Title: Genome composition predicts physiological response to environmental change in polyploid salamanders

Keywords: metabolism, total resistance to water loss, unisexual, salamander, polyploid, respiration efficiency ratio

Word count: 6371

Elements of manuscript: abstract, introduction, methods, results, discussion, references, table (1), and figures (5)

Article type: main article

Short title: Subgenomes predict unisexual physiology

Isabella J. Burger[1], Michael Itgen[2], Lynn Tan[2], Parker Woodward[2], Linet Rivas-Moreno[2], Tamyra Hunt[2], Hailey R. Ready[2], Xochitl G. Martin Geronimo[2], Robert D. Denton[2], Eric A. Riddell[1]

[1]Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

[2]Department of Biology, Marian University, Indianapolis, IN 46222

Abstract

Multi-trait analyses can be used to measure the differential performance of phenotypic traits in species complexes. Hybridization within these complexes can result in a mismatch between mitochondrial and nuclear DNA that may lead to reduced performance and acclimation capacity in hybrids. To test the effect of this mismatch on physiology, we compared physiological performance and acclimation capacity of metabolic rate (VCO_2) and total resistance to water loss (r_T) between two sexual *Ambystoma* species and a closely related unisexual lineage. We also separated unisexuals by their unique biotypes to determine how physiology varies with subgenomic composition. We found that unisexual biotypes exhibited phenotypes more like their related sexual species than to other unisexuals. We also found a trade-off between r_T and VCO_2, with increasing r_T resulting in a decrease in VCO_2. This trade-off was stronger in unisexuals than either sexual species, indicating that unisexuals may be more sensitive to environmental change. While we did not find evidence for mitonuclear mismatch, our results show that genomic composition of hybrids is more indicative of trait performance than other metrics (e.g., ploidy). Multi-trait analyses are imperative for understanding variation in phenotypic diversity, providing insight into how this diversity affects species responses to environmental change.

Introduction

The formation of hybrids plays a key role in the evolution and ecology of speciation (Arnold, 1992). Once hybrids are formed, they can positively or negatively impact parental species through the degree of gene flow, competition, or the reinforcement of species boundaries (Arnold, 1992; Barton, 2001; Coyne and Orr, 2004; Edelman et al., 2019). The outcome of these interactions can depend on the differential fitness of phenotypic traits between the hybrids and parental species (Barton, 2001; Thompson et al., 2021; Moran et al., 2021). When hybrids' traits are better fit for their environment, by exhibiting beneficially intermediate phenotypes (i.e. heterosis), they can outcompete one or both parental species (Schluter, 2000; Birchler et al., 2006; Hessenauer et al., 2020). Alternatively, phenotypic traits can be negatively impacted through hybridization when intermediate traits perform poorly in the parental habitats. Further, hybrids can be disadvantaged by Dobzhansky-Muller genetic incompatibilities (DMIs), in which the interactions between divergent alleles can result in reduced performance and, in some cases, hybrid lethality (Dobzhansky, 1982; Arnold and Martin, 2010; Thompson et al., 2023; Moran et al., 2024). Thus, the success of a hybrid can be determined by the performance of their phenotype relative to the parental lineages (Barton, 2001; Thompson et al., 2021). Physiological traits are particularly important for understanding hybrid performance because they are highly integrated, covary with other behavioral and life history traits, and often encompass a broad suite of traits that impact an organism's performance and fitness (Ricklefs and Wikelski 2002; Wikelski et al. 2003; Martin et al. 2006).

Metabolic rate and water loss are two important physiological traits that covary in terrestrial animals (Woods and Smith, 2010). Specifically, these traits are linked by the properties of respiratory surfaces (lungs, skin, etc.) that facilitate the diffusion of gases, like oxygen and

carbon dioxide. Respiratory surfaces are thin and highly vascularized, which enables oxygen to diffuse rapidly across the tissue (Czopek, 1962; Feder and Burggren, 1985). However, the properties that facilitate oxygen uptake also result in high rates of water loss (Feder and Burggren, 1985). This relationship between gas exchange and water loss results in a trade-off: physiological changes to reduce water loss also limit an organism's ability to breathe (Addo-Bediako et al. 2001; Woods and Smith, 2010; Riddell et al. 2018). By lowering water loss rates (e.g. as a response to desiccation), organisms often experience a concurrent reduction in metabolic rate due to lower rates of gas exchange (Anderson, 1970; Riddell et al. 2018). This reduction in metabolic rate might lower an organism's ability to fuel energetically demanding activities, such as foraging, reproduction, and territory-defense (Burggren and Vitalis, 2005; Biro and Stamps, 2010; Sadowska et al., 2015; Sokolova, 2021). This trade-off may also be unidirectional because changes in metabolic rate may occur without affecting an organism's physiological resistance to water loss. Additionally, the strength of the trade-off can vary across species (Burger et al., 2024), which can provide insight into the underlying mechanisms and evolutionary potential of these traits. For example, the absence of a trade-off suggests that these two traits could be decoupled, meaning that they can respond (and potentially evolve) to the environment independently (Agrawal, 2022). Alternatively, a strong trade-off suggests that these traits are tightly linked, limiting their ability to independently respond to change. Understanding these linkages may help to predict organismal and evolutionary responses to environmental change.

When measuring the relationship between gas exchange and water loss, the use of multiple metrics can provide a better understanding of how changing environmental conditions impact whole organism performance. Organisms that can maintain a high metabolic rate despite

any changes to water loss rates would have a greater capacity to simultaneously avoid desiccation while maintaining their ability to do work (Auer et al. 2015; Levy et al. 2016; Burger et al. 2024). These organisms (high gas exchange, low water loss) exhibit more efficient respiration because they have an increased capacity for exchanging vital gases while limiting desiccation risk (Burger et al. 2024). This respiration efficiency can be measured via the water-gas exchange ratio (WGER), which is calculated as the rate of gas exchange per unit water loss (Burger et al. 2024). The WGER provides a separate measure for analyzing water loss and gas exchange that can explain additional aspects of the relationship between these two traits. The trade-off between gas exchange and water loss determines how physiological changes to minimize water loss (e.g., vasoconstriction of the skin, breathing rate) affect an organism's capacity to breathe. Alternatively, the WGER measures how much gas an organism exchanges for each unit of water lost, providing an index of an organism's respiration efficiency (Burger et al., 2024). These physiological indices provide additional metrics for comparing performance between hybrids and parental lineages.

The process of allopolyploidization–hybridization events that result in offspring with more than two sets of chromosomes–can have direct consequences for physiological traits due to the potential consequences of the interactions among divergent subgenomes (Gregory and Mable, 2005; Balao et al., 2011; Mable, 2013; Wertheim et al., 2013; Moran et al., 2024). First, polyploids possessing divergent nuclear haplotypes can benefit from increased heterozygosity and the masking or deletion of deleterious alleles, minimizing the effects of harmful mutations (Comai, 2005). Alternatively, intergenomic incompatibilities can occur between the different nuclear haplotypes and/or between the haplotypes and the mitochondria. Interactions between nuclear haplotypes and mitochondria produce a 'mitonuclear mismatch' between nuclear- and

104 mitochondrial-encoded proteins (Sharbrough et al., 2017). At its most extreme, mitonuclear
105 mismatch causes a reduction in ATP production and an increase in reactive oxygen species,
106 causing oxidative stress (Harrison and Burton 2006, Toews and Brelsford, 2012; Wolff et al.
107 2014). These intergenomic incompatibilities can lead to decreased performance (Sackton et al.
108 2003; Comai, 2005; Lee-Yaw et al. 2014; Denton et al. 2017; Rank et al. 2020; Klabacka et al.,
109 2022; Moran et al., 2024) and fitness in hybrid offspring, such as reduced survivorship,
110 development, and fecundity (Yee et al. 2013; Wolff et al. 2014). While it is established that
111 mitonuclear mismatch can result in reduced performance, studies that test for associations
112 between mitonuclear mismatch and physiological performance/acclimation capacity are limited
113 by the number of naturally occurring study systems that are tractable for the necessary
114 experiments.

115 A secondary consequence of allopolyploidization is its effects on DNA content and,
116 consequently, cell size (Gregory, 2001; Balao et al., 2011). The total amount of DNA within a
117 cell (i.e., genome size) is strongly correlated with the size of the cell; therefore, polyploidization
118 causes a universal increase in cell size throughout the organism (Gregory, 2001). Large genome
119 and cell size have been hypothesized to drive the evolution of physiological traits through the
120 scaling effects of lower surface-area-to-volume ratios in larger cells (Szarski, 1983; Vinogradov
121 1995; Kozłowski et al., 2003). It has been shown that large genomes (and by proxy cell sizes)
122 have been correlated with higher resistance to water loss rates, which is a measurement of a
123 physiological response to minimize water loss and desiccation risk (Johnson et al., 2021).
124 However, there has been conflicting evidence whether cell-size-mediated differences in
125 metabolic rate scale upward to reduce the metabolic costs of tissues and organisms
126 (Monnickendam and Balls 1973; Licht and Lowcock, 1991; Uyeda et al., 2017; Gardner et al.,

127 2020; Johnson et al., 2021; Itgen, 2022). Thus, it remains unclear if polyploidization has a
128 consistent effect on organismal physiology across taxa.

129 In this study, we measured the thermal sensitivity and acclimation capacity of metabolic
130 rate, resistance to water loss rate, respiration efficiency (measured via the WGER), and the trade-
131 off between resistance to water loss and gas exchange in the unisexual *Ambystoma* complex (see
132 *Study species* in Methods). Specifically, we compared traits between two sexual, diploid
133 *Ambystoma* species (Jefferson salamander [*A. jeffersonianum*] and blue-spotted salamander [*A.*
134 *laterale*]) and unisexual, polyploid salamander hybrids (Figure 1C-E). Unisexual salamanders
135 undergo frequent genome introgression with sexual species and show signs of reduced endurance
136 (Denton et al., 2017). Thus, we predicted that unisexual salamanders would have lower
137 metabolic rates, respiration efficiency, and acclimation capacity compared to *A. jeffersonianum*
138 and *A. laterale* due to the combination of polyploidy and mitonuclear mismatch. Alternatively,
139 we predict that unisexuals will exhibit higher resistance to water loss rates due to their increased
140 ploidy and thus larger cell sizes. Analyzing multiple facets of an organism's ecophysiology will
141 allow us to develop a more comprehensive understanding of the effects of hybridization and
142 environmental variation on whole-organism performance.

