RESEARCH ARTICLE

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Assessing hybrid vigour using the thermal sensitivity of physiological trade-offs in tiger salamanders

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Abstract

1. Hybridization between species affects biodiversity and population sustainability in numerous ways, many of which depend on the fitness of the hybrid relative to the parental species. Hybrids can exhibit fitter phenotypes compared to the parental lineages, and this 'hybrid vigour' can then lead to the extinction of one or both parental lines.

2. In this study, we analysed the relationship between water loss and gas exchange to compare physiological performance among three tiger salamander genotypes—the native California tiger salamander (CTS), the invasive barred tiger salamanders (BTS) and CTS × BTS hybrids across multiple temperatures (13.5°C, 20.5°C and 23.5°C). We developed a new index of performance, the water-gas exchange ratio (WGER), which we define as the ratio of gas exchange to evaporative water loss ($\mu L\ VO_2/\mu L\ H_2O$). The ratio describes the ability of an organism to support energetically costly activities with high levels of gas exchange while simultaneously limiting water loss to lower desiccation risk. We used flow through respirometry to measure the thermal sensitivity of metabolic rate and resistance to water loss of each salamander genotype to compare indices of physiological performance.

3. We found that temperature had a significant effect on metabolic rate and resistance to water loss, with both traits increasing as temperatures warmed. Across genotypes, we found that hybrids have a higher WGER than the native CTS, owing to a higher metabolic rate despite having a lower resistance to water loss.

4. These results provide a greater insight into the physiological mechanisms driving hybrid vigour and offer a potential explanation for the rapid spread of salamander hybrids. More broadly, our introduction of the WGER may allow for species- or lineage-wide comparisons of physiological performance across changing

environmental conditions, highlighting the insight that can be gleaned from multitrait analysis of organism performance.

KEYWORDS
gas exchange, genotype, hybridization, metabolism, respiration efficiency, salamander, trade-offs, water loss

1 | INTRODUCTION

Hybridization between species affects population sustainability and biodiversity both positively and negatively. Hybridization can increase genetic diversity and adaptive potential by forging new genetic combinations and replacing deleterious or previously lost alleles (Abbott et al., 2013; Chan et al., 2019; Rieseberg, 2009). This higher adaptive potential could lower extinction risk by allowing species to expand their ranges and minimize the effects of environmental change (Adavoudi & Pilot, 2022; Becker et al., 2013; Frankham, 2015; Hamilton & Miller, 2016; Hoffmann & Sgró, 2011). Hybrids can also evolve into a distinct species with unique morphological traits and habitat requirements (Abbott et al., 2013; Adavoudi & Pilot, 2022; Donovan et al., 2010; Rieseberg, 2009). In contrast to these beneficial effects, hybridization can result in genetic swamping, outbreeding depression and loss of local adaptation that might increase extinction risk (Adavoudi & Pilot, 2022; Colella et al., 2019; Grobler et al., 2018; Todesco et al., 2016). Hybridization can also exacerbate invasiveness and potentially threaten native parental species or other native taxa (Brasier et al., 1999; Ellstrand & Schierenbeck, 2000; Facon et al., 2005; Hall, 2016; Kent, 1988). Furthermore, rare, endemic species can be disproportionally impacted by breeding with introduced species, especially if the resulting hybrids outperform the parental lineages (Allendorf et al., 2004; Muhlfeld et al., 2014; Rhymer & Simberloff, 1996; Todesco et al., 2016).

The outcome of hybridization on biodiversity depends on the fitness of the hybrid relative to the parental species. Hybrid vigour occurs when a hybrid exhibits phenotypes with higher performance than both parental lineages, resulting in higher fitness (Birchler et al., 2006). In some cases, hybrid vigour may be readily observable, particularly for traits related to morphology or life history. For example, some hybrids are larger at birth, have higher growth rates and are larger at first reproduction than either parental morph (Facon et al., 2005). Hybridization has also resulted in higher lifetime fecundity in hybrids relative to the parental lineages (Szucs et al., 2012). Similar results have been observed in plants, with hybridization in corn (Jones, 1919) and rapeseed (Shen et al., 2017) producing hybrids with higher yields. Other cases of hybrid vigour, however, may be subtler and less readily detectable, such as those related to physiological performance (Cooper & Shaffer, 2021).

