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Sex Reversal and Temporary Pseudohermaphroditism: Complex Sexual Development in the Central Bearded Dragon (Squamata: *Pogona vitticeps*)

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Statement of Authorship

The research carried out in the course of this investigation and the results presented in this report are, except where acknowledged, the original work of the author, and all research was conducted during the Honours program.

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Abstract

Sex determination in reptiles is typically controlled either by sex chromosomes (genetic sex determination) or by temperature (temperature dependent sex determination). But it is not always so simple: *Pogona vitticeps* (Central Bearded Dragon) can switch from GSD to TSD within one generation, as high temperatures induce genetic males to reverse their sex and develop as females. This unusual trait makes possible to assess both genetic and temperature influences on sexual development in this species. To do this, I designed four treatments where eggs from concordant (ZW) or sex reversed (ZZf) mothers were incubated at either high (36˚C) or low temperatures (28˚C). This provided developmental baselines for both genetic and temperature controlled development, including sex reversal. I describe embryogenesis of *P.* vitticeps for the first time using a multidisciplinary approach that incorporated morphological staging analyses, gonadal histology, scanning electron microscopy, and embryonic genotyping. I found that regardless of the sex determining cue (genetic or temperature), female development is characterised by a prolonged period of temporary pseudohermaphroditism (TPH) where female gonads (ovaries) are present at the same time as masculinised genitalia (bilobed hemipenes, reduced hemipenes and hemiclitores). This may be because ovarian hormone secretion is delayed, or the genitalia is unreceptive to the hormones. This finding is contrary to the commonly held assumption that gonad differentiation and genital development are closely linked. Hence, this research provides unprecedented insight into embryogenesis under both TSD and GSD in *P. vitticeps,* revealing that female sexual development is considerably more complex than currently appreciated.

Key words: sex differentiation; sex determination; sex reversal; gonads; genitalia; embryonic development; *Pogona vitticeps.*

1. Introduction

1.1. Sexual Development in Squamates

One of the most fundamental aspects of any sexually reproducing organism is its phenotypic sex, as this profoundly influences many aspects of its life history, and eventual reproductive success (Norris and Lopez 2011). In vertebrates, the development of sexual phenotypes is a highly conserved developmental process resulting in the growth of gamete-producing endocrine glands (gonads) and external genitalia (Andrews 2004; Kohno et al. 2014). In squamates, a diverse vertebrate radiation that includes lizards and snakes, sexual development is controlled by a variety of mechanisms resulting from a dynamic evolutionary history. These can be broadly categorised into temperature dependent sex determination, and genetic sex determination (Morrish and Sinclair 2002; Valenzuela and Lance 2004; Rhen et al. 2005; Norris and Lopez 2011; Bachtrog et al. 2014; Kohno et al. 2014; Matsumoto and Crews 2016).

Under temperature dependent sex determination (TSD), the sex of the embryo is determined by the temperature it is exposed to during development. TSD is found in all crocodiles, many turtles, and some lizards (Pieau et al. 1998; Harlow 2000; Ezaz et al. 2009; Wise et al. 2009; Holleley et al. 2015; Gomez-Saldarriaga et al. 2016). The exact molecular mechanisms of TSD is still unknown, but is thought to act during the thermosensitive period (TSP), the developmental period when temperature can influence sex. The TSP typically spans the middle third of development, but can vary in different lineages (Ezaz et al. 2009; Wise et al. 2009; Rhen and Schroeder 2010; Matsumoto and Crews 2012; Merchant-Larios and Diaz-Hernandez 2013; Holleley et al. 2015; Gomez-Saldarriaga et al. 2016; Sun et al. 2016). Temperature can have highly variable effects on sex determination even in closely related species, suggesting that there may be a variety of undescribed thermally sensitive genetic sex determination mechanisms (Alder 1981; Webb and Smith 1984; Webb et al. 1987; Etchberger et al. 1991; Packard et al. 1991; Crews and Bergeron 1994; Ewert et al. 1994; Viets et al. 1994; Crews 1996; Crews et al. 1996; Valenzuela 2008; Warner and Shine 2008; Matsumoto et al. 2013; McCoy et al. 2015; Gomez-Saldarriaga et al. 2016; Matsumoto and Crews 2016; Sun et al. 2016).

Compared to the diversity of squamates with TSD, fewer species are known to possess a genetic sex determining (GSD) system (most snakes and some lizards; Viets et al. 1994; Raman 2002; Valenzuela 2008; Wapstra and Warner 2010). Thus far, research on GSD in squamates has focused on comparing genes controlling the development of sexual phenotypes with better studied mammalian systems (Western et al. 1999; Valleley et al. 2001; Shoemaker et al. 2007; Gredler et al. 2015; Leal and Cohn 2015). While there are some genetic similarities between squamates and mammals, there are also critical differences. Thus, much remains to be learnt about the genetic underpinnings of squamate sexual development (Andrews 2004; Leal and Cohn 2014; Gredler et al. 2015).

1.2. Squamate Sexual Development: Gonads and Ducts

Regardless of the mechanism (GSD or TSD), the process by which bipotential gonads are committed to a cellular fate (primary sex determination), and differentiate into either ovaries or testes, is driven by conserved molecular processes (Raman 2002; Doddamani 2006; DeFalco and Capel 2009; Antonio-Rubio et al. 2015). A key function of differentiated gonads is to secrete sex-specific steroid hormones, which prompts the development of other sexual characteristics, such as the Wolffian and Müllerian ducts (Norris et al. 1987; Wake 1992; Wibbels et al. 1999; Greenbaum and Carr 2001; Barske and Capel 2008; Gredler et al. 2014). The Wolffian ducts give rise to the epididymis and vasa deferentia in males, and typically regress in females (Forbes 1956; Alder 1981; Neaves et al. 2006; Norris et al. 1987; Austin 1988; Sever 2011; Shaw and Renfree 2014). In males, the Müllerian ducts begin to regress soon after testis differentiation due to the secretion of the anti-Müllerian hormone (Raynaud et al. 1970; Fox 1977; Raynaud and Pieau 1985; Wibbels et al. 1999; Greenbaum and Carr 2001; Raman 2002; Neaves et al. 2006; Gredler et al. 2014; Antonio-Rubio et al. 2015). In females, the Wolffian ducts regress while the Müllerian ducts continue to develop, giving rise to the oviducts, and a portion of the cloaca (Fox 1977; Norris et al. 1987; Wibbels et al. 1999; Neaves et al. 2006; Antonio-Rubio et al. 2015). Because female development is understudied, the hormonal activity driving the regression of the Wolffian ducts and development of the Müllerian ducts remains unknown (Pieau et al. 1999; Gredler et al. 2014; Martinez-Torres et al. 2015).

1.3. Squamate Sexual Development: Genitalia

The embryonic development of genitalia in squamates tends to be a highly conserved process, but mature genitalia exhibit considerable morphological diversity, and evolve faster than other traits (Raman 2002; Leal and Cohn 2014; Gredler et al. 2014; Tschopp et al. 2014; DeFalco and Capel 2015; Klaczko et al. 2015). Owing to the effects of gonadal hormones on the genitalia, vertebrates typically have a very short delay between gonadal differentiation and the development of sexually dimorphic genitalia (Raynaud and Pieau 1985; Beck and Wade 2008; Gredler et al. 2014). For example, hemipenes (paired copulatory organs characteristic of squamates) of mature males are commonly used for taxonomic identification because of their morphological diversity, even between closely related species (Arnold 1984; Siegel 2011; Nunes et al. 2012; Gredler et al. 2014; Klaczko et al. 2015). Mature female squamates typically exhibit small hemiclitores, but their genital structures are poorly understood (Raynaud and Pieau 1985; Bohme 1995; Neaves et al. 2006; Kasperoviczus 2011; Valdecantos and Lobo 2015). There is a growing body of evidence suggesting that female genital morphologies may prove to be similarly diverse, displaying a range of genital structures, including rudimentary hemipenes (Dufaure and Hubert 1961; Raynaud and Pieau 1985; Wake 1992; Neaves et al. 2006), hemiclitores (Bohme 1995; Kasperoviczus 2011; Martinez-Torres et al. 2015; Valdecantos and Lobo 2015), exact miniatures of hemipenes (Telemeco 2015), and even longer hemipenes and associated musculature than males (Hoge et al. 1959; Hardy 1970).

1.4. Squamate Evolutionary Dynamics: Sex Determining Mechanisms

The evolutionary history of sex determining mechanisms (SDMs) is remarkably diverse in squamates when compared with mammals. Sex chromosomes have independently evolved in many lineages, including transitions from TSD to GSD occurring within short evolutionary timeframes (Barske and Capel 2008; Hugall et al. 2008; Valenzuela 2008; Ezaz et al. 2009a and b; Pokorna and Kratochvil 2009). Phylogenetic analyses have sought to untangle the evolutionary history of SDMs in squamates, but this is problematic owing to the unresolved ancestral relationships. These issues are further compounded as squamate sex chromosomes are often morphologically similar to other chromosomes. It has only been with the development of high resolution cytogenetic techniques that cryptic sex chromosomes have become detectable, so it is likely

evolutionary transitions from TSD to GSD are more prevalent than is currently appreciated (Ezaz et al. 2009; Matsubara et al. 2014; Pokorna 2014a and b; Holleley et al. 2016; Rovatsos et al. 2016; Tomaszkiewicz et al. 2016).

1.5. Challenging the GSD-TSD Dichotomy

The most unusual SDMs in squamates is seen in two species from evolutionarily disparate lineages; *Pogona vitticeps* (the Central Bearded Dragon) and *Bassiana duperreyi* (the Eastern Three-Lined Skink). Whilst it has been convenient to categorise SDMs into two discrete categories (GSD vs TSD), these two species suggest the reality is far more complex. Both species have sex chromosomes, but environmental extremes can induce sex reversal in the laboratory and the wild (Shine et al. 2002; Quinn et al. 2007; Radder et al. 2008; Holleley et al. 2015; Holleley et al. 2016, see Figure 13). In *B. duperreyi*, low temperatures (approximately 16˚C) masculinise genetically female (XX) individuals (Shine et al. 2002; Radder et al. 2008; Quinn et al. 2007). The opposite occurs in *P. vitticeps* as high temperatures (>32˚C) feminise genetically male (ZZ) individuals (Quinn et al. 2007; Holleley et al. 2015; Holleley et al. 2016, see Figure A1).

It has been argued that sex-reversal may be a significant contributing factor to the evolution of diverse SDMs in squamates because it provides a mechanism that drives transitions from GSD to TSD (Holleley et al. 2015, 2016). This is because in *P. vitticeps* and *B. duperreyi*, matings between the sex-reversed and concordant individuals, which are both homogametic (both sex chromosomes are the same: XX or ZZ), yield offspring whose sex is completely determined by temperature. This allows a complete transition from GSD to TSD within one generation, which is likely a critical evolutionary process explaining some of the variety of SDMs in squamates (Holleley et al. 2015, 2016; see Figure A1).

1.6. Towards an Understanding of Squamate Sexual Development

Here I present the first study to characterise and compare the developmental effects of different incubation temperatures and maternal genotypes in *P. vitticeps* (the Central Bearded Dragon). This will reveal for the first time the developmental patterns associated with temperature induced sex reversal. As a method for categorising development into discrete stages, I developed a staging table for this species that incorporates numerous

descriptions of morphological characters with histological examination of the gonads. It is also the first to consider the developmental effects of GSD and TSD in the same species. Because neither SDMs have been shown to differentially affect sexual development in other species, I assume that *P. vitticeps'* unusual sex determining system will also have no effect. On this basis, I tested the following predictions using this staging table, combined with gonadal histology, and scanning electron microscopy (SEM):

1) Staging will accurately describe gross embryonic development. I expect that individuals at higher temperatures will develop and consume their yolk more quickly, but the sequence of staging events will remain unchanged.
2) Onset, timing, and order of gonad and genital developmental sequences will not be affected by the cue for sexual development (genotypic or temperature dependent sex determination).
3) In both males and females, sexually dimorphic genital and duct development will closely follow gonad differentiation, as in other squamates.

2. Methods

2.1 Breeding and Incubation Treatments

In order to assess if there are any developmental differences between GSD and TSD, I set up crosses between ZW (concordant) and ZZ (sex-reversed) females, with ZZ males. When eggs were laid, they were collected and separated into the four following experimental treatments, which cover all offspring phenotypes resulting from combinations of high and low temperatures (28˚C, 36˚C) and maternal genotypes (ZZ, ZW; see Figure 1):

1) 28ZW treatment (n = 59): Eggs from concordant (ZW) females incubated at 28˚C provide a baseline for normal development in *P. vitticeps*. Under these conditions, sex is genetically determined (males have two Z chromosomes, females have a Z and a W chromosome).
2) 36ZW treatment (n = 50): Eggs from concordant (ZW) females incubated at 36˚C will yield both concordant (ZWf) and sex reversed (ZZf) females, allowing the development of these two female phenotypes to be compared at the same temperature.
3) 36ZZ treatment (n = 122): As sex reversed ZZf mothers (i.e. sex reversed genetic males) yield offspring whose sex is determined exclusively by temperature, eggs from ZZf females incubated at 36˚C are expected to yield 100% sex reversed female offspring. This allows sex-reversed specimens obtained from the two maternal phenotypes to be compared.
4) 28ZZ treatment (n = 23): Eggs from ZZf females incubated at 28˚C yield 100% concordant male (ZZ) offspring. This allows the development of males between the two maternal phenotypes at this temperature to be compared.

In total, 254 eggs were incubated and sampled. 122 eggs were sampled in the 36ZZ treatment between 6 and 42 days post-oviposition (dpo). 59 eggs were sampled in the 28ZW treatment from 7 and 70 dpo, and another 50 were sampled in the 36ZW treatment from 6 and 45 dpo. 23 eggs between 15 and 70 dpo were sampled in the 28ZZ treatment (see Figure 1). In total, 221 eggs were obtained from the University of Canberra's (UC)

captive breeding colony, and an additional 33 eggs were obtained from a private reptile breeder (10 sampled in the 36ZW treatment, 23 in the 28ZW treatment; see A.3.1. in Appendix for ethics permits details). All eggs were incubated in damp vermiculite (four parts vermiculite to five parts water by weight) in temperature controlled incubators with minimal temperature fluctuations outside of the set range (<1˚C range, excluding fluctuations arising from examination of the eggs). All embryos were sampled by making an incision in the egg, and extracting the embryo and intact yolk. Following the procedure used by Sanger et al. (2008) for Anole lizards, all embryos from the second half of the incubation period were humanely euthanized by an intracranial injection of 100µl of sodium pentobarbitone (60mg/ml).

2.2. Sampling Protocol

Eggs from any given mother (n= 17 breeding females) were were systematically sampled across development following a two-phase design (see Figure A2):

Phase 1: Eggs were initially evenly sampled across the entirety of development (every 3 days at 36˚C, and every 5 days at 28 ˚C) in order to establish baseline data on developmental timing.

Phase 2: Based on critical periods of development identified in Phase 1, more frequent sampling and/or higher replication was required to establish the timing and order of developmental events. As it was not possible to predict how many eggs would be laid during the breeding season, this approach ensured that eggs were allocated for the most essential sampling days. This was important given low egg yields from concordant females during the 2016 breeding season (four clutches). Fewer eggs were allocated to the 28ZZ treatment because the 36ZZ treatment was considered more important for the purposes of this study.

2.3. Molecular Sex Identification in Embryonic Samples

The genotypic sex of all embryos resulting from ZW x ZZ parental crosses was determined using a polymerase chain reaction (PCR)-based molecular sex test that amplifies a W-chromosome-specific size polymorphism (Holleley et al. 2015). A ZZ x ZZ

parental cross necessarily yields ZZ individuals, thus only a subset of these individuals were randomly selected and genotyped for confirmation (46% of ZZ offspring genotyped).

