OXFORD

# Plastic mechanisms for unraveling a universal trade-off between water loss and respiration

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## Abstract

Phenotypic expression is often constrained by functional conflicts between traits, and the resulting trade-offs impose limits on phenotypic and taxonomic diversity. However, the underlying mechanisms that maintain trade-offs or allow organisms to resolve them via phenotypic plasticity are often challenging to detect. The trade-off between gas exchange and water loss across respiratory surfaces represents a fundamental trade-off that constrains phenotypic diversity in terrestrial life. Here, we investigate plastic mechanisms that mitigate this trade-off in lungless salamanders that breathe exclusively across their skin. Our field and laboratory experiments identified plastic responses to environmental variation in water loss and oxygen uptake, and gene expression analyses identified putative pathways that regulate this trade-off. Although the trade-off was generally strong, its strength covaried with environmental conditions. At the molecular level, antagonistic pleiotropy in multiple biological pathways (e.g., vasoconstriction and upregulation of aerobic respiration) putatively produce the trade-off, while other pathways mitigate the trade-off by affecting a single trait (e.g., oxygen binding affinity, melanin synthesis). However, organisms are likely to encounter novel trade-offs in the process of bypassing another. Our study provides evidence that alternative pathways allow organisms to mitigate pleiotropic conflicts, which ultimately may allow greater phenotypic diversity and persistence in novel environments.

**Keywords:** functional conflicts, constraints, metabolic rate, respiration, plasticity, *Plethodon*

## Introduction

Ecological and evolutionary processes are universally dictated by trade-offs (Garland, 2014). Trade-offs occur because organisms are highly integrated systems such that not all biological processes can be optimized at the same time due to underlying genetic correlations, shared biological pathways, finite resources, or physical constraints (Cohen et al., 2012; Garland et al., 2022; Mauro & Ghalambor, 2020; Stearns, 1989). Though trade-offs impose limits on biological design and phenotypic expression, they also provide opportunities for organisms to express alternative combinations of phenotypes and life-history strategies across the fitness landscape (Agrawal, 2020; Meyer et al., 2015; Ricklefs & Wikelski, 2002; Sheftel et al., 2018; van Noordwijk & de Jong, 1986).

The evolutionary constraints imposed by trade-offs can either be largely fixed (e.g., those governed by physical or chemical laws) or flexible (e.g., those sensitive to resource availability). In turn, these differences influence the stability of trade-offs over time and across different environments (Arnold, 1992). At one end of the spectrum, fixed trade-offs are thought to impose strong evolutionary constraints on the ability of traits to evolve independently in response to natural selection. Examples include biophysical constraints, such as bite force and vocal performance trade-offs in bill shape (Herrel et al., 2009), force-velocity trade-offs in muscle fiber composition (Garland et al., 2022), and genetic effects such as antagonistic pleiotropy and linkage (Mauro & Ghalambor, 2020). In such instances, when environments select for opposing strategies, the responses are expected to be constrained along a trade-off axis (Etterson & Shaw, 2001; Ghalambor et al., 2003). On the other end, flexible trade-offs are often context dependent and can vary across individuals based on their condition (e.g., the allocation of resources between competing traits; van Noordwijk & de Jong, 1986) or across changing environments (Norin & Metcalfe, 2019). In these flexible cases, variation in the strength of the trade-off can arise from environmentally driven phenotypic plasticity on the organismal scale, often achieved through physiological changes such as acclimation (Havird et al., 2020; Kelly et al., 2012; Ricklefs & Wikelski, 2002; Terblanche & Hoffmann, 2020). If traits linked by trade-offs can be regulated via phenotypic plasticity, organisms may have greater flexibility to express novel combinations of allowable phenotypes, albeit at the cost of alternative trade-offs (Sinervo & Svensson, 1988; Tikhonov et al., 2020). This combination of plasticity, integration, and conflicting functions make physiological traits chief candidates for promoting or limiting diversity and adaptation (Feder et al., 2000; Garland & Carter, 1994).