Metabolic rate and water loss are two fundamental aspects of organismal physiology that shape performance, survival and reproduction. Animals with lower water loss and metabolic rates, for instance, may exhibit higher survivorship because of a lower desiccation risk and reduced energetic costs, respectively (Anderson, 1970; Riddell &

Sears, 2015). These traits can also be intrinsically linked in an organism due to the requirements for gas exchange (Porter & Gates, 1969; Woods & Smith, 2010). The respiratory surface of an organism must be moist for oxygen to dissolve into the liquid and diffuse into the underlying tissue, but wetter surfaces are also more prone to desiccation (Maina, 2002). These two traits, metabolic rate and water loss, are correlated across a wide variety of taxa, spanning evolutionary timescales (Woods & Smith, 2010). They are also linked within individuals, such that a reduction in water loss rate induces a simultaneous reduction in gas exchange (Riddell et al., 2018). Thus, there is a clear trade-off between these two traits such that physiological responses to avoid desiccation by lowering water loss rates across respiratory surfaces subsequently limit the ability of an organism to breathe (Addo-Bediako et al., 2001; Woods & Smith, 2010). This trade-off between gas exchange and water loss can be stronger in 'leaky' organisms, such as wet-skinned amphibians, in which water loss rates are high relative to the amount of oxygen consumed (Feder, 1978, 1983, 1988). Amphibians that can decouple these traits by limiting water loss while maintaining respiration would likely have a greater capacity to fuel energetic demand for foraging, growth, territory defence and reproduction without simultaneously enhancing desiccation risk (Burggren & Vitalis, 2005; Feder, 1983; Riddell & Sears, 2015, 2020). Thus, expressing the amount of gas exchange to water loss as a ratio (referred to as the water-gas exchange ratio (WGER) from here on) likely provides an effective physiological index to assess hybrid vigour.

In this study, we investigated physiological performance in the California tiger salamander (*Ambystoma californienses*; CTS), the barred tiger salamander (*A. mavortium*; BTS) and their hybrid by measuring and comparing metabolic and water loss rates of the three genotypes. Hybridization between the BTS and CTS began approximately 80 years ago as a result of human-mediated introduction of the BTS into CTS habitat, and hybrids continue to spread throughout the Salinas Valley of California, endangering native CTS (Cooper & Shaffer, 2021; Fitzpatrick & Shaffer, 2007a; Riley et al., 2003). In this system, experiments have revealed hybrid vigour in thermal tolerances, survival and growth (Cooper & Shaffer, 2021; Fitzpatrick & Shaffer, 2007a; Johnson et al., 2013; Ryan et al., 2009), suggesting that hybrid expansion is likely to continue. To further investigate the physiological differences between these three genotypes, we evaluated the thermal sensitivity of metabolic rate, water loss rate, resistance to water loss and the WGER in hybrids and both parental lineages. We assessed the trade-off between metabolic rate and resistance to water loss within each genotype and measured the time spent breathing across the skin (as opposed to the lungs) to understand the reliance on cutaneous respiration (Whitford & Hutchison, 1965). We hypothesized

that hybrids would lose less water per unit of oxygen consumed relative to the native CTS (i.e., higher WGER) and that hybrids will be less sensitive to temperature than CTS, providing a potential mechanism underlying the spread of invasive alleles.

2 | MATERIALS AND METHODS

2.1 | Animal collections and rearing

Eggs and recently hatched larvae were collected in February and March of 2015 and 2016 from populations throughout California. BTS, CTS and hybrids were collected from 20, 2 and 7 ponds, respectively, as part of an in-depth research project on the hybrid system (Carter, 2018). Most populations have been part of a repeated tissue sampling effort that validated each genotype (Fitzpatrick & Shaffer, 2007b; Riley et al., 2003; Wayne & Shaffer, 2016). The remaining populations not involved in the effort are part of genetically intact CTS populations outside the Salinas Valley (San Luis and Merced National Wildlife Refuge). Eggs were individually reared in deli cups with dechlorinated water and transferred to 1-L plastic containers once hatching occurred. Young larvae were fed live brine shrimp ad libitum, and older larvae were housed in 6-L containers filled with a modified Holtfreter's solution and fed live California blackworms until metamorphosis. This variation in feeding was consistent across genotype. Metamorphs were moved to plastic containers lined with dampened paper towels and fed crickets supplemented with calcium. Paper towels were replaced weekly, and each container was fully sterilized monthly with 1% bleach. All salamanders were reared under temperature and lighting conditions that reflected natural cycles in central California.

Animal collections, housing and experimentation were carried out in accordance with USFWS Federal Recovery Permit TE-094642-9, California Scientific Collecting Permit SC-13203, and University of Tennessee Animal Care and Use Protocol 2310.