143 **Materials and methods**

144 *Study species*

145 Unisexual *Ambystoma* are an all-female salamander lineage that is the oldest recognized
146 unisexual vertebrate group, persisting for ~5 million years (Bi and Bogart, 2010). Unisexuals
147 reproduce via kleptogenesis, in which they must "steal" sperm from a congeneric male to
148 stimulate egg development (Bogart et al. 2007; Gibbs and Denton, 2016). The genetic material
149 from males can then either be discarded (offspring are clones of mother) or incorporated into the

150 chromosome array of the embryos. This process has resulted in a range of polyploidy biotypes,
151 ranging from diploid to pentaploid and combining genomes from five different species
152 (Robertson et al. 2006; Bi and Bogart, 2010, Bogart et al. 2007). While frequent genome
153 introgression from sexual species into unisexual populations has produced highly variable
154 combinations of nuclear genomes (Bogart, 2019; Gibbs and Denton, 2016), the mitochondrial
155 genome is strictly inherited matrilineally and monophyletic (Hedges et al., 1992; Spolsky et al.,
156 1992). The lack of co-evolutionary history between the mitochondrial and nuclear genomes
157 likely has energetic consequences for unisexual salamanders by reducing endurance relative to
158 sympatric sexual species (Denton et al. 2017). In contrast, measurements of oxygen consumption
159 and water loss did not differ in at least one population of unisexual salamanders (Licht and
160 Bogart, 1990). However, no study has compared unisexual biotypes with related sexual species
161 across their range (Figure 1) to make comprehensive comparisons and understand if unisexual
162 biotypes exhibit phenotypes more like the sexual species to which they are most genetically
163 similar. In this study, we compare a suite of physiological traits between *A. jeffersonianum, A.*
164 *laterale* and unisexuals from the same populations with different biotypes (LJ, LLJ, LLJ, LJJJ) to
165 determine if mitonuclear mismatch affected unisexual physiological performance.

166 *Animal collections and housing*

167 We collected animals between February and April 2020, 2022, and 2023 during breeding
168 migrations. We collected *Ambystoma jeffersonianum* from Litchfield, CT; Campbell, KY; and
169 Delaware, OH; *A. laterale* from Calumet, WI; Marquette, MI; Livingston, MI; Windham, CT;
170 and Addison, VT; and unisexuals from each location (Figure 1B, see figure for sample sizes). We
171 captured sexually mature individuals using a combination of nighttime surveys, terrestrial drift
172 fences, bucket traps, and/or cover boards, depending on the approved collection methods for

each state. We acquired scientific collection permits where required (KY:SC2311177, MI:FSCP12302022102023, OH:18-164/SRP23-01, VT:SR-2023-03, WI:SRLN-23-46) and worked with local field personnel when possible to help state agencies as part of larger survey efforts.

We housed salamanders in individual clear boxes (19 x 33 x 10 cm) within refrigeration units set at 12-14˚C immediately following collection. Each container contained moist paper towels that were changed weekly and a PVC hide. We fed animals 1-2 appropriately sized European Nightcrawlers (*Eisenia hortensis*) per week. We covered the front glass of the refrigeration units except for narrow slits that would let in ambient light from the room during normal building hours (~9am-5pm). We weighed each animal once per month and checked animals each day to monitor for any signs of stress. Prior to the initiation of this study, we acquired approval from IACUC (protocol #RD-2021-1R). In May 2023, we transported salamanders to [university redacted for double-blind review] for physiological experiments. For transport, we stored individuals in smaller plastic containers lined with dampened paper towels. Total transport lasted approximately 7.5 hours, at which time we immediately removed salamanders from the vehicle and transferred them to a walk-in environmental chamber (2.5 x 3 m) set to 14˚C for the duration of the experiment (except for the acclimation trials – see *Physiological experiments*). We kept individuals in the same plastic containers described above. We ensured that lights were kept off in the housing unit, but salamanders were exposed to passive light through a small access window during the day. We made all efforts to keep the housing at the experimental laboratory as similar as possible to that of the location where animals were housed after capture.

Genetic analysis

We clipped a small tissue sample from the tail of each captured animal to determine their genetic identity. First, we sequenced a highly variable portion of the mitochondrial genome and compared it to known reference sequences for multiple *Ambystoma* species to determine species identity (primers F-THR and R-651; McKnight and Shaffer, 1997; Bogart et al. 2007). For individuals that were identified as unisexuals, we then used fragment length data from three microsatellite loci to determine their specific ploidy and subgenome composition (Teltser and Greenwald, 2015). Subgenome composition refers to the different nuclear genomes in the unisexuals. For example, an LLJ individual would have three subgenomes–two from *A. laterale* and one from *A. jeffersonianum.* These subgenomes make up the separate unisexual biotypes (LLJ, LJJ, etc.).

Study design

We conducted physiological experiments from May to July 2023. Prior to experimentation, we separated salamanders into five batches of 28 individuals. We further divided these batches into groups of seven, as only seven salamanders could be placed in the flow-through system at a time. We randomized each group by site and species to ensure equal representation in each batch. Salamanders were given one week at 14˚C to adjust to laboratory conditions prior to taking physiological measurements in the flow-through system (description below).

We exposed each salamander to three acute temperature treatments in the flow-through system that represent a typical range of spring temperatures they would experience during active periods in the wild: 6˚C, 14˚C, and 22˚C (Lowcock et al., 1991; Hoffman et al., 2018; Mitchel et al., 2020). To ensure that salamanders reached thermal equilibrium prior to the run, we placed a group in the incubator (Percival, Inc.; Model #I-36VL) at the experimental temperature for one hour prior to physiological measurements. We ran two groups (14 salamanders) a day, and

individuals were given a rest day after each run to allow time for rehydration. We determined if a salamander was rehydrated by monitoring their mass prior to and following the respirometry trials. Therefore, it took two days to run a full batch at one temperature.

After the initial physiological measurements at each temperature, we moved the salamanders to a separate incubator (Percival, Inc.; Model #I-36VL) set to 20˚C for 20 days to evaluate their capacity to acclimate by adjusting physiological traits. During this time, we increased the rate of feeding to twice per week. We conducted the same physiological measurements following this 20-day period to determine acclimation capacity of each individual. We conducted all physiological measurements between 7:00 and 17:00 to measure physiological traits when salamanders would be inactive in the field (McNab, 1997). Additionally, we stopped feeding salamanders one week prior to experimental treatments to ensure a post-absorptive state. We ensured each individual was at rest during the trials using a Reolink Network Video Recorder (Model RLN8-410) to monitor and record salamander activity. We checked that salamanders were inactive through visual inspection of the data output and verification using the video recordings. Activity is characterized by erratic peaks in metabolic rate. Using this information, we removed all trials where activity was clear and verified inactivity in the other trials using the recorded videos. We removed all trials where the salamanders were active from the final dataset.

Flow-through system

In preparation for the physiological trials, we placed salamanders on a hardwire mesh platform in a glass chamber (20 x 3.6 cm; volume *c*. 207 mL). Our primary objective was to understand physiological responses to temperature; thus, the mesh restricted the salamander's ability to behaviorally reduce water loss by curling onto themselves. We placed chambers in a temperature- and humidity-controlled incubator (Percival, Inc.; Model #I-36VL) and connected

to a flow-through system (Sable Systems International (SSI), Las Vegas, NV). We delayed physiological measurements by 30 minutes once the chambers were connected to allow the salamanders to come to rest in the new environment.

For the 14˚C and 22˚C treatments, we used a bench pump (PP-2; SSI) to pull air from the incubator. We also adjusted the relative humidity at each temperature to ensure a similar vapor pressure deficit (VPD) across the treatments. The VPD is the difference between the saturated vapor pressure (the amount of water the air can hold) and actual vapor pressure (the amount of water in the air). As temperature increases, saturation vapor pressure increases, potentially exposing organisms to higher VPDs (and thus greater evaporative demand) (Riddell et al. 2017, Weaver et al. 2022). By maintaining the same VPD across temperature treatments, we ensured that variation in water loss rates could be attributed to organismal responses to temperature and not the effects of humidity. We adjusted humidity across these temperatures to maintain a VPD of 0.75 ± 5% kPa. We were unable to use the incubator to control humidity at 6˚C, so we split the airstream being pulled by the pump into two columns: one pulling air straight from the incubator and one pulling air from the incubator and through a column of Drierite™. We adjusted the ratio of these two columns until we reached the target VPD (0.75 kPa).

After air was pumped out of the incubator, it passed through a flow-through manifold (SSI) and separated into different streams with a continuous flow rate of 100 $mL \cdot min^{-1}$. These streams passed through the chambers containing the salamanders, as well as through an additional, empty chamber that we used to collect baseline data. A multiplexer (MUX8; SSI) cycled through the eight chambers (seven experimental and one baseline), and excurrent air samples from each passed through a water-vapor analyzer (RH300; SSI) and a carbon dioxide analyzer (CA-10A; SSI) to measure water vapor and CO_2, respectively, in volts. We calibrated

265 the analyzers once a month throughout the duration of the experiment to ensure accurate data
266 collection. We calibrated the CA-10 by passing N_2 gas and a known concentration of CO_2 gas
267 through the analyzer to zero and span the system, respectively. We calibrated the RH-300 by
268 passing N_2 gas through the system to set zero and using our humidity-controlled incubator to set
269 the span. The empty chamber was sampled between each salamander measurement to correct for
270 any drift throughout the duration of the experiment. We measured individuals three times at five-
271 minute intervals over a three-and-a-half-hour period.

272 We connected each instrument to a universal interface (UI-3; SSI) that uploaded the
273 voltage data to Expedata. We then linearly transformed the data in Expedata using equations
274 provided by the manufacturer (SSI). We used lag- and drift-corrections to correct for the time
275 delay in gas exchange/water loss and for drift that occurred during the experiment, respectively.
276 We also corrected the flow rate and carbon dioxide measurements for barometric pressure and
277 water vapor pressure of the excurrent air (Lighton 2008). We then transformed CO_2 values from
278 percentages into partial pressure by dividing the corrected data by 100. We measured the
279 asymptote of water loss values and used z-transformations to calculate stable water loss
280 measurements. We used equations 3-5 in Riddell et al. (2017) and 10.4 in Lighton (2008) to
281 calculate evaporative water loss (EWL), total resistance to water loss (r_T), and metabolic rate
282 (VCO_2) using the collected data. We measured total resistance to water loss as the inverse of
283 water loss, while accounting for the evaporative demand of the air (Riddell et al. 2017). Lastly,
284 we averaged VCO_2, r_T, and EWL values across the measurements when salamanders were at rest.

285 *Statistical analysis*

286 We ran three groups of analyses on the collected data: analysis of the traits, change in the traits,
287 and the trade-off between resistance to water loss and gas exchange. For the trait analyses, we

ran three separate linear mixed effects models (LMER) to determine how VCO_2, r_T, and respiration efficiency (WGER) varied throughout the experiment (*A. laterale*: n = 34; *A. jeffersonianum*: n = 18; unisexual: n = 80). We included mass as a covariate and species, temperature treatment, and time period (before and after acclimation) as factors in each separate analysis. We also included interactions between species, temperature treatment, and time period to assess differences between species. We included time period as an effect in these analyses to account for the variation introduced by this treatment; however, we restricted our discussion of the effects of acclimation to the change in trait analyses (see below). VCO_2 and WGER values were log-transformed to meet assumptions of normality. For the change in the trait, we conducted additional analyses that used the change in VCO_2, r_T, and respiration efficiency between acclimation periods as the response variables (ΔVCO_2 [*A. laterale*: n = 32; *A. jeffersonianum*: n = 18; unisexual: n = 76], Δr_T [*A. laterale*: n = 32; *A. jeffersonianum*: n = 18; unisexual: n = 75], and ΔWGER [*A. laterale*: n = 32; *A. jeffersonianum*: n = 18; unisexual: n = 75], respectively). For these analyses, we only included data from individuals that had a value for both pre- and post-acclimation. We also removed time period as a factor since we calculated the change in traits across the time periods. We conducted these two groups of analyses (trait and change in the trait) to explicitly test our hypotheses on respiration efficiency and acclimation capacity. Additionally, we used a separate linear regression to evaluate the trade-off between the change in VCO_2 and the change in r_T [*A. laterale*: n = 32; *A. jeffersonianum*: n = 18; unisexual: n = 75]. All models included mass as a covariate and salamander ID nested inside batch number as a random effect. We also included year collected as a fixed effect, but it was not significant for any analysis on average trait or change in trait ($p > 0.05$). We did not include location as it was highly confounded with species.