Extracting DNA from embryos is challenging because of their small size, limited blood volumes, and my need to preserve morphological traits intact for staging. Thus, I developed a novel and non-invasive approach where embryonic blood from the inside of the eggshell was swabbed onto an FTA® Elute Micro Card (Whatman) immediately upon dissecting the egg. DNA was extracted following the manufacturer's instructions with a protocol adapted for automated high throughput analysis on the Eppendorf EPmotion 5075 liquid handling platform. Following the conditions from Holleley et al. (2015), and using primers H2 and F (Quinn et al. 2009), 3mm diameter FTA card punches were washed in water, boiled for 30 minutes (100˚C) in a total volume of 100ul water, and then vortexed for 2 minutes to release the DNA from the card. This test is diagnostic for the presence of the W chromosome. Two bands amplify in ZW individuals whereas a single control band amplifies in ZZ individuals. Animals showing genotype–phenotype discordance were classified as sex-reversed. I also quantified the genotyping error rates by comparing results from blood taken directly from the embryonic heart to the non-invasively sampled eggshell swabs. Duplicate PCR tests were also run, and maternal contamination was investigated by checking phenotypically male embryos (obligate ZZ genotype) for W chromosome contamination. M. Castelli at the University of Canberra assisted with running some of the PCRs, and quantified the DNA concentrations.

2.4. Specimen Storage

Embryos were kept in 10% neutral buffered formalin fixative for a minimum of 24 hours, before being rinsed and stored in 70% ethanol (EtOH) for long term storage.

2.5. Morphological Staging System

Every specimen and yolk were weighed separately for analysis of embryo growth and yolk absorption rates. I developed a staging system for *P. vitticeps* based on Sanger's et al. (2008) staging system for *Anolis spp*, which was also used by Melville et al. (2016) for the limb development of *P. vitticeps*. I also included characters from Wise et al (2009) comprehensive staging system for the leopard gecko (*Eublepharis macularis*). I also

described gonad and genital development, which are rarely included in staging systems (see Table A1 for summary). My new staging system allows the timing of the development of any given trait to be compared across treatments, despite each treatment having different developmental timeframes. Specimens obtained from a commercial breeder were not used to establish pigmentation development, as it may be influenced by selective breeding for the pet trade.

2.6. Gonad Histology

A subset of specimens expected to exhibit important gonadal phenotypes were dissected (see A.3.1. in Appendix), and processed following standard hematoxylin and eosin (H&E) staining procedure at the University of Queensland's School of Biomedical Sciences Histology Facility (Cardiff et al. 2016). As these tissues stained readily, I modified the standard procedure so each rack was dipped in eosin only 10 times, instead of being submerged for two minutes, and were left in hematoxylin for three minutes.

2.7. Scanning Electron Microscopy (SEM) of External Genitalia

I used SEM (Field Emission Scanning Electron Microscope JEOL JSM-7001 F at the University of Queensland's Centre for Microscopy and Microanalysis) to assess the microscopic structures on the developing genitalia of both males in females. This included the presence or absence of a characteristic male trait, the sulcus spermaticus. This structure is used to direct sperm into the females' cloaca during mating, and is thus an indicator of the extent of genital masculinisation during development (Gredler et al. 2014). In preparation for SEM, the complete genitalia were dissected from eight specimens (two control males, five sex-reversed and one concordant female with hemipenes or reduced hemipenes). Specimens were then dehydrated by transferring from 70% to 90% EtOH for an hour, and then two lots of 100% EtOH for an hour each. The specimens were then critical point dried, mounted, and coated with iridium before being viewed under SEM and photographed.

2.8. Embryo Growth Analyses

Embryo growth was estimated using the relationship between weight (g) over time (age, days post-oviposition), an exponential relationship whose curves were fitted for each

treatment using the *nls* function in R version 3.2.2. Using the *nlme* package to construct non-linear mixed effects models to compare curve parameters between all treatments, and between populations (specimens from UC's captive breeding program and from the breeder) I used the following equation:

(Embryo Weight~exp(a+b*Age), where a= slope, b= intercept

Note that while slope and intercept are terms usually associated with linear analyses, they can be used for non-linear exponential models.

Log embryo weight and log yolk weight were plotted against age (days post oviposition, dpo) as this allowed the crossover period between embryo growth and yolk consumption in each of the treatments to be easily identified in order to assess if any differences occurred between the treatments.

2.8.1. *Comparison of Age and Stage across Development*

In order to quantify how well age as a function of stage explained embryo growth (defined as embryo weight over age), four separate linear models were run for each treatment, yielding R^2 values to indicate the fit of each of the models.

3. Results

3.1. Staging

The staging criteria developed for *P. vitticeps* based on Sanger's et al. (2008) work on *Anolis spp.* lizards provided detailed descriptions of morphological characters diagnostic of *P. vitticeps* development (see Table 1 and Figure 2). Although toe lamellae defined stage 13, a characteristic that *P. vitticeps* does not possess, it was still possible to assign this stage based on brain and eyelid characteristics. Often *P. vitticeps* embryos showed a combination of traits across two stages, and so were denoted as 0.5 of a stage (see Table 1 and Figure 2). Because of this, staging is most accurate when describing organogenesis and limb development, and becomes less accurate as the embryos grow because the morphological changes become less discrete. For each of the treatments, stage as a function of age explained embryo growth very well (36ZZ: P = <0.0001, R^2 = 0.97; 36ZW: P = <0.0001, R^2 = 0.96; 28ZW: P = <0.0001, R^2 = 0.97; 28ZZ: P = <0.0001, R^2 = 0.98), and temperature did not influence the order of the development of non-sexual phenotypes.

3.2. Male Sexual Development: Overview

In male *P. vitticeps* embryos (28ZW treatment: 22 males, 52%; 28ZZ: 23 males. Total: 45 male specimens), gonadal and genital development follows the general developmental patterns described below (see Figures 5, 6, 7, 8, and 12).

Genital development begins as small paired phallic swellings form on either side of the developing cloaca (28ZZ: stages 6 to 8, n = 6; 28ZW: stages 5.5 to 8, n = 12) while the bipotential gonads are beginning to form in the 28ZW treatment (stage 6), and while the testes in the 28ZZ treatment have already differentiated (stage 7; see Figures 5 and 7). The swellings increase in size until they achieve a club-shaped appearance, and are enclosed by the now distinct anterior and posterior cloacal lips (28ZZ: stages 9 to 11, n = 4; 28ZW: stages 9 to 10, n = 4; see Figure 5b). This club shape becomes more pronounced as development progresses until the spermatic furrow deepens, bifurcating the distal tips of each hemipenis, creating the distinct bilobed appearance of mature hemipenes (28ZZ: stages 13 to 18, n = 13; 28ZW: stages 12 to 18, n = 10; see Figures 5c, 6a, and 6c). Ongoing development of the hemipenes is characterised by an increasingly folded appearance, which considerably enlarges the bilobes. The hemipenes of one stage 18 (60 dpo)

specimen was investigated under SEM, revealed that the sulcus spermaticus was only just beginning to form, extending ventrally from the base of the hemipenes (see Figure 6a and b). In all male specimens, the hemipenes were consistently everted, however in both treatments a total of four stage 18 specimens exhibited no everted hemipenes. It is unclear as to whether they were simply folded within the vent as the specimens approached hatching, or were truly absent. In the 28ZW treatment, one stage 17 (55 dpo) specimen exhibited reduced hemipenes, while two stage 18 (65 dpo and 70 dpo) specimens exhibited hemiclitores and a genital ridge respectively (see Figures 5 and 6).

3.2.1. Male Gonad and Duct Development

The bipotential gonads initially develop towards the posterior end of the mesonephros (anterior section of the embryonic kidney), and exhibit an elongated shape as they move to an anterior position (see Figure 7a). Once at the anterior-most portion of the mesonephros, the gonads become extensively attached to it, and develop a rounder shape. Defined cortex and medullary layers are present just prior to differentiation (see Figure 7b). Testes differentiation is characterised by the reduction of the cortex and the proliferation of the medulla, within which seminiferous tubules form (see Figure 7f). Both the Wolffian (WD) and Müllerian (MD) ducts were present in all specimens sectioned. They both initially develop along the outside of the mesonephros (anterior portion of the embryonic kidney) and developed in a craniocaudal direction. The MDs are dense, darkly stained circles with a lumen surrounded by epithelial cells and mesenchymal cells separated by basal lamina. As they extend posteriorly, lumen becomes reduced, as does attachment to the mesonephros or metanephros, and mesenchymal cell layers increase. While they initially start on the outside of the kidney, the posterior development of these mesenchymal cell layers extends as far as the connective tissues between the lower metanephros, below the proctodaeum (posterior region of the cloaca; see Figure 8). The WDs, which are comprised of simple cuboidal epithelium, develop just within the margins of the mesonephros. As they extend caudally, they develop as a series of elongated tubules extending through the metanephros. Some small tubules with lumen may also be present alongside the MDs within the proctodaeum (see Figure 8).

3.3. Female Sexual Development: Temporary Pseudohermaphroditism

Unlike male development, female sexual development is a two-stage process characterised by the internal and external components of the urogenital system developing at disparate rates, regardless of whether the sex determination cascade is initiated by sex chromosomes or temperature. This creates a period during development when female embryos exhibit both ovaries and masculinised genitalia (hemipenes, reduced hemipenes and hemiclitores) concurrently. During this period of ambiguous sexual development embryos are classed as temporary pseudohermaphrodites (TPHs) because they exhibit both male and female characteristics, but only for a defined period of time before becoming completely female (characterised by a genital ridge). I observed variation in the timing and persistence of a given genital phenotype between the treatments, which influences the duration of the TPH phase. This demonstrates inter-individual variation, and also considerable developmental plasticity both within and across the experimental treatments (see Figures 4 and 12).

3.3.1. Female Sexual Development: Overview

In general, the development of the genitalia, bipotential gonads, Wolffian, and Müllerian ducts in females follows the same pattern described for males. The crucial difference is that while the ovaries have differentiated (reduced medulla and proliferating cortex with oogonia) by stage 8 (see Figure 7d), the genitalia continue to masculinise until distinctly bilobed hemipenes have formed, defining the beginning of the TPH phase (see Figures 4, 5, and 6). After a period of hemipenis retention, the overall length of the hemipenes decreases, but the lobes are retained until the length is considerably reduced. The lobes then completely reduce so that only two small swellings characteristic of hemiclitores persist on the genital ridge. As embryos approach hatching (stage 18) the hemiclitores reduce completely so that only the genital ridge is present (see Figures 5 and 6).

3.3.2. ZW Concordant Female Development (ZW offspring at 28˚C and 36˚C)

The genitalia initially develop as paired swellings (28ZW: stages 5.5 to 8, n = 8; 36ZW: stages 6 to 10, n = 8), and then continues to increase in size, eventually developing a club shaped appearance (28ZW: stages 9 to 12, n = 4; 36ZW: stages 10 to 13, n = 5; see Figures

5a and b). During this time, the bipotential gonads have formed (28ZW: stages 6 to 7; n = 2; 36ZW: stage 6, n = 1; see Figure 7a and b), before differentiating into ovaries from stage 9 in both treatments (see Figure 7d). The genitalia continues to develop into hemipenes (28ZW: stages 11 to 14, n = 4; 36ZW: stages 12 to 16, n = 3; see Figures 5c and 6g) before regressing to reduced hemipenes (28ZW: stages 11.5 to 17, n = 7; 38ZW: stages 13.5 to 15, n = 11; see Figures 5d and 6h), and hemiclitores (38ZW: stage 17, n = 1; see Figure 5e). The mature female phenotype is not present until close to hatching and is characterised by a genital ridge absent of any protrusions (28ZW: stages 15 to 18, n = 3; 36ZW: stage 18, n = 4, see Figure 5f).

At both high and low temperatures, concordant female development is characterised by a TPH phase that persists for approximately 9 stages (see Figure 4). There was a stage 16 (33 days post-oviposition) specimen that had hemipenes, despite all other specimens around the same age and/or stage having either heavily reduced hemipenes, hemiclitores, or genital ridges (see Figures 5 and 6). This specimen likely had some developmental issues as it also had atypical ovarian morphology as it retained extensive attachment to the mesonephros, and the medullary tissue had a directional rather than random arrangement (see Figure 7e).

3.3.3. ZZ Sex Reversed Female Development (ZZ offspring at 28˚C, sex reversed ZW offspring at 36˚C)

Embryos with a ZZ genotype, including those undergoing temperature mediated sex reversal (36ZZ, n=122; 36ZW treatment, n= 50) follow a developmental pattern very similar to concordant (ZW) females. Between stages 5 to 8, specimens exhibit either ongoing development of the cloacal region (36ZZ: stage 7, n= 2; 36ZW: stage 5, n = 1), or paired phallic swellings (36ZZ: stages 6 to 8, n = 20; 36ZW: stages 6 to 8, n = 17). 36ZZ stage 8 specimens may also exhibit appendages taking on a club-shaped appearance (n = 11), which persists through stages 9 to 12 (n = 28; see Figure 5). By stage 9, ovaries were present in both treatments (36ZZ: n = 8; 36ZW: n = 9; see Figure 7d). In the 36ZZ treatment, stage 11 to 12 specimens can also exhibit bilobed hemipenes (n = 10; see Figure 5c and 6g). From stages 12.5 to 16, specimens begin to exhibit reduced hemipenes that become progressively smaller as the embryos grow (n = 35; see Figures 5d and 6h). Specimens

towards the end of development (stages 16.5 to 18) exhibited hemiclitores (n = 12; see Figure 5e) and genital ridges (n = 5; see Figure 5f). Two stage 18 (34 and 36 dpo) specimens retained hemipenes. In the 36ZW treatment, no sex reversed specimens exhibited club shaped appendages, and the only two specimens that exhibited hemipenes also had testes, so did not sex reverse (stage 11, 17 dpo and stage 13, 21 dpo). One specimen exhibited reduced hemipenes (stage 15, 30 dpo), while two stage 18 (39 dpo) specimens exhibited genital ridges.

While ovaries were observed between stages 8 to 9 in the 36ZZ treatment (n = 2), interestingly, ovotestes were also found in this treatment in two stage 9 and 9.5 (14 dpo) specimens (see Figures 4 and 7c). This is a gonadal phenotype characterised by a cortex that looks identical to that of a typical ovary, including the presence of oogonia. Instead of the medulla consisting of loose, randomly arranged cells and connective tissues, the cells have condensed into rudimentary seminiferous tubules, occasionally with lumen, akin those seen in normal testis. Ovaries are consistently observed again from stage 9.5 onwards (n = 5, see Figure 4).

3.4. Comparison of Embryo Growth and Yolk Consumption

In all treatments, embryo growth follows an exponential growth curve whose slopes at any given time do not significantly differ *within* the two temperature groups: 36˚C, P = 0.54; 28˚C, P = 0.17. However, *between* the two temperature groups, the slopes differ significantly: P = <0.0001 (see Figure 9). Early in development, yolk weight is highly variable and not clearly related to embryo weight. However, later in development yolk is rapidly consumed once all major developmental events, such as organogenesis, have finished and the embryo prepares for hatching. This creates a crossover period that coincides with stages 13 to 17 (40 to 55 dpo) in the 28ZW treatment, and 14 to 17 (45 to 55 dpo) in the 28ZZ treatment. In the 36ZW treatment, this period coincided with stages 13.5 to 18 (24 to 35 dpo), and stages 17 to 18 (28 to 38 dpo) in the 36ZZ treatment (see Figure 10).

3.5. Comparison of Growth Between Populations

Because my study used individuals from two breeding populations, before pooling data from these two sources, I checked for evidence of differences in embryo growth and

yolk consumption that might be explained by local adaptation. A comparison of both of these factors in the 36ZW and 28ZW treatments found no significant difference (*a*, slope; *P* = 0.6, 0.8 and *P* = 0.2, 0.2 respectively; see Figure 11).

3.6. Genotyping Error Rates

In 23 specimens, a comparison of genotypes from blood taken directly from the embryonic heart to the eggshell swabs had a 78% concordance rate. I also conducted duplicate PCR tests on seven individuals (five from an eggshell smear, and two from the embryos heart), which had a 14% concordance rate. Finally, of the four sexually differentiated male specimens histologically processed in the 28ZW treatment, two had testes, and both of these specimens had a ZZ genotype, hence there was no evidence of maternal contamination.