2.2 | Physiological experiments

Physiological experiments were conducted in December 2015 and 2016, with animals being run during their collection year. All salamanders (n = 56 CTS, 22 BTS and 96 hybrids; Table 1) were 9–10 months during their respective trials, yet the weights of each genotype varied (BTS = 27.5 ± 0.7 g; hybrids = 13.3 ± 0.3 g; CTS = 12.6 ± 0.3 g). Individuals were acclimated to laboratory conditions (18°C) for 10 days prior to experimentation and were randomly assigned to three temperature treatments representing major life history events for tiger salamanders in central California: (*i*) the average substrate temperature during winter breeding migration (13.5°C), (*ii*) the average burrow temperature during the summer (20.5°C) and (*iii*) the average substrate temperature during early summer dispersal (23.5°C). To ensure that salamanders reached thermal equilibrium in their treatments, individuals were placed in an incubator (Percival, Inc.;

TABLE 1 Replication statement.

Scale of inference	Scale at which the factor of interest is applied	Number of replicates at the appropriate scale
Genotype	Individual	56 California tiger salamanders, 22 barred tiger salamanders and 96 hybrids

Note: The scale at which this study was conducted and the number of replicates at this scale.

Model #I-36VL) at the experimental temperatures for 2 h prior to physiological measurement.

To measure physiological traits, each salamander was placed on a hardwire mesh platform in an acrylic chamber (16 × 3.5 cm; volume *c.* 153 mL). Salamanders can behaviorally limit water loss by curling in on themselves to reduce surface area (Heatwole, 1960). These platforms restricted the salamander's ability to curl onto themselves while also mimicking their posture during activity. The chambers were placed in a temperature-controlled incubator (Percival, Inc.; Model #I-36VL) and connected to a flow-through system (Sable Systems International (SSI), Las Vegas, NV) to measure the partial pressure of O_2 and water vapour at the three temperature treatments. Once salamanders were placed in the respirometry chambers, measurements were delayed for 30 min to allow salamanders sufficient time to come to rest in the new surroundings. Air was initially pushed through a filter located in the incubator using a subsampler pump (SS4; SSI) and was then passed through a dewpoint generator (DG4; SSI) to control for vapour pressure. Humidity was adjusted across temperature treatments to keep the vapour pressure deficit (VPD) at 0.5 kPa. Air was then separated into different streams with flow rates at 300 mL·min^{-1} using a flow-through manifold (SSI) and continuously passed through the chambers. Chambers were cycled through using a multiplexer (MUX8; SSI), and air samples from each individual were passed through a water-vapour analyser (RH300; SSI) and a dual differential oxygen analyser (Oxzilla; SSI). The airstream was scrubbed of water vapour and carbon dioxide prior to passing through the Oxzilla. Individuals were measured in triplicate over a two-hour period, and values were averaged across the three measurements to calculate the average metabolic rates (VO_2) and water loss rates.

Baseline data were collected between measurements by sampling air that was passed through an empty chamber to correct for any drift during the experiment. Each instrument analog output was connected to a universal interface (UI-3; SSI), and transformed voltage outputs were continuously measured using Expedata (SSI). These voltage outputs were then used in calculations described by Riddell et al. (2017) and Lighton (2008) to determine total resistance to water loss and VO_2 (Appendix S1).

We also calculated a novel index of performance, the WGER (µL VO_2/µL H_2O), to determine additional physiological differences

between genotypes and temperatures. This parameter was measured by taking the ratio of VO_2 ($\mu L\ VO_2$/h) to evaporative water loss ($\mu L\ H_2O$/h). We assumed that individuals with higher oxygen intake per unit of water loss are more efficient from a gas exchange perspective; therefore, a higher WGER indicates a higher level of efficiency, as such individuals can fuel more activity per rate of water loss. To ease interpretability of units, we elected to express the ratio using water loss rate (μL/h) instead of resistance to water (s/cm). The ratio is similar to a ratio often used in studies on endotherms (Ramirez et al., 2022), but this ratio is interpreted specifically to heat balance. While we acknowledge that the trade-off between metabolic rate and resistance to water loss and the WGER are similar, they provide insight into different aspects of performance. Both parameters include metabolic rate and water loss rate; however, calculations of resistance to water loss control for the evaporative demand of the air and thus do not reflect the absolute water loss rate. In other words, an organism with the same resistance to water loss can experience vastly different water loss rates in different environments. Therefore, the analysis on the trade-off between resistance to water loss and metabolic rate assesses the effect of the changes of resistance to water loss on gas exchange. In contrast, the WGER incorporates the absolute water loss rate and the metabolic rate into a single value for each individual. Therefore, the WGER provides better information on the efficiency of respiration (i.e., the efficiency of oxygen intake per gram of water lost). This ratio can then be compared within an individual (e.g., at different environmental conditions) or across individuals, populations, or species to provide insight into variation in gas exchange efficiency, as well as the relationship between WGER and other metrics of fitness and performance.

