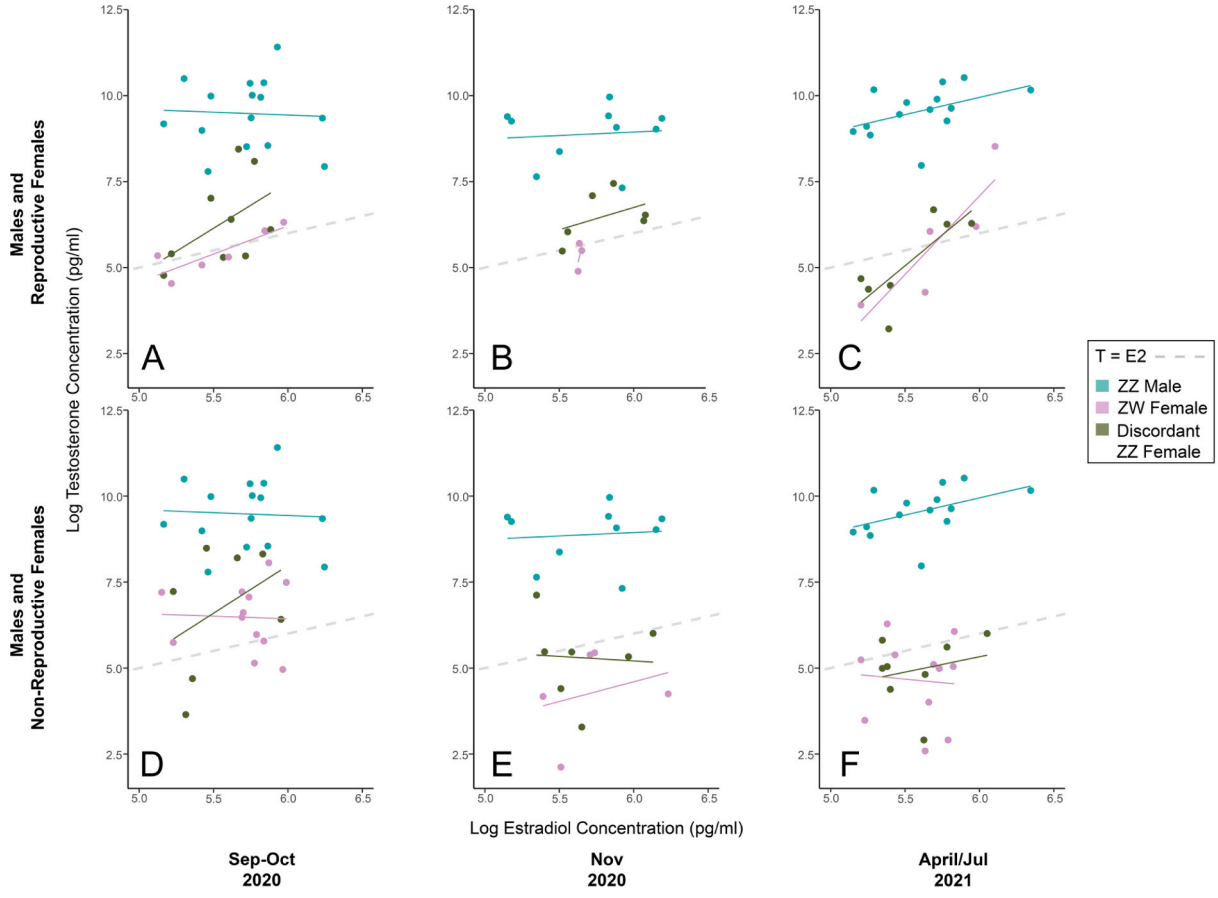

Fig. 2. Relationship between an individual's testosterone (T) and estradiol (E2) measurements from each sample, at each sampling period. The top panel (A-C) shows the T and E2 measurements of reproductive females, contrasted against those of males. The bottom panel (D-F) shows the T and E2 measurements of non-reproductive females, contrasted again with those of males for reference. Each point shows an individual's measurement, with each sampling point presented separately. Lines of best fit are shown for each sex-class (ZZm, ZWf, or discordant ZZf) at each time point. The dashed grey lines show a relationship where concentrations of T and E2 are equal, with individuals below the line thus higher in E2 than T, and vice versa. Note that the model for reproductive females used 'days since last clutch' rather than sampling point. Residual correlation coefficients from the multiresponse models were used to test whether T and E2 concentrations covaried within individual samples, after accounting for the effect of model predictor variables. E2 concentrations were not significantly correlated in samples from males and non-reproductive females (Model residual correlation estimate = 0.21 [-0.08, 0.48]) but were significantly correlated in samples from reproductive females (Model residual correlation estimate = 0.53 [0.12, 0.79]).

