Metabolic consequences of sex-reversal in two lizard species: a test of the like genotype and like phenotype hypotheses

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Short running title: Energetic consequences of sex-reversal

Abstract

Vertebrate sex is typically determined genetically, but in many ectotherms sex can be determined by genes (Genetic Sex Determination: GSD), temperature (Temperature-dependent Sex Determination: TSD), or interactions between genes and temperature during development. Temperature dependent sex determination may involve GSD systems with either male or female heterogamety (XX/XY or ZZ/ZW) where temperature overrides chromosomal sex determination to cause a mismatch between genetic sex and phenotypic sex (sex-reversal). In these temperature-sensitive lineages, phylogenetic investigations point to recurrent evolutionary shifts between genotypic and temperature-dependent sex determination. These evolutionary transitions in sex determination can occur rapidly if selection favours the reversed sex over their concordant phenotypic sex. To investigate the consequences of sex-reversal on offspring fitness, we measured two energy-driven traits often linked to fitness (metabolism and growth-rate) and 6-month survival in two species of reptile with different patterns of temperature-induced sex-reversal. Male sex-reversal occurs in *Bassiana duperreyi* when chromosomal females (femaleXX) develop male phenotypes ($male_{SR}XX$), while female sex-reversal occurs in *Pogona vitticeps* when chromosomal males (maleZZ) develop female phenotypes ($female_{SR}ZZ$). We show metabolism in $male_{SR}XX$ was like that of maleXY, that is, reflective of phenotypic sex and lower than genotypic sex. In contrast, for *Pogona vitticeps*, $female_{SR}ZZ$ metabolism was intermediate between maleZZ and femaleZW metabolic rate. For both species, our data indicate differences in metabolism become more apparent as individuals become larger. Our findings provide some evidence for a fitness advantage from sex-reversal in both species but do not exclude energetic processes as a constraint on the distribution of sex-reversal in nature.

*Keywords**:** energetics, sex determination, sex-reversal, Pogona vitticeps, Bassiana duperreyi*

1 | Introduction

Sex-determination in vertebrates is highly variable, ranging from genotypic sex determination (GSD) where sex is established by sex chromosomes, to environmental sex determination (ESD) where sex is primarily influenced by prevailing environmental conditions (Bull, 1983). For some species, these pathways of reproductive development are not mutually exclusive, but can co-occur (Bachtrog et al., 2014; Sarre et al., 2004). In a few well-studied species, GSD systems with either male (XX/XY) or female heterogamety (ZZ/ZW) are influenced by incubation temperature (Temperature-dependent sex determination; TSD) (Holleley et al., 2015; Noble et al., 2018; Quinn et al., 2009). In these GSD species, when conditions experienced during critical developmental stages exceed a threshold temperature that overrides genetic sex-determining mechanisms. This temperature override, commonly referred to as sex reversal, causes a discordance between phenotypic and genotypic sex (Holleley et al., 2015; Quinn et al., 2009; Radder et al., 2009). Theoretical models predict that when sex-reversed individuals have a greater fitness advantage populations can rapidly loose the heterogametic sex chromosome (XY or ZW) and result in pure TSD lineages within a few generations (Grossen et al., 2011; Holleley et al., 2015; Schwanz et al., 2020). Consequently, these TSD lineages should become widely established in free-living populations where environmental conditions favour their emergence. However, sex-reversal in some species is not distributed across ecotypes in natural systems as would be, suggesting that free-living animals experience costs associated with sex-reversal that are not accounted for in theoretical models predicted (Castelli et al., 2021; Wild et al., 2022). Therefore, quantifying the costs and benefits of sex-reversal will help clarify patterns of sex-reversal in wild populations and will provide insight into the mechanisms that may inhibit or accelerate evolutionary transitions in sex-determining systems (Bachtrog et al., 2014; Sarre et al., 2004).

Of crucial importance for individual growth, reproduction, and survival (i.e., fitness traits) is energy expenditure (Angilletta, 2009; Bradshaw, 1997), which can be estimated by measuring metabolic rates. Metabolism is inextricably linked to patterns of growth, reproduction and survival across individuals and so is a fundamental currency for understanding fitness effects (Peterson et al., 1999; White et al., 2022). Such estimates provide a crucial link between individual life history traits (growth rates, developmental rates, and age at maturity) and population processes (growth rates, carrying capacity, and rates of competition) in a variety of taxa (Brown et al., 2004; Ernest et al., 2003; Burger et al., 2021; Savage et al., 2004). Importantly, strategies used to secure, allocate and expend energy can vary considerably between phenotypic sexes (Arnqvist et al., 2022; Boratyński et al., 2010; Codding et al., 2011; Geffroy, 2022) and may play a driving role in establishing fitness differences in sex-reversed individuals and their phenotypic counterparts. Exploring how sex-reversal impacts metabolism and other traits that relate to energy use will provide insight into how the costs or benefits vary across sex classes. These data will provide valuable information that may help understand distributional patterns of sex-reversal in natural populations.

Identifying the costs and benefits of sex-reversal allows predictions of the conditions under which selective advantages of sex-reversal might lead to rapid transitions in sex-determining mechanisms and help provide an understanding of variation in its frequency across wild populations. Three plausible phenotypic/genetic patterns may manifest that can influence the evolution of sex-reversal in nature (Fig. 1 – e.g., metabolism):

(1) there is no difference in metabolism, growth, or survival among different genotype-phenotype combinations such that males, females, and sex-reversed individuals are indistinguishable (Null);
(2) sexes are phenotypically similar with discordant sex-reversed individuals (e.g. female$_{SR}$ ZZ) and concordant individuals of the same *phenotypic* sex (e.g. female ZW) exhibiting similar metabolic rate, growth, and/or survival (Like Phenotype); or
(3) sexes are phenotypically different with discordant sex-reversed individuals (e.g. female$_{SR}$ ZZ) and concordant individuals of the same *chromosomal* sex (e.g. male ZZ) exhibiting similar metabolic rate, growth, and/or survival (Like Genotype).

Evidence for the Like Phenotype would suggest that metabolic differences between phenotypic sexes (i.e., male vs. female) are driven by hormonal mechanisms or sexually-antagonistic selection that leads to sexual dimorphism in traits such as morphology or physiology (Cox et al., 2017; Eyer et al., 2019; Lipinska et al., 2015; van Doorn & Kirkpatrick, 2010). Alternatively, support for the Like Genotype would imply that sex-linked genes are involved in the expression of traits linked with metabolism, energy use, and potentially other fitness-related endpoints (Charlesworth & Charlesworth, 1980; Fisher, 1931; Harrison et al., 2015). Under the scenario where selection for the Like Genotype is favoured, an increase in the frequency of XX or ZZ individuals within a population would occur and would rapidly increase the chances of a transition from GSD to TSD (Grossen et al., 2011; Schwanz et al., 2020). To date, no studies have explored the metabolic consequences of sex-reversal in any other vertebrate even though metabolism is essential to various fitness-related aspects (Geffroy, 2022; Somjee et al., 2022).