To test the mode of respiration, we also measured the time spent breathing across the skin, as opposed to across lungs. In the data output, peaks in VO_2 indicate pulmonary respiration, whereas the flat, stable readings between indicate cutaneous respiration. In order to measure the time an individual spent breathing across the skin, we summed the total time between VO_2 peaks for each measurement and averaged these values for each individual (Figure S1). We ensured that individuals were resting during these trials by visually monitoring activity via an access window. Furthermore, as a result of previous pilot studies, we found that activity is characterized by erratic peaks in metabolic rates, which are different from the smooth, single peaks indicating pulmonary respiration; therefore, we were able to determine whether individuals were resting or active by also inspecting the data output. Except for a few individuals we removed from the study due to urination, all salamanders appeared to be resting due to stable water loss rates and a lack of erratic peaks in metabolic rate. All individuals included in the data analysis were in a state of rest.

2.3 | Statistical analysis

We used separate type II analyses of covariance (ANCOVAs) to understand the effect of mass, genotype and temperature on three response variables: metabolic rate, resistance to water loss and

WGER. To analyse the trade-off between metabolic rate and resistance to water loss, we calculated the residuals of metabolic rate and resistance to water loss regressed against mass to account for the effect of body size. We then used a type II ANCOVA to analyse the effect of genotype, temperature and resistance to water loss on metabolic rate to assess this trade-off. Values for metabolic rate, resistance to water loss and WGER were log-transformed to normalize data. To analyse reliance on skin respiration, we first changed total time spent breathing across the skin to the proportion of time breathing across the skin by dividing each value by the maximum time of the measurement period (400 s). Due to the presence of zeros, we used a quasibinomial logistic regression to analyse the proportion data. For all analyses, a genotype by temperature treatment interaction was included as an interaction effect and mass was included as a covariate. We ran Shapiro Wilkes and Levene's tests to test for normality and equal variance, respectively, along with visual inspection of residual plots. We also ran omega squared (ω^2) analyses to measure the effect sizes of each variable in the ANCOVAs, which was calculated using the following equation:

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

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. All data are shown as mean $\pm$ SEM. We calculated these values using the effects package in R, which returns an adjusted mean that incorporates the effects of all terms in the model. We conducted all statistical analyses in R (version 4.0.5).

We also presented the untransformed data and statistical analyses (using the same model structure) for metabolic rate in the Supplement to illustrate patterns in the raw data that may reveal insight into physiological performance (Appendix S2; Figure S3).

3 | RESULTS

3.1 | Metabolic rate (VO_2)

Mass ($F_{1,164} = 29.52, p < 0.001, \omega^2 = 0.36$), temperature ($F_{2,164} = 13.12, p < 0.001, \omega^2 = 0.12$) and genotype ($F_{2,164} = 6.64, p = 0.002, \omega^2 = 0.06$) had a significant effect on metabolic rate (Figure 1). In particular, larger salamanders had a higher VO_2 (Figure S2), and individuals exhibited the lowest VO_2 at 13.5°C compared to the warmer treatments (Figure 1b). Hybrids had a higher VO_2 than BTS and CTS, with the difference being greater for CTS than for BTS (Figure 1a). We did not find a significant interaction between temperature and genotype on metabolic rate ($F_{4,164} = 0.86, p = 0.49, \omega^2 = 0.00$).

3.2 | Resistance to water loss

Temperature ($F_{2,164} = 120.20, p < 0.001, \omega^2 = 0.58$) and genotype ($F_{2,164} = 10.73, p < 0.001, \omega^2 = 0.10$) influenced resistance to