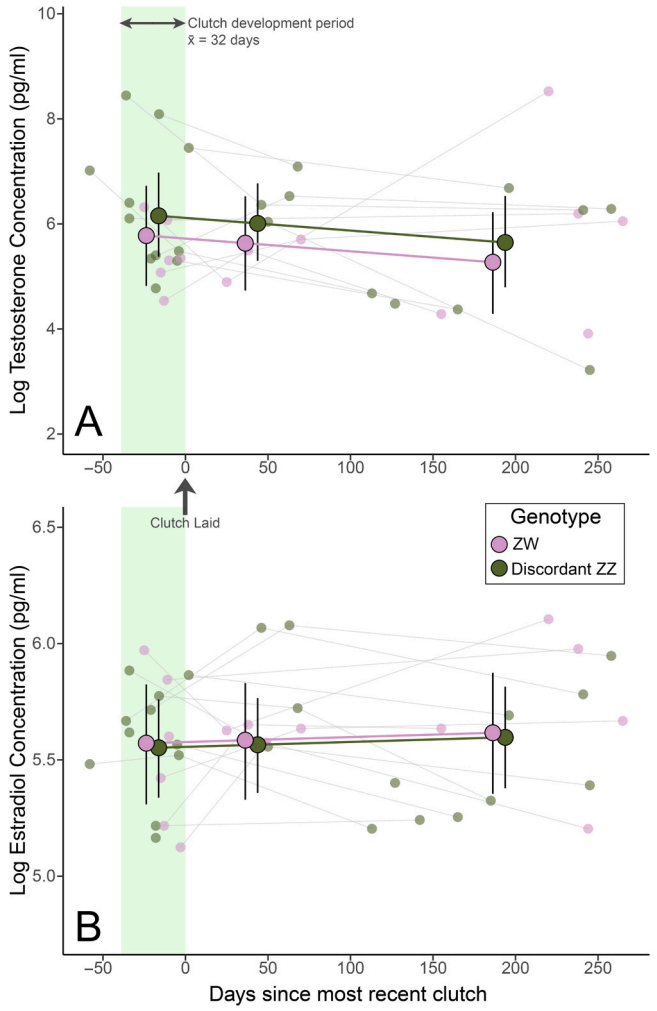

Fig. 3. Relationship between the hormone concentrations of reproductive ZZ and ZW females and their inferred reproductive state, for testosterone (A) and estradiol (B). Individual points show hormone measurements taken in one of the three sampling periods from the main analysis. Grey lines link repeated measurements in the same individual. The X axis shows inferred reproductive state through an index of days since most recent clutch, which is negative for females who would go on to lay a clutch after that sample was taken. The period highlighted in green from –32 to 0 days represents the likely period in which sampled females were actively developing a clutch of eggs, derived from the mean interclutch period recorded in females from this study (26–39 days, n = 12). The bolded points show the predicted mean hormone concentrations for each genotype at −20 days since most recent clutch, 40 days, and 190 days, respectively. Predicted means are based on the posterior distribution from a Bayesian mixed-effects model accounting for certain missing data and ELISA plate number. Note that the Y axis scaling is different for each hormone as changes in estradiol were much smaller in magnitude than those for testosterone.