Water loss and gas exchange are two tightly linked physiological processes due to functional constraints inherent to

terrestrial life. Both $O_2$ and $CO_2$ are exchanged with the atmosphere through permeable respiratory tissues that are highly vascularized, thin, and saturated with body water (Riddell et al., 2024; Toledo & Jared, 1993; Tracy, 1976). Being thin and saturated, respiratory tissue is also prone to experiencing high water loss rates, thus producing a fundamental trade-off between the capacity to breathe and the capacity to retain water (Tattersall, 2007; Woods & Smith, 2010). This fundamental trade-off constrains phenotypic diversity among terrestrial plants and animals (Woods & Smith, 2010), illustrating its potential to limit independent evolutionary change in these two traits (Riddell & Sears, 2020; Silvertown et al., 2015). However, there are notable evolutionary innovations that mitigate this trade-off. Nasal turbinates in birds and mammals recapture respiratory water in the nasal passage during exhalation (Geist, 2000). In some plants, CAM photosynthesis allows the capture of $CO_2$ during the night when temperatures are lower, reducing the costs of evapotranspiration (Heyduk, 2022). Comparative studies on amphibians have found the trade-off between gas exchange and water loss to be absent in some species and present in others (Burger et al., 2024; Riddell et al., 2024). Thus, investigating the trade-off in organisms with elevated hydric costs of respiration may reveal mechanisms through which the two traits are inherently linked or, alternatively, the capacity for them to be independently regulated.

The strength of any trade-off depends on the degree to which traits are independently regulated. Water loss and gas exchange tend to be linked because both processes are often regulated by the degree of blood flow to the respiratory surface (Feder & Burggren, 1985). Furthermore, physiological pathways that regulate aerobic and anaerobic metabolism may lower metabolic demand when organisms are threatened by water imbalance (Guppy & Withers, 1999; Storey & Storey, 1990, 2004). As these traits change through plastic or evolutionary processes, changes in one trait tend to have opposing effects on the other due to shared underlying mechanisms such as cutaneous blood flow regulation, skin thickness, or lipid content (Riddell et al., 2018a, 2024; Woods & Smith, 2010). Consequently, this trade-off is likely to reflect antagonistic pleiotropy as regulatory genes that increase the performance of one trait might have a simultaneous reduction in performance in the other (Mauro & Ghalambor, 2020; Rose, 1982). These types of gene-trait interactions likely weaken the ability of organisms to independently regulate each trait, thereby limiting the potential for independent evolutionary change. However, there are several alternative pathways that may allow organisms to regulate each trait independently. For instance, regulating oxygen binding affinity of hemoglobin may affect metabolic rate without impacting water loss (Han et al., 2011). Similarly, the synthesis of cutaneous pigments such as melanin may limit water flux across tissues (Fernandez & Koide, 2013; Ramniwas et al., 2013; Välimäki et al., 2015) without compromising gas exchange. These and other pathways may allow for selection to act on phenotypic changes that occur in one trait independently of a related trait. Therefore, an exploratory approach such as transcriptomic analyses may uncover pathways that influence the independent expression of constrained phenotypes. Furthermore, identifying co-expression networks can reveal the extent to which such pathways may be independently expressed or constrained, and consequently their potential for resolving antagonistic pleiotropy.

Here, we investigated the strength of the trade-off between respiration and water loss as well as its underlying mechanisms in field-based and laboratory studies in a species of lungless salamander (*Plethodon metcalfi*). The trade-off is particularly strong in this lineage because, being lungless, water loss and gas exchange are restricted to the same respiratory surface—the skin (Riddell et al., 2018a). Specifically, *Plethodon* salamanders rely exclusively on cutaneous respiration to breathe (Gatz et al., 1974), which suggests they have a particularly limited capacity to independently regulate water loss and gas exchange. Consistent with the trade-off, laboratory experiments on lungless salamanders have demonstrated simultaneous reductions in water loss and oxygen consumption when acclimating to temperature and humidity (Riddell et al., 2018a). Gene expression analyses suggest that salamanders lower water loss rates through vasoconstriction and vasoregression (Riddell et al., 2019), similar to the mechanisms used by other amphibians (Burggren & Vitalis, 2005). Therefore, the regulation of skin vascularity constitutes a putative mechanism underlying the interdependence of water loss and gas uptake. However, the extent to which mechanisms are associated with both water loss and gas uptake or only a single trait has yet to be explored.