To estimate the energetic and fitness consequences of sex-reversal and to determine if sex-reversed individuals are more similar to their reproductive phenotype or chromosomal complement, we measured two energy-driven traits often linked to fitness (metabolism and growth) and 6-month survival in two lizard species that demonstrate sex-reversal (*Pogona vitticeps* & *Bassiana duperreyi*). Sex-reversal in *B. duperreyi* occurs when chromosomal females (female XX) develop male phenotypes [male$_{SR}$ XX] (Dissanayake, Holleley, Deakin, et al., 2021; Quinn et al., 2009), whereas sex-reversal in *P. vitticeps* occurs when chromosomal males (male ZZ) develop female phenotypes [female$_{SR}$ ZZ] (Holleley et al., 2015; Quinn et al., 2007). In both species, this phenomenon has been documented in the wild (Dissanayake et al., 2020; Holleley et al., 2015; Wild et al., 2022), with evidence indicating both species are sensitive to climate change (Dissanayake, Holleley, & Georges, 2021; Holleley et al., 2015; Schwanz et al., 2020). Similarities between phenotypic forms (female$_{SR}$ ZZ and female ZW in *P. vitticeps*; or male$_{SR}$ XX and male XY in *B. duperreyi*) in these traits would suggest a lack of fitness differences imparted by sex-reversal, while similarities between genotypic forms (female$_{SR}$ ZZ and male ZZ in *P. vitticeps*; or male$_{SR}$ XX and female XX in *B. duperreyi*) would indicate the potential for differences in fitness between discordant and concordant sex. How these traits vary across sex can provide valuable information on the ecological significance of sex-reversed individuals and help determine mechanisms affecting the evolution of sex chromosomes in vertebrate species.

2 | Materials and methods

2.1| Lizard collection and husbandry

Bassiana duperreyi –Twenty-five *B. duperreyi* nests with a total of 40 eggs (1-4 eggs per nest) were opportunistically located in November 2020 by flipping rocks, logs, and other cover objects at two field locations within the Brindabella Range (Mount Ginini – 1640 m

a.s.l., 35°31’29.6“S 148°46’58.7”E; Piccadilly Circus – 1240 m a.s.l., 35°21’42.0“S 148°48’12.5”E). These sites were selected because of high frequencies of sex-reversal previously documented within these populations (Dissanayake, Holleley, Deakin, et al., 2021). The number of eggs per nest was recorded, and temperature dataloggers (iButton® model DS1921G; accuracy± 1°C) were placed at the core of each nest to monitor nest temperatures. Each nest was maintained in natural conditions for 9-10 weeks at each location, and the mean nest temperatures (Mount Ginini – 18.94°C ± 0.98 & Piccadilly Circus – 20.42°C ± 0.84; Fig. S1) were monitored to ensure approximately 90% of the development period passed in natural conditions (Shine et al., 2002). Therefore, sex-reversal in *B. duperreyi* occurred in natural nest sites due to exposure to sex-reversing low temperatures (<20°C) *in situ*. The eggs were then collected, placed in moist vermiculite, and transported back to the University of Canberra. Eggs were placed in incubators (LabWit, ZXSDR1090) that maintained 23°C, which produces a balanced sex ratio (Shine et al., 2002). *See* Dissanayake et al 2021 for the study site description and further detail regarding general egg collection methods.

Phenotypic sex was determined by squeezing the tail base to evert the hemipenes (Harolow, 1996) for 3-to-7-day old hatchlings and was checked again by hemipene transillumination after 5 weeks (Dissanayake, Holleley, & Georges, 2021). Blood from the tail of each individual was collected on Whatman FTATM Elute Micro Card (CAT No. WB120410). Lizards were housed individually in plastic containers (0.35x0.25x0.15m). Each tub contained cardboard tubes and paper egg carton pieces for hides. Full-spectrum UV bulbs and heat bulbs were placed alternating between tubs to create a thermal gradient in each tub (heat from one side, UV from the other). Hatchlings were fed live, gut-loaded crickets once per day *ad libitum* and twice per week the crickets were dusted with calcium powder. Hatchlings were provided with shallow water dishes that were replenished daily, and they were misted twice per day with water.

Pogona vitticeps – The University of Canberra (UC) maintains a breeding colony of adult *P. vitticeps*, where breeding enclosures are comprised of one male (male ZZ) to either three sex-reversed females (female$_{SR}$ ZZ) or three concordant females (female ZW). During the summer of 2020–2021, females were allowed to lay eggs, which were collected within 2h of deposition. Eggs (n= 96) from 15 clutches were randomly allocated to either 28°C (n= 43; no sex-reversal expected) or 34°C (n = 53; reversal of 50% of ZZ genotypes expected) in temperature-controlled incubators (LabWit, ZXSDR1090) on moist vermiculite. Thus, sex-reversal in *P. vitticeps* occurred because of exposure to sex-reversing high temperatures (> 32°C) during incubation. Once hatchlings emerged, the determination of phenotypic sex and blood sampling followed the same protocols as for *B. duperreyi*. Hatchlings were housed in plastic tubs (0.8x0.5x0.35m; 5 individuals per tub), and in addition to crickets, finely grated vegetables were introduced to the diet beginning at 6-7 weeks post-hatch.

2.2| Genotyping

Genotypic sex was determined for both *B. duperreyi* and *P. vitticeps* using polymerase chain reaction (PCR)-based molecular sex tests from extracted DNA collected from blood samples. DNA was extracted from tissue samples. DNA purity was determined using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and quantified using the Qubit 2.0 Fluorometric Quantitation (Invitrogen, Life technologies, Sydney, N.S.W., Australia). The sex-reversal status was determined for *B. duperreyi* by using PCR as described by Dissanayake et al. (Dissanayake et al., 2020), where the genotypic sex was identified based on Y-specific markers allowing identification of XX and XY samples. No XY females were observed, which is consistent with previous observations that

recombination and/or mutation involving these loci is negligible and does not affect the accuracy of genotypic sex assignment. Genotypic sex (ZZ/ZW) for *P. vitticeps* was determined using a PCR-based molecular sex test that amplifies a W-chromosome-specific size polymorphism (Holleley et al., 2015), in which two bands amplify in ZW individuals and one control band amplifies in ZZ individuals. No ZW males were observed. All PCR products were visualized on a 1.5% agarose gel using SYBR Safe (Life Technologies, Carlsbad, USA), and all molecular sex tests were conducted blind to the phenotypic sex of the individuals. For both species, sex class accounted for genotype and phenotype and when genotype–phenotype discordance occurred individuals were classified as sex-reversed (Holleley et al., 2015).

2.3 | Respirometry

Metabolic rate (MR) was defined as the rate of oxygen consumption ($\dot{V}O_2$, mL min^{-1}) of post-absorptive animal using a stop flow respirometry system (Stable System FMS, Las Vegas NV, USA). A gas analyser sub-sampler was used to pump outside air scrubbed of CO_2 (using soda lime, Chem-Supply, AUS) and water vapour (using Drierite, W. A. Hammond Drierite Co. Ltd) to a mass flow controller that regulated flow rate to a nominal value of 130 ml min^{-1} (*B. duperreyi*) or 250 ml min^{-1} (*P. vitticeps*). After passing through the mass flow controller, air was pushed through an airtight cylindrical respirometry chamber, with dimensions designed specifically for each species (*B. duperreyi*: 75x20mm; *P. vitticeps*: 200x40mm). Air was pushed into the chamber and then through a flow meter ensuring that flow rates were constant. Air was then rescrubbed of water vapor, using Drierite, before being passed through H_2O and O_2 gas analysers. The fractional concentration of O_2 in the ex-current air (FO_2) was recorded at a frequency of 1 Hz. Following the manufacture protocols, both H_2O and O_2 analysers were calibrated prior to experiments.