water loss, with resistance increasing with temperature (Figure 2b). CTS had the highest resistance, followed by hybrids and then BTS (Figure 2a). Mass also affected resistance to water loss, with larger individuals having higher resistances ($F_{1,164} = 582.40$, $p < 0.001$, $\omega^2 = 0.90$). The interaction between temperature and genotype was not significant ($F_{4,164} = 0.19$, $p = 0.94$, $\omega^2 = 0.00$).
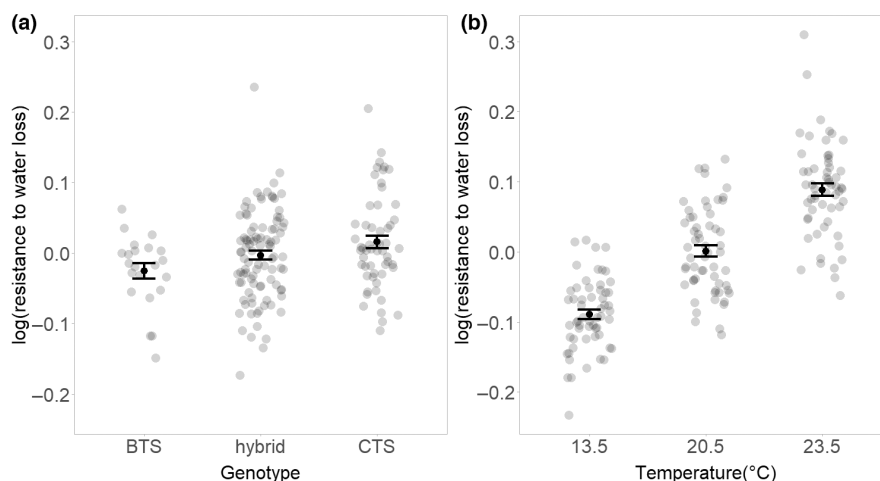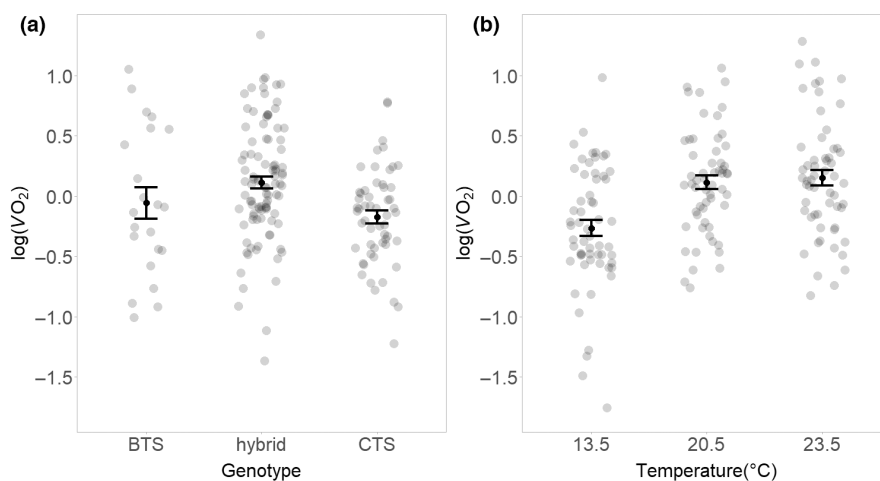
3.3 | Trade-offs between gas exchange and water loss

We found a clear trade-off between VO_2 and resistance to water loss, with VO_2 decreasing as resistance increased ($F_{1,164} = 21.29$, $p < 0.001$, $\omega^2 = 0.00$) (Figure 3). There was also a significant difference in this trade-off for genotype ($F_{2,164} = 5.77$, $p = 0.004$, $\omega^2 = 0.07$, Figure 3a) and temperature ($F_{2,164} = 25.17$, $p < 0.001$, $\omega^2 = 0.22$, Figure 3b). On average, BTS had the strongest trade-off

between resistance to water loss and metabolic rate, followed by hybrids then CTS (Figure 3a). The cooler treatment temperature (13.5°C) had the strongest trade-off (Figure 3b), followed by the other two treatments (20.5°C and 23.5°C). There was no effect of the interaction between temperature and genotype ($F_{4,164} = 0.98$, $p = 0.42$, $\omega^2 = 0.00$).
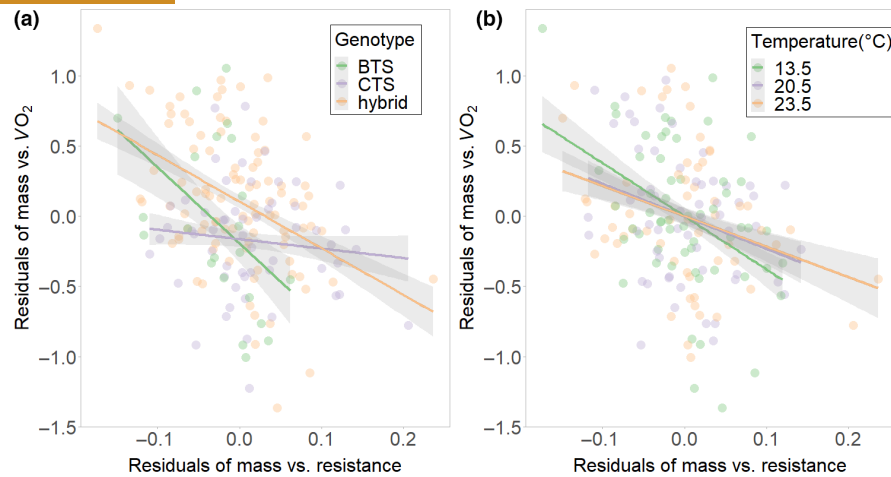
3.4 | Reliance on skin respiration

There was no effect of temperature ($F_{2,164} = 1.5$, $p = 0.22$), mass ($F_{1,164} = 0.42$, $p = 0.51$), or the interaction between genotype and temperature ($F_{4,164} = 0.62$, $p = 0.65$) on time spent breathing exclusively across the skin. However, genotype influenced the duration of skin respiration ($F_{2,164} = 7.89$, $p = 0.0003$), with CTS spending more time breathing solely across the skin compared to hybrids and BTS (Figure 4).