We evaluated the strength and consistency of the trade-off between water loss and oxygen uptake in *P. metcalfi* throughout their active season in individuals recently collected from nature to understand whether salamanders are capable of mitigating the trade-off. We also combined acclimation experiments in the laboratory with gene expression analysis to identify gene-trait associations underlying the independent and dependent regulation of water loss and gas uptake. We did this by focusing on the subset of genes with expression patterns correlated with changes in both oxygen uptake and water loss (i.e., pathways consistent with the trade-off) or with expression correlated with one trait but uncorrelated with the other. Thus, our study tests whether organisms are constrained by adjusting phenotypes along a single axis consistent with a fixed trade-off or can escape trade-offs by regulating traits independently.

## Methods

This study is divided into two components (*seasonal acclimatization* and *laboratory acclimation*) that measured oxygen uptake and skin resistance to water loss in response to changing environmental conditions. The "*seasonal acclimatization study*" investigates variation in the correlation between oxygen uptake and skin resistance to water loss in salamanders recently collected from nature throughout their active season (May to October), and thus identifies the strength of the trade-off as salamanders respond to environmental change in nature. The "*laboratory acclimation experiment*" investigates plasticity in oxygen uptake and skin resistance in response to controlled temperature and humidity in the laboratory, combined with gene expression analyses on the skin. Thus, the laboratory experiment helps to identify how these traits are linked from an organismal and mechanistic perspective while minimizing variation due to the environmental history experienced in nature. In both studies, we used the volume of oxygen uptake ($VO_2$) as a metric of oxygen uptake, and skin resistance to water loss ($r_i$) as the metric of water loss. Skin resistance to water loss is defined as the ratio between the water vapor density gradient and the cutaneous water loss

rate by the surface area of the organism (Riddell et al., 2017; Spotila & Berman, 1976). Including the gradient controls for the evaporative demand of the air and thus isolates the physiological changes in water loss. A flowchart summarizing the two experimental designs can be found in the Supplementary Materials (Supplementary Figure S1).

## Seasonal acclimatization study

We analyzed oxygen uptake and water loss rates in 181 salamanders divided into five time points between May and September 2015 (May, early July, late July, August, and September). Animals were collected from the Balsam Mountain Range in the Nantahala National Forest (35° 20′ N, 83° 4′ W) across an elevational gradient spanning 1,200 and 1,600 m. Collection occurred in sites randomly generated on QGIS v2.1 and at least 100 m away from roads. The collection of oxygen uptake and water loss data in the laboratory is described below (*Respirometry trials*). To ensure that salamanders were in a postabsorptive state, we maintained them at 15 °C and in the absence of food for 1 week prior to respirometry trials. We also measured temperature and humidity throughout the season to understand abiotic the conditions salamanders experienced. We randomly generated 15 coordinates within the collection sites to deploy temperate-humidity loggers (Hygrochrons; Maxim Integrated), which were placed on the surface of the forest floor in a hardwire mesh frame to protect the logger from moving. Data were collected every 20 min ($n = 2,939$). At each time point, we report the temperatures (°C) and vapor pressure deficits (VPD; kPa), which describe the difference between the amount of moisture in the air and the amount it can hold when saturated. From a biophysical perspective, the VPD is the primary driver of evaporative water loss rates and describes the evaporative demand of the air (Riddell et al., 2017). To calculate VPD from humidity data, we used the equations described by Riddell et al. (2017).

## Laboratory acclimation experiment

In May 2016, we collected 120 salamanders to investigate the potential for plasticity in the trade-off between oxygen uptake and skin resistance to water loss in controlled laboratory conditions. For salamanders to become accustomed to laboratory conditions, we kept them for 1 month in individual containers (17 cm × 17 cm × 12 cm) with moist paper towels. Salamanders were maintained in an incubator (Percival, Inc.; Model #I-36VL) that cycled through a temperature regime that ranged between 8.5 and 15 °C, mimicking temperatures they experienced above and below ground. After this period, we collected baseline measurements of oxygen uptake and skin resistance to water loss using respirometry trials (see below). We then evenly distributed the 120 individuals across four experimental groups consisting of temperature and humidity exposures in a 2 × 2 factorial design (temperature cycles ranging either between 8.5 and 15 °C, or between 15 and 22.5 °C; vapor pressure deficits of either 0.2 or 0.4 kPa).