3.7. DNA Concentrations

Results from DNA concentration quantifications showed that the smears from the embryonic blood supply had less DNA (11ng/µL, *SD* ± 19, n = 65) than blood taken directly from the embryonic heart (22ng/µL, *SD* ± 19, n = 19). Both of these embryonic sample types yielded less DNA than blood sampled from adult dragons (43ng/µL, *SD* ± 43, n = 37).

4. Discussion

4.1. Novel Developmental Pathways in P. vitticeps Sexual Development

My investigation has revealed a delay between gonad differentiation and the development of mature genitalia in both male and female dragons, which is highly unusual for squamates. In male development (28ZZ treatment), this delay extended across six stages, while in the 28ZW treatment, the delay was only two stages. Female dragon development proved to be considerably more complex than males, also exhibiting a delay between gonad and genital development. These findings directly contradict the common assumption that the timing of gonad and genital development is closely linked (Raynaud and Pieau 1985; Holmes and Wade 2005; Gredler et al. 2014, 2015).

Preliminary scanning electron images showed that the sulcus spermaticus is only starting to develop in a stage 18 (60 dpo) male, and wasn't present in the stage 18 (32 dpo) sex reversed female I examined. This suggests that the sulcus develops very late in development, and likely only fully develops post-hatching, which contradicts the assumption that female hemipenis regression occurs shortly after the development of the sulcus spermaticus (Raynaud and Pieau 1985; Holmes and Wade 2005; Gredler et al. 2014, 2015).

The sexual development of both concordant and sex reversed females was characterised by a period of hemipenis retention and regression long after the ovaries had differentiated, resulting in a prolonged but temporary period of pseudohermaphroditism (TPH phase). This differs from true hermaphroditism where an individual possesses the gonads and genitalia of each sex at maturity due to abnormal development (Dirckx 2011). It is important to note that these specimens were not developing abnormally, as my data suggests that asynchronous development of the sex organs in females under both genetic and temperature influences is part of normal development for this species.

As I predicted, growth patterns and the temporal sequence of developmental events were unaffected by sex reversal: all phenotypic females developed in the same way regardless of the primary trigger for the female cascade (sex chromosomes or

temperature). The only difference I observed between concordant and sex-reversed female specimens was the presence of ovotestes in the 36ZZ treatment (see Figure 12 for a schematic diagram of sexual development). However, ovotestes were rare (0.02% of specimens in the 36ZZ treatment) and may not have been detected in the 36ZW treatment due to lower sample sizes. Similar to previous research, I found that the threshold for sex reversal is higher among offspring from ZW mothers than from sex reversed ZZ mothers (Holleley et al. 2015). In the 36ZZ treatment, 100% of ZZ offspring developed as phenotypic females (n = 122), whereas in the 36ZW treatment 62% developed as females (n = 31). Two specimens in the 36ZW treatment exhibited male only characteristics (testes, hemipenes, ZZ genotype), supporting evidence found by Holleley et al. (2015) that sex reversal under these conditions is not 100%. These data suggest that there is variation in the threshold for sex reversal. If this variation is heritable, it could be an evolutionary mechanism that stabilises sex-ratios against female bias, as skewed ratios typically detrimentally affect population viability (Hulin 2009; Mitchell and Janzen 2010; Neuwald and Valenzuela 2011).

4.2. Staging

The staging table I developed for this study is one of the first to incorporate genitalia and histological sections of the gonads with typical morphological characters. It is one of the few systems to document development at different temperatures, and is the first to include the process of temperature-mediated sex-reversal, making it the most comprehensive staging tables available for an agamid lizard (see Table A1 for overview). As expected, neither temperature nor maternal influences caused any changes in the timing of developmental events. In fact, stage rather than age often proved far more relevant for determining the timing of the development of phenotypic features, including the gonads. This also means that for future studies my staging system will allow targeted sampling of sexual phenotypes with fewer replicates across more days, instead of heavily replicating across increments of several days, which increases the likelihood of capturing the appropriate stage.

Staging is often criticised because there is no standard practice, it usually does not take into account the effects of incubation temperature, differences between field and laboratory raised animals, and often uses small sample sizes (Werneburg 2009; Dormer et

al. 2016). Despite these issues, my study shows staging remains the best method for categorising development to facilitate comparisons within and between species. It proved invaluable in this study because it accounts for differences in incubation periods between the treatments, and was shown to account for embryo growth extremely well (see Figure 3). My staging table can now be used to facilitate ongoing research about the developmental processes involved in sex determination in *P. vitticeps*, particularly as it is emerging as a model organism in fields such as developmental and evolutionary biology, genetics, endocrinology, and behaviour (Witten 1983; Viets et al. 1994; Tattersall and Gerlach 2005; Quinn et al. 2007; Schaerlaeken et al. 2008; Ezaz et al. 2009a and b; Khan et al. 2010; Kis et al. 2015; Holleley et al. 2015; Li et al. 2016).

4.3. Delayed Gonad and Genital Development in Male P. vitticeps

In both treatments in which males are produced (28ZZ and 28ZW), there was a delay between testes differentiation and hemipenis development, which was more pronounced in the 28ZZ treatment. Currently, the results from the 28ZW treatment show that hemipenis maturation occurs relatively soon (two stages) after testes differentiation. This slight delay could be explained by it simply taking longer for the hemipenes to grow, though their development is likely being driven by hormone secretions from the testes. However, as so few specimens exhibited these phenotypes, further research with larger sample sizes would be required to determine the accuracy of the time frames identified in this study. Such work is also required for the 28ZZ treatment, which exhibited a much greater delay (seven stages), though it remains unclear as to whether this disconnect would still exist had replication been higher during this period. Future research will either find that the hemipenes do develop soon after testes differentiation, which would support the assumption that these developmental events are closely linked (Raynaud and Pieau 1985, Homes and Wade 2005; Gredler et al. 2014, 2015). Alternatively, this research will confirm that there is indeed a considerable delay between these events, which would suggest that either it simply takes a long time for the hemipenes to grow, or that there is a delay in hormone secretions from the testes, and that once secretion begins hemipenis growth rapidly progresses. If the current trend of a longer delay in the 28ZZ treatment when compared with the 28ZW persists, this raises the exciting possibility that GSD and TSD have differential effects on the development of sexual phenotypes in male *P. vitticeps.*

4.4. Female Development Characterized by Temporary Pseudohermaphroditism

The most striking result in my study was the discovery of discordant timing between gonad differentiation and genital maturation in all females, regardless of the cue for sexual development (temporary pseudohermaphroditism, TPH). I hypothesise that the TPH phase occurs either due to a delay in the receptiveness of the genitalia to steroid hormones secreted by the ovaries, or by a delay in ovarian hormone secretion. Though these hypotheses may not be mutually exclusive, I will discuss each separately below, as they have significant implications for scientific understanding of the evolution of squamate sexual phenotypes.

4.4.1. Hypothesis 1: Delayed Genital Receptiveness to Ovarian Hormones

It is assumed that secondary sex characteristics develop soon after gonad differentiation due to the secretion of sex specific hormones from the gonads, driving the development of either male or female phenotypes (Raynaud and Pieau 1985; Neaves et al. 2006; Holmes and Wade 2005; Gredler at al. 2015). So how do female *P. vitticeps* continue to develop hemipenes long after ovarian differentiation? Assuming that the ovaries are hormonally active (Raynaud and Pieau 1985; Holmes and Wade 2005; Rhen et al. 2005; Beck and Wade 2008; Gredler et al. 2014), this delay must occur because the genitalia are not receptive to these hormones. The resulting propensity for maleness supports the generally held assumption that the homogametic sex (ZZ males in this species) is the default developmental pathway, and an overriding suite of molecular events is required to switch the embryo onto a female developmental trajectory (Raynaud and Pieau 1985; Mittwoch 1998; Wilhelm et al. 2007; Hersmus et al. 2008). Should this hypothesis prove correct, further experimentation is required to explain why this propensity for maleness is seen in the genitalia but not the gonads. It will also be essential to quantify ovarian secretions during development, what receptors they act on, and what triggers the late onset hormone sensitivity in the genitalia.

In further support of this hypothesis, it seems likely that, at least in the 36ZZ treatment, the gonads are endocrinologically active given the presence of ovotestes. This is because the only conditions which have been shown to induce ovotestes are when levels of sex steroid hormones are such that neither male or female gonadal phenotypes are

repressed or developed to their typical extent (Crews et al. 1994; Pieau et al. 1998). As ovotestes were only observed in the 36ZZ treatment, it is possible they developed as a result of a period of antagonism between the masculinizing effects of these specimens' ZZ sex chromosomes, and the feminizing effects of high temperatures, which affected hormone levels. In order to untangle the genetic and hormonal underpinnings of ovotestes development, this period of antagonism will prove crucial for future work that seeks to better understand the interactions between gene expression, hormones, and temperature during sex reversal. If ovotestes are shown to result from this antagonism, it is possible that TSD species which exhibit ovotestes when incubated at the pivotal temperature may in fact have cryptic sex chromosomes that exert a greater influence when not overridden by the effects of more extreme temperatures (Sarre et al. 2004). Given TSD species are vulnerable to climate change skewing population sex ratios, a mechanistic understanding of sex determination will allow measures to be taken to mitigate these effects (Hulin 2009; Mitchell and Janzen 2010; Neuwald and Valenzuela 2011).

4.4.2. Hypothesis 2: Delayed Ovarian Hormone Secretion

In female *P. vitticeps* embryos, the delay between ovarian development and the maturation of the genitalia may occur because the ovaries are not secreting hormones early in development, or the levels are too low to exert an effect. Comparative studies of genital and gonadal development in other squamates may provide support for this hypothesis. Of the 26 staging papers available for squamates, only two describe the development of both of gonads and genitalia for two evolutionarily disparate species (Imbricate Alligator Lizard, *Barisia imbricata*, and the Ocellated Skink, *Niveoscincus ocellatus*). Though not defined as such by the authors, female development in these two species is also characterised by temporary pseudohermaphroditism (TPH), though the characteristics of TPH differ greatly between the three species (Neaves et al. 2006; Martinez-Torres et al. 2015; see Figure 13 and Table A1). Thus there are three squamate species with sufficient data to identify delayed hemipenis regression, but only one species (*Anolis carolinensis*) where hemipenis regression occurs almost immediately after gonadal differentiation (Holmes and Wade 2005; Gredler et al. 2015). While evidence is currently limited, this suggests that delayed hemipenis regression (resulting in a TPH phase), which

has been hypothesised to occur due to delayed ovarian hormone secretion, may be a common feature of female development in squamates (Martinez-Torres et al. 2015).

Further evidence supporting this hypothesis is the persistence of the Wolffian ducts in female *P. vitticeps*. Given duct development has been shown to be influenced by gonad hormones (Raynaud et al. 1970; Fox 1977; Raynaud and Pieau 1985; Wibbels et al. 1999; Greenbaum and Carr 2001; Raman 2002; Neaves et al. 2006; Antonio-Rubio et al. 2015), it is possible that the Wolffian ducts are persisting in females due to an absence of the hormones required to trigger their regression. Ultimately, it is clear that the molecular processes associated with female sexual development are poorly understood, and are likely to be a very complex suite of thermally sensitive molecular cascades that require much more attention.

4.5. Embryo Growth and Yolk Consumption

As expected, temperature increased the rate of development so that embryos in the 36˚C treatments grew more quickly than their 28˚C counterparts (Raynaud and Pieau 1985; Webb et al. 1987; Dormer et al. 2016). Yolk and embryo weight were not closely correlated early in development in all treatments, which was not expected, but may be explained in several ways (see Figures 9 and 10). As the embryos only energy source, the yolk must be consumed during the entirety of development. Because the embryo is much smaller relative to the yolk early in development, its consumption would be negligible until they become approximately the same weight. The embryos' yolk consumption would then have a greater impact on the size of the yolk, explaining the pronounced decrease in yolk weight towards the end of development. Alternatively, it is possible that the embryo truly consumes little yolk until towards the end of development. This may be because it is more efficient to consume as much yolk as possible prior to hatching either because the embryos growth is accelerated, or requires more energy to develop biologically 'expensive' materials. Future research taking into consideration the effects of temperature and maternal type (concordant or sex reversed) on embryo metabolism and energetics is required to better understand these trends seen in embryo growth and yolk consumption.

4.6. Embryonic Genotyping

As this is the first study to attempt to obtain genotypes from the embryonic blood supply, I applied existing methods that have been developed for large quantities of blood from adult dragons (Quinn et al. 2009; Holleley et al. 2015). This means that there are considerable error rates associated with the genotyping results because of low DNA levels in the blood supply. Work is ongoing to optimise the protocol for lower DNA concentrations, which will reduce error rates. Once the protocol has been modified, this novel method will prove useful for future studies like mine, which require confident determination of genotypic sex. It is more time efficient because of the difficulties in obtaining a blood sample from a minute embryonic heart. It also means the embryo is not tampered with in any way, yielding more accurate measurements of weight and morphology.

4.7. Implications of Findings and Avenues for Future Research

I have found that, contrary to commonly held assumptions about squamate sexual development, both males and females exhibited a delay between gonad differentiation and genital maturation, regardless of the developmental queue. I have provided two new hypotheses (delayed ovarian hormone secretion and delayed genital hormone receptiveness) to explain this developmental pattern (TPH) in female *P. vitticeps.* Much more research is required to understand which, or perhaps both, of these hypotheses are responsible for the TPH phase. This research into the genetic and hormonal mechanisms driving this developmental pattern, and how it may be influenced by temperature, will make it possible to reach a mechanistic understanding of sexual development in female *P. vitticeps.*

Understanding these hormonal and genetic mechanisms is critical as temperature has been documented to affect offspring sex ratios of numerous squamate species, there is increasing concern that climate change will jeopardise population viability of TSD species by skewing sex ratios (Hulin 2009; Mitchell and Janzen 2010; Neuwald and Valenzuela 2011). Hence, greater knowledge of how temperature influences sexual development is crucial if any attempts to mitigate detrimental effects of climate change are to be made. This is why it is essential to describe the influence of temperature on the development of

sexual phenotypes in *P. vitticeps*, an emerging model organism with particularly relevant sex determining modes.

My study has demonstrated novel developmental pathways associated with female development in *P. vitticeps*. There is an undiscovered reservoir of developmental diversity in female squamates, a knowledge gap exacerbated by a traditional focus on male development. My study paves the way for future research on the hormonal and genetic underpinnings of sexual development, as well as broader implications for the evolution of SDMs and sexual phenotypes in squamates, particularly in the context of climate change.