Metabolic rate was measured within 3 weeks of hatching for all individuals. After a minimum of 24 h fasting period, body mass (0.01g) was measured of each individual lizard using a digital sale (Ohaus SP-202) before and after being placed in the respirometry chamber. Two incubators (LabWit, ZXSDR1090) were used to control the temperature of outside air being pulled into the respirometry system and then flowed through to the second incubator that controlled the temperature ($\pm$ 1°C) in which animals in chambers were placed. Incubator temperatures were held at a constant temperature relevant to the thermal preference for each species (*B. duperreyi* 34°C (Du et al., 2010); *P. vitticeps* 33°C (Greer, 1989). At approximately 17:00 lizards were placed in respirometry chambers inside a dark incubator and remained in the chambers overnight for the duration of the experiment. As such, these animals were mainly in a quiescent state, but some activity may have occurred within the chamber. Each respirometry trial lasted approximately 14 h, and to ensure that animals were habituated within chambers, the first 2 h of data were discarded from analysis. The system contained seven chambers that lizards were placed in individually and one empty chamber designated as a control. The O_2 consumption of each lizard was measured continuously for 5 min over a 70 min sampling window, and this sampling sequence was repeated every 70 min for the duration of the experiment. Immediately following each individual lizard measurement, the control chamber recorded for 5 min as a baseline of O_2. During each 70 min sampling window O_2 depletion for each individual was identified using the R package "metabR" (github.com/daniel1noble/metabR) and O_2 depletion was averaged for each individual across the night to represent MR. The rate of O_2 depleted by an individual was calculated following Eq. 4.21 *in* Lighton, 2008):

$$\dot{V}O_2 mL min^{-1} = \frac{\%O_2(V_{Chamber} - V_{lizard})}{t}$$

where the rate of O_2 is the maximum percentage of O_2 a sample below that baseline; $V_{chamber}$ is the volume of the chamber (*B. duperreyi*: 23.56 mL; *P. vitticeps*: 251.33 mL); V_{lizard} was calculated as an average between the pre- and post-measurement mass of each individual, and t is the duration of time the chamber was sealed between air samples taken (70 min). The mass of each lizard was used as a proxy for its volume (1g = 1 ml) because of their high correlation and increased accuracy and precision in mass measurements (Friesen et al., 2017).

2.4 | Growth and survival

Measurements of snout-to-vent length (SVL) and mass were used to estimate growth rates. SVL and mass were initially measured during respirometry experiments and remeasured 6 months after the initial measurements. Growth rate was calculated by subtracting initial measurements (SVL or mass) from the final remeasurement and dividing the elapsed time between measurements. SVL growth rate was recorded in mm/d for both species, and mass growth rate was recorded in centigrams (cg/d) for *B. duperreyi* and (g/d) for *P. vitticeps*. The survival rate of hatchlings was determined by documenting the frequency of mortality between the hatch date and 6 months post-hatch date for both species.

2.5 | Statistical analysis

All statistical analysis were conducted using the R environment, ver. 4.1.0 (www.r.-project.org). Bayesian linear mixed effect models from the package *brms* (Bürkner, 2017) were used to analyse O_2 data for each species. Default priors and 4 MCMC chains of 5000 were run with a burn in of 1000 and a thinning interval of 5 for the "brms" models. All models were checked for proper mixing and convergence by visually inspecting trace plots. For each species two models were fitted, the first in which homoscedasticity of the data was assumed and the second in which heteroscedasticity was accounted for within the data. The first model for estimating metabolism was fitted using the following structure:

$$MR_{ijk} = \left(\beta_0 + id_j + d_k\right) + \beta_1 \cdot Sex_{Female} + \beta_2 \cdot Sex_{Male} + \beta_3 \cdot Sex_{SR} + \left(\beta_4 + \beta_{(id_{ij})} \cdot time_z\right) + \beta_5 \cdot log\ Mass_{sc} \cdot Sex_{Female} + \beta_6 \cdot log\ Mass_{sc} \cdot Sex_{Male} + \beta_7 \cdot log\ Mass_{sc} \cdot Sex_{SR} + e_{ijk}$$

where MR_{ijk} is the metabolic rate ($log\ \dot{V}O_2 \cdot mL^{-1} \cdot min^{-1}$) for measurement *i* (i = 1 to N_m, number of measurements) on individual *j* (j = 1 to N_{id}, number of individuals) and day *k* (k = 1 to N_d, number of days). Contrasts for the different sex classes ($\beta_1 - \beta_3$), where Sex_{Female} and Sex_{Male} are for concordant sexes and Sex_{SR} sex-reversed animals, respectively. A linear slope β_4 was estimated for measurement time ($time_z$, z-transformed) and a random intercept (id_j) and slope for $time_z$ ($\beta_{id_{ij}}$) were included for individual *j* across measurement occasions. A linear slope for log transformed mass ($log\ Mass_{sc}$, centered on mean, sc) and mass scaling relationships were estimated separately for the different sex classes (i.e., $\beta_5 \cdot log\ Mass_{sc} \cdot Sex_{Female}$, $\beta_6 \cdot log\ Mass_{sc} \cdot Sex_{Male}$, and $\beta_7 \cdot log\ Mass_{sc} \cdot Sex_{SR}$ respectively). Deviations were sampled from a multivariate normal distribution ($\sim MVN([0,0], ID)$, where ID is a (co)variance matrix with a random intercept and slope variance and their covariance. A random-effect for day (d_k) ($\sim N(0, \sigma_k^2)$) was also included in the model to account for variation across days in metabolic rate. In all models, we retained data for each measurement throughout the night to improve analytical power. Given that animals were quiescent, our MR data is expected to be representative of Standard Metabolic Rate (SMR). Nonetheless, some movement did occur in our chambers. As such, we also fit the same models described above but kept the lowest 10% of oxygen consumption values during trials – data that should be

quite close to SMR. We found no changes in our results when using the full dataset compared to the dataset that only used the lowest 10% (*see* Fig. S2; Tables S2 & S3 in *Supp*). Therefore, all $\dot{V}O_2$ measurements from trials (MR) were kept for further analysis.

Differences in growth rates were compared across sex class using Bayesian linear mixed effect models. Growth rate of SVL and mass were analysed as a function of initial size (or mass) measurements, sex class and their interaction. Fisher's exact tests were used to determine if there was an association between sex class and frequency of hatchling mortality after six months.

For all Bayesian models, posterior estimates were from multiple chains, and we present posterior means and their 95% credible intervals. To test for the Like Genotype (genotype - sex-reversed) or Like Phenotype (phenotype - sex-reversed) framework for each species, contrasts were calculated by subtracting the posterior distributions of each sex class. To test if the magnitude of these differences varied significantly, probabilities of parameter estimates were considered statistically significant when the 95% CIs did not include 0, and the pMCMC values calculated by *MCMCglmm* were less than 0.05 (Hadfield, 2010). Data, code, and additional resources are available at https://github.com/daniel1noble/energy_sex_reversal.git.

3 | Results

3.1 | Energetic consequences of sex-reversal

Bassiana duperreyi - A total of 760 measurements for 40 individuals ($male_{SR}$ XX: n = 13, female XX: n = 15, male XY: n = 12) were recorded. There was a strong scaling relationship between log metabolic rate and log mass (Table 1) that varied significantly by sex class (significant interaction between sex class × logmass – Fig. 2A). Sex-reversed male XX *B. duperreyi* had a scaling relationship that was most like their phenotypic counterparts (male XY - $male_{SR}$ XX; pMCMC = 0.33; Table 3) and lower than their genotypic counterparts (female XX - $male_{SR}$ XX; pMCMC < 0.01). For phenotypic males ($male_{SR}$ XX & male XX), the scaling relationship between logmass and metabolism changed similarly across differently sized individuals (Fig. 2B). Pairwise comparisons across sex class indicated no differences in body mass across our treatments (Fig. 2A; Table S3). The homogeneous variance model was the most parsimonious ([heteroscedastic model – homoscedastic model] loo: -5.5, SE = 6.87), accounting for 77% (95% CI:0.75 - 0.78) of the variation in metabolic rate.