On each night during the acclimation experiment, we moved salamanders into activity enclosures (17 cm × 17 cm × 12 cm) that ensured salamanders experienced ambient humidity in the incubator. The enclosures consisted of dry soil as substrate and a hardwire mesh roof to allow air from the incubator to circulate. Exposures happened in a window of three hours at any point between 2100 and 0600 hours for five consecutive nights per week (followed by one night of rest) for four consecutive weeks. Each individual was weighed to the nearest 0.001 g before and after each bout of exposure. We monitored the body mass lost after each exposure period to ensure no animal lost more than 10% of their baseline mass, in which case that individual would not participate in another exposure period until recovery. This outcome occurred in 1.1% of all exposures. To maintain all salamanders in a postabsorptive state, they were not fed during the acclimation experiment. At the end of the acclimation experiment, we euthanized each individual for gene expression analysis and selected eight individuals from each of the four experimental groups ($n = 32$) for sequencing. Because the skin is directly involved in cutaneous respiration and resistance to water loss, we dissected a portion of the dorsal skin (approximately 0.5 cm$^2$) for total RNA extraction (see *RNA sequencing*). We note that salamander skin (and amphibian skin in general) is extremely thin, with the epidermis typically being about one- to two-cell layers thick (Lillywhite, 2006). These samples included both the epidermis and underlying dermis, which contains glands as well as dermal vasculature. We also note that analyzing gene expression at the end of the experiment limits our ability to detect acute regulatory mechanisms responsible for acclimation through time (Bonzi et al., 2021), but our analysis leveraged individual variation in the plasticity of physiological traits to draw conclusions about the potential underlying mechanisms. Thus, our analysis uses a continuum of plasticity among individuals (ranging from individuals with limited plasticity to high plasticity) to identify putative mechanisms involved in physiological plasticity.

## Respirometry trials

We used the same respirometry system for the seasonal acclimatization and laboratory acclimation studies. We measured oxygen consumption and skin resistance to water loss using a flow-through respirometry system (Sable Systems Int. [SSI], Las Vegas, NV). In our system, temperature was controlled using a Percival incubator and maintained at 18 °C while a subsample pump (SS4; SSI) constantly pumped air through a bubbler bottle which saturated the airstream. This saturated airstream then continued through a dew point generator (DG4; SSI) that controlled the water vapor content (vapor pressure deficit = 0.5 kPa). Then, the airstream was separated using a flow manifold (SSI) that also allowed adjustment of flow rates (180 ml/min). These adjusted airstreams were then passed through acrylic chambers (16 × 3.5 cm; volume c. 153 ml) containing the salamander, suspended over hardwire mesh. This chamber design was implemented to promote stereotypical posture during activity and to minimize the potential for posture to confound water loss rates. Although air constantly flowed through all chambers, data collection was cycled through one chamber at a time using a multiplexer (MUX8; SSI). Out of a given chamber, the airstream passes through a water vapor analyzer (RH300; SSI) and lastly through a flow meter and a dual differential oxygen analyzer (Oxzilla; SSI). The conversion of raw data outputs from the respirometry system into physiological traits ($VO_2$, ml/min; $r_s$, s/cm) is described by Riddell et al. (2018b). Both studies were approved by the Institute for Animal Care and Use Committee (IACUC) at Clemson University (#2014-024) with approvals from the North Carolina Wildlife Resource Commission (#16-SC00746), the US Fish and Wildlife Service (#16-SC00746), and Nantahala National Forest.

## Statistical analyses

We determined the strength, consistency, and changes in the trade-off using linear regressions with oxygen uptake ($VO_2$) as a function of resistance to water loss ($r_i$). We used this approach due to the established connections between limiting water loss through perfusion, which also limits oxygen uptake (Burggren & Moallf, 1984; Burggren & Vitalis, 2005). Linear regressions also allow us to include body mass as a covariate, given its established relationship with oxygen uptake (Hayes, 2001). For the seasonal acclimatization study, we analyzed the relationship between $VO_2$ and $r_i$ for each time point separately due to high multicollinearity between time point and resistance to water loss (as determined by generalized variance inflation factor (GVIF) > 100, Fox & Monette, 1992).