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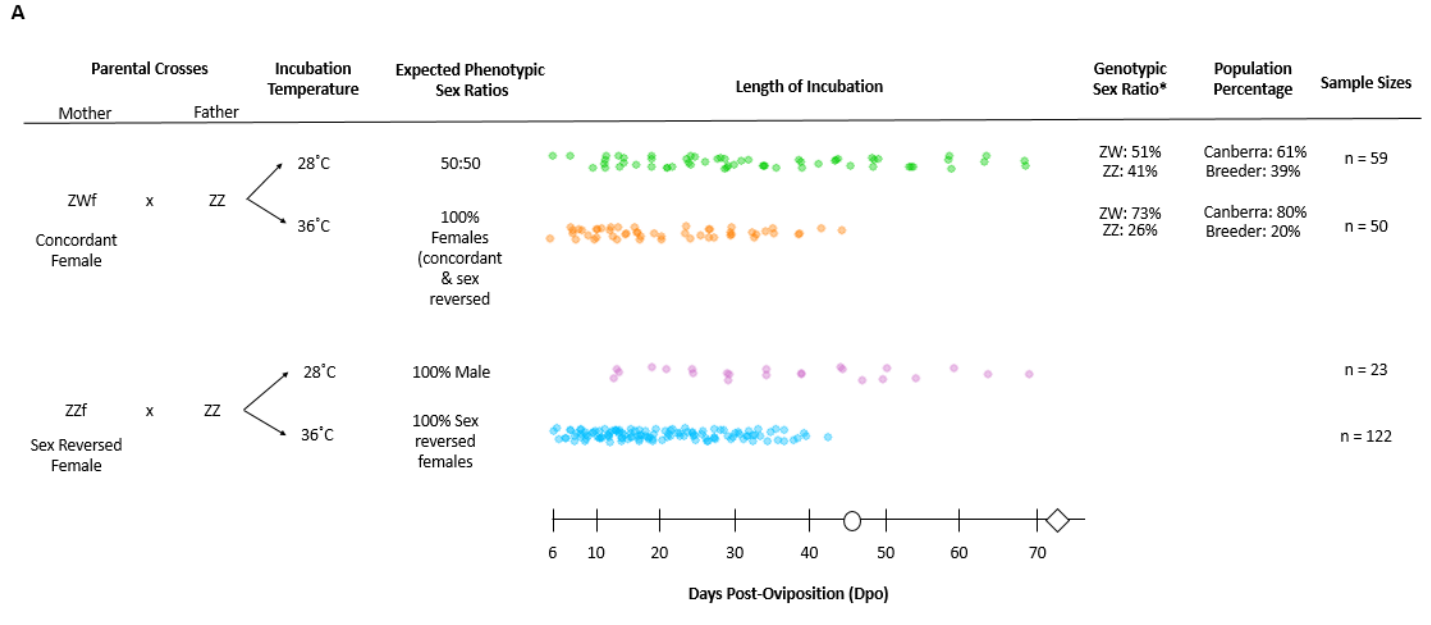
7. Figures and Tables



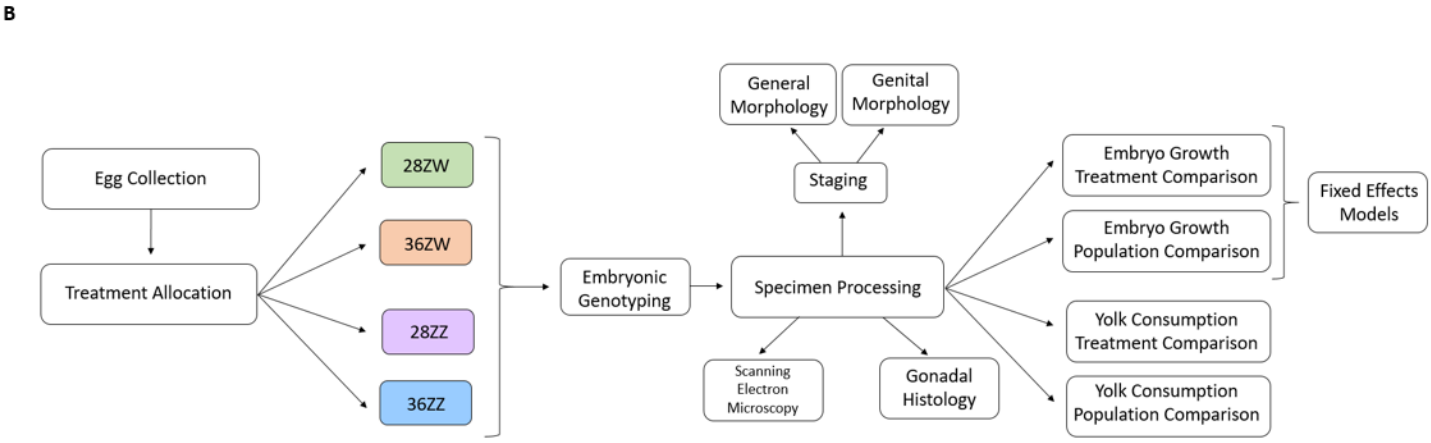


Figure 1: Experimental design (A) and methods workflow (B) encompassing all procedures used in this project. Circle denotes approximate day of hatching for eggs incubated at 36˚C (46.7±1.6), and diamond for eggs incubated at 28 ˚C (73±3.5) based on estimates from Holleley et al. (2015). Note that only a subset of specimens were used for scanning electron microscopy and gonadal histology, and that the reported genotypic sex ratios are not finalised.

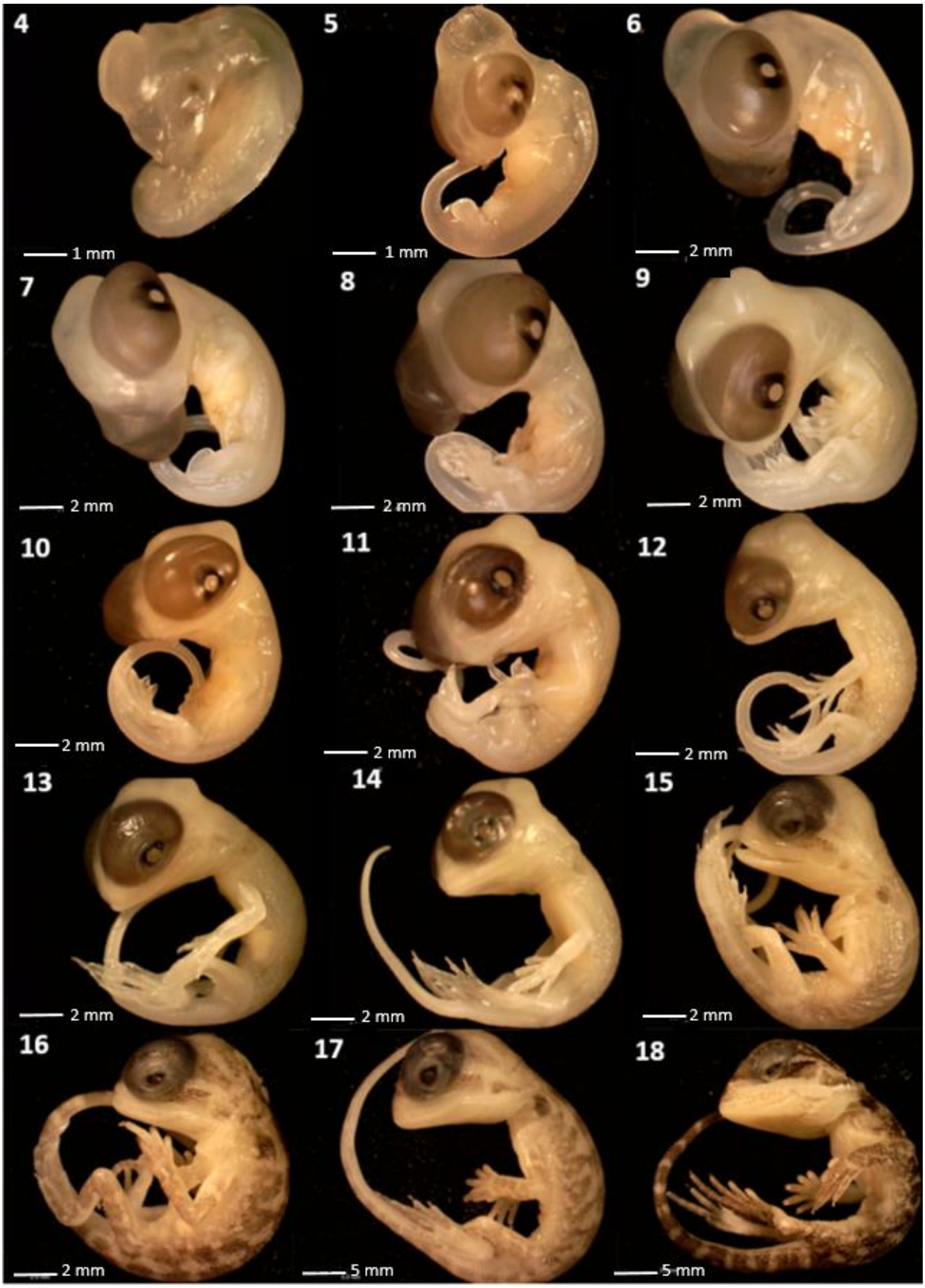


Figure 2: Developmental staging series for *P. vitticeps* modified from Sanger et al. (2008) staging system for *Anolis* lizards depicting the complete range of stages observed across all treatments.

Table 1: Developmental staging table for *P. vitticeps* based on Sanger et al. (2008) staging system for *Anolis* lizards. It also includes more detail about certain morphological traits not described for *Anolis*, as well as a general characterisation of genital and gonad development.

Stage	Description
4. Early Limb Bud	*Limbs:* Hind and forelimb buds are well defined and approximately the same size. *Cranial:* The mesencephalic bulge is large and translucent with slightly delineated margins where joined to the lower developing cranium. *Eye:* There is some faint pigmentation around the developing pupil, and the eyes are only slightly protuberant. *Thorax:* Three pharyngeal arches are present. All organs, aside from the embryonic kidney, are developing outside of the body cavity, and are not yet distinguishable.
5. Late Limb Bud	*Limbs:* Both the hind and forelimb buds have increased in size and begin to show a slight pinching between the length of the limb and the developing hand. *Cranial:* The mesencephalic bulge remains large and translucent, but now is smoothly attached to the lower developing cranium. *Eye:* The eyes have become much larger and protuberant, taking on a light brown colouration with diffuse black pigmentation surrounding the developing pupil. *Thorax:* The heart is internalised, while the intestines remain herniated. *Genitalia:* The cloaca begins to form as a small indentation between the hindlimb buds.
6. Paddle Shaped Limb Bud	*Limbs:* Both the hind and forelimbs have a distinct paddle, or spade-like shape, but no delineated phalanges. *Cranial:* The mesencephalic bulge becomes slightly less protuberant and translucent. *Eye:* The eyes continue to increase in size, while the black pigmentation around the pupil becomes less diffuse. *Thorax:* The intestines are almost completely enclosed within the body cavity. *Genitalia:* The cloaca continues to become more defined as very small genital swellings begin to form on either side of the cloacal opening, between the hindlimbs. *Gonads:* The bipotential gonads have formed along the anterior portion of the metanephros, and begin moving towards the mesonephros

7. Digital Plate	*Limbs:* Both the hind and forelimbs have become obviously proximodistally segmented, increase in width and become slightly pointed at the apex. Faint digit condensations are visible. *Cranial:* The mesencephalic bulge reduces further and becomes less translucent. There is also some definition of the presumptive paired brain swellings. *Eye:* The eyes become more protuberant and the eyelid beings to form as a thin, translucent covering of skin around the ventral margin of the eye. *Thorax:* All organs have become completely internalised. *Genitalia:* The genital swellings increase in size and the anterior and posterior cloacal lips start to develop.
8. Digital Condensations	*Limbs:* The phalangeal bones have condensed, the interdigital webbing becomes slightly reduced, and the limb joints become more distinct. *Cranial:* The mesencephalic lobes become more obviously delineated. *Eye:* The overall appearance of the eye remains unchanged from the previous stage, but the eyelids continue to envelop more of the eye. *Genitalia:* The genital swellings increase in size, and start to take on a slightly club-like shape. The anterior and posterior cloacal lips continue to become more defined.
9. Early Digital Web Reduction	*Limbs:* The interdigital webbing continues to reduce so that the distal tips are freed, while the elbow joint becomes more distinct, making the limbs flex at approximately 90 degrees. *Cranial:* Four mesencephalic lobes have become delineated, and the pineal eye is visible. The two posterior lobes are slightly more protuberant than the two anterior lobes. *Eye:* The eyelid now covers approximately three-quarters of the eye. *Scales:* Epidermal papillae first become visible, particularly along the dorsal surface. *Genitalia:* The genitalia continues to grow and develop an increasingly club-like shape, and the cloacal lips continue to thicken. *Gonads:* The ovaries have differentiated exhibiting a distinct medulla and cortex with proliferating oogonia.
10. Digital Webbing Partially Reduced	*Limbs:* The digital webbing continues to reduce to approximately half the length of the phalanges. *Scales:* Epidermal papillae develop along the dorsal surface, and margin of the presumptive beard. The epaulettes also start to form. *Genitalia:* The genitalia now have a club shaped appearance, but are not yet bilobed. *Gonads:* The testes have differentiated exhibiting a reduced cortex and medulla with proliferating seminiferous tubules.

11. Digital Webbing Completely Reduced	*Limbs:* The phalanges are no longer joined by any interdigital webbing, and there is a slight pinching at the distal tips of each phalange. *Cranial:* The posterior mesencephalic bulges become more protuberant. *Eye:* The eyelid thickens, creating an almond-like shape around the eye. The pigmentation darkens, and the black pigmentation condenses around the pupil. *Scales:* The epidermal papillae become more prominent along the dorsal surface and margins of the presumptive beard. Very faint pigmentation is visible on the developing epaulettes. *Genitalia:* Both sexes now exhibit bilobed hemipenes.
12. Digital Pad	*Limbs:* The phalanges become more elongated with some joint definition and increased pinching at the distal tips, but the claws remain transparent. *Eye:* They eyelid now covers the eye up to around the margins of the black pigmentation around the pupil *Scales:* Scale analgen are present along the margins of beard and side of body, and dorsal surface. Epidermal papillae develop on the dorsal surface along with faint, scattered pigmentation on the developing epaulettes. *Genitalia:* Bilobed hemipenes continue to develop in both sexes
13. Toe Lamellae	*Limbs:* The claws are well defined and are no longer transparent. All limb joints become well defined. *Eye:* The eyelid surrounds the pupil and beings to thicken at its anterior margins, but remains transparent, underneath which the eyes' darkening pigmentation is still visible. *Scales:* Epidermal papillae are now evident across the entire body. Scale analgen increase in number and become more defined along the beard margins, sides of body, and dorsal surface. Very faint, scattered melanophores appear on the developing epaulettes, extending caudally from the margins of the eye and ear hole. Patterning develops along the dorsal surface and the pigmentation slightly darkens. *Genitalia:* Bilobed hemipenes continue to develop, however in some females they begin to reduce in length but retain their bilobed appearance.
14. Scale Analgen	*Limbs:* The limbs no longer change in shape. *Cranial:* The mesencephalic bulges begin to reduce, and the area of the presumptive parietal bone becomes less translucent. *Eye:* The anterior margins of the eyelid continue to thicken. *Scales and Pigmentation:* Scale analgen now cover most of the body, and light pigmentation appears on limbs, tail and cranium, while the dorsal patterning continues to slightly darken.

	Genitalia: The hemipenes continue to regress in females, and remain unchanged in males.
15. First Full Scales	*Cranial:* The mesencephalic lobes continue to reduce so that the head has an even dome shape in profile. The presumptive parietal bones continue to develop, further reducing the transparency of the skull. *Eye:* The eyelids' anterior margins thicken further, more closely enclosing the pupil, but remain transparent. The whole eye darkens so there is no delineation between pigmentation around the pupil and the rest of the eye. The eyelid encloses the pupil and begins to form a distinct margin *Scales and Pigmentation:* Pigmentation has darkened considerably from pervious stage, and distinct patterns cover the entire dorsal surface. The claws also darken. Overlapping scales cover most of the dorsal surface, including the tail and limbs. Presumptive spines develop along the beard and sides of body. *Genitalia:* In females, the genitalia continues to regress so that either the bilobed appearance is retained, or has disappeared so that the genitalia resemble small, even swellings characteristic of hemiclitores. In some specimens bilobed hemipenes are retained.
16. Fully Developed Scales	*Cranial:* The parietal bone continues to develop so that the mesencephalic lobes are less visible. *Eye:* The eyelids continue to thicken over the whole eye, and have a well-defined almond shape around the pupil. *Scales and Pigmentation:* Fully developed scales are now common across the body, particularly along the beard and dorsal surface. Scales begin to become more prominent on the phalanges and eyelids. Pigmentation continues to darken over the whole body, so that distinct patterns are now obvious. *Genitalia:* In females, the genitalia generally continues to regress to hemiclitores, however there are still some specimens that retain bilobed hemipenes or regressed hemipenes.
17. Pigmentation	*Cranial:* The parietal bone is well developed so that the cranium has a smooth dome-like appearance in profile and the mesencephalic lobes are barely visible. *Eye:* The eyes become less protuberant, but the pigmentation and eyelid morphology remains unchanged. *Scales and Pigmentation:* Scales continue to develop across the body, and are now commonly found to be overlapping. Pigmentation darkens across all dorsal surfaces. *Genitalia:* Most female specimens now exhibit either regressed hemipenes or hemiclitores.