Fig. 4a–b). Hormone profiles of eggs from the same clutch were more similar than profiles of eggs from different clutches, although there were large confidence intervals on the effect size (bootstrapped intra-class correlation coefficient of maternal identity: Testosterone; R = 0.438, CI = [0.000, 0.746], **p = 0.022,** Estradiol; R = 0.453, CI = [0.000, 0.748], **p = 0.011**; Fig. 4c–d).

There was a positive association between yolk testosterone and estradiol levels from individual eggs (slope: adjusted $R^2 = 0.531$, **p < 0.001**; Fig. S5.1). We found a negative association between maternal estradiol levels and average yolk estradiol levels (adjusted $R^2 = 0.502$, **p = 0.030**; Fig. 4f). However, there was no association between maternal testosterone levels and clutch average testosterone levels (adjusted R^2 = -0.082, p = 0.519; Fig. 4e).

4. Discussion

Our results provide the first description of hormonal profiles of *Pogona vitticeps* across a breeding season and suggest that concordant ZW and discordant ZZ females do not differ in sex steroid profiles. Past studies have found that discordant female *P. vitticeps* (ZZf) display ZZ male-like behavioural and physiological traits (Li et al., 2016; Wild et al., 2023). Although male-type traits can be driven by testosterone (Sakata et al., 2003; Ketterson et al., 2005; Crews et al., 2009; Golinski et al., 2014; Cox et al., 2015), we found no differences in testosterone levels between ZZ and ZW females across a breeding season. Likewise, we found no differences in yolk hormone levels between clutches produced by ZZ and ZW females. As expected, males had higher testosterone levels than ZZ and ZW females across all timepoints. Together, these results suggest that discordant ZZ females have sex steroid profiles that are like-phenotype rather than like-genotype. Our results add to a small but growing number of studies that have found the like-phenotype sex steroid profiles in discordant individuals. Differences in behavioural and physiological traits between discordant individuals and their concordant phenotypic counterparts do not appear linked to differences in circulating sex-steroid concentrations. This seems to persist across systems with varied taxonomy, heterogamety, and sex reversal triggers (Table 1.).

In our study, discordant ZZ females had similar hormonal profiles to concordant ZW females and, thus, endogenous sex steroid levels are unlikely to be solely responsible for behavioural differences between female genotypes (ZZ/ZW) in *P. vitticeps*. However, sex steroids could still modulate trait differences if ZZ and ZW females display different hormone receptor profiles that change the way individuals respond to sex steroids without altering circulating hormones. Differences in receptor profiles could be driven by organizational effects where exposure to sex steroids at key developmental stages programs an individual's future sensitivity to those hormones (Goel and Bale, 2008; Arnold, 2009; Lagunas et al., 2022). Discordant ZZ female *P. vitticeps* begin development as male and then transition to the female development pathway, which involves a temporary ovotestis structure (Whiteley et al., 2022). In reptiles and other vertebrates, embryonic gonads can produce sex steroids and exposure to sex steroids during development can have masculinising effects that are sustained into adulthood (De Ridder et al., 2002; Crews et al., 2009; Arnold, 2009; Huber et al., 2017). It is currently unknown if the ovotestes in *P. vitticeps* produce androgens and, if so, whether prenatal exposure to elevated levels of androgens has masculinizing effects. In laboratory conditions, sex reversal in *P. vitticeps* is induced through exposure to consistent temperatures of 32 °C and above. However, in natural settings, *P. vitticeps* are likely to experience fluctuating temperatures during development (Castelli et al., 2021a), which can affect development rate, and, in turn, the amount of time sex-reversed females retain ovotestes (Whiteley et al., 2017; Whiteley et al., 2022). To determine the functional implications of ovotestes in free-living organisms, future research should investigate how varying incubation temperatures influence their development, and whether or not ovotestes secrete androgens during development.