For the laboratory acclimation experiment, we assessed the strength of the trade-off before and after laboratory acclimation, and plasticity in the trade-off over the course of the experiment ($n = 120$). For the first model, we regressed initial $VO_2$ on initial $r_i$ before acclimation, and for the second model, we regressed final $VO_2$ on final $r_i$ after the experimental acclimation. Lastly, we regressed the change in $VO_2$ ($\Delta VO_2$) on the changes in $r_i$ ($\Delta r_i$) after the experimental acclimation. We selected $r_i$ as the independent variable as changes in resistance to water loss are suspected to cause changes in gas exchange, but not vice versa. For all models, body mass was included as a covariate. We also included interactions between treatments (temperature × humidity) and skin resistance for the second and third models to test if the strength of the trade-off changed in response to the experimental treatments. Due to the lack of an effect of the treatments and high collinearity between treatments and skin resistance (see Supplementary Table S1), we report results from the simplified model (without the interactions with treatment).

## RNA sequencing

We extracted total RNA from the dorsal skin of 32 salamanders immediately after the final respirometry measurements in the laboratory acclimation study. We euthanized the salamanders by immersion in liquid nitrogen and removed dorsal skin tissue using a flame-sterilized razor blade. We then immersed the tissue samples in TRIzol reagent (Item#: 15596026; Thermo Fisher Scientific, 168 Third Avenue, Waltham, MA 02451), which was then homogenized using a motorized tissue homogenizer with sterilized pestle (Item#: UX-44468-25; Argos Technologies Inc., 625 E. Bunker Ct., Vernon Hills, IL 60061). Tissue homogenates were stored at –80 °C until further downstream processing.

To purify samples for library preparation, we used the RNeasy Mini Kit for total RNA (ID: 74136; Qiagen, 1001 Marshall St., Redwood City, CA 94063), and we reduced DNA contamination by treating the samples with DNase. We quantified total RNA using a Qubit Fluorometer (Thermo Fisher Scientific) and assessed RNA integrity using Agilent Bioanalyzer to ensure sufficient RNA of high quality in each sample and prepared libraries using the Illumina TruSeq mRNA Stranded Kit (Product#: RS-122-2101, Illumina, Inc., 5200 Illumina Way, San Diego, CA 92122). We then used the Qubit Fluorometer to quantify the amount of cDNA in each sample prior to sequencing.

We sequenced single-end 100-bp reads across four lanes of an Illumina HiSeq2500 platform at the Genomics Facility at Cornell University. To avoid lane biases, individuals from each treatment were distributed randomly and evenly across the four sequencing lanes. After sequencing, we trimmed sequencing adapters from the raw reads using Trimmomatic (v. 0.36) and evaluated error probabilities for all reads using ConDeTri (v. 2.3), which removed low-quality bases based on default parameters. After removing low-quality reads, we used FastQC (version 0.11.5) to ensure our sequences had high-quality scores and found the averages and whiskers were above 30 at each position in the read. The *P. metcalfi* de novo transcriptome was assembled using Trinity (v. 2.4.0) using standard transcript expression normalization, and is further described by Riddell et al. (2019) and was based on 48 tissue samples (36 skin and 12 heart samples).

## Gene-trait associations

We used Pearson's correlations to assess the relationship between individual variation in transcript expression and the plasticity of metabolic rate and skin resistance. The purpose was to group genes based on whether they were correlated with only one trait or both. We used two thresholds for the approach: a higher correlation threshold (99th quantile of absolute correlations) to evaluate individual genes correlated with each or both traits and a lower threshold (90th quantile of absolute correlations) to investigate functional enrichments on larger sets of genes. For genes strongly aligned with the trade-off, we selected genes that were above the 99th quantile of absolute correlations both with $\Delta VO_2$ ($|r|_{\Delta VO2} > Q_{99}$) and $\Delta r_i$ ($|r|_{\Delta ri} > Q_{99}$). We evaluated genes in this set individually for potential associations with processes that might affect both water loss and oxygen uptake. We also tested for antagonistic pleiotropy by assessing the sign of the relationship between the gene and trait of interest. Evidence of negative pleiotropy would be indicated by a positive relationship between the expression of a gene on one trait but a negative relationship for the other trait. Next, we selected a larger set of genes for a GO enrichment analysis. These were genes above the 90th quantile of absolute correlations with $\Delta VO_2$ and $\Delta r_i$ ($|r|_{\Delta VO2} > Q_{90}$ and $|r|_{\Delta ri} > Q_{90}$).