Pogona vitticeps - A total of 1365 measurements for 96 individuals ($female_{SR}$ ZZ: n = 28, female ZW: n = 30, male ZZ: n = 38) were recorded. There was a strong scaling relationship between log metabolic rate and log mass (Table 2) that varied significantly by sex class (significant interaction between sex class × logmass - Fig. 2C). Sex-reversed female *P. vitticeps* ($female_{SR}$ ZZ) had a scaling relationship that was overall higher than their genotypic counterparts (male ZZ - $female_{SR}$ ZZ; pMCMC < 0.01), but lower than their phenotypic counterparts (female ZW - $female_{SR}$ ZZ; pMCMC = 0.04; Table 3). As $female_{SR}$ ZZ got larger, the mass scaling relationship of metabolism was more like ZZmales than ZW females (Fig. 2D). Pairwise comparisons of body mass across sex class in *P. vitticeps* indicated no differences in body mass across treatments (Fig. 2C; Table S4). The heteroscedasticity variance model was the most parsimonious ([heteroscedastic model – homoscedastic model] loo: -189.8, SE = 33.96), accounting for 84% (95% CI:0.83 - 0.85) of the variation in metabolic rate.

3.2 | Effects of sex-reversal on growth and survival

Growth rates for both SVL and mass supported the Null prediction among *B. duperreyi,* where there were no detectible differences across sex class (Table 3; Table S5). Similarly, in *P. vitticeps* the Null prediction was supported when comparing SVL and mass growth rates

across sex class (Table 3; Table S5). Sex-reversed male *B. duperreyi* had the lowest rates of survival (77%; Table S6) in comparison to concordant females (87%) and concordant males (100%), but this relationship was non-significant ($p = 0.29$). Similarly, sex-reversed *P. vitticeps* individuals had the lowest rates of survival (75%; Table 3) in comparison to concordant females (83%) and concordant males (95%), but this relationship was also not significant ($p = 0.06$).

4 | Discussion

In this study, we examined two species with different modes of sex-reversal to test whether metabolic, growth, and survival differed for sex-reversed individuals and how these individuals compare to their phenotypic or genotypic sex. Metabolic responses differed between the two species, with clear support for the Like Phenotype when males reverse sex ($male_{SR}$ XX; *Bassiana duperreyi*) and equivocal support for each prediction when females reverse sex ($female_{SR}$ ZZ; *Pogona vitticeps*). For both species, regardless of whether individuals reversed sex, phenotypic females required more energy than phenotypic males as individuals grew larger. Higher energy requirements for phenotypic females may partly be driven by energy allocation towards reproduction investment (Congdon, 1989; Hayward & Gillooly, 2011). While sex-reversed animals appeared to have reduced survival, albeit not significantly so, there is no clear evidence in either species for growth advantages over their phenotypic sex. It is possible that within ZZ/ZW GSD systems, hatchling $female_{SR}$ ZZ experience competing energetic demands associated with both their phenotype and genotype due to high energy demands documented in phenotypic females (Geffroy, 2022). If these patterns occur in adult individuals in natural populations, energetic processes may have varying impacts on the species' life-history traits, which could provide insight into what constrains the distribution of sex-reversal in nature.

This is the first study in any vertebrate species to estimate the metabolic consequences of temperature-induced sex reversal. In both GSD systems in this study, concordant females had higher mass scaling relationships in metabolism than concordant males (Tables 1 & 2), but we showed that metabolic scaling relationships of sex-reversed individuals differed depending on the GSD system. In the ZZ/ZW system, larger sex-reversed females ($female_{SR}$ ZZ;> +1.5SD above mean mass) have lower metabolism (15%) than concordant females (female ZW) and appear to be more like concordant males (male ZZ; Fig. 2D). This surplus in energy reserves for $female_{SR}$ ZZ may explain why adult $female_{SR}$ ZZ *P. vitticeps* are more active, aggressive, and larger than female ZW (Holleley et al., 2015; Li et al., 2016). For example, if larger phenotypic females have similar amounts of energy intake in the ZZ/ZW system, and all other aspects of the energy budget are the same, $female_{SR}$ ZZ would have more residual energy than female ZW to allocate to production and activity after resting metabolic costs have been paid. However, these “male-like” phenotypes may also be a selective disadvantage for $female_{SR}$ ZZ in the wild because $female_{SR}$ ZZ are known to have high mortality and lower fecundity rates than female ZW (Wild et al., 2022). Different strategies of energy allocation between ZZ and ZW individuals across ontogeny may explain previously observed differences in morphology and behaviours as adults.

The lack of differences observed in metabolic rates between male XY and $male_{SR}$ XX *B. duperreyi* suggests that this species likely has little or no selection for sex-reversal during early development. However, geographic range, habitat, and behaviours can notably affect metabolic rates within species (Angilletta, 2001; Sears, 2005). Sex-reversal in *B. duperreyi* is linked to changes in elevational gradients where high elevations, with cooler temperatures, increase the frequency of sex-reversed males (Dissanayake, Holleley, Deakin, et al., 2021). Additionally, hatchling phenotypes - morphology, locomotor performance, growth rates,

survival, cognitive ability - are significantly influenced by incubation temperatures in *B. duperreyi* (Amiel & Shine, 2012; Flatt et al., 2001; Shine et al., 1997; Shine & Harlow, 1996). It is possible that selection on metabolism does occur, but these differences are subtle depending on the environment or population of *B. duperreyi* being sampled. For populations at higher elevations, we would predict higher temperature dependence for physiological processes, such as metabolic rate. Additionally, lizard populations at higher elevations would have limited time to achieve body temperatures at physiological optimums or acclimation responses to temperature may differ in lower populations (Jameson Jr et al., 1977; Tsuji, 1988). One alternative explanation for not capturing differences between male$_{SR}$ XX and male XY in our metabolic measurements is that the temperature selected for our metabolic experiments was not at an ecologically relevant body temperature hatchling lizards actively select for in natural settings to assimilate energy. Behaviours and physiological processes of hatchling *B. duperreyi* are affected by mean temperature, the variance of temperature within each day, and temperature differences across months (Shine, 2002, 2004; Shine et al., 1997; Shine & Elphick, 2001; Shine & Harlow, 1996). Local adaptations in other physiological traits have been postulated as a mechanism for explaining the distribution of sex-reversal in other species (Castelli et al., 2001). Further insight could be made by examining how metabolic rates vary along a gradient of temperatures and how local adaptations in physiological traits influence selection processes of sex-reversal in *B. duperreyi* and other species that undergo sex-reversal.

The frequency of sex-reversal in *P. vitticeps* occurs across a large part of its range, but neither latitude nor climate explains the distribution of sex-reversal (Castelli et al., 2021). Resource availability is a limiting factor for many adult lizards in arid and semi-arid environments (Bradshaw, 1997; Congdon, 1989; Kearney & Porter, 2004) occupied by *P. vitticeps* (Greer, 1989). Resource competition in these systems has been shown to drive selection towards lower metabolic rates when resources are scarce and vice versa when resources are high (Arnqvist et al., 2022; Mueller & Diamond, 2001). The unpredictable resource pulses (high rainfall events/high productivity vs. drought/low productivity) of this region shape demographic processes for other species (Kwok et al., 2016; Letnic & Dickman, 2010; Noy-Meir, 1973). Selection may favour female$_{SR}$ ZZ when resources are limited, pushing the species along a physiologically constrained path (Burton et al., 2011; Ricklefs & Wikelski, 2002). Low energy requirements documented in female$_{SR}$ ZZ hatchlings may provide a competitive advantage during drought conditions, allowing female$_{SR}$ ZZ to persist within populations that experience unpredictable stochastic environmental changes. The role resource availability may play in explaining the distribution of sex-reversal in this species and others requires further investigation.