18. Near Hatching	*Cranial:* The partial bone has formed, and is now covered in scales and darkly pigmented. The pineal eye is still visible. *Eye:* The eyes are significantly less protuberant, but there is still some dark pigmentation visible under the eyelid. *Scales and Pigmentation:* The scales are almost completely developed, and the pigmentation patterns look much like those seen in hatchlings. *Genitalia:* All genitalia has completely regressed in females so that only the genital ridge is present within the vent. Males possess large bilobed hemipenes.

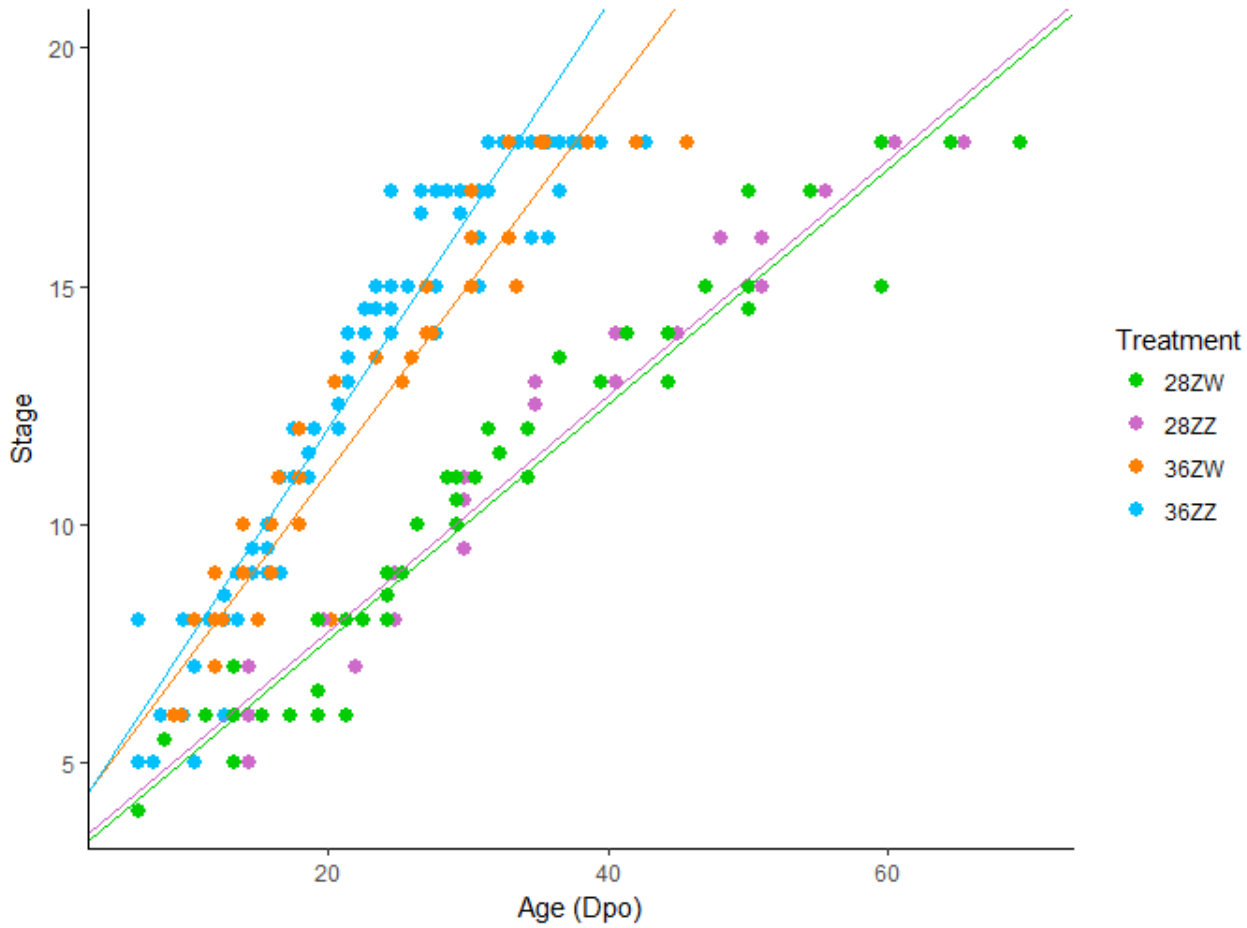


Figure 3: The linear relationship between age and stage for each of the four treatments demonstrating that any given stage can occur across several days, and that numerous stages can be present at any given age.

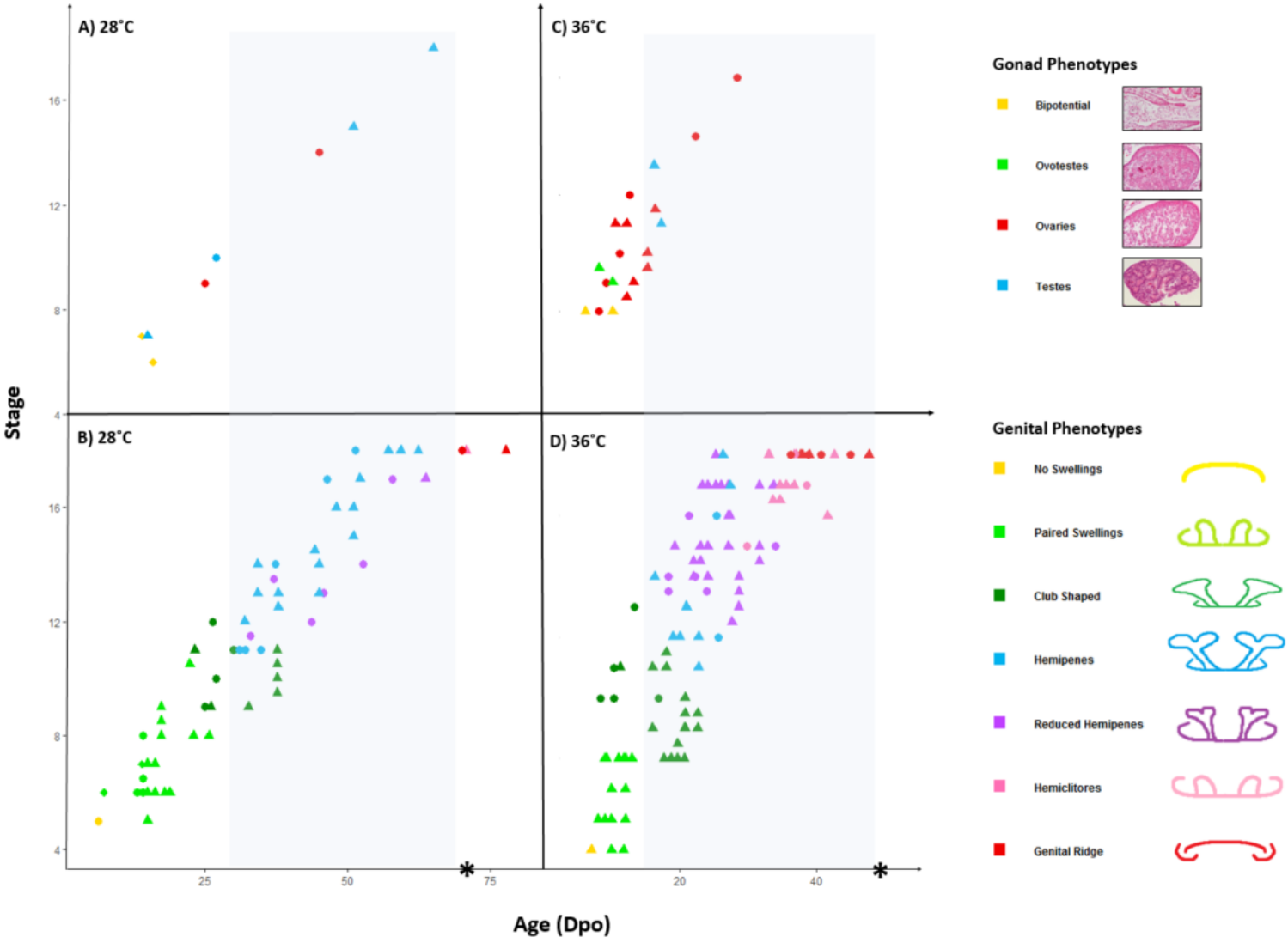


Figure 4: Timeline of genital and gonad development at 28˚C (28ZW and 28ZZ treatments, panels A and B) and 36˚C (36ZW and 36ZZ treatments, panels C and D) for age (days post-oviposition) and stage. Sexual phenotype is indicated by colour as per the legend, and sexual genotype is indicated by shape (triangles = ZZ specimens, circles = ZW specimens, diamonds = unknown). The grey shading defines the period of temporary pseudohermaphroditism during female development, which persists for approximately 9 stages in the 28ZW, 36ZW and 36ZZ treatments. The black asterisks denote approximate time of hatching (73±3.5 dpo at 28˚C and 46.7±1.6 dpo at 36˚C; Holleley et al. 2015).

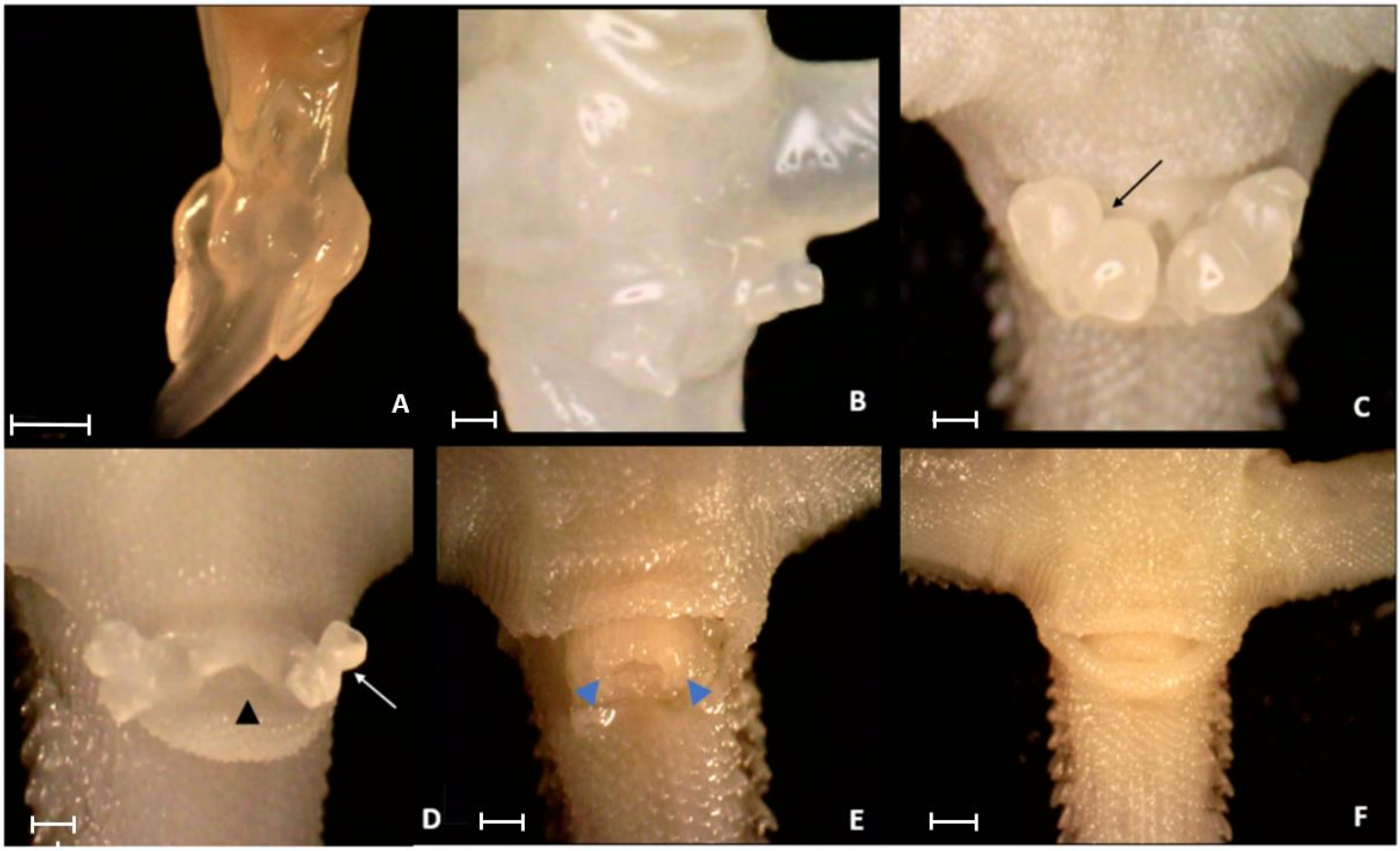


Figure 5: Genital phenotypes observed in females. Development progresses from rounded paired swellings between the hindlimbs (A), club-shape (B), bilobed hemipenes (C, phallic ridge: black arrow), which becomes increasingly accentuated as the phallic ridge deepens (black and white arrows), regressed bilobed hemipenes (D, black arrowhead: cloacal opening), hemiclitores (E, blue arrowheads) to the genital ridge (F). The anterior and posterior cloacal lips in specimen E were removed to expose the hemiclitores. Scale bar = 1mm.