We were also interested in whether VCO_2, r_T, and respiration efficiency differed between the unisexual biotypes. Therefore, we ran additional analyses on the traits above but included biotype as a fixed effect. Due to limited sample sizes of the diploid (n = 2) and tetraploid (n = 2) unisexuals, we only analyzed the triploid biotypes (analysis of traits: LLJ: n = 39; LJJ: n = 36; trade-offs: LLJ: n = 37; LJJ: n = 34). In these models, we treated biotype (*A. jeffersonianum* [JJ], *A. laterale* [LL], LJJ, and LLJ), temperature, and time period as factors. For the change in the trait analyses (ΔVCO_2 = LLJ: n = 37; LJJ: n = 35; Δr_T= LLJ: n = 37; LJJ: n = 34; ΔWGER = LLJ: n = 37; LJJ: n = 34), we removed time period as a factor (similar to the above analysis). We also included mass as a covariate and the same random effects as the models described above. Sample size of *A. jeffersonianum* and *A. laterale* were the same as above for all biotype analyses.

Additionally, we calculated ω^2 to measure the effect size of each variable in each analysis using the following equation:

$$\omega^2 = \frac{SS_{treatment} - df_{treatment}\ x\ MS_{error}}{SS_{total} + MS_{error}}$$

where $SS_{treatment}$ is the sum of squares for the parameter, $df_{treatment}$ is the degrees of freedom for the parameter, MS_{error} is the mean square error, and SS_{total} is the total sum of squares (Olejnik and Algina, 2003). We also calculated marginal and conditional R^2 to describe the variation accounted for by the fixed effects and the variation accounted for by all effects in the model (fixed and random), respectively. We show all data as mean ± SEM using the *effects* package in R, which returns an adjusted mean incorporating the effects of all variables in the model. We conducted all analyses in R (version 4.3.1). Table 1 reports the outputs from the above linear mixed effects models.

Results

Metabolic rate (VCO_2)

Our results indicated that VCO_2 was positively related to mass, with larger individuals having higher metabolic rates. We also found that VCO_2 increased with temperature, and *A. jeffersonianum* had a higher VCO_2 than *A. laterale* and unisexuals. The interaction between temperature and species also influenced VCO_2, with *A. jeffersonianum* having the highest sensitivity to the changes in temperature (Figure 2A). Moreover, 77% of the variance was explained by our treatments and mass as indicated by the marginal R^2. In the ΔVCO_2 analysis, salamanders reduced metabolic rate by 16% in responses to exposure to warm temperatures (Figure 2B). Additionally, ΔVCO_2 was positively related to mass and was greatest at 22˚C. There was also an effect of the interaction between temperature and species on ΔVCO_2, with *Ambystoma jeffersonianum* having the lowest change in metabolic rate at 6˚C but the highest change at 22˚C (Figure 2B). We found a marginal R^2 of 12%, with an additional 27% of the variation being explained by individual ID (conditional R^2 = 39%).

When we separated unisexuals by their triploid biotype (LJJ, LLJ), we found that VCO_2 increased with mass and temperature (Figure 2C). We also found that biotypes had different metabolic rates across temperatures, with unisexual biotypes exhibiting metabolic rates most similar to the sexual species with which they share the greater number of chromosome sets (e.g. LJJ similar to *A. jeffersonianum* (JJ); Figure 2C). For the ΔVCO_2 analysis, ΔVCO_2 was positively related to mass and was greatest at 22˚C. We also found a significant effect of the interaction between temperature and biotype on ΔVCO_2, with ΔVCO_2 varying across biotypes and temperatures (Figure 2D).

Total resistance to water loss (r_T)

For total resistance to water loss (r_T), we found larger individuals and warmer temperatures had a higher r_T (Figure 3A). Additionally, the interaction between temperature and species affected r_T, with *A. laterale* having the lowest thermal sensitivity of resistance (Figure 3A). We also found that our treatments and mass explained 97% of the variation in our data. When analyzing Δr_T, we found that total resistance to water loss declined by 2% over the course of the acclimation experiment across all treatments (Figure 2B). We also found that Δr_T was greater in *A. jeffersonianum* than *A. laterale* or unisexuals (Figure 3B). Mass and treatments accounted for 11% of the variation, as shown by the marginal R^2 value, with an additional 25% being explained by individual ID (conditional R^2 = 36%).

When we separated unisexuals by their biotype, we found that r_T was positively related to body mass, with larger individuals having the highest resistance, and r_T increased with temperature (Figure 3C). The interaction between biotype and temperature also influenced r_T, with LJJ and *A. jeffersonianum* (JJ) having the highest thermal sensitivity of resistance (Figure 3C). *Ambystoma jeffersonianum* (JJ) also had a higher Δr_T value than any other group (Figure 3D).

Respiration efficiency (WGER)

Respiration efficiency and mass were positively related, as were efficiency and temperature (Figure 4A). Furthermore, *A. jeffersonianum* had the highest respiration efficiency. We also found that the interaction between temperature and species influenced efficiency, with *A. laterale* having the least variation in efficiency across temperatures (Figure 4A). Our treatments and mass accounted for 83% of the variation, based on the marginal R^2. In the ΔWGER analysis, we found that salamanders were 14% less efficient following the acclimation period (Figure 4B), and they were most efficient at 22˚C. Additionally, the interaction between temperature and species

378 affected efficiency, with *Ambystoma jeffersonianum* having the greatest fluctuation in ΔWGER
379 across temperatures (Figure 4B). Lastly, we found a marginal R^2 of 17%, with an additional 23%
380 of the variation being explained by individual ID (conditional R^2 = 40%).

381 When we separated the unisexual group by biotype, we found that larger individuals had
382 the highest respiration efficiency, and respiration efficiency increased as temperatures warmed
383 (Figure 4C). We also found that the interaction between temperature and biotype was significant.
384 Specifically, *A. jeffersonianum* and LJJ exhibited similar efficiencies, as did *A. laterale* and LLJ
385 (Figure 4C). For ΔWGER, we found that all values were negative, indicating that individuals had
386 lower respiratory efficiency following the acclimation treatment (Figure 4D). Additionally,
387 ΔWGER was positively related to both mass and temperature (Figure 4D). We also found an
388 effect of the interaction between biotype and temperature on ΔWGER, with acclimation capacity
389 varying by biotype and temperature (Figure 4D).

390 *Trade-offs between gas exchange and water loss*

391 We also found a trade-off between the change in r_T and VCO_2, in which the change in resistance
392 was negatively related to the change in VCO_2 (Figure 5A-B). Additionally, we found that
393 unisexuals exhibiting the strongest trade-off between the two traits (Figure 5A), yet there was no
394 variation by temperature (Figure 5B). When separating unisexuals by biotype, we also found a
395 significant trade-off between the change in r_T and VCO_2 (Figure 5C). However, we did not find
396 any variation in the strength of the trade-off by biotype (Figure 5C) or temperature.

397 **Discussion**

398 The unisexual salamander system combines three potential consequences of hybridization that
399 may negatively impact metabolic processes: divergent nuclear haplotypes, mitonuclear

400 mismatch, and polyploidy. Our initial predictions were that unisexuals, regardless of biotype,
401 would have poorer physiological performance due to these potential consequences of
402 allopolyploidization. However, we found that unisexual polyploids mimicked the most
403 representative species in their sub-genome composition, indicating that the divergent nuclear
404 haplotypes, mitonuclear mismatch, and polyploidy did not reduce physiological performance of
405 unisexual salamanders as we predicted. Our study is the first to include both sexual species that
406 contribute genomes to the unisexual lineage (*A. laterale* and *A. jeffersonianum*) and subsequently
407 the first to show that subgenome composition plays a larger role than ploidy in estimating
408 physiological performance. These results highlight the importance of including the performance
409 of the parental species when measuring and interpreting the performance of hybrid offspring.

410 Accounting for the subgenome composition in hybrids is necessary for understanding
411 variation in hybrid performance across traits. Unisexual *Ambystoma* exhibit certain phenotypes
412 that surpass congenerics (Saccucci et al. 2016), but they have other phenotypes that are inferior
413 to sexual species, possibly due to mitonuclear mismatch (Denton et al. 2017). Alternatively, the
414 unisexual *Ambystoma* salamanders in this study broadly display intermediate values between the
415 sexual species and mirror the values of their major subgenome when comparing unique biotypes
416 (i.e. LLJ is more like *A. laterale* than LJJ). The inconsistencies of unisexual performance
417 compared to sexual congenerics across these studies may be due to the differences in the
418 genomic array of the unisexuals measured. While the unisexual lineage is very old (Bogart et al.
419 2007; Bi and Bogart 2010), it readily adds genomic variation from nearby species into its
420 genome (Bi et al. 2008; Gibbs and Denton 2016; Denton et al. 2018). Therefore, variation in
421 unisexual performance across different traits (e.g., metabolism and total resistance to water loss,
422 this study; tissue regeneration, Saccucci et al. 2016; and endurance/dispersal rates, Denton et al.

423 2017) likely depends on the genomic composition of each individual and the relative
424 performance of the sexual species from which each genome was received. Additionally, our
425 study measured resting metabolic rate as opposed to active or maximal metabolic rate. Active
426 metabolic rate relies on a higher rate of ATP production to fuel the greater energetic demand
427 (Clarke and Fraser, 2004); therefore, mitonuclear mismatch may have more of an impact on
428 active organisms (as seen in Denton et al., 2017) since it reduces the production of ATP.
429 Therefore, accounting for the genomic array of the unisexuals and the physiological traits being
430 measured is necessary to understand hybrid performance across studies.