There has been little to no attention focused on the energetic and fitness consequences associated with genotype and phenotype mismatches and how these sex-reversed individuals compare to their phenotypic or genotypic sex. The lack of clear evidence for differences in metabolism and fitness-related traits for sex-reversed individuals (male$_{SR}$ XX or female$_{SR}$ ZZ) over their concordant phenotypic sex (male XY or female ZW) in our study provides insight into possible targets of selection on hatchling phenotypes for species that undergo sex-reversal. In particular, the data indicate that the magnitude of metabolic differences vary across sexes as individuals get larger (Fig. 2), possibly affecting the fitness consequences of sex-reversal at different times in the life cycle and could be further exacerbated by local environmental conditions. Investigating ontogenetic changes of sex-reversal will provide promising insights into the consequences of such effects. While egg incubation differed between the species for logistical reasons – for *B. duperreyi*, 90% occurred in the field while

in *P. vitticeps* all eggs were incubated in the laboratory – we do not expect this difference to impact the relative differences we observed between sex-reversed and concordant individuals. However, it may have resulted in some differences in patterns observed between the two species (beyond their different genetic sex-determining systems), although we think this is unlikely. In both species, incubation temperatures mimicked nest temperatures documented in the wild (Castelli et al., 2020; Dissanayake et al., 2021), and all hatchlings were reared under common laboratory conditions for the first 6-months of life when all measurements were taken. Although we did not detect a significant difference in survivorship, in both species, sex-reversed hatchlings had a higher frequency of mortality over a 6-month period than the other sexes. High mortality has been previously observed in sex-reversed individuals in laboratory experiments (Mikó et al., 2021) and in the wild (Wild et al., 2022). Further investigation is required to understand the cause of this low survivorship and the demographic consequences these results have for the emergence of sex-reversal (Cotton & Wedekind, 2009). Overall, the lack of explicit support in our data for the Like Genotype in metabolism, growth, or survivorship reveals clues on the mechanisms that drive sex-reversal in nature

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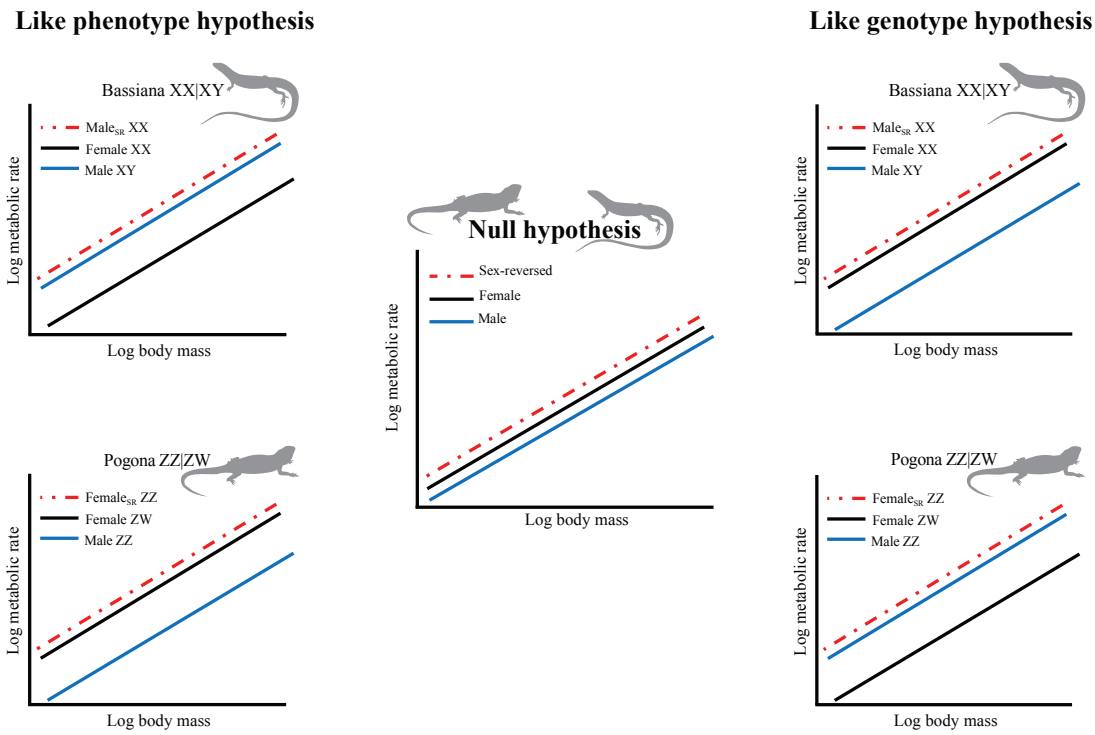

Figure 1. The Like Phenotype/Genotype Framework for testing the metabolic consequences of sex-reversal for ZZ/ZW and XX/XY genetic sex determination systems with different patterns of genetic sex determination. Colours indicate sex class for each species. Body mass and metabolic rates have been log transformed to approximate linear relationships. Each pattern of genetic sex determination contains three competing hypotheses for the relationship between body mass and metabolic rates: Null – no differences; Like Phenotype – similarities between reversed sex and concordant phenotype; Like Genotype – similarities between reversed sex and concordant genotype.