FIGURE 1   (a) The residuals of log-transformed metabolic rates regressed against mass and temperature for the three genotypes (BTS—Barred tiger salamander, hybrid and CTS—California tiger salamander) with hybrids exhibiting the highest metabolic rate and CTS exhibiting the lowest. (b) The residuals of log-transformed metabolic rates regressed against mass and genotypes across the three different temperature treatments with the lowest rate occurring at the coolest temperature. Means are shown with standard error.
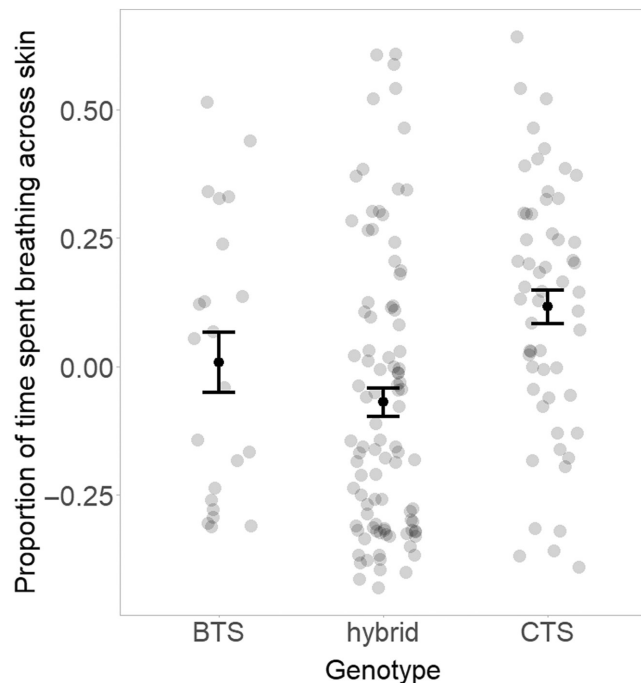

FIGURE 2   (a) The residuals of log-transformed resistance to water loss rates regressed against mass and temperature for the three genotypes (BTS—Barred tiger salamander, hybrid and CTS—California tiger salamander), showing that CTS exhibited the highest resistance to water loss rate and BTS the lowest, with hybrids exhibiting an intermediate response. (b) The residuals of log-transformed resistance to water loss rates regressed against mass and genotype across the three different temperature treatments with resistance to water loss increasing as temperatures increase. Means are shown with standard error.

FIGURE 3 (a) The residuals of metabolic rate (VO_2) and resistance to water loss regressed against mass for the three genotypes (BTS—Barred tiger salamander, hybrid and CTS—California tiger salamander), demonstrating that BTS exhibited the strongest trade-off and CTS exhibited the weakest. (b) The residuals of metabolic rate (VO_2) and resistance to water loss regressed against mass across the three temperature treatments with the strongest trade-off occurring at the coolest temperature. Means are shown with standard error.

FIGURE 4 The residuals of the proportion of time (s) spent breathing across the skin regressed against mass and temperature for the three genotypes (BTS—Barred tiger salamander, hybrid and CTS—California tiger salamander). CTS spent the most time breathing across the skin, followed by BTS and hybrids. Means are shown with standard error.
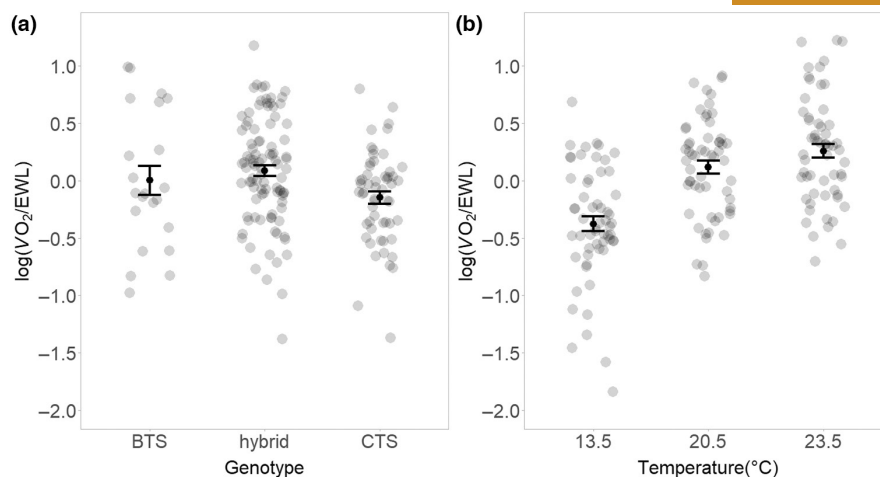
3.5 | Water-gas exchange ratios