Organisational effects stemming from embryonic exposure to sex steroids and sex-specific gene expression could also drive behavioural differences between concordant and discordant individuals through changes in neural development (Goel and Bale, 2008; Williams and Carroll, 2009; Filová et al., 2013). The influence of sex chromosomes in particular may be more influential in brain sexual dimorphism than has been previously assumed. Recent studies with sex reversed transgenic mouse models (*Mus musculus*) have highlighted how neural development can proceed according to genetic sex, rather than gonadal sex, in sex-reversed individuals (Arnold, 2020). For instance, aromatase expression is higher in XY (male-type) than XX (female-type) neurons in the amygdala and stria terminalis (regions known to regulate fear,

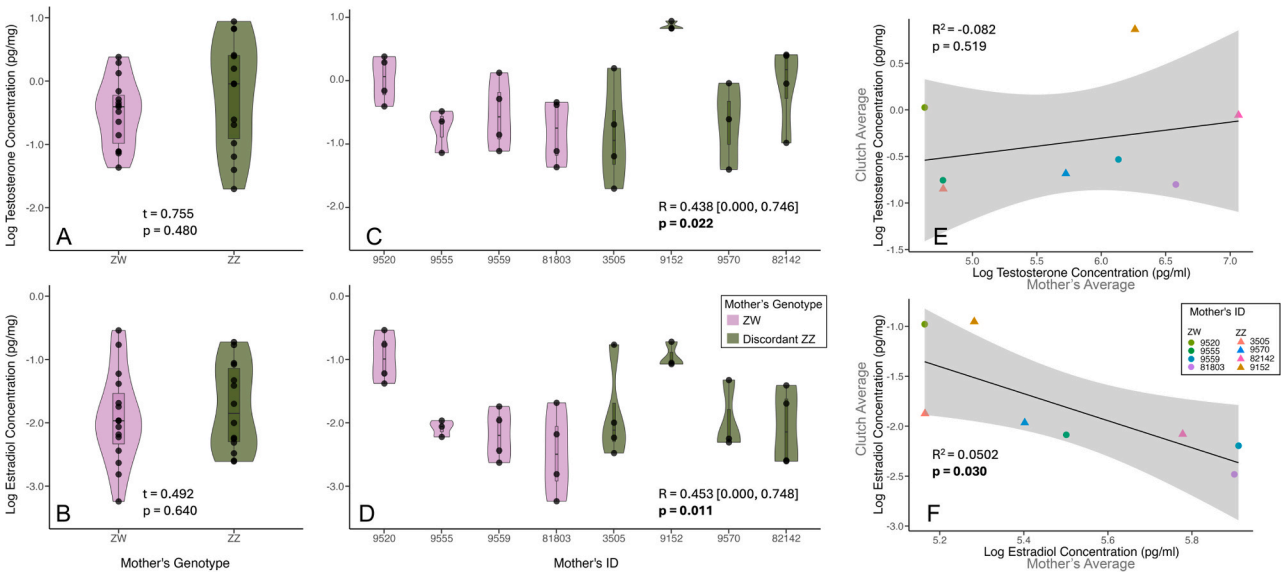

Fig. 4. Sex steroid profiles of egg yolks from discordant ZZ and concordant ZW mothers. A). Measurements of testosterone in the yolk of 3–4 eggs from the clutches of four ZW and four SR ZZ female *Pogona vitticeps*, grouped by maternal genotype. Points show individual egg measurements, violin plots the density distribution, and boxplots the medians, lower and upper quartiles of each group. B). As for A, but for estradiol. C.) Yolk testosterone measurements grouped by clutch, labelled with mother's identity and genotype. D.) As C, but for estradiol. E.) Regression of mother's mean testosterone measurement (calculated from successful measurements, maximum n = 3; see Fig. S5.2) against that of her clutch's mean testosterone measurement, as indicated by the boxplot in C. Grey shading shows 95 % confidence interval. F.) As E, but for estradiol.

aggression, and social responses; Lebow and Chen, 2016; Lischinsky and Lin, 2020). This facilitates sex-specific differences in estrogen signalling and drives sexual dimorphism in certain brain regions (Cisternas et al., 2015; Cisternas et al., 2017; Cisternas et al., 2018). The same mouse models have demonstrated a role for both gonadal and genetic sex in regulating brain androgen and estrogen receptor expression (Cisternas et al., 2015; Cisternas et al., 2017; Cisternas et al., 2018; Arnold, 2020). In *P. vitticeps,* neural changes during development could affect regulatory pathways that affect androgen uptake or sensitivity, potentially driving male-like behaviours, morphology and physiology in discordant females (Li et al., 2016; Cox et al., 2009).