431 Only when considering the tradeoff between resistance to water loss and metabolic rates
432 do unisexual salamanders show any distinction from the two sexual species. As unisexual
433 salamanders became more resistant to water loss, they experienced a greater reduction in
434 metabolic rate compared to the parental lineages. Anatomically, this difference in the strength of
435 the trade-off may be due to differences in cell size since the unisexuals (polyploids) have larger
436 cells than the sexual species (diploids; Uzzell 1964; Wilbur 1971; Licht and Bogart 1987). Large
437 cell size has a potentially complex relationship with these physiology traits–one that does not
438 consistently scale across levels of biology (Kozłowski et al., 2004; Johnson et al. 2021; Itgen,
439 2022; Cadart et al., 2023). Similar body sizes and different cell sizes result in unisexuals being
440 comprised of fewer, larger cells compared to sexual species, which may have consequences for
441 the efficiency of metabolic processes in tissues and organs (Fankhauser, 1945; Hanken and
442 Wake, 1993; Itgen et al., 2022). Larger and fewer cells could result in decreased metabolic and
443 water loss rates due to an increase in the thickness of the respiratory surfaces and a reduced
444 surface-area-to-volume ratio (Vinogradov 1994; Monnickendam and Balls 1973; Cadart et al.,
445 2023). However, we found no evidence for this in our study system since polyploid unisexuals

446 did not exhibit lower water loss or metabolic rates compared to the diploid sexual species. Other
447 consequences of allopolyploidization – mitonuclear mismatch and nuclear discordance – may
448 also influence the trade-off; however, disentangling these effects would require investigation at
449 the cellular level. Therefore, our results do not support any influence from polyploidy on the
450 trade-off, pointing to alternative mechanisms shaping the regulation of gas exchange and water
451 loss.

452 Alternatively, variation in the strength of the tradeoff between unisexuals and sexual
453 species may be related to using different mechanisms for controlling water and gas flux across
454 respiratory surfaces. One of the proposed mechanisms for reducing water loss in drier
455 environments is through vasoconstriction, which would decrease both water loss rates and rates
456 of gas exchange (Burggren and Moalli, 1984; Riddell et al., 2019). However, there are other
457 mechanisms that may affect one trait without affecting the other. Regulation of oxygen binding
458 affinity may affect gas flux without influencing resistance to water loss (Wood, 1971). Similarly,
459 some organisms increase the production of melanin to waterproof skin surfaces, which would be
460 unlikely to affect gas flux (Han et al., 2011; Fernandez & Koide, 2013; Ramniwas et al., 2012;
461 Välimäki et al., 2015). Our results suggest that sexual species use mechanisms that allow the
462 independent regulation of each trait (that is, resistance to water loss and metabolic rate are
463 decoupled), whereas unisexual species use mechanisms that affect both gas flux and water loss
464 (such as, vasoconstriction). By using processes that couple gas flux and water loss, unisexuals
465 experience a reduction in the capacity to fuel energetically demanding tasks as a consequence of
466 increasing resistance to water loss (Burggren and Vitalis, 2005; Biro and Stamps, 2010;
467 Sadowska et al., 2015; Sokolova, 2021), potentially affecting their persistence through
468 environmental change. Additional investigation into the effects of allopolyploidization on trait

regulation may provide insight into the degree at which hybridization affects organismal performance.

The stronger trade-off in unisexuals poses a paradox between their reduced capacity to do work as they respond to environmental change and their continued persistence across millennia. In addition to exhibiting a stronger trade-off, unisexuals have lower fecundity (Uzzell 1969), are avoided by sexual males in mate-choice trials (Dawley and Dawley, 1986), and have lower endurance and dispersal rates (Denton et al., 2017) compared to sexual species. Along with the "theft" of genomic material from sympatric species (Charney 2011), these combined effects suggest that unisexuals should be outcompeted by sexual species and not be able to persist. However, unisexuals exhibit faster growth rates (Licht and Bogart, 1987), have a competitive advantage over certain congenerics as larvae (outcompete *A. laterale* larvae but not *A. texanum*; Brodman and Krouse, 2007), and can potentially drive sexual populations to extinction (Clanton, 1934). Unisexuals also require the presence of sympatric sexual species to reproduce, with unisexual populations declining when sexual species are absent (Bogart et al., 2017). In fact, the persistence of unisexuals may even rely on cyclical patterns of population fluctuations between competing unisexuals and sexual species (Greenwald et al., 2016). Understanding the coexistence between sexual and unisexual species as well as the limits of their geographic ranges may require linking physiology to organismal performance across ecological and environmental contexts, especially when considering future climate scenarios and that the species in this study are considered threatened or protected in many parts of their range (Canada's Species at Risk Act, 2002; Illinois DNR, 2020; Ohio DNR, 2022;).

Species responses to environmental change may be partly explained by the environmental conditions experienced throughout their geographic range (Navas, 1996; Wilson, 2001; Lancaster

492 et al., 2020). *Ambystoma laterale* is the most cold-tolerant salamander in North America (Storey
493 and Storey 1992) and is primarily found in higher latitudes and previously glaciated regions
494 (Figure 1B), so higher performance at cooler temperatures may be an adaptive advantage for this
495 species. We observed higher metabolic rates, resistance to water loss, and respiration efficiency
496 in *A. laterale* at cool temperatures, which might facilitate prolonged activity while minimizing
497 desiccation risk at temperatures they experience more regularly. *Ambystoma laterale* also have
498 the most widespread geographic range and therefore experience a broader thermal gradient
499 (Figure 1B). Their lower thermal sensitivity to acute changes in temperature (Figures 2-4) may
500 lessen any negative effects of higher thermal variation by minimizing energetic costs at different
501 temperatures. Alternatively, *A. jeffersonianum* is subject to warmer average temperatures, which
502 could explain their higher performance at the warmer temperatures. *Ambystoma jeffersonianum*
503 also have a much smaller geographic range (Figure 1B), providing a potential explanation for
504 their relatively higher thermal sensitivity of physiological traits. Unisexuals experience a
505 transitional climate, overlapping both *A. laterale* and *A. jeffersonianum* ranges; therefore, their
506 intermediate phenotypes may be the result of evolving in this geographic region (Figure 1B) or
507 simply the additive effects of their sub-genomes (McElroy et al. 2017). These interspecific
508 differences and the environmental conditions that explain them could inform predictions on how
509 environmental change will affect species physiological responses, performance, and fitness.

510 Including phenotypic measurements of parental species is imperative for an accurate
511 interpretation of hybrid performance. In this study, we discovered that subgenome composition
512 was highly indicative of hybrid trait response, with biotypes performing more like the parental
513 species of their major subgenomes. Although we found no effect of allopolyploidization on the
514 traits studied here, measuring other metrics of performance, such as maximal metabolic rate,

515 could better elucidate the effects of allopolyploidization on hybrid performance. Therefore,
516 further investigation into phenotypic variation and the mechanistic drivers of this variation could
517 give insight into not only the persistence of certain lineages throughout millennia, but also the
518 persistence and vulnerability of various species towards current and future environmental
519 change.

References

Addo-Bediako, A., S. L. Chown, and K. J. Gaston. 2001. Revisiting water loss in insects: a large scale view. Journal of Insect Physiology 47.

Agrawal A. A. 2022. A scale-dependent framework for trade-offs, syndromes, and specialization in organismal biology. Ecology 101:e02924.

Anderson, J. F. 1970. Metabolic rates of spiders. Pergamon Press 33:51-72.

Arnold, M. L. 1992. Natural hybridization as an evolutionary process. Annual Review of Ecology and Systematics 23:237-261.

Arnold, M. L., and N. H. Martin. 2010. Hybrid fitness across time and habitats. Trends in ecology & evolution 25:530–536.

Auer, S. K., K. Salin, G. J. Anderson, and N. B. Metcalfe. 2015. Aerobic scope explains individual variation in feeding capacity. Biology letters 11:20150793.

Balao, F., J. Herrera, and S. Talavera. 2011. Phenotypic consequences of polyploidy and genome size at the microevolutionary scale: a multivariate morphological approach. The New phytologist 192:256–265.

Barton, N. H. 2001. The role of hybridization in evolution. Molecular ecology 10:551–568.

Bi, K., and J. P. Bogart. 2010. Time and time again: unisexual salamanders (genus Ambystoma) are the oldest unisexual vertebrates. BMC evolutionary biology 10:1-14.

Bi, K., J. P. Bogart, and J. Fu. 2008. The prevalence of genome replacement in unisexual salamanders of the genus Ambystoma (Amphibia, Caudata) revealed by nuclear gene genealogy. BMC evolutionary biology 8:158.

Birchler, J. A., H. Yao, and S. Chudalayandi. 2006. Unraveling the genetic basis of hybrid vigor. Proceedings of the National Academy of Sciences of the United States of America 103:12957-12958.

Biro, P. A. and J. A. Stamps. 2010. Do consistent individual differences in metabolic rate promote consistent individual differences in behavior? Trends in Ecology & Evolution 25:653–659.

Bogart J.P. 2019. A family study to examine clonal diversity in unisexual salamanders (genus *Ambystoma*). Genome 62:549-561.

Bogart, J. P, J. E. Linton, and A. Sandilands. A population in limbo: unisexual salamanders (genus Ambystoma) decline without sperm-donating species. Herpetological Conservation and Biology. 12:41-55.

Bogart, J. P., K. Bi, J. Fu, D. W. Noble, and J. Niedzwiecki. 2007. Unisexual salamanders (genus Ambystoma) present a new reproductive mode for eukaryotes. Genome 50:119-136.

554 Brodman, R., and H.i D. Krouse. 2007. How blue-spotted and small-mouthed salamander larvae
555 coexist with their unisexual counterparts. Herpetologica 63:135–43.

556 Burger, I. J., E. T. Carter, L. M. Magner, M. M. Muñoz, M. W. Sears, B. M. Fitzpatrick, and E. A.
557 Riddell. 2024. Assessing hybrid vigour using the thermal sensitivity of physiological
558 trade-offs in tiger salamanders. Functional Ecology 38:143-152.

559 Burggren W. W. and R. Moalli. 1984. 'Active' regulation of cutaneous gas exchange by capillary
560 recruitment in amphibians: experimental evidence and a revised model for skin
561 respiration. Respiration Physiology 55:379-392.

562 Burggren, W. W. and T. Z. Vitalis. 2005. The interplay of cutaneous water loss, gas exchange and
563 blood flow in the toad, Bufo woodhousei: Adaptations in a terrestrially adapted
564 amphibian. Journal of Experimental Biology 208:105–112.

565 Cadart, C., J. Bartz, G. Oaks, M. Z. Liu, and R. Heald. 2023. Polyploidy in Xenopus lowers
566 metabolic rate by decreasing total cell surface area. Current Biology 33:1744-1752.e7.

567 Canada's Species at Risk Act. 2002. An Act respecting the protection of wildlife species at risk in
568 Canada: List of Wildlife Species at Risk. Minister of Justice.

569 Charney, N. D. 2011. Relating hybrid advantage and genome replacement in unisexual
570 salamanders. Evolution 66:1387-1397.

571 Clanton, W. 1934. An unusual situation in the salamander *Ambystoma jeffersonianum* (green).
572 Occassioanl papers of the museum of zoology, University of Michigan 290.

573 Clarke, A. and K. P. P. Fraser. 2004. Why does metabolism scale with temperature? Functional
574 Ecology 18:243–251.

575 Comai, L. 2005. The advantages and disadvantages of being polyploid. Nature Reviews Genetics
576 6:836–846.

577 Coyne, J. A., and H. A. Orr. 2004. Speciation: a catalogue and critique of species
578 concepts. Philosophy of biology: an anthology 272-92.

579 Czopek, J. 1962. Vascularization of respiratory surfaces in some caudata. Copeia 1962:576-587.

580 Dawley, E. M., and R. M. Dawley. 1986. Species discrimination by chemical cues in a unisexual-
581 bisexual complex of salamanders. Journal of Herpetology 20:114.