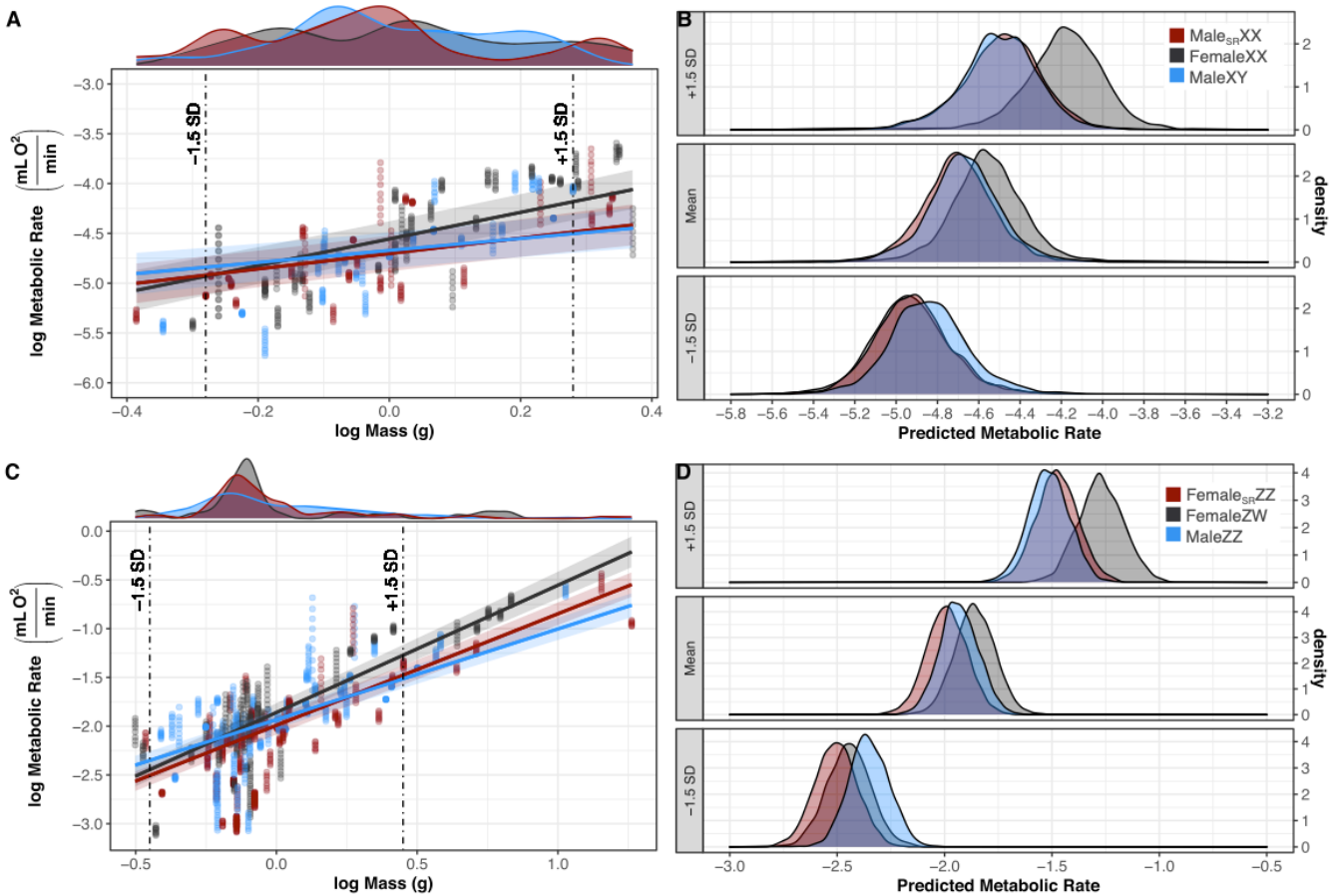

Figure 2. Comparison of log metabolic rate ($\dot{V}O2$ mL min^{-1}) across log body mass (g) by sex class for *Bassiana duperreyi* (A-B) and *Pogona vitticeps* (C-D). Sex-reversed individuals ($male_{SR}$ XX or $female_{SR}$ ZZ) are denoted by red colour, phenotypic females (female XX or female ZW) are denoted in black, phenotypic males (male XY or male ZZ) are denoted in blue. Fitted lines were obtained from predicted values from the brms model for each species and confidence bands were constructed from the SE of prediction values for each sex (A,C). Density plots above each regression plot denote the distribution in body mass (log mass) by sex for each species. To visualize how log metabolic rate changes across log body mass, panels (B and D) show the distribution of predicted metabolic rate at three areas of log body mass (mean, +1.5SD, and -1.5SD) denoted by the dash-dot line in panels A and C for each sex and species

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Table 1. Model coefficients for testing whether sex affects the slope of metabolic rate for *Bassiana duperreyi*, where the intercept is concordant females. Metabolic rate and mass were log transformed and time was z-transformed. Columns l-95% CI and u-95% CI, are the lower and upper bound of the 95% credible interval for each parameter, estimated from the posterior distribution.

Parameter	Estimate	l-95% CI	u-95% CI
Fixed Effects			
Intercept (FemaleXX)	**-4.56**	**-4.90**	**-4.20**
Male$_{SR}$XX	-0.15	-0.32	0.02
MaleXY	-0.12	-0.29	0.06
logMass	**1.34**	**0.87**	**1.81**
ztime	0.01	-0.02	0.05
Male$_{SR}$XX:logMass	**-0.56**	**-0.90**	**-0.23**
MaleXY:logMass	**-0.74**	**-1.07**	**-0.41**
Random Effects			
Lizard Identity (id)			
Intercept	**0.25**	**0.19**	**0.33**
Slope	**0.09**	**0.07**	**0.13**
Sampling Session (day)			
Intercept	**0.38**	**0.17**	**0.83**
Residuals	**0.26**	**0.25**	**0.28**

Table 2. Model coefficients form hetero testing whether sex affects the slope of metabolic rate for *Pogona vitticeps,* which heteroscedasticity was accounted for within the data. The intercept is concordant females. Metabolic rate and mass were log transformed and time was z-transformed. Columns l-95% CI and u-95% CI, are the lower and upper bound of the 95% credible interval for each parameter, estimated from the posterior distribution.

Parameter	Estimate	l-95% CI	u-95% CI
Fixed Effects			
Intercept (FemaleZW)	**-1.86**	**-2.04**	**-1.67**
Female$_{SR}$ZZ	-0.13	-0.28	0.03
MaleZZ	-0.07	-0.22	0.07
logMass	**1.30**	**1.11**	**1.49**
ztime	**0.06**	**0.04**	**0.08**
Female$_{SR}$ZZ:logMass	**-0.16**	**-0.32**	**-0.01**
MaleZZ:logMass	**-0.37**	**-0.55**	**-0.21**
Random Effects			
Lizard Identity (id)	0.22	0.18	0.27
Intercept	**0.30**	**0.25**	**0.35**
Slope	**0.07**	**0.06**	**0.09**
Sampling Session (day)			
Intercept	**0.28**	**0.19**	**0.42**
Residuals			
Sigma_Intercept	**-1.60**	**-1.64**	**-1.56**
Sigma_logMass	**-1.40**	**-1.54**	**-1.26**
Sigma_ztime	**0.22**	**0.18**	**0.27**

Table 3. Posterior distributions for log metabolic rate (Log MR) and growth rate (SVL or mass) estimates when testing if sex-reversed individuals show support for Like Genotype or Like Phenotype Framework for *Bassiana duperreyi* and *Pogona vitticeps*. Estimates were comparisons from subtracting the model posterior distribution of the median for sex-reversed individuals by either their phenotypic or genotypic counterparts, depending on the framework being tested.

Species	Test	Contrast	Estimate	l-95% CI	u-95% CI	pMCMC Value
B. duperreyi	Log MR	$Male_{SR}$ XX - Male XY	0.18	-0.17	0.53	0.33
		$Male_{SR}$ XX - Female XX	**-0.56**	**-0.90**	**-0.23**	**< 0.01**
	SVL (mm/d)	$Male_{SR}$ XX - Male XY	0.01	0.00	0.02	0.29
		$Male_{SR}$ XX - Female XX	0.00	-0.01	0.02	0.39
	Mass (cg/d)	$Male_{SR}$ XX - Male XY	0.00	-0.02	0.01	0.73
		$Male_{SR}$ XX - Female XX	-0.01	-0.02	0.01	0.24
P. vitticeps	Log MR	**$Female_{SR}$ ZZ - Female ZW**	**-0.16**	**-0.32**	**-0.01**	**0.05**
		$Female_{SR}$ ZZ - Male ZZ	**0.21**	**0.09**	**0.32**	**< 0.01**
	SVL (mm/d)	$Female_{SR}$ ZZ - Female ZW	0.00	-0.02	0.02	0.94
		$Female_{SR}$ ZZ - Female ZW	-0.01	-0.02	0.01	0.48
	Mass (g/d)	$Female_{SR}$ ZZ - Female ZW	-0.03	-0.12	0.05	0.40
		$Female_{SR}$ ZZ - Male ZZ	-0.08	-0.18	0.00	0.06

Supplementary Analysis, Figures, and Tables

To determine if standard metabolic rate (SMR) resulted in different conclusions compared to if we used all metabolic measurements taken over night, we refit our models using only SMR. We defined SMR as the lowest 10% of values of oxygen consumption rate during our overnight trials. For both species this resulted in the removal of nearly 90% of our data and resulted in higher sampling error (unsurprisingly). Nonetheless, this did not change the overall results. Below we provide the detailed results and corresponding figures and tables using SMR for each species.

Bassiana duperreyi - Once SMR data (lowest 10% of metabolic rate) were removed, we had a total of 83 measurements for 40 individuals ($male_{SR}$ XX: *n = 13*, female XX: *n = 15*, male XY: *n = 12*). There was a strong scaling relationship between log metabolic rate and log mass (Table S1). Sex-reversed male XX *B. duperreyi* had a scaling relationship that was most like their phenotypic counterparts (male XY - $male_{SR}$ XX; pMCMC = 0.26; Table S1; Fig. S1) compared to their genotypic counterparts (female XX - $male_{SR}$ XX; pMCMC = 0.07). The homogeneous variance model was the most parsimonious ([heteroscedastic model – homoscedastic model] loo: -1.31, SE = 2), accounting for 73% (95% CI:0.63 - 0.8) of the variation in metabolic rate.