Although there is accumulating evidence that sex steroid levels do not drive trait differences in animals with flexible modes of sex determination (Table 1), it is possible discordant behaviour is regulated by other steroid hormones. Although we found no differences in estradiol and testosterone levels between concordant and discordant females, we did not measure progesterone or androgenic or estrogenic metabolites (e.g., dihydrotestosterone or estrone). These hormones / metabolites are known to regulate behavioural and physiological traits in other species (Ball and Balthazart, 2007; Sze and Brunton, 2020; Aspesi et al., 2025). We further found that estradiol levels did not change over the breeding season for either ZW or ZZ females, suggesting that estrogenic metabolites or progesterone may be more important for driving female reproductive physiology (and possibly discordant traits) in *P. vitticeps* (Amey and Whittier, 2000; Zena et al., 2020). Future studies could use gas chromatography-mass spectrometry to comprehensively evaluate differences in sex steroids (including progesterone) and estrogenic and androgenic metabolites across reproductive stages in *P. vitticeps* (de Kock et al., 2018).

Glucocorticoids (i.e., 'stress hormones') are another class of steroids that can influence behaviour and physiology in sex-specific ways potentially explaining differences between concordant and discordant individuals (Jennings et al., 2000; Veyrunes et al., 2024). In the African pygmy mouse (*Mus minutoides*), discordant XY females and concordant XY males have lower baseline corticosterone levels than concordant XX females (Veyrunes et al., 2024). Lower glucocorticoid levels have been linked to decreased anxiety and increased aggression in XY males and females, in comparison with XX females (Saunders et al., 2016; Heitzmann et al., 2023). Yet, glucocorticoid profiles do not vary between concordant and discordant individuals in all systems. For example, cortisol levels in discordant XX male Nile tilapia (*Oreochromis niloticus*) are similar to concordant XY males (Dussenne et al., 2022). Similarly, in *P. vitticeps*, previous work by Castelli et al. (2021b) found no differences in baseline corticosterone between adult ZZ males, ZW females, and ZZ females. However, the two female genotypes could differ in patterns of elevated (stress-induced) corticosterone and/or levels of corticosteroid-binding globulin (CBG) that affect the availability of corticosterone to target tissues (Breuner et al., 2020). In these ways, variation in ZZ and ZW female *P. vitticeps* glucocorticoid responses could lead to their divergent behaviour, including the increased boldness of ZZ females (Li et al., 2016).

In addition to steroids, other classes of hormones could drive discordant traits; for example, the nonapeptide hormones arginine vasotocin (AVT) and the mammalian homologue arginine vasopressin, are important in regulating social and aggressive behaviours (Wilczynski et al., 2017; Campos and Belkasim, 2021; Kabelik et al., 2022). AVT neurons show sex- and dominance-specific patterns in terms of size and abundance in different brain regions (Wilczynski et al., 2017; Lischinsky and Lin, 2020). Unique AVT neuron patterns have already been observed in discordant individuals from two species, both of which show differences from concordant individuals in their aggressive and social behaviours. Discordant XX male Nile tilapia (*Oreochromis niloticus*) show fewer or larger AVT neurons in some areas of their hypothalamus compared to concordant XY males and XX females (Dussenne et al., 2020). Heitzmann et al. (2023) observed a similar trend for discordant XY female African pygmy mice (*Mus minutoides*) to have lower numbers of arginine vasopressin neurons, also in an area of the hypothalamus, compared to concordant XY males and XX females. As juveniles, discordant ZZ female *P. vitticeps* are bolder and more exploratory than both concordant ZZ males and ZW females (Li et al., 2016). This could be evidence of unique interactions between female-type hormone profiles (as we observed here) and masculinised brain structures resulting from the ZZ genotype.