582 Denton, R. D., K. R. Greenwald, and H. L. Gibbs. 2017. Locomotor endurance predicts
583 differences in realized dispersal between sympatric sexual and unisexual
584 salamanders. Functional Ecology 31:915-926.

585 Denton, R. D., A. E. Morales, and H. L. Gibbs. 2018. Genome-specific histories of divergence
586 and introgression between an allopolyploid unisexual salamander lineage and two
587 ancestral sexual species. Evolution; international journal of organic evolution 72:1689-
588 1700.

589 Dobzhansky T. 1982. Genetics and the Origin of Species. New York, NY: Columbia University
590 Press.

591 Edelman, N. B., P. B. Frandsen, M. Miyagi, B. Clavijo, J. Davey, R. B. Dikow, G. García-
592 Accinelli, et al. 2019. Genomic architecture and introgression shape a butterfly radiation.
593 Science 366:594-599.

594 Fankhauser, G. 1945. The Effects of Changes in Chromosome Number on Amphibian
595 Development. The Quarterly review of biology 20:20-78.

596 Feder, M. E. and W. W. Burggren. 1985. Skin breathing in vertebrates. Scientific American
597 253:126-143.

598 Fernandez, C. W., and R. T. Koide. 2013. The function of melanin in the ectomycorrhizal fungus
599 *Cenococcum geophilum* under water stress. Fungal Ecology 6:479–86.

600 Gardner, J. D., M. Laurin, and C. L. Organ. 2020. The relationship between genome size and
601 metabolic rate in extant vertebrates. Philosophical transactions of the Royal Society of
602 London. Series B, Biological sciences 375:20190146.

603 Gibbs, H. L., and R. D. Denton. 2016. Cryptic sex? Estimates of genome exchange in unisexual
604 mole salamanders (Ambystoma sp.). Molecular ecology 25:2805-2815.

605 Greenwald, K. R., R. D. Denton, and H. L. Gibbs. 2016. Niche partitioning among sexual and
606 unisexual *Ambystoma* salamanders. Ecosphere 7.

607 Gregory, T. R. 2001. The Bigger the C-Value, the Larger the Cell: Genome Size and Red Blood
608 Cell Size in Vertebrates. Blood Cells, Molecules, and Diseases 27: 830–843.

609 Gregory, T. R., and B. K. Mable. 2005. Polyploidy in animals. The evolution of the genome.
610 Academic Press 427-517.

611 Han, S. Mi, J. H. Yeo, Y. H. Cho, and S. C. Pak. 2011. Royal jelly reduces melanin synthesis
612 through down-regulation of tyrosinase expression. The American Journal of Chinese
613 Medicine 39:1253-1260.

614 Hanken, J., and D. B. Wake. 1993. Miniaturization of Body Size: Organismal Consequences and
615 Evolutionary Significance. Annual review of ecology and systematics 24:501–519.

616 Harrison, J.S. and R. S. Burton. 2006. Tracing hybrid incompatibilities to single amino acid
617 substitutions. Molecular Biology and Evolution 23:559–564.

618 Hedges, S. B., J. P. Bogart, and L. R. Maxson. 1992. Ancestry of unisexual salamanders. Nature
619 356:708-710.

620 Hessenauer, P., A. Fijarczyk, H. Martin, J. Prunier, G. Charron, J. Chapuis, L. Bernier, et al.
621 2020. Hybridization and introgression drive genome evolution of Dutch elm disease
622 pathogens. Nature ecology & evolution 4:626–638.

Hoffmann, K., M. Hunter, A. J. K. Calhoun, and J. Bogart. 2018. Post-breeding migration and habitat of unisexual salamanders in Maine, USA. Journal of Herpetology 52:273–81.

Illinois Endangered Species Protection Board. 2020. Checklist of Illinois endangered and threatened animals and plants. Illinois Department of Natural Resources,

Itgen, M. W. 2022. Evolutionary increase in genome size drives changes in cell biology and organ structure. Doctoral dissertation, Colorado State University.

Itgen, M. W., G. R. Natalie, D. S. Siegel, S. K. Sessions, and R. L. Mueller. 2022. Genome size drives morphological evolution in organ-specific ways. Evolution; international journal of organic evolution 76:1453–1468.

Johnson, B. B., J. B. Seale, and J. P. Sparks. 2021. Genome size influences adaptive plasticity of water loss, but not metabolic rates, in lungless salamanders. The Journal of Experimental Biology 224:242196.

Klabacka, R. L., H. A. Parry, K. N. Yap, R. A. Cook, V. A. Herron, L. M. Horne, M. E. Wolak, J. A. Maldonado, M. K. Fujita, A. N. Kavazis, J. R. Oaks, and T. S. Schwartz. 2022. Reduced mitochondrial respiration in hybrid asexual lizards. 199:719-728.

Kozłowski, J., M. Konarzewski, and A. T. Gawelczyk. 2003. Cell size as a link between noncoding DNA and metabolic rate scaling. Proceedings of the National Academy of Sciences of the United States of America 100:14080–14085.

Lancaster, L. T. and A. M. Humphreys. 2020. Global variation in the thermal tolerances of plants. Proceedings of the National Academy of Sciences 117:13580–13587.

Lee-Yaw, J. A., C. G. Jacobs, and D. E. Irwin. 2014. Individual performance in relation to cytonuclear discordance in a northern contact zone between long-toed salamander (Ambystoma macrodactylum) lineages. Molecular Ecology 23:4590-4602.

Levy, O., T. Dayan, W. P. Porter, and N. Kronfeld-Schor. 2016. Foraging activity pattern is shaped by water loss rates in a diurnal desert rodent. The American Naturalist 188:205-218.

Licht, L. E., and J. P. Bogart. 1987. Comparative Size of Epidermal Cell Nuclei from Shed Skin of Diploid, Triploid and Tetraploid Salamanders (Genus Ambystoma). Copeia 1987:284–290.

———. 1990. Comparative rates of oxygen consumption and water loss in diploid and polyploid salamanders (genus ambystoma). Comparative biochemistry and physiology. Part A, Physiology 97:569–572.

Licht, L. E., and L. A. Lowcock. 1991. Genome size and metabolic rate in salamanders. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 100:83–92.

Lighton, J. R. 2008. Measuring metabolic rates: a manual for scientists. Oxford University Press.

Lowcock, L. A., H. Griffith, and R. W. Murphy. 1991. The *Ambystoma laterale-jeffersonianum* complex in central Ontario: ploidy structure, sex ratio, and breeding dynamics in a bisexual-unisexual community. Copeia 1991:87.

Mable, B. K. 2013. Polyploids and hybrids in changing environments: winners or losers in the struggle for adaptation? Heredity 110:95–96.

Martin I., B. Lynn, D. Hasselquist, and M. Wikelski. 2006. Investment in immune defense is linked to pace of life in house sparrows. Oecologia 147:565-575.

McElroy, K. E., R. D. Denton, J. Sharbrough, L. Bankers, M. Neiman, and H. L. Gibbs. 2017. Genome expression balance in a triploid trihybrid vertebrate. Genome Biology and Evolution 9:968-980.

McKnight, M. L., and H. B. Shaffer. 1997. Large, rapidly evolving intergenic spacers in the mitochondrial DNA of the salamander family Ambystomatidae (Amphibia: Caudata). Molecular biology and evolution 14:1167-1176.

McNab, B. K. 1997. On the utility of uniformity in the definition of basal rate of metabolism. Physiological Zoology 70:718–720.

Mitchel, K. B. 2020. Temporal changes in unisexual and sexual *Ambystoma* salamander populations in Southesast Michigan. Master's thesis. Eastern Michigan University.

Monnickendam, M. A., and M. Balls. 1973. The relationship between cell sizes, respiration rates and survival of amphibian tissues in long-term organ cultures. Comparative biochemistry and physiology. A, Comparative physiology 44:871–880.

Moran, B. M., C. Payne, Q. Langdon, D. L. Powell, Y. Brandvain, and M. Schumer. 2021. The genomic consequences of hybridization. eLife 10.

Moran, B. M., C. Y. Payne, D. L. Powell, E. N. K. Iverson, A. E. Donny, S. M. Banerjee, Q. K. Langdon, et al. 2024. A lethal mitonuclear incompatibility in complex I of natural hybrids. Nature 626:119–127.

Navas, C. A. 1996. Metabolic physiology, locomotor performance, and thermal niche breadth in neotropical anurans. Physiological Zoology 69:1481–1501.

Ohio Division of Wildlife. 2022. Ohio's Listed Species: Wildlife that are considered to be endangered, threatened, species of concern, special interest, extirpated, or extinct in Ohio (Report No. 5356). Ohio Department of Natural Resources.

Olejnik, S., and J. Algina. 2003. Generalized eta and omega squared statistics: measures of effect size for some common research designs. Psychological methods 8:434.

Ramniwas, S., B. Kajla, K. Dev, and R. Parkash. 2012. Direct and correlated responses to laboratory selection for body melanisation in *D. melanogaster* : support for melanism-desiccation resistance hypothesis. Journal of Experimental Biology jeb.076166.

695 Rank, N. E., P. Mardulyn, S. J. Heidl, K. T. Roberts, N. A. Zavala, J. T. Smiley, and E. P.
696 Dahlhoff. 2020. Mitonuclear mismatch alters performance and reproductive success in
697 naturally introgressed populations of a montane leaf beetle. Evolution 74:1724-1740.

698 Ricklefs, R. E., and M. Wikelski. 2002. The Physiology/Life-History Nexus. Trends in Ecology
699 & Evolution 17:462–68.

700 Riddell, E. A., E. K. Apanovitch, J. P. Odom, and M. W. Sears. 2017. Physical calculations of
701 total resistance to water loss improve predictions of species range models. Ecological
702 Monographs 87:21-33.

703 Riddell, E. A., E. Y. Roback, C. E. Wells, K. R. Zamudio, and M. W. Sears. 2019. Thermal cues
704 drive plasticity of desiccation resistance in montane salamanders with implications for
705 climate change. Nature Communications 10: 4091.

706 Riddell, E. A., J. McPhail, J. D. Damm, and M. W. Sears. 2018. Trade-offs between water loss
707 and gas exchange influence habitat suitability of a woodland salamander. Functional
708 Ecology 32:916-925.

709 Robertson, A. V., C. Ramsden, J. Niedzwiecki, J. Fu, and J. P. Bogart. 2006. An unexpected
710 recent ancestor of unisexual Ambystoma. Molecular Ecology 15:3339-3351.

711 Saccucci, M. J., R. D. Denton, M. L. Holding, and H. L. Gibbs. 2016. Polyploid unisexual
712 salamanders have higher tissue regeneration rates than diploid sexual relatives. Journal of
713 zoology 300:77–81.

714 Sackton T.B., R. A. Haney, and D. M. Rand. 2003. Cytonuclear coadaptation in Drosophila:
715 disruption of cytochrome c oxidase activity in backcross genotypes. Evolution 57:2315–
716 2325.

717 Sadowska, J., A. K. Gebczynski, K. Paszko, and M. Konarzewski. 2015. Milk Output and
718 Composition in Mice Divergently Selected for BMR. Journal of Experimental Biology
719 jeb111245.