Across all genotypes, individuals had the highest WGER at 23.5°C, with efficiency lowering at cooler temperatures ($F_{2,164} = 29.24$, $p < 0.001$, $\omega^2 = 0.25$) (Figure 5b). We also found that mass ($F_{1,164} = 20.41$, $p < 0.001$, $\omega^2 = 0.36$) had a significant effect on WGER, with larger individuals having a higher efficiency. Genotype also influenced the WGER, with hybrids and BTS having a similar WGER and CTS having the lowest ($F_{2,164} = 4.43$, $p = 0.01$, $\omega^2 = 0.04$) (Figure 5a). The interaction between treatment and genotype did not influence the WGER ($F_{4,164} = 1.06$, $p = 0.38$, $\omega^2 = 0.001$).

4 | DISCUSSION

Hybridization is one of the leading threats to rare, endemic taxa (Todesco et al., 2016). Thus, investigating the mechanisms behind the effects of hybrids is imperative for predicting potential outcomes. In this study, we found that hybrid salamanders had a higher metabolic rate, lower resistance to water loss and less reliance on skin respiration compared to CTS. We also introduced a new measure of physiological performance based on the exchange of water and gas across respiratory surfaces—WGER. We defined the WGER as the ratio of oxygen consumption to water loss (μL VO_2/μL H_2O) of an organism. Because the WGER consists of these two traits, an increase in WGER could be the result of an increase in metabolic rate and/or a decrease in the water loss rate. Hybrids had a higher metabolic rate compared to CTS, and CTS had a lower water loss rate compared to hybrids. In theory, these differences could result in a similar WGER between genotypes; however, hybrids exhibited a higher WGER. We found that the higher WGER was the result of the difference in metabolic rate between the two genotypes being greater than the difference in water loss rate. This greater respiratory efficiency could provide insight into mechanisms underlying the hybrid advantage in this system, such as their greater dispersal ability (Johnson et al., 2010). With a higher WGER, hybrids might be able to support the energetic costs associated with dispersal while simultaneously minimizing the risk of desiccation. Conversely, CTS appear to be relatively resistant to desiccation risk due to their high resistance to water loss.

FIGURE 5 (a) The residuals of log-transformed water-gas exchange ratio (WGER: shown as the ratio of oxygen consumed to water lost) regressed against mass and temperature for the three genotypes (BTS—Barred tiger salamander, hybrid and CTS—California tiger salamander). Hybrids exhibited the highest respiratory efficiency and CTS the lowest respiratory efficiency. (b) The residuals of log-transformed WGER regressed against mass and genotype across the three different temperature treatments with the lowest respiratory efficiency occurring at the coolest temperature. Means are shown with standard error.

However, their lower WGER suggests a reduced ability to support energetically demanding traits, such as dispersal and competition. This hybrid advantage in WGER may explain other instances of hybrid vigour and may further uncover how the hybrid swarm continues to spread throughout the Salinas Valley.

Even though hybrids may be more efficient from a gas exchange perspective, we found that their thermal sensitivity of metabolic rate is similar to that observed in CTS. Both hybrids and CTS had an increase in metabolic rate from 13.5°C to 20.5°C, followed by a steady rate from 20.5°C to 23.5°C (Figure S3). This similar physiological response could be attributed to the genetic similarity between hybrids and CTS, which have high levels of introgression (Fitzpatrick & Shaffer, 2007a). As hybrids repeatedly backcross with CTS, CTS genes would become more prevalent throughout the genome in hybrids. In this study, salamanders with >40% non-native ancestry were considered hybrids; therefore, hybrids could have up to 60% genetic similarity to CTS, providing a potential explanation for the similar thermal sensitivity. Additional investigation into the hybrid genome could uncover the genetic mechanisms between these similarities and differences among genotypes.