Consistent with our results showing no differences in steroid levels between ZW and ZZ females, we found no differences in yolk steroid levels in their clutches (Fig. 4a–b). Despite this, we found that yolk

steroid levels varied due to maternal identity (estradiol and testosterone) and were negatively associated with maternal levels (estradiol only) such that mothers with relatively high estradiol laid eggs with relatively low estradiol (Fig. 4f). Maternal sex steroids can be transferred to developing eggs from the surrounding follicle wall as well as from neighbouring follicles and maternal circulation (Groothuis and Schwabl, 2008; von Engelhardt and Groothuis, 2011b). The most parsimonious hypothesis to explain the relationship between maternal and yolk steroids predicts a positive correlation between steroid levels because of the lipophilic properties of steroids and egg yolk (i.e., the *physiological epiphenomenon hypothesis*; Groothuis and Schwabl, 2008). However, there is mixed support for this hypothesis with studies finding that maternal and yolk hormones can be positively correlated (Badyaev et al., 2005), negatively correlated (Williams et al., 2005), or not correlated (Marshall et al., 2005). Negative correlations between maternal and yolk hormones suggest that females can facultatively regulate steroid deposition in eggs, potentially leading to trade-offs between the optimal steroid levels for mothers and their offspring (i.e., the *flexible distribution hypothesis*; Groothuis and Schwabl, 2008). Embryonic exposure to estradiol and other steroids can have sustained effects on physiology, behaviour, and neural function (von Engelhardt and Groothuis, 2011b; Mouton and Duckworth, 2021). Together, our results suggest the potential for hormone-mediated maternal effects in *P. vitticeps* to be driven by variation in maternal estradiol levels, even though maternal estradiol levels do not appear to vary between ZZ and ZW females.

Although we did not find a correlation between maternal and yolk testosterone levels, we did find a positive correlation between yolk testosterone and yolk estradiol levels (Fig. S5.1). In yolk, estradiol can be derived from testosterone by the steroidogenic enzyme aromatase (Lance, 2009; Singh et al., 2020). In our experiment, it is possible that we did not find a correlation between maternal and yolk testosterone levels due to the embryonic conversion of testosterone to estradiol. The metabolism and conversion of steroids to their derivatives can be affected by the temperature embryos experience during development (Elf et al., 2002). It is noteworthy that eggs in our experiment were all housed at a standard ambient temperature (22 °C) in the < 3 h prior to collection. Our results suggest that ZW and ZZ females do not have different levels of endogenous hormones as adults (Fig. 1). However, it is possible that developing ZZ females are exposed to different profiles of yolk hormones than ZW females due to temperature-mediated metabolism of yolk hormones, as sex reversal in *P. vitticeps* occurs when males are exposed to elevated temperatures during development (Quinn et al., 2007). This is supported by evidence in other reptiles for incubation temperature regulating responses to steroid hormones in adulthood (Crews et al., 2009). Future studies could test this hypothesis by measuring yolk hormone levels from ZW and ZZ eggs exposed to different temperatures during development and genotype specific expression in aromatase levels.

Here, we provide the first evidence in *P. vitticeps* that sex-reversed ZZ females do not differ from non-sex reversed ZW females in sex steroid profiles. Our results suggest that estradiol and testosterone do not underlie trait differences between concordant and discordant females in *P. vitticeps* described in past studies. However, sex steroid metabolites, glucocorticoids, and hormone receptor expression could regulate trait differences and future studies that examine other endocrine traits are warranted. Additionally, future studies should focus on understanding how organisational genetic sex effects act on the gonads and brain to shape trait differences between concordant and discordant individuals. Understanding the mechanisms that underlie trait development and expression in discordant individuals will allow for a better understanding of how and why sex reversal evolves and help predict how environmental conditions such as climate change shape population-level effects in species with sex reversal (Schwanz et al., 2020).

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CRediT authorship contribution statement

Naomi E. Laven: Writing – review & editing, Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Data curation. **Phillip R. Pearson:** Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Kristoffer H. Wild:** Writing – review & editing, Supervision, Conceptualization. **Daniel W.A. Noble:** Writing – review & editing, Supervision, Formal analysis. **Ondi L. Crino:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2025.114754.

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