720 Schluter, D. 2000. The Ecology of Adaptive Radiation. OUP Oxford.

721 Sharbrough, J., J. L. Conover, M. Fernandes Gyorfy, C. E. Grover, E. R. Miller, J. F. Wendel, and
722 D. B. Sloan. 2022. Global Patterns of Subgenome Evolution in Organelle-Targeted Genes
723 of Six Allotetraploid Angiosperms. Molecular biology and evolution 39.

724 Sokolova, I. 2021. Bioenergetics in environmental adaptation and stress tolerance of aquatic
725 ectotherms: linking physiology and ecology in a multi-stressor landscape. Journal of
726 Experimental Biology jeb236802.

727 Spolsky, C. M., C. A. Phillips, and T. Uzzell. 1992. Antiquity of clonal salamander lineages
728 revealed by mitochondrial DNA. Nature 356:706-708.

729 Storey, K. B., and J. M. Storey. 1992. Natural freeze tolerance in ectothermic vertebrates. Annual
730 review of physiology 54:619–637.

Szarski, H. 1983. Cell size and the concept of wasteful and frugal evolutionary strategies. Journal of theoretical biology 105:201–209.

Teltser, C., and K. R. Greenwald. 2015. Survivorship of ploidy-variable unisexual Ambystoma salamanders across developmental stages. Herpetologica 71:81-87.

Thompson, K. A., Y. Brandvain, J. M. Coughlan, K. E. Delmore, H. Justen, C. R. Linnen, D. Ortiz-Barrientos, et al. 2023. The Ecology of Hybrid Incompatibilities. Cold Spring Harbor perspectives in biology.

Thompson, K. A., M. Urquhart-Cronish, K. D. Whitney, L. H. Rieseberg, and D. Schluter. 2021. Patterns, Predictors, and Consequences of Dominance in Hybrids. The American naturalist 197:E72–E88.

Toews, D. P., and A. Brelsford. 2012. The biogeography of mitochondrial and nuclear discordance in animals. Molecular ecology 21:3907-3930.

Uyeda, J. C., M. W. Pennell, E. T. Miller, R. Maia, and C. R. McClain. 2017. The Evolution of Energetic Scaling across the Vertebrate Tree of Life. The American naturalist 190:185–199.

Uzzell, T. M. 1964. Relations of the Diploid and Triploid Species of the Ambystoma jeffersonianum Complex (Amphibia, Caudata). Copeia 1964:257-300.

Välimäki, P. S. M. Kivelä, J. Raitanen, V-M. Pakanen E. Vatka, M. I. Mäenpää, N. Keret, and T. Tammaru. 2015. Larval melanism in a geometrid moth: promoted neither by a thermal nor seasonal adaptation but desiccating environments. Journal of Animal Ecology 84:817-828.

Vinogradov, A. 1995. Nucleotypic effect in homeotherms: body-mass-corrected basal metabolic rate of mammals is related to genome size. Evolution 49:1249-1259.

Weaver, S. J., H. Edwards, T. McIntyre, S. M. Temple, Q. Alexander, M. C. Behrens, ... and E. N. Taylor. 2022. Cutaneous evaporative water loss in lizards is variable across body regions and plastic in response to humidity. Herpetologica 78:169-183.

Wertheim, B., L. W. Beukeboom, and L. van de Zande. 2013. Polyploidy in animals: effects of gene expression on sex determination, evolution and ecology. Cytogenetic and Genome Research 140:256-269.

Wikelski, M., L. Spinney, W. Schelsky, A. Scheuerlein, and E. Gwinner. 2003. Slow pace of life in tropical sedentary birds: a common-garden experiment on four stonechat populations from different latitudes. Proceedings of the Royal Society of London. Series B: Biological Sciences 270:2383-2388.

Wilbur, H. M. 1971. The ecological relationship of the salamander ambystoma laterale to its all-female, gynogenetic associate. Evolution; international journal of organic evolution 25:168–179.

767 Wilson, R. S. 2001. Geographic variation in the thermal sensitivity of jumpint performance in the
768 frog *Limnodynastes peronii*. Journal of Experimental Biology 204:4227-4236.

769 Wolff, J.N., E. D. Ladoukakis, J. A. Enriquez, and D. K. Dowling. 2014. Mitonuclear
770 interactions: evolutionary consequences over multiple biological scales. Philosophical
771 Transactions of the Royal Society B: Biological Sciences 369:20130443.

772 Wood, S. C. 1971. Effects of metamorphosis on blood respiratory properties and erythrocyte
773 adenosine triphosphate level of the salamander *Dicamptodon ensatus (eschscholtz*).
774 Respiration Physiology 12:53–65.

775 Woods, H. A., and J. N. Smith. 2010. Universal model for water costs of gas exchange by
776 animals and plants. Proceedings of the National Academy of Sciences of the United
777 States of America 107:8469–8474.

778 Yee W. K. W., K. L. Sutton, and D. K. Dowling. 2013. In vivo male fertility is affected by
779 naturally occurring mitochondrial haplotypes. Current Biology 23:R55–56.

780

Table 1: Results for linear models from the trait value analyses, change of trait analyses, and trade-off analysis*.

Trait	Fixed Effects	Trait value analysis			Change in trait analysis		
		F statistic (df)	P value	Effect size (ω^2)	F statistic (df)	P value	Effect size (ω^2)
Metabolic rate – by species	Mass	359.22 (1,141)	< 0.001	0.71	4.50 (1,122)	0.03	0.03
	Temperature	735.97 (2, 556)	< 0.001	0.67	15.26 (2, 200)	< 0.001	0.12
	Species	3.24 (2, 119)	0.04	0.03	0.12 (2,112)	0.88	< 0.001
	Acclimation	116.65 (1, 567)	< 0.001	0.13	-	-	-
	Species × Temperature	15.15 (4, 552)	< 0.001	0.09	3.12 (4, 195)	0.02	0.04
	Species × Acclimation	3.40 (2, 557)	0.03	0.01	-	-	-
	Temperature × Acclimation	4.64 (2, 556)	0.01	< 0.001	-	-	-
	Species × Temperature × Acclimation	1.78 (4, 553)	0.13	< 0.001	-	-	-
Metabolic rate – by biotype	Mass	231.98 (1,140)	< 0.001	0.62	4.92 (1,115)	0.03	0.03
	Temperature	841.99 (2,530)	< 0.001	0.75	13.19 (2,192)	< 0.001	0.12
	Biotype	2.49 (3,114)	0.06	0.02	0.15 (3, 108)	0.93	< 0.001
	Acclimation	133.75 (1, 549)	< 0.001	0.18	-	-	-
	Biotype × Temperature	26.65 (6,530)	< 0.001	0.22	3.26 (6,191)	0.004	0.06
	Biotype × Acclimation	3.64 (3, 532)	0.01	0.02	-	-	-
	Temperature × Acclimation	5.27 (2, 530)	0.005	0.01	-	-	-
	Biotype × Temperature × Acclimation	1.80 (6, 529)	0.10	< 0.001	-	-	-
Total resistance to water loss – by species	Mass	8187.17 (1,125)	< 0.001	0.98	1.78 (1,125)	0.19	< 0.001
	Temperature	2561.67 (2,516)	< 0.001	0.88	1.73 (2,184)	0.18	< 0.001
	Species	2.51 (2,108)	0.09	< 0.001	10.43 (2,115)	< 0.001	0.14
	Acclimation	2.03 (1, 519)	0.15	< 0.001	-	-	-
	Species × Temperature	39.46 (4,508)	< 0.001	0.23	1.44 (4,181)	0.22	< 0.001
	Species × Acclimation	0.78 (2, 511)	0.46	< 0.001	-	-	-
	Temperature × Acclimation	1.58 (2, 511)	0.21	< 0.001	-	-	-
	Species × Temperature × Acclimation	0.61 (4, 510)	0.66	< 0.001	-	-	-

Total resistance to water loss – by biotype	Mass	4038.57 (1,134)	< 0.001	0.97	1.49 (1,114)	0.22	< 0.001
	Temperature	3277.0 (2,487)	< 0.001	0.93	1.01 (2,188)	0.37	< 0.001
	Biotype	1.86 (3,109)	0.14	0.02	4.69 (3,112)	0.004	0.09
	Acclimation	4.52 (1, 506)	0.03	< 0.001	-	-	-
	Biotype × Temperature	57.84 (6,485)	< 0.001	0.41	1.47 (6,189)	0.19	0.01
	Biotype × Acclimation	1.20 (3, 486)	0.31	< 0.001	-	-	-
	Temperature × Acclimation	2.06 (2,484)	0.13	< 0.001	-	-	-
	Biotype × Temperature × Acclimation	0.87 (6, 483)	0.52	< 0.001	-	-	-
Respiration efficiency – by species	Mass	250.80 (1,143)	< 0.001	0.63	3.10 (1,90)	0.08	0.02
	Temperature	1359.96 (2, 520)	< 0.001	0.80	25.77 (2,168)	< 0.001	0.21
	Species	3.29 (2,119)	0.04	0.03	0.15 (2,89)	0.86	< 0.001
	Acclimation	107.18 (1, 527)	< 0.001	0.13	-	-	-
	Species × Temperature	17.53 (4,515)	< 0.001	0.11	2.49 (4,164)	0.04	0.03
	Species × Acclimation	3.26 (2,517)	0.04	0.01	-	-	-
	Temperature × Acclimation	2.51 (2, 517)	0.08	< 0.001	-	-	-
	Species × Temperature × Acclimation	1.37 (4, 516)	0.24	< 0.001	-	-	-
Respiration efficiency – by biotype	Mass	154.05 (1,142)	< 0.001	0.52	3.99 (1,99)	0.04	0.03
	Temperature	1573.61 (2,493)	< 0.001	0.86	22.64 (2,164)	< 0.001	0.21
	Biotype	2.48 (3,112)	0.06	0.03	0.22 (3,84)	0.88	< 0.001
	Acclimation	130.66 (1, 512)	< 0.001	0.19	-	-	-
	Biotype × Temperature	29.76 (6,493)	< 0.001	0.25	2.40 (6,164)	0.03	0.05
	Biotype × Acclimation	3.26 (3, 494)	0.02	0.02	-	-	-
	Temperature × Acclimation	3.65 (2, 492)	0.03	< 0.001	-	-	-
	Biotype × Temperature × Acclimation	1.25 (6, 491)	0.28	< 0.001	-	-	-
Tradeoff – by species (VCO_2)	Total resistance to water loss	-	-	-	16.70 (1,250)	< 0.001	0.03
	Temperature × Total resistance to water loss	-	-	-	1.38 (2,206)	0.25	< 0.001
	Species × Total resistance to water loss	-	-	-	3.62 (2,243)	0.03	0.02
	Total resistance to water loss	-	-	-	17.95 (1,237)	< 0.001	0.05

Tradeoff – by biotype (VCO_2)	Temperature × Total resistance to water loss	-	-	-	0.03 (2,211)	0.96	< 0.001
	Biotype × Total resistance to water loss	-	-	-	1.41 (3,227)	0.23	< 0.001

*Results for linear models from the trait value analyses and change of trait analyses for the effects of mass, temperature, species/biotype, and the interaction of species/biotype and temperature on metabolic rate, total resistance to water loss, respiration efficiency, and the trade-off between metabolic rate and total resistance to water loss.