The physiological trade-off between gas exchange and resistance to water loss impacts rates of energy acquisition and desiccation in terrestrial organisms (Woods & Smith, 2010). We found a strong, negative correlation between resistance to water loss and metabolic rate across all temperatures, consistent with patterns observed across a broad range of taxa (Woods & Smith, 2010), including other salamanders (Riddell et al., 2018). For genotypes, the severity of this trade-off between gas exchange and moisture conservation varied. Specifically, the trade-off was strongest in BTS, with high resistance individuals also exhibiting the lowest metabolic rate. Hybrids exhibited a weaker trade-off between these traits, and no trade-off was observed in CTS, indicating

that resistance to water loss and metabolic rate may be decoupled in CTS. We also found that CTS spent more time breathing across their skin (rather than lungs) compared to BTS or hybrids, which likely contributes to the higher resistance to water loss by reducing water loss from pulmonary ventilation. These results suggest that a higher reliance on skin respiration might facilitate decoupling of water loss rates and respiration. Two main pathways for regulating the flux of water and gases across amphibian skin have been proposed: (1) regulation of the vasculature in the skin to control blood flow and (2) regulation of the lipid barrier (Burggren & Moallf, 1984; Feder & Burggren, 1985; Riddell et al., 2019). To decouple water loss and gas exchange, CTS may have a different composition of ceramides (waterproofing lipids) in the skin, which have been shown to reduce water loss in other taxa (Lillywhite, 2004). CTS might also regulate integumentary gas exchange via the structure of vasculature and perfusion. In contrast to CTS, the stronger trade-off in BTS and hybrids could be linked to their stronger reliance on pulmonary ventilation, which should increase total evaporative water loss rates. Additionally, there may be a link between resistance to water loss and rates of rehydration. Organisms that become more water-tight may limit the amount of water they can absorb. There may also be additional behavioural differences between genotypes that could alter rates of water loss, subsequently affecting resistance rates. Further investigation into these mechanisms could provide a more proximate explanation behind the decoupling of gas exchange and water loss in CTS and the consequences for performance under changing environmental conditions.

The WGER is a new physiological metric that can further untangle the mechanisms behind hybrid vigour and provide additional insight into the phenotypic differences between genotypes. Furthermore, because this trade-off between gas exchange and water loss occurs across terrestrial animals and plants (Woods

& Smith, 2010), the WGER could likewise be applied on a much broader phylogenetic scale. The WGER might explain phenotypic variation between species and/or genotypes across terrestrial taxa. For example, hybrids of two populations of the tansy ragwort flea beetle (*Longitarus jacobaeae*) have a higher lifetime fitness than both parental lines (Szucs et al., 2012). Comparing the respiratory efficiencies of these genotypes may provide a mechanism for this fitness advantage, as having a higher rate of gas exchange per unit water loss may allow for more energetically demanding tasks while limiting desiccation risk and allow for fitness-based activities across a wider range of ambient environmental conditions. Our results suggest that more studies should be developed to determine if WGER is related to fitness or activities that affect fitness, such as competitive ability, desiccation avoidance or reproductive potential. Results from these studies would provide additional insight into the mechanisms behind phenotypic variation, allowing for broader comparisons within and among taxa.

A major conservation concern is that hybridization between CTS and BTS has the potential to eradicate pure CTS genotypes from the Salinas Valley and surrounding areas (Cooper & Shaffer, 2021; Riley et al., 2003). Hybrids can tolerate higher temperatures, develop faster (Cooper & Shaffer, 2021; Johnson et al., 2010) and reduce survivability and size of native CTS larvae (Ryan et al., 2009), likely contributing to the geographic expansion of the hybrid swarm. Although hybrids had a lower resistance to water loss than CTS, we found that hybrids are more efficient at fueling energetic demand while minimizing moisture loss than CTS. This higher WGER could explain the hybrid vigour observed in this system. Given that annual mean temperatures in the Salinas Valley and surrounding areas are estimated to increase by 2100 (Pierce et al., 2018), improved hybrid performance at warm temperatures may favour their geographic expansion and facilitate the genetic swamping of CTS. More investigation into the genomic differences, decoupling of water loss and gas exchange, and ecological relevance of physiological performance could further explain the phenotypic variation found in our study. Furthermore, our introduction of the WGER opens up a new avenue of research and another lens with which to explore traits that shape performance. Incorporating the WGER into studies across terrestrial taxa could allow for broad-scale comparisons of respiration efficiency and physiological performance, both across taxa and changing environmental conditions. These integrated, multitrait analyses could improve our understanding of phenotypic variation between species, while continuing to untangle the effects of a changing environment on organismal physiology.

AUTHOR CONTRIBUTIONS

Evin T. Carter, Michael W. Sears, Benjamin M. Fitzpatrick and Eric A. Riddell conceived the ideas and designed methodology; Evin T. Carter, Lexie M. Magner and Eric A. Riddell collected the data; Isabella J. Burger and Eric A. Riddell analysed the data; Isabella J. Burger, Martha M. Muñoz and EAR led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1: Resistance to water loss and metabolic rate calculations.

Appendix S2: Results of untransformed physiological data for metabolic rate.

Figure S1: Measuring time spent breathing across skin using metabolic rate (VO_2) output.

Figure S2: Log-transformed metabolic rate (VO_2) across mass (g).

Figure S3: Effect of genotype and temperature on metabolic rates.