Pogona vitticeps - Once SMR data (lowest 10% of metabolic rate) were filtered we had a total of total of 146 measurements for 96 individuals ($female_{SR}$ ZZ: *n = 28*, female ZW: *n = 30*, male ZZ: *n = 38*) were recorded. There was a strong scaling relationship between log metabolic rate and log mass (Table S2). Sex-reversed female *P. vitticeps* ($female_{SR}$ ZZ) had a scaling relationship that was overall higher than their genotypic counterparts (male ZZ - $female_{SR}$ ZZ; pMCMC = 0.61; Fig S1), but lower than their phenotypic counterparts (female ZW - $female_{SR}$ ZZ; pMCMC = 0.64; Table 2).The heteroscedasticity variance model was the most parsimonious ([heteroscedastic model – homoscedastic model] loo: -16.4, SE = 5.03), accounting for 86% (95% CI:0.79 - 0.92) of the variation in metabolic rate.

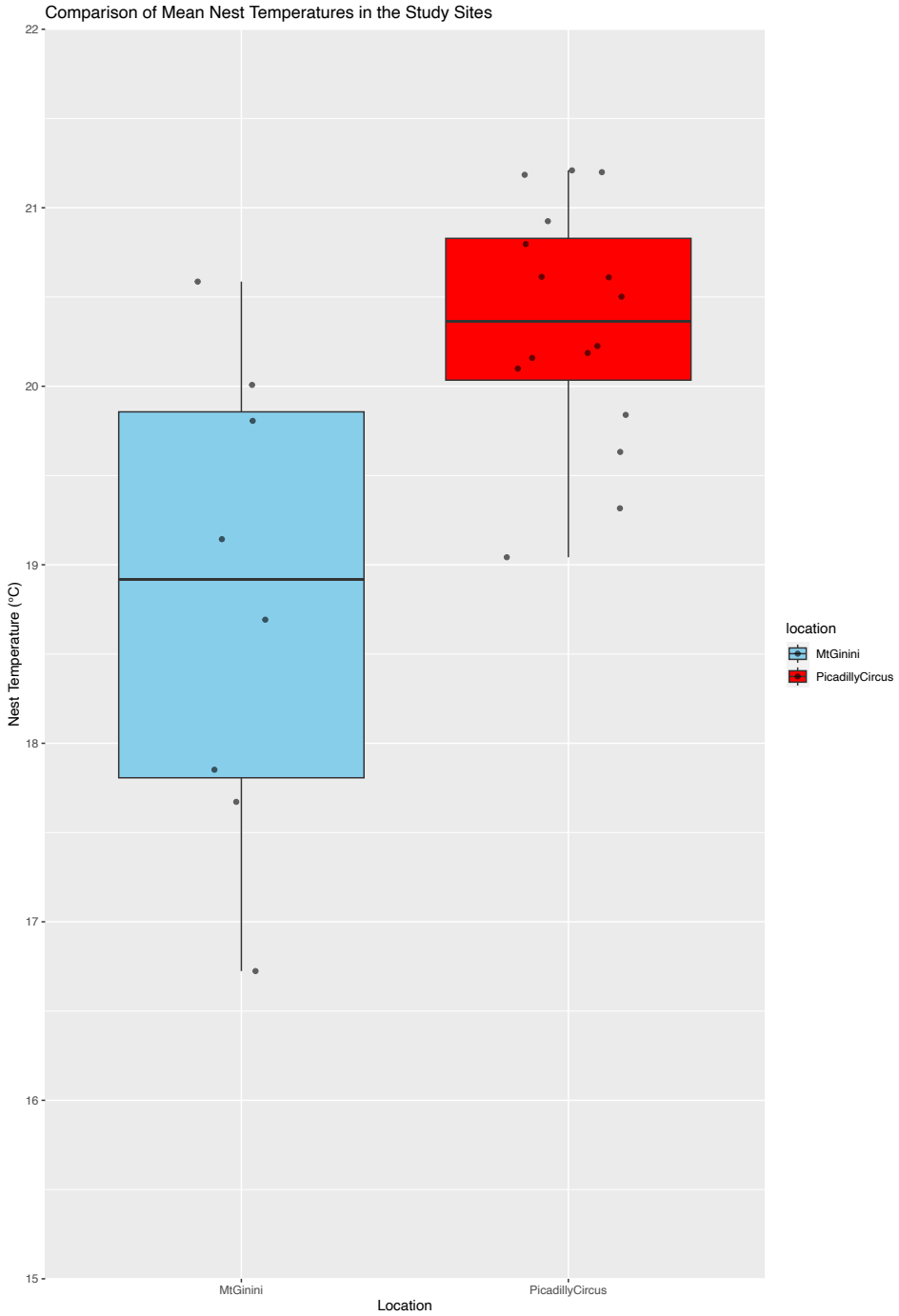

Figure S1. Comparison of mean nest temperatures between Piccadilly Circus and Mt Ginini.