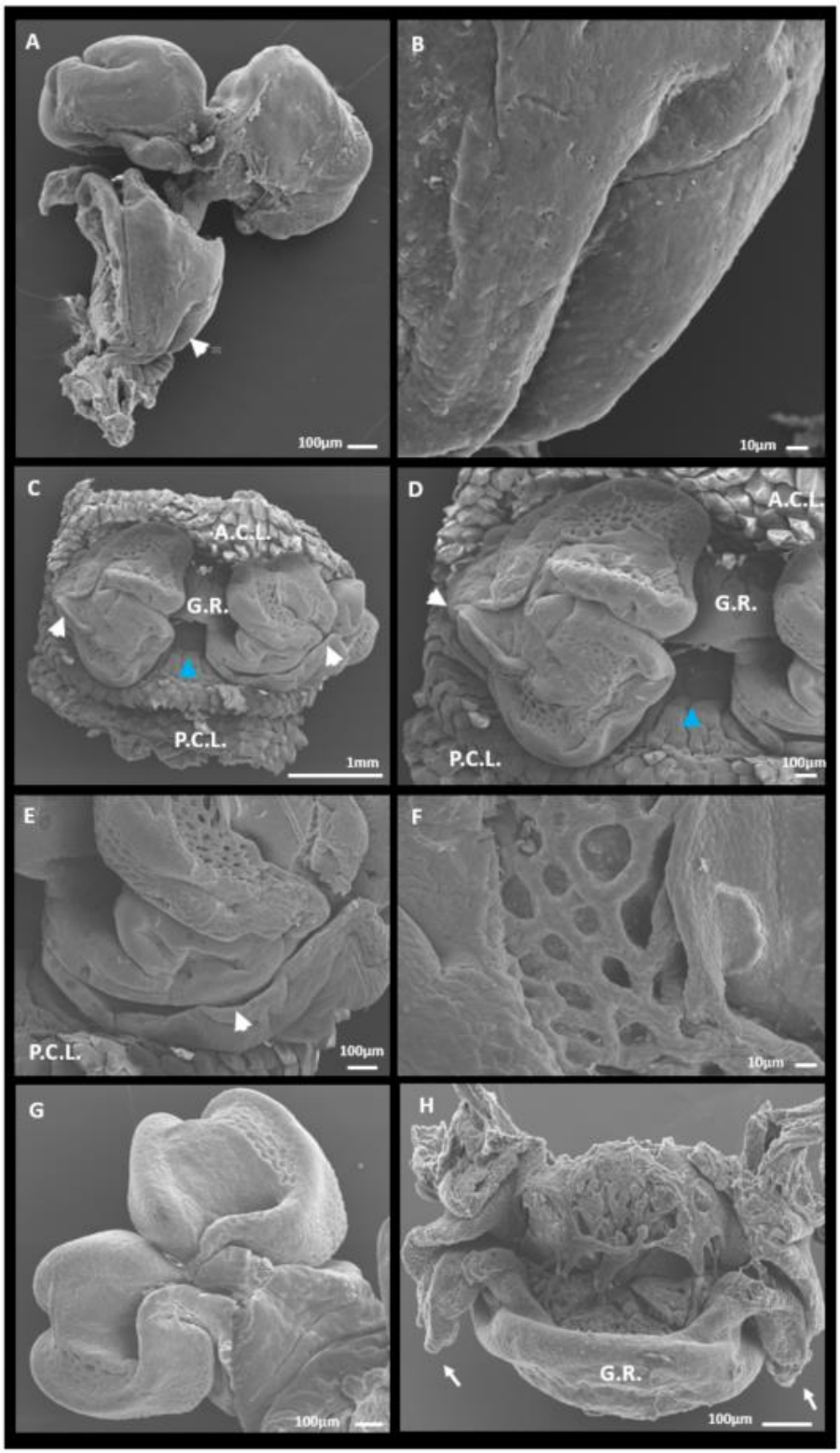


Figure 6: Scanning electron micrographs of a subset of genital phenotypes observed during the development of *P. vitticeps*. A) Single bilobed hemipenis of a stage 18 (60 dpo) male in the 28ZW treatment showing the early stages of sulcus spermaticus development. B) Close up view of the developing sulcus spermaticus from specimen in A. C) Complete genital region of a stage 17 (55 dpo) male in the 28ZZ treatment with the bilobed hemipenes potentially exhibiting sulcus spermaticus, the cloacal opening, genital ridge, and anterior and posterior cloacal lips. D) Zoomed in on single hemipenis from specimen in C. E) Zoomed in on potential sulcus spermaticus extending around the base of the hemipenis from specimen in C and D. F) Zoomed in on the surface of hemipenis showing numerous uncharacterised invaginations. G) Single bilobed hemipenis from a stage 18 sex reversed female with uncharacterised invaginations on the hemipenis. D) Reduced hemipenes of a stage 15 sex reversed female extending over the genital ridge. White arrowheads: sulcus spermaticus, blue arrowheads: cloacal opening, G.R.: genital ridge, A.C.L.: anterior cloacal lip, P.C.L: posterior cloacal lip, white arrows: reduced hemipenes.

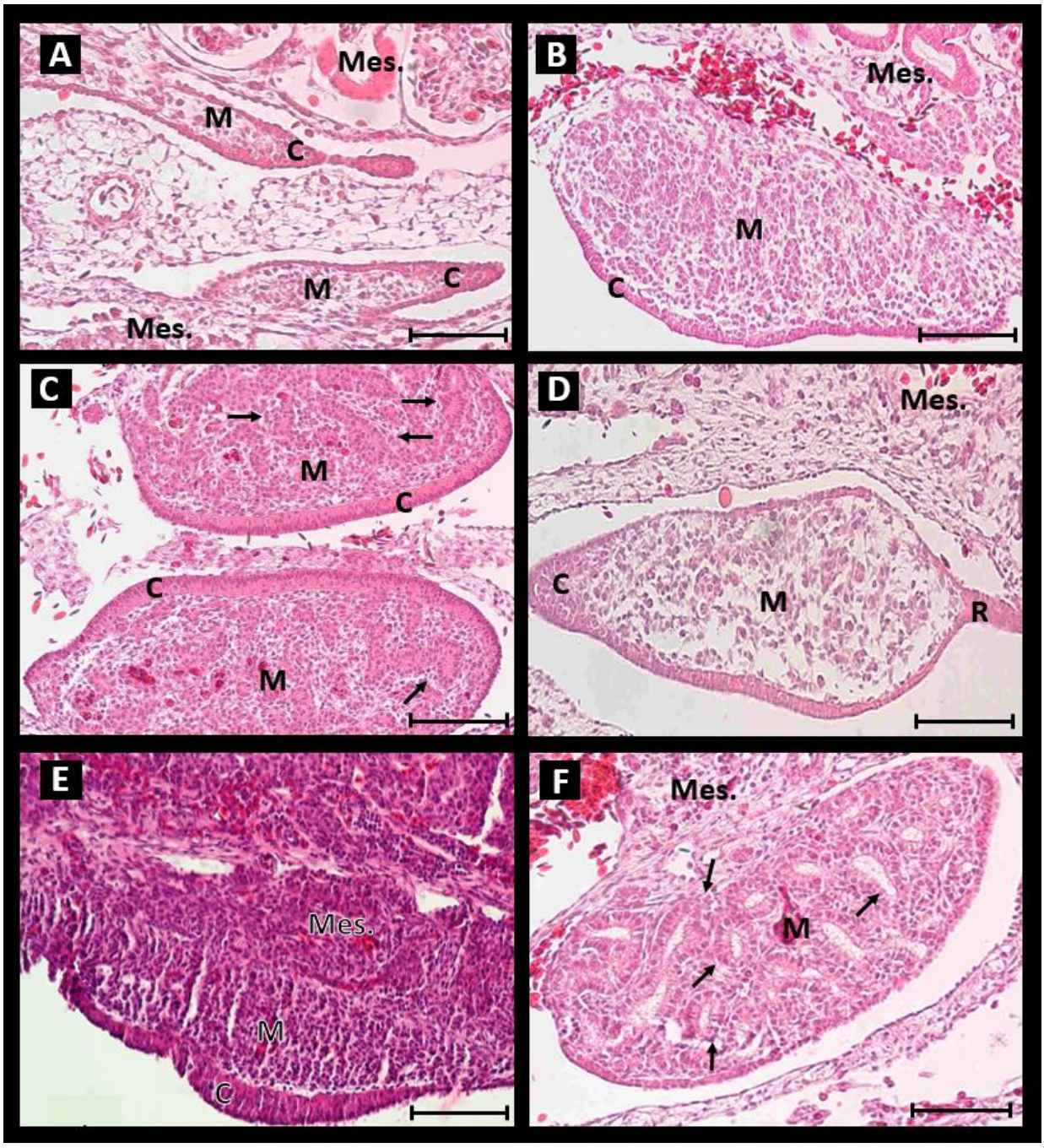


Figure 7: Histological sections of embryonic *P. vitticeps* urogenital system. A: bipotential gonads with developing cortex and medullary regions as it is moving towards the anterior mesonephros. B: bipotential gonad with thin cortex and dense medullary tissues once it has reached its final position on the anterior portion of the mesonephros. C) ovotestes from the 36ZZ treatment showing a proliferating cortex with oogonia, a medulla with numerous rudimentary seminiferous tubules. D: differentiated ovary with a reducing medulla, cortex proliferating with oogonia, and attachment to the mesonephros via the rete cord. E: Atypical ovary found in a concordant stage 16 specimen in the 36ZW treatment exhibiting dense, longitudinally arrange medullary tissues, an elongated shape, and extensive attachment to the mesonephros. F: differentiated testes with a reducing cortex, medulla with developing seminiferous tubules, and extensive mesonephric attachment. All specimens were stained with haematoxylin and eosin and sectioned 6µm thick. M = Medulla, C = Cortex, Mes. = Mesonephros, R = Rete Cord, black arrows = seminiferous tubules.

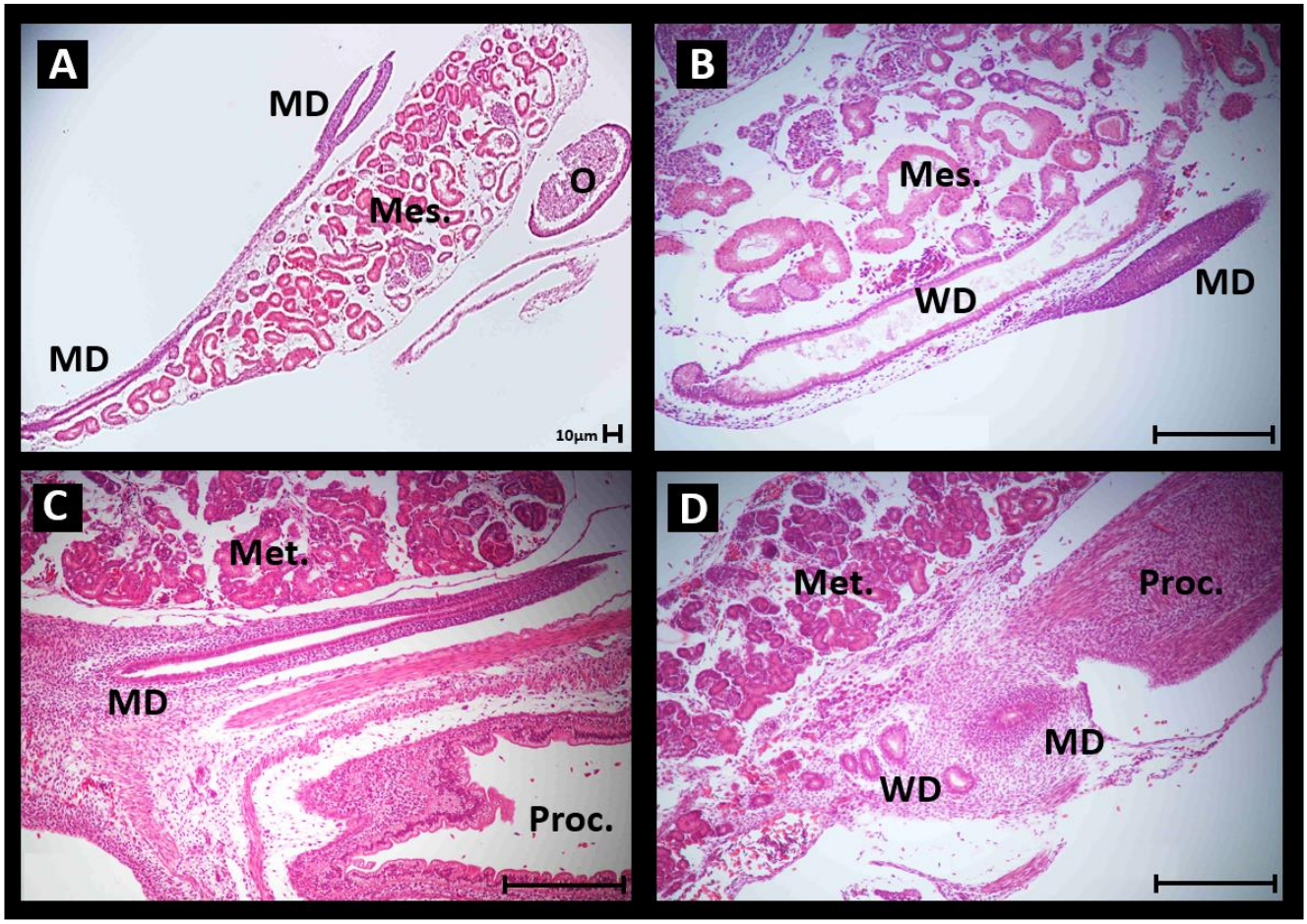


Figure 8: Histological sections of embryonic *P. vitticeps* urogenital system. A: left mesonephros with a Müllerian duct extending along its entire length, and an unattached ovary with clear cortex and medulla differentiation. B: a Wolffian duct with extensive lumen within the outer layer of epithelial cells surrounding the mesonephros, to which a Müllerian duct is attached. G: posterior end of a Müllerian duct embedded within the connective tissues between the proctodaeum and metanephros. H: Müllerian and Wolffian tubules embedded within the connective tissues below the proctodaeum and beside the metanephros. All specimens were stained with haematoxylin and eosin and sectioned 6µm thick. Mes. = Mesonephros, Met. = metanephros, MD = Müllerian duct, WD = Wolffian duct, O = ovary, Proc. = proctodaeum.

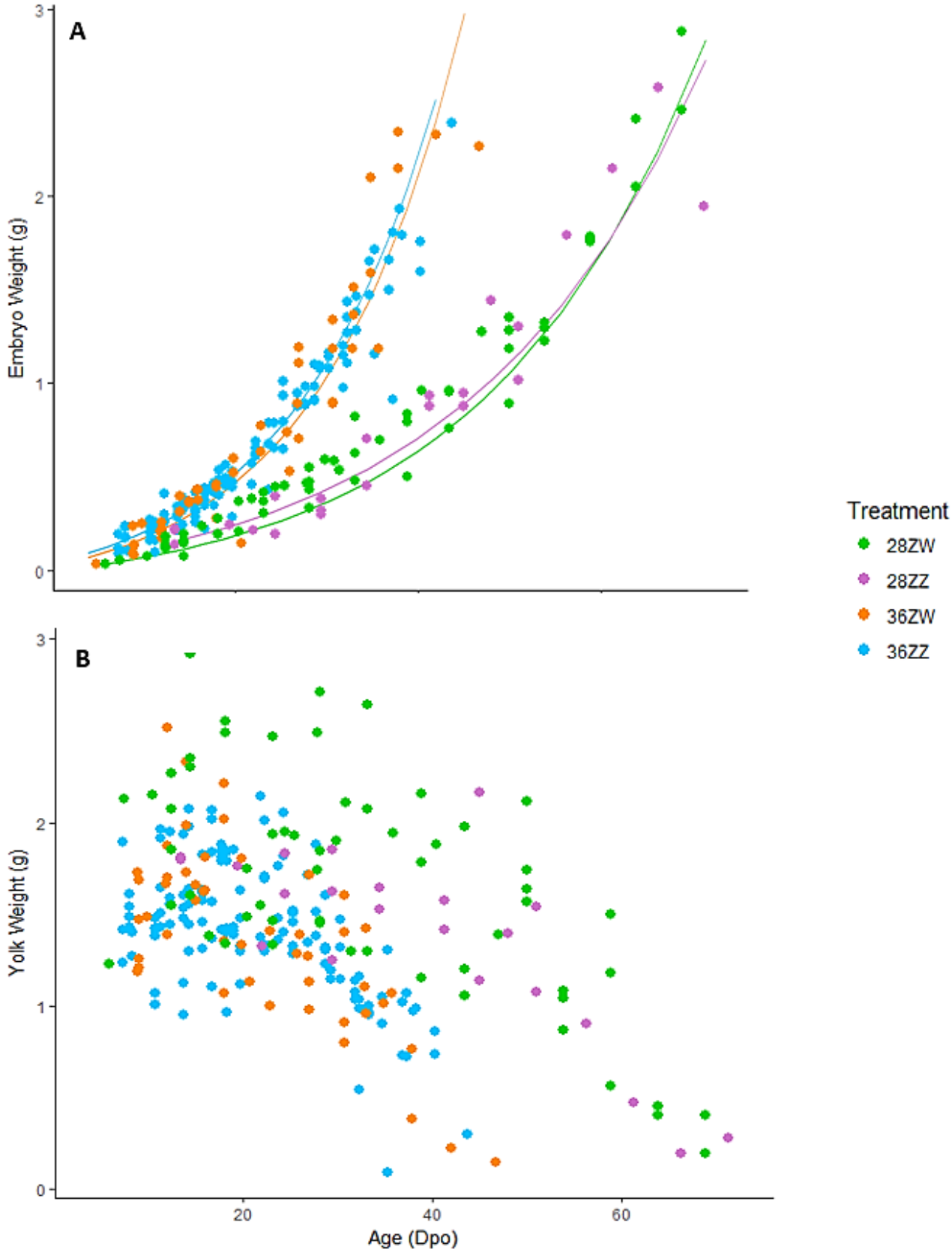


Figure 9: Embryo growth (A) and yolk consumption (B, weight, g) over time (age, days post-oviposition) for each experimental treatment.