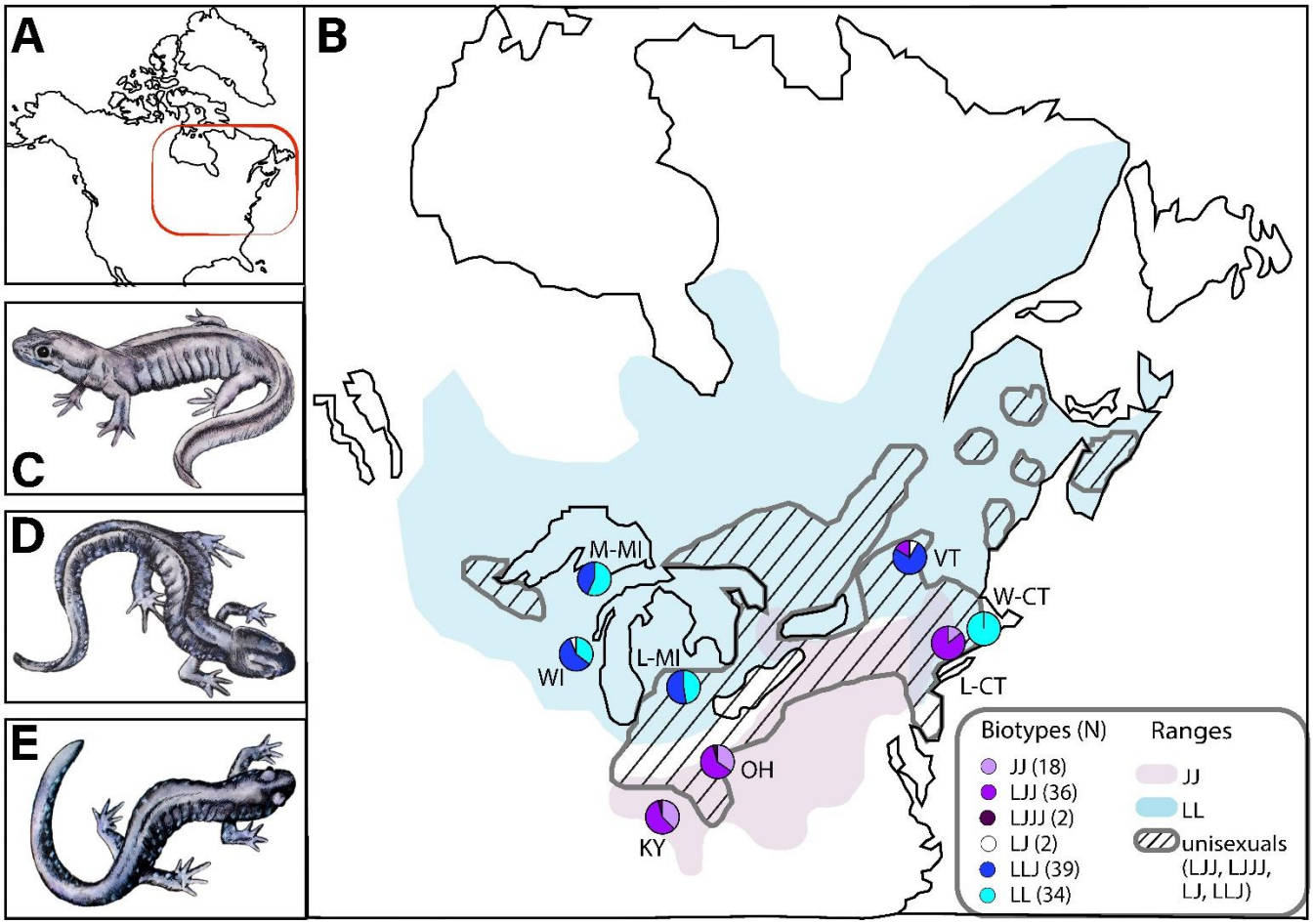

Figure 1 Inset of United States and Canada with the area of interest outlined (A). Area of interest showing distribution, sample size (N), and range of different biotypes across collection sites. M-MI: Marquette, Michigan; L-MI: Livingston, Michigan; WI: Calumet, Wisconsin; KY: Campbell, Kentucky; OH: Delaware, Ohio; VT: Addison, Vermont; W-CT: Windham, Connecticut; L-CT: Litchfield, Connecticut; JJ: *Ambystoma jeffersonianum;* LJ, LJJ, LLJ, and LJJJ: different *Ambystoma* unisexual biotypes; LL: *Ambystoma laterale.* Pie charts indicate relative numbers of individuals collected at each site (B). Pictures of *Ambystoma jeffersonianum* (C), unisexual polyploid (D), and *Ambystoma laterale* (E). Illustrations provided by Katie Garrett.

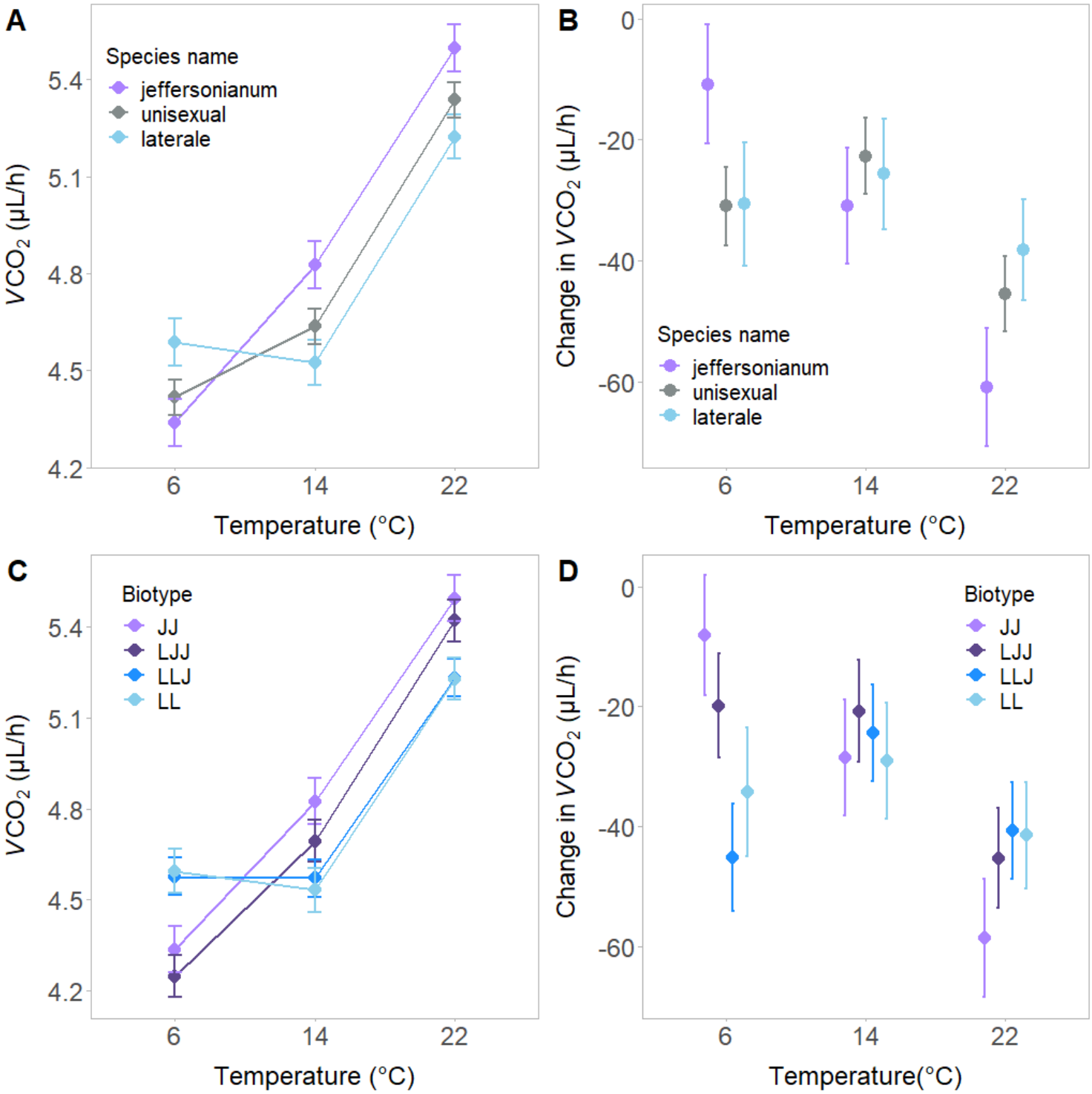

Figure 2 Variation in metabolic rate (VCO_2) across temperature and species/biotype. The effects of temperature on log-scaled VCO_2 separated by species (A) and biotype (C). The effects of

temperature on the change in $\dot{V}CO_2$ separated by species (B) and biotype (D). Negative values in acclimation plots (B &D) show that salamanders acclimated by lowering their $\dot{V}CO_2$ following the acclimation treatment. Data for all panels are shown as the adjusted mean ± SEM, incorporating the effects of all variables in the model.