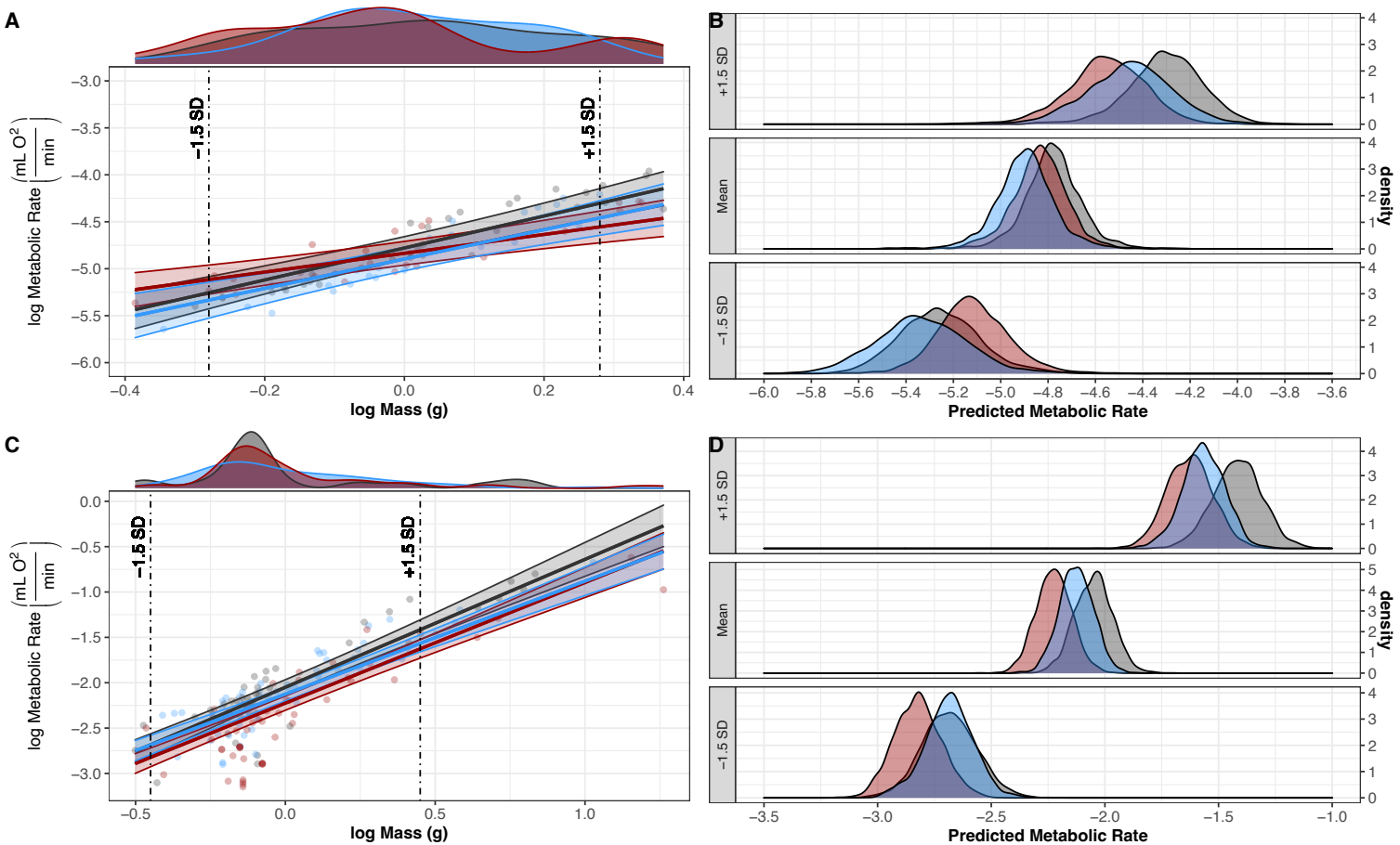

Figure S1. Comparison of log standard metabolic rate ($\dot{V}O2$ mL min^{-1}) across log body mass (g) by sex class for *Bassiana duperreyi* (A-B) and *Pogona vitticeps* (C-D). Sex-reversed individuals ($male_{SR}$ XX or $female_{SR}$ ZZ) are denoted by red colour, phenotypic females (female XX or female ZW) are denoted in black, phenotypic males (male XY or male ZZ) are denoted in blue. Fitted lines were obtained from predicted values from the brms model for each species and confidence bands were constructed from the SE of prediction values for each sex (A,C). Density plots above each regression plot denote the distribution in body mass (log mass) by sex for each species. To visualize how log metabolic rate changes across log body mass, panels (B and D) show the distribution of predicted metabolic rate at three areas of log body mass (mean, +1.5SD, and -1.5SD) denoted by the dash-dot line in panels A and C for each sex and species

Table S1. Model coefficients for testing whether sex affects the slope of standard metabolic rate for *Bassiana duperreyi*, where the intercept is concordant females. Metabolic rate and mass were log transformed and time was z-transformed. Columns l-95% CI and u-95% CI, are the lower and upper bound of the 95% credible interval for each parameter, estimated from the posterior distribution.

Parameter	Estimate	l-95% CI	u-95% CI
Fixed Effects			
Intercept (FemaleXX)	**-4.78**	**-5.02**	**-4.52**
$Male_{SR}XX$	-0.06	-0.22	0.10
MaleXY	-0.12	-0.28	0.05
logMass	**1.70**	**0.93**	**2.52**
ztime	0.01	-0.06	0.08
$Male_{SR}XX$:logMass	-0.70	-1.52	0.08
MaleXY:logMass	-0.14	-1.09	0.85
Random Effects			
Lizard Identity (id)			
Intercept	0.08	0.00	0.19
Slope	0.07	0.00	0.18
Sampling Session (day)			
Intercept	**0.38**	**0.17**	**0.83**
Residuals	**0.26**	**0.21**	**0.32**

Table S2. Model coefficients form hetero testing whether sex affects the slope of metabolic rate for *Pogona vitticeps,* which heteroscedasticity was accounted for within the data. The intercept is concordant females. Metabolic rate and mass were log transformed and time was z-transformed. Columns l-95% CI and u-95% CI, are the lower and upper bound of the 95% credible interval for each parameter, estimated from the posterior distribution.

Parameter	Estimate	l-95% CI	u-95% CI
Fixed Effects			
Intercept (FemaleZW)	**-2.05**	**-2.22**	**-1.89**
Female$_{SR}$ZZ	-0.17	-0.34	0.02
MaleZZ	-0.08	-0.22	0.06
logMass	**1.41**	**1.08**	**1.76**
ztime	0.00	-0.07	0.07
Female$_{SR}$ZZ:logMass	-0.08	-0.42	0.31
MaleZZ:logMass	-0.17	-0.53	0.19
Random Effects			
Lizard Identity (id)			
Intercept	**0.18**	**0.11**	**0.26**
Slope	**0.11**	**0.01**	**0.23**
Sampling Session (day)			
Intercept	**0.22**	**0.13**	**0.35**
Residuals			
Sigma_Intercept	**-1.67**	**-2.01**	**-1.39**
Sigma_logMass	**-1.51**	**-2.35**	**-0.53**
Sigma_ztime	-0.11	-0.39	0.15

Table S3: BRMS model coefficients for each respective species when testing mass differences across sex class for animals used in respirometry experiments. Mass was log-transformed and lower and upper bounds were derived from the 95% credible interval for each parameter, estimated from the posterior samples.

Species	Sex	n	Estimate	l-95% CI	u-95% CI	Contrast	pMCMC Value
B. duperreyi	Female XX	15	0.04	-0.02	0.11	Female XX-Male$_{SR}$ XX	0.45
	Male$_{SR}$ XX	13	-0.02	-0.10	0.05	Male$_{SR}$ XX-Male XY	0.91
	Male XY	12	0.02	-0.06	0.09	Male XY-Female XX	0.70
P. vitticeps	Female ZW	30	0.01	-0.10	0.11	Female ZW-Female$_{SR}$ ZZ	0.48
	Female$_{SR}$ ZZ	28	0.06	-0.04	0.16	Female$_{SR}$ ZZ-Male ZZ	0.44
	Male ZZ	38	0.01	-0.08	0.10	Male ZZ-Female ZW	0.96

Table S4: BRMS Model coefficients for SVL and mass growth rate estimates across sex class *Bassiana duperreyi*. Growth rate was calculated by dividing the change in growth (SVL or mass) between the initial measurement and subsequent remeasurement by the total number of days elapsed. Due to the small size and rate of change in grams, mass was converted to centigrams (cg). Animals were remeasured between 3 and 6 months post hatch.

Growth rate	Covariate	Estimate	l-95% CI	u-95% CI
mass(cg/d)	Intercept (FemaleXX)	0.09	-0.33	0.51
	$Male_{SR}XX$	0.35	-0.24	0.94
	MaleXY	0.21	-0.50	0.91
	mass(cg)	0.00	-0.01	0.01
	$Male_{SR}XX$:mass(cg)	-0.01	-0.02	0.01
	MaleXY:mass(cg)	0.00	-0.02	0.01
SVL(mm/d)	Intercept (FemaleXX)	0.16	0.01	0.30
	MaleSRXX	-0.14	-0.46	0.19
	MaleXY	0.02	-0.16	0.20
	SVL(mm)	0.00	-0.01	0.00
	$Male_{SR}XX$:SVL(mm)	0.00	-0.01	0.02
	MaleXY:SVL(mm)	0.00	-0.01	0.01

Table S5: BRMS Model coefficients for SVL and mass growth rate estimates across sex class for *Pogona vitticeps*. Growth rate was calculated by dividing the change in growth (SVL or mass) between the initial measurement and subsequent remeasurement by the total number of days elapsed. Animals were remeasured between 3 and 6 months post hatch.

Growth rate	Covariate	Estimate	l-95% CI	u-95% CI
SVL(mm/d)	Intercept (FemaleZW)	0.20	-0.14	0.54
	$Female_{SR}ZZ$	-0.01	-0.77	0.73
	MaleZZ	-0.28	-0.89	0.35
	SVL(mm)	0.00	-0.01	0.01
	$Female_{SR}ZZ$:SVL(mm)	0.00	-0.02	0.02
	MaleZZ:SVL(mm)	0.01	-0.01	0.02
mass(g/d)	Intercept (FemaleZW)	0.22	0.13	0.32
	$Female_{SR}ZZ$	0.09	-0.15	0.33
	MaleZZ	-0.13	-0.30	0.03
	mass(g)	-0.01	-0.04	0.02
	$Female_{SR}ZZ$:mass(g)	-0.03	-0.12	0.05
	MaleZZ:mass(g)	0.05	0.00	0.10

Table S6: Frequency of mortality across sex class for *Bassiana duperreyi* and *Pogona vitticeps*. These measurements were recorded from initial hatch date to 6 months post-hatch date.

Species	Sex Class	Alive	Dead
B. duperreyi	XXf	13	2
	XXm	10	3
	XYm	12	0
P. vitticeps	ZWf	25	5
	ZZf	21	7
	ZZm	36	2