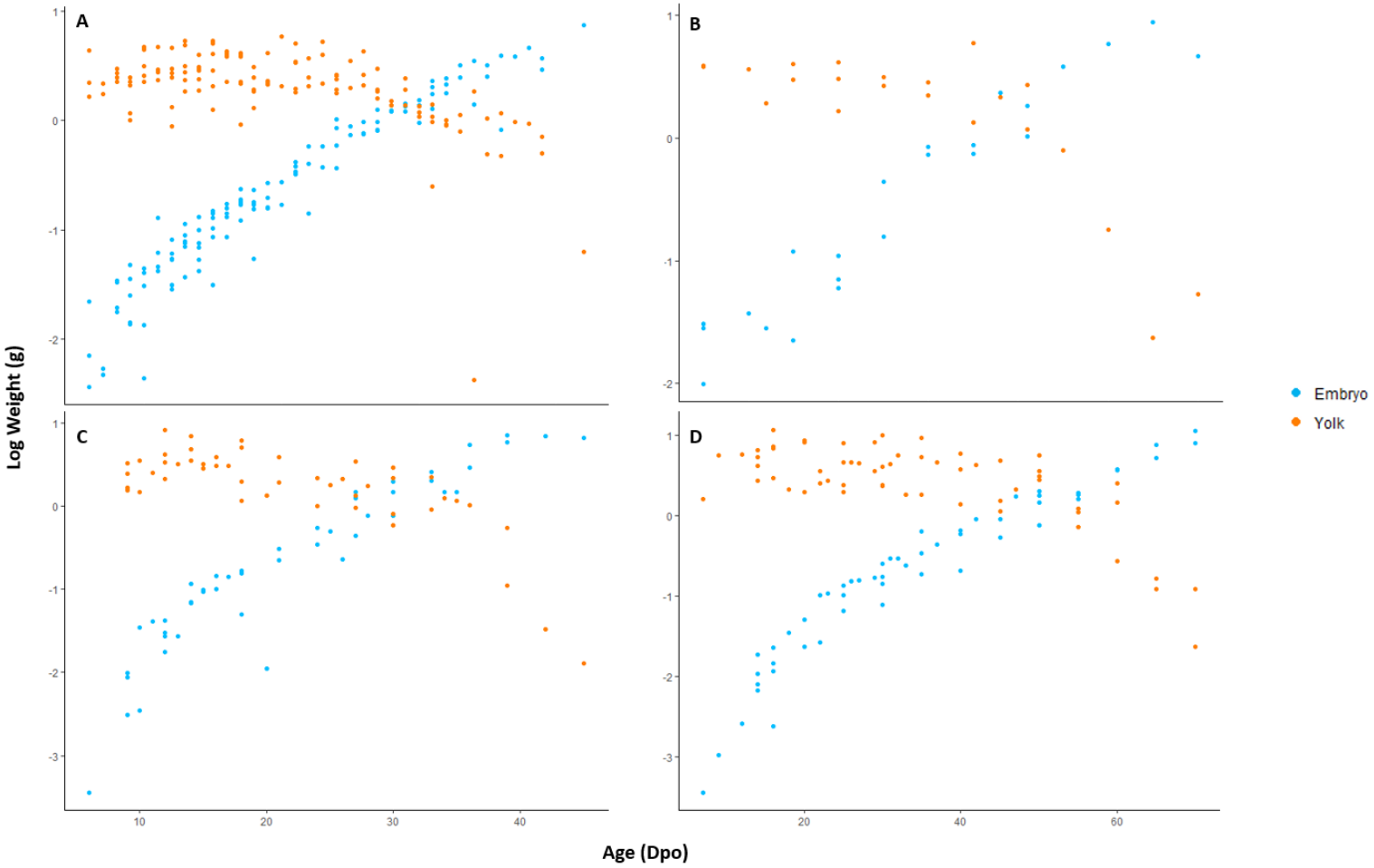


Figure 10: Crossover of log embryo and yolk weight (grams) over time (age, days post-oviposition) for each experimental treatment. A: 36ZZ. B: 28ZZ. C: 36ZW. D: 28ZW.

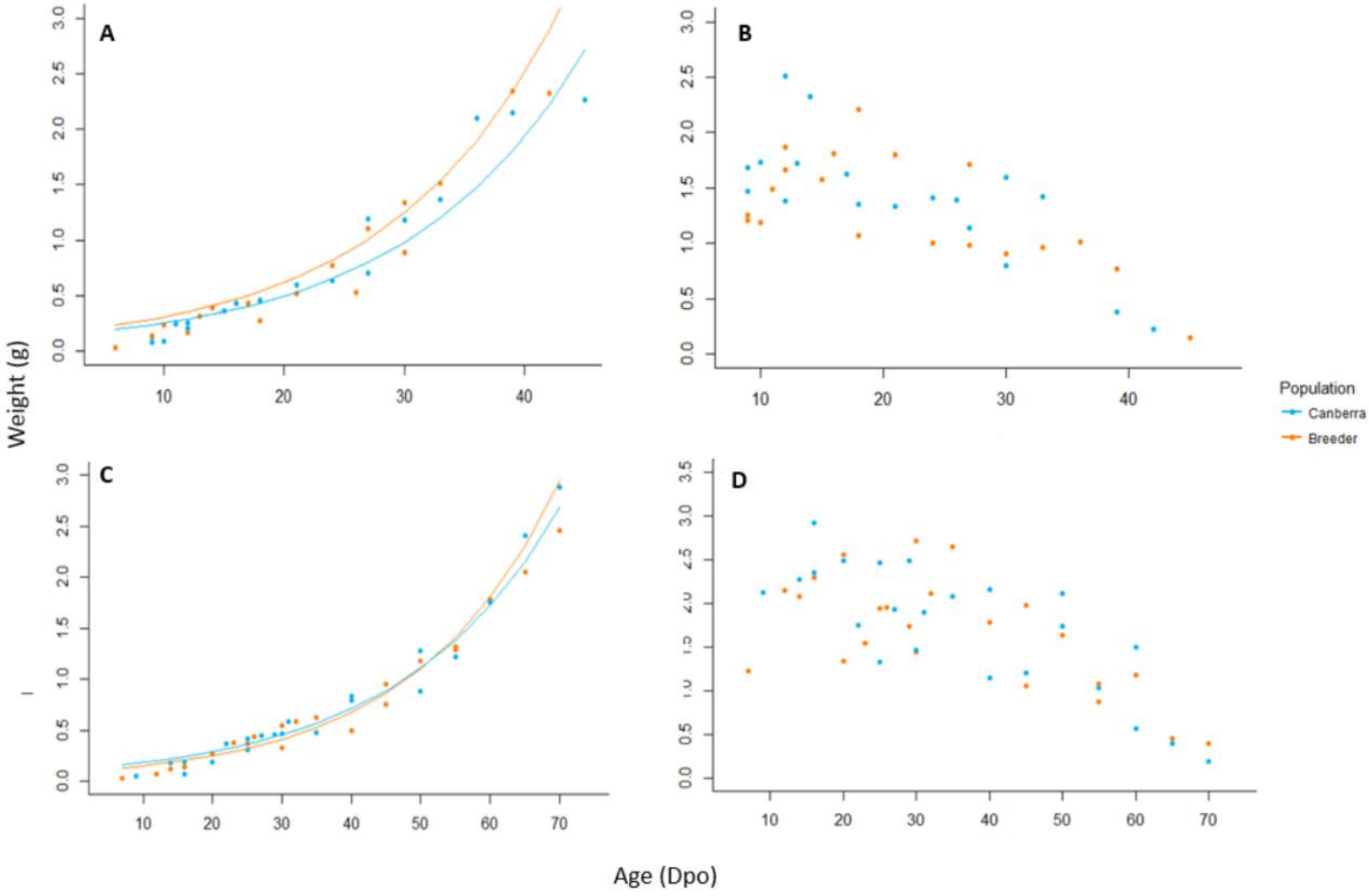


Figure 11: Comparison between populations for embryo weight (A, C) and yolk weight (B, D) over time (age, days post-oviposition) for the 36ZW (A, B) and 28ZW (C, D)

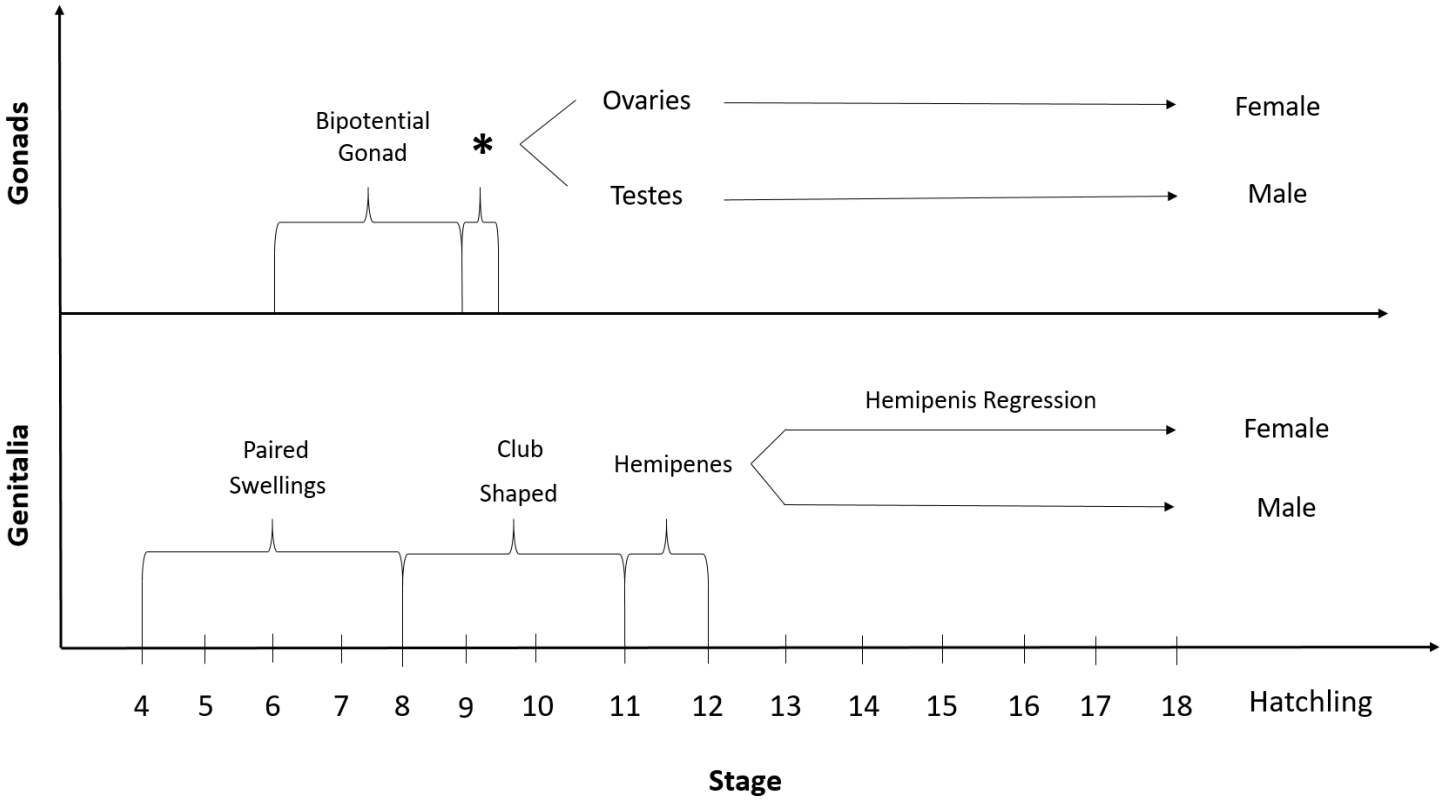


Figure 12: Schematic of the process of sexual development in males and female (concordant and sex-reversed) *P. vitticeps*. After a period of antagonism (black asterisk) during sex-reversal characterised by the presence of ovotestes, the gonads have differentiated by stage 9 in all sexes. In males, the genitalia matures soon after and remain unchanged for the rest of development. In females, hemipenes are developed by stage 11, and begin to regress at stage 13. It is not until stage 18 (near or at hatching) that mature female genitalia (genital ridge) are observed in both concordant and sex-reversed specimens. Developmental event timeframes (based on new staging system, see Figure 2 and Table 1) are approximations only.

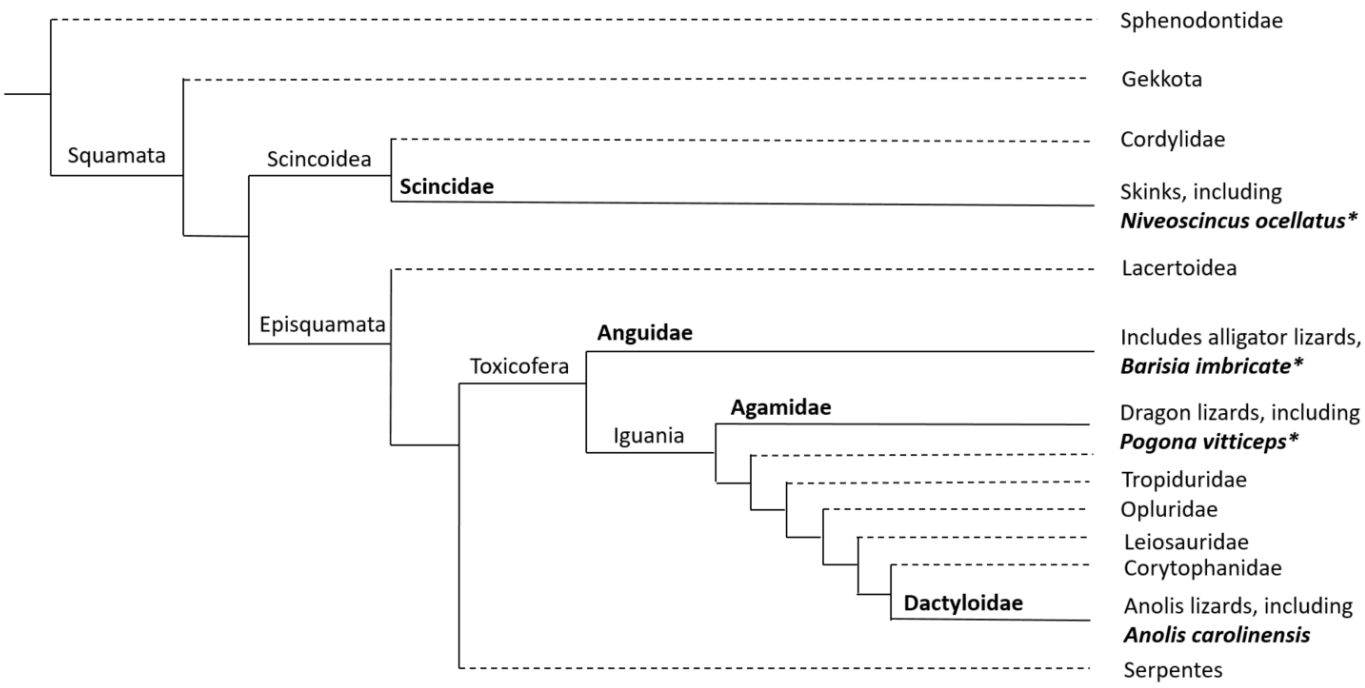


Figure 13: Simplified phylogenetic relationships between key squamate families adapted from Pyron et al. (2013). The three squamate species that exhibit temporary pseudohermaphroditism (TPH, denoted by a black asterisk) belong in these evolutionarily disparate families: Scinidae (the Ocellated Skink, *Niveoscincus ocellatus*), Anguidae (the Imbricate Alligator Lizard, *Barisia imbricata*), and Agamidae (the Central Bearded Dragon, *P. vitticeps*).