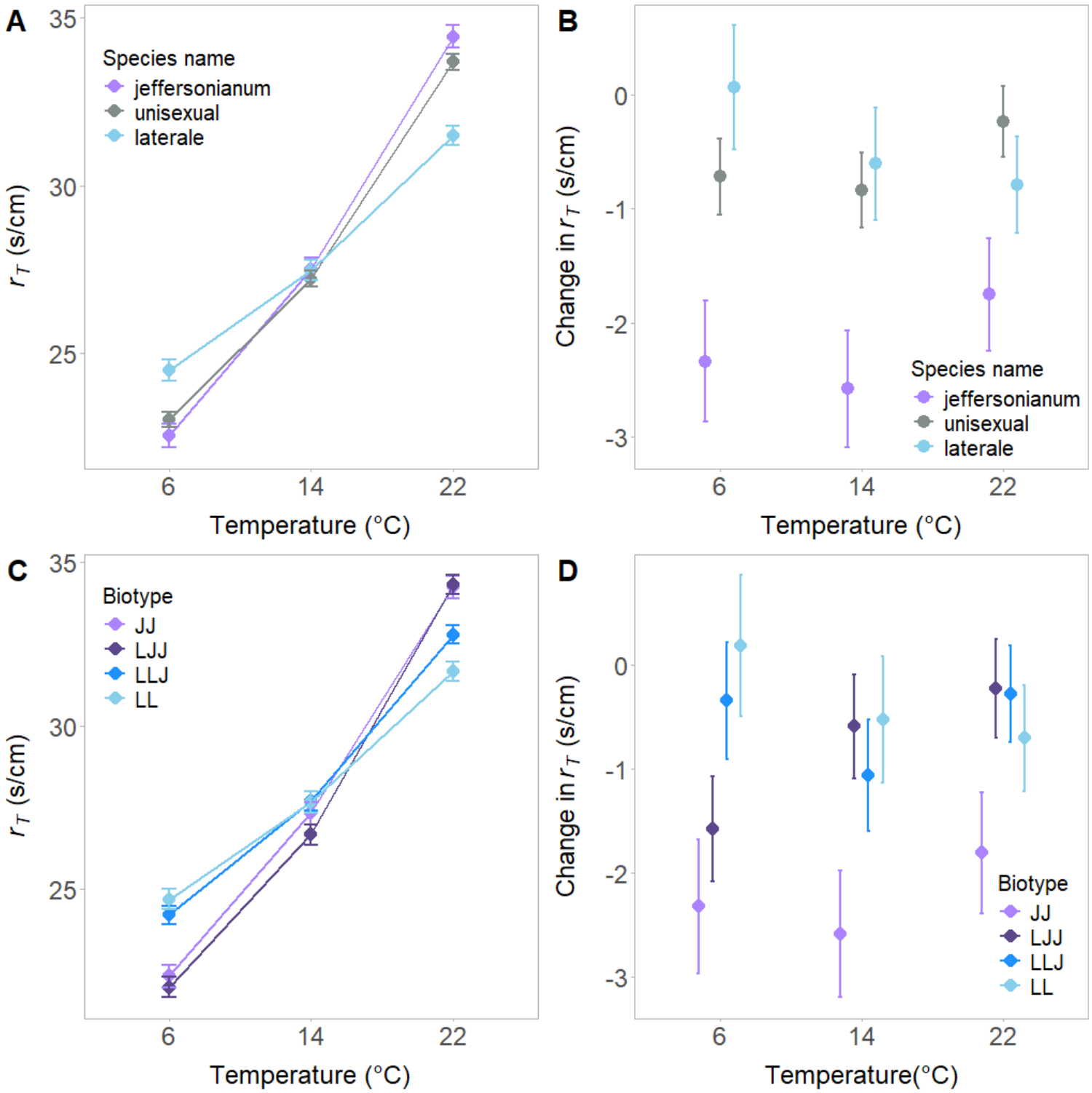

Figure 3 Variation in total resistance to water loss (r_T) across temperature and species/biotype. The effects of temperature on r_T separated by species (A) and biotype (C). The effects of

temperature on the change in r_T separated by species (B) and biotype (D). Negative values in acclimation plots (B &D) show that salamanders acclimated by lowering their r_T following the acclimation treatment. Data for all panels are shown as the adjusted mean ± SEM, incorporating the effects of all variables in the model.

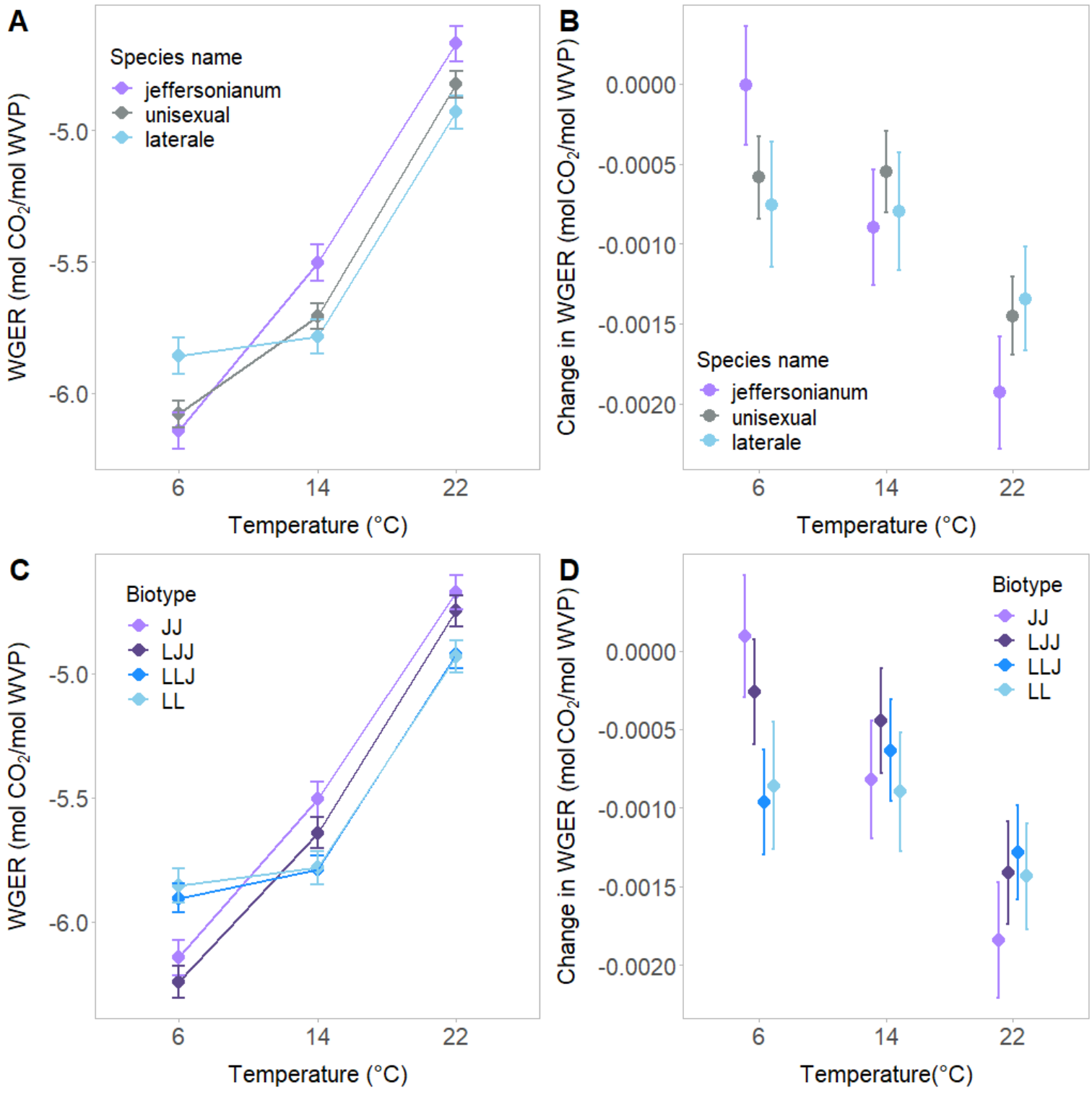

Figure 4 Variation in respiration efficiency (WGER) across temperature and species/biotype. The effects of temperature on WGER separated by species (A) and biotype (C). The effects of

temperature on the change in WGER separated by species (B) and biotype (D). Negative values in acclimation plots (B &D) show that salamanders acclimated by lowering their WGER following the acclimation treatment. Data for all panels are shown as the adjusted mean ± SEM, incorporating the effects of all variables in the model.

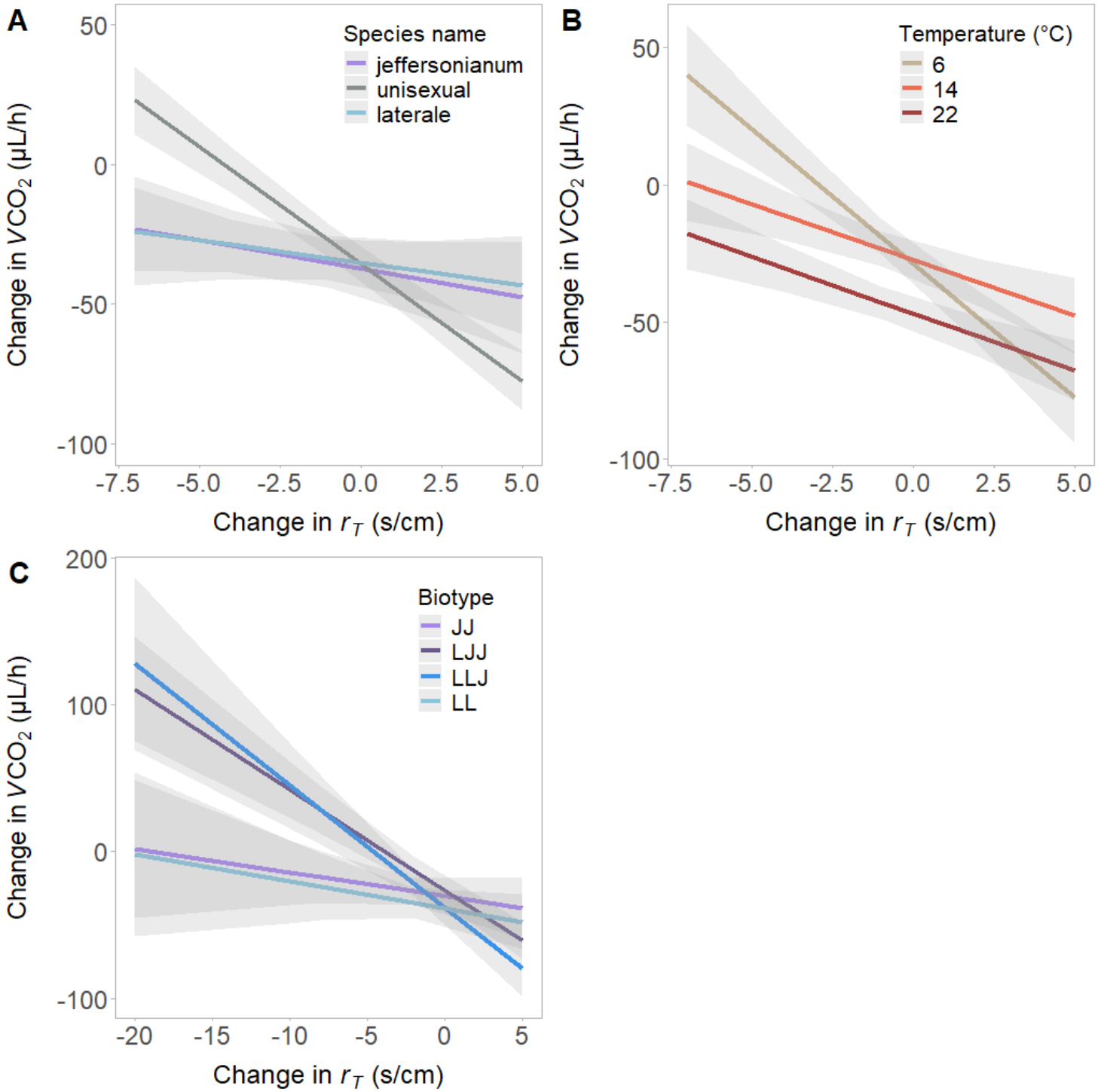

Figure 5 Trade-off between the acclimation of metabolic rate (VCO_2) and change in total resistance to water loss (r_T) across temperature and species/biotype. Unisexuals exhibited the strongest trade-off between VCO_2 and r_T (A). There was no difference in the strength of the trade-off across temperatures, yet 6˚C had the greatest slope (B). There was no difference in the

strength of the trade-off between unisexuals and sexual species when separated into their unique biotypes, yet LJJ and LLJ exhibited a greater slope (C). Data for all panels are shown as adjusted mean ± SEM, incorporating the effects of all variables in the model.