8. Appendix

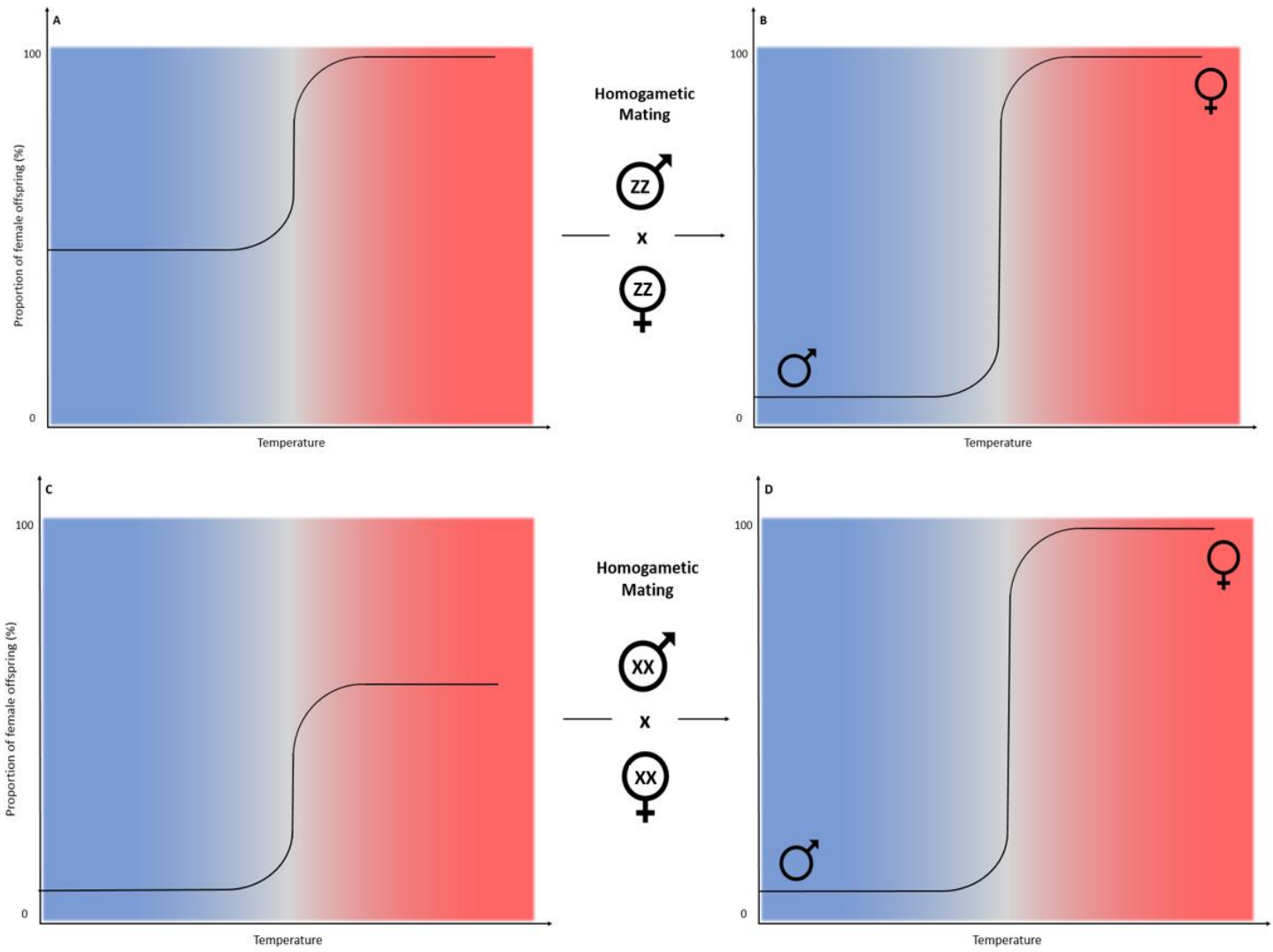


Figure A1: Schematic diagrams for the processes of sex reversal, and resulting matings between sex reversed and concordant individuals, in *P. vitticeps* (A and B) and *B. duperreyi* adapted from Deveson et al. (2017). A depicts the high temperature induced sex reversal that results in the feminization of genetically ZZ males. When these sex-reversed individuals mate with concordant ZZ males, their offspring exhibit a TSD pattern where males are produced at low temperatures, and females at high temperatures (B). In *B. duperreyi*, low temperatures cause genetic XX females to develop as males (C). When these sex reversed XX males mate with concordant XX females, their offspring sex in determined by the same TSD pattern as *P. vitticeps* (D).

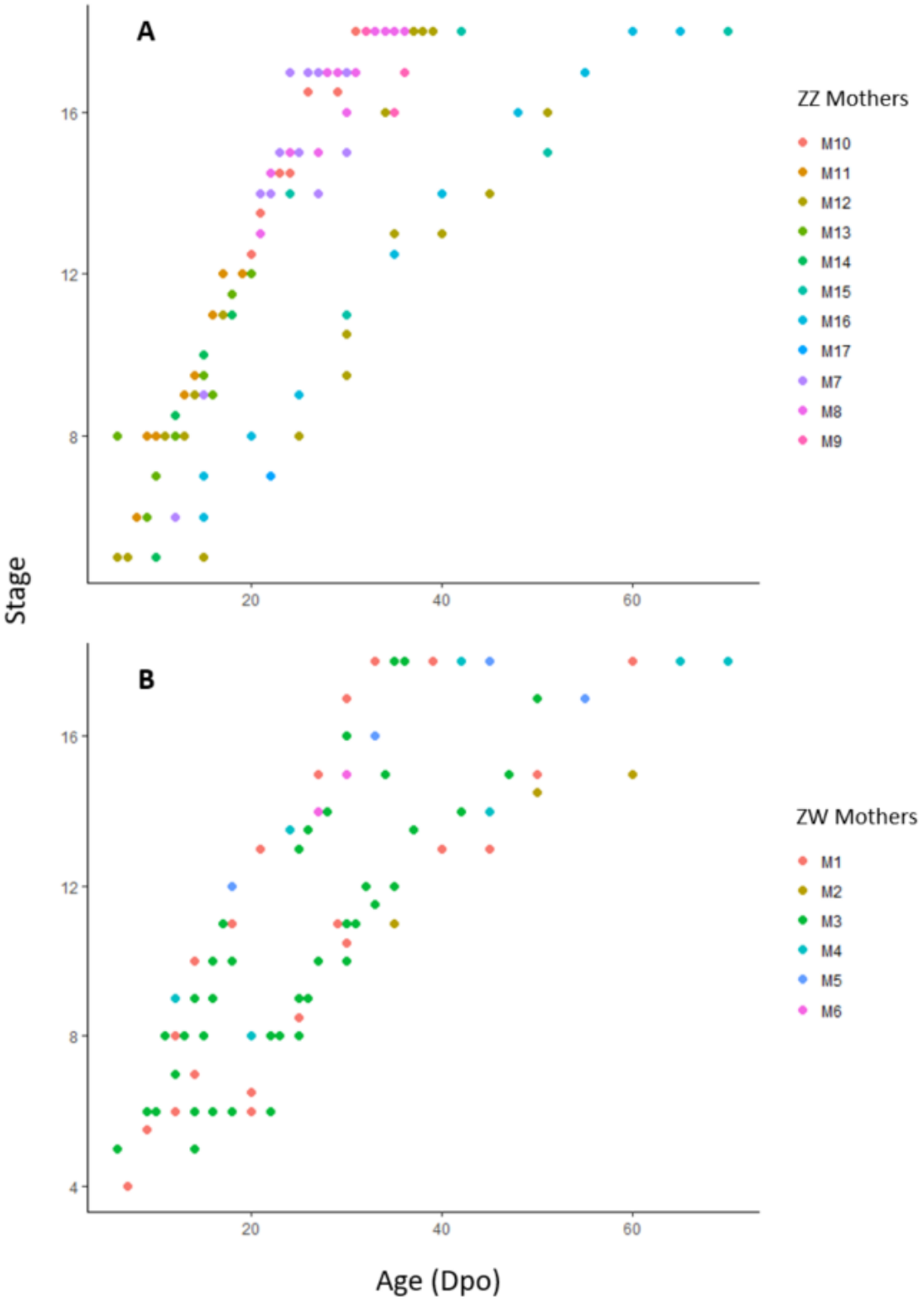


Figure A2: Allocation of eggs from each mother over time (age), and the stage of this embryo for each mother's genotype (A = ZZ, B = ZW). Eggs from mothers 1 and 2 were received from the breeder and eggs from mothers 3 to 17 were from the University of Canberra's breeding colony.

A3. Supplemental Methods

A.3.1. Dissecting the Urogenital system of Embryonic P. vitticeps

To avoid decalcification where possible, the urogenital system is completely dissected for immediate histological processing. The specimen is anchored to wax using dissection pins, and is sexed. Often the hemipenes are already everted, but can be manually exposed by gently squeezing the base of the tail with forceps. This method can also slightly evert the females' hemiclitores. This species is easily sexed near hatching as the male hemipenes are significantly larger than the hemiclitores, and develop a phallic ridge that results in two distinct bulges forming on the end of each hemipenis. However, due to the temporary hermaphroditism (TPH) phase in females, it is essential to genotype specimens prior to dissection otherwise it is not possible to distinguish males and females earlier in development.

The external structures left on the specimen as completely as possible. In order to do this, an incision is made from the posterior cloacal lip extending ventrally as high as the sternum. The skin can either be cut away or pinned to the side. The gastrointestinal system is located anterior to the urogenital system, as are the fat bodies, all of which can be carefully removed by creating an incision through the cloaca anterior to the cloacal lip. These organs can either be removed completely or pinned to one side. This will expose the backbone along which a small paired organ with a yellowish tinge is visible. In older specimens, the kidney presents as a lobular, well vascularised structure that is anteriorly separated, but joins together slightly below the cloaca and into the base of the tail. This is the embryonic kidney to which the gonads are attached. The ovaries and testes are not morphologically distinguishable and have a whitish colour. They are attached to mesonephros (anterior section of the kidney) on either side of the spinal column.

From these organs an obvious connection extends towards the cloaca. At this stage, in order to expose the metanephros (posterior section of the kidney), the pelvic girdle must be severed down the middle. The hind limbs, and associated joints and muscles, can be removed or pinned to the side.

To remove the entire urogenital system intact, expose the posterior end of the kidney, and carefully sever all connective tissue around the sides of the organ. Using forceps to gently lift up the kidney, all connective tissue underneath can be cut away. Once the area where the kidney separates into two lobes is reached, gently lift up the connection between the gonads and the kidney, and continue to sever the connective tissue, repeating on the other side until the entire system can be removed.

A.3.2. Animal Ethics

In total, 221 eggs were obtained from the University of Canberra's (UC) captive breeding colony under animal ethics permit CEAE 15-21. An additional 33 eggs were obtained from a private breeder (10 sampled in the 36ZW treatment, 23 in the 28ZW treatment) under animal ethics permit SBS/295/16.

Table A1: Categorisation of literature on developmental staging of reptiles, including what staging methods were used and their associated author/s. Where possible, any details regarding the timing of gonad and genital development was included. Only papers describing development under normal conditions with no experimental manipulations were included. Any papers that were not written in English with no translation were omitted. It should be noted that often a sexual characteristic was described for the first time, but this was not necessarily the earliest stage of gonadal development. Such instances are marked with an asterisk. NA denotes that these details were not included in the paper, while N denotes that male characteristics were included but female characteristics were excluded, and dpo denotes days post-oviposition.

Author	Species	Staging Methodology	Gonad/Genital Character	Stage/dpo	Female Development
Dufaure and Hubert 1961	*Lacerta vivipara*	Developed new system	Early penis development	Stage 31	Genital but not gonadal development
Zehr 1962	*Thamnophis sirtalis sirtalis*	Developed new system	Cloacal mound can be seen	Stage 23	N
Hubert and Dufaure 1968	*Vipera aspis*	Dufaure and Hubert (1961)	Cloacal region develops small swellings	Stage 35	Genital but not gonadal development
Muthukkaruppan et al. 1970	*Calotes versicolor*	Dufaure and Hubert (1961)	The early penis rudiment is present	Stage 32*	Genital but not gonadal development
Lemus et al. 1981	*Liolaemus tenuis tenuis*	Lemus (1967)	A well-developed phallus is present in both sexes	Stage 37 (21 dpo)*	Genital but not gonadal development
Austin 1988	*Sceloporus undulates*	Dufaure and Hubert (1961)	Undifferentiated mesenchymal tissue	Stages 24-30	Gonadal but not genital development
Doddamani, 1994	*Calotes versicolor*	Muthukkaruppan et al. 1970	Genital ridge on either side of the dorsal mesentery	Stage 27 (oviposition)	Gonadal but not genital development
Jackson, 2002	*Naja kaouthia*	Dufaure and Hubert (1961) and Zehr (1962)	NA	NA	NA

Neaves et al. 2006	*Niveoscincus ocellatus*	Dufaure and Hubert (1961)	Hemipenes Sexual Differentiation	Prior to Stage 1 Stage 5	**Both genital and gonadal development**
Boughner et al. 2007	*Python sebae*	Developed new system	Stage 3*	Hemipenes are visible medial to the limb bud	N
Sanger et al. 2008	*Anolis* (genus)	Developed new system based on limb development	Stage 10	Hemipenes obvious in males	N
Noro et al. 2009	*Paroedura pictus*	Sanger et al. (2008)	Genital buds distinctly bulging at the roots of the hind limbs	4 dpo	N
Wise et al. 2009	*Eublepharis macularius*	Dufaure and Hubert (1961), Muthukkarruppan et al. (1970), Hamburger and Hamilton (1951), Sanger et al. (2008a); Noro et al. (2009)	NA	NA	NA
Boback et al. 2011	*Boaedon fuliginosus*	Werneburg (2009),Zehr (1962), Hubert and Dufaure (1968), Jackson (2002), Boughner et al. (2007)	The hemipenial apparatus first becomes visible	Stage 4 (9-13 dpo)	N
Roscito and Rodrigues 2011	*Nothobachia ablephara*	Werneburg (2009)	Buds of the cranial lip of the cloaca present	Stage 3, (10-12 dpo)*	Genital but not gonadal development
Roscito and Rodrigues 2011	*Calyptommatus sonebrachiatus*	Werneburg (2009)	Hemipenis buds are small, primordia of the cranial lips of the cloaca are present.	Stage 6 (12-14 dpo)*	Genital but not gonadal development

Gregorovicova et al. 2012	*Varanus indicus*	Dpo, Lemus et al. (1981), Sanger et al. (2008), Noro et al. (2009), Wise et al. (2009)	Cloacal swelling	Stage 8, Dpo 43	N
Khanoon and Evans 2014	*Naja h. haje*	Zehr (1962), Hubert and Dufaure (1968)	Distinct cloacal mound and hemipenis analgen	Stage 1 (day of oviposition)	N
Leal and Cohn 2014	*Python regius*	Bougher et al. (2007) and Raynaud (1972)	External genital outgrowth	Stage 1	N
Antonio-Rubio et al. 2015	*Sceloporus aeneus*	Dufaure and Hubert (1961), Austin (1988)	Visible germ cells	Stage 30 (gonadal stage 1)	Gonad but not genital development
Gredler et al. 2015	*Anolis carolinensis*	Sanger et al. (2008)	Phallic swelling forms proximally on the posterior-ventral side of the hindlimb bud	Stage 4	Genital but not gonadal development
Khanoon 2015	*Tarentola annularis*	Hamburger and Hamilton (1951), Dufaure and Hubert (1961), Muthukkarruppan et al. (1970), Wise et al. (2009)	NA	NA	NA
Kovtun and Sheverdyukova 2015	*Natrix natrix*	Zehr (1962)	NA	NA	NA
Martinez-Torres et al. 2015	*Barisia imbricata*	Dufaure and Hubert (1961)	Formation of gonadal ridge	Stage 33-34	**Both genital and gonadal development**
Py-Daniel et al. 2017	*Tropidurus torquatus*	Dufaure and Hubert (1961), Sanger et al. (2008), Wise et al. (2009), Werneburg 2009	Primordium of external genitalia present as a ventral-proximal swelling	Stage 29	Genital development but not gonad development
Khanoon and Zahradnicek 2017	*Psammophis sibilans*	Zehr (1962), Dufaure and Hubert (1968)	NA	NA	NA