To identify putative pathways underlying the independent regulation of skin resistance and gas uptake, we determined which genes were correlated with changes in one physiological trait while being uncorrelated to changes in the other trait. We investigated individual genes that were above the 99th quantile of absolute correlations with $r_i$ ($|r|_{\Delta ri} > Q_{99}$) and below the $Q_{90}$ for $|r|_{\Delta VO2}$ and, reciprocally, genes with $|r|_{\Delta VO2} > Q_{99}$ and $|r|_{\Delta ri} < Q_{90}$. Lastly, we used a lower threshold to obtain a larger set of putative genes for a GO enrichment analysis. These sets were genes with $|r|_{\Delta ri} > Q_{90}$ and $|r|_{\Delta VO2} < Q_{90}$, and $|r|_{\Delta VO2} > Q_{90}$ and $|r|_{\Delta ri} < Q_{90}$. These analyses assume a continuous relationship between transcript abundance and phenotypic expression. However, threshold effects induced by low-level or discrete changes in gene expression on phenotypes (Reid & Acker, 2022) may not be captured by this approach.

## Weighted gene co-expression network analysis

Gene co-expression networks can reveal functional relationships among genes that reflect known biological pathways and networks (Campbell-Staton et al., 2018). We used a weighted gene co-expression network analysis (WGCNA) to identify networks of genes with correlated expressions that are then assigned into modules of co-expression (Zhang and Horvath, 2005). Modules were constructed via pairwise Pearson's correlations between genes using the *blockwiseModules* function with a threshold of $R^2 > 0.8$. The co-expression similarity
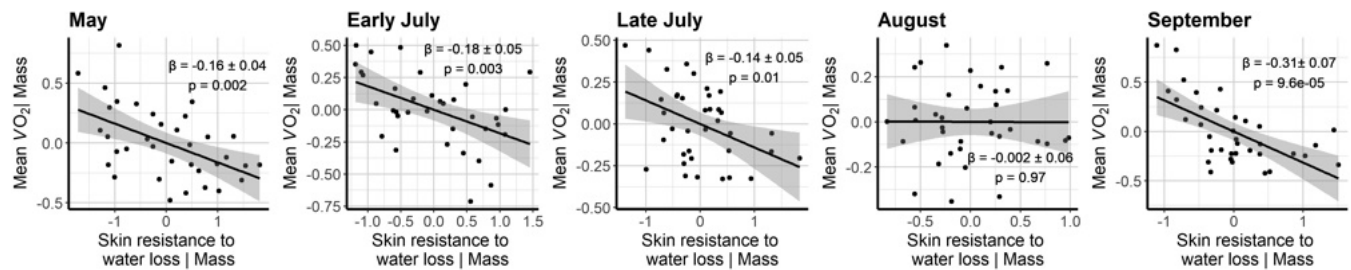
**Figure 1.** Fluctuating strength of the trade-off between skin resistance and water loss throughout the active season in *P. metcalfi*. During August, no significant association between the two traits was observed. Plots show partial residuals that account for individual body mass.

values were then transformed into an adjacency matrix using the soft thresholding approach. We then used a soft threshold of 4 to maximize the scale-free topology and mean connectivity among genes. Next, we defined a dendrogram of the gene networks using average linkage hierarchical clustering coupled with a gene dissimilarity matrix. Finally, highly correlated modules were merged using the Dynamic Tree Cut approach with a height cut of 0.25 (Zhang & Horvath, 2005). These modules allow us to assess whether candidate genes (identified in the correlation-based analysis above) are controlled by a shared mechanism (i.e., if they belong to the same co-expression module) or expressed independently (i.e., if they belong to many different modules). If candidate genes are assigned to different modules, we can infer that the trait is regulated by multiple independent pathways. Conversely, candidate genes in the same module would suggest the trait is potentially regulated as part of an integrated pathway. To provide an additional (but independent) analysis relating gene expression to phenotypic variation, we used a principal components analysis to summarize individual variation in phenotypic change in $VO_2$ and $r_i$ and then assessed the correlation between the first two principal components and the eigenvalues of the WGCNA modules. In this analysis, the first principal component (PC1) encompasses the variation consistent with the trade-off (the correlation between the change in $VO_2$ and $r_i$). The second principal component encompasses the variation orthogonal to the trade-off and thus describes the independent changes in both $VO_2$ and $r_i$. We note that PC2 is not capable of determining which genes are associated with the independent regulation of $VO_2$ or $r_i$ (as done in the correlation analysis above), as they are both integrated into the same principal component.

After removing all genes with less than 10 counts across 28 samples, we retained 9,346 genes for the WGCNA. We then normalized all gene counts using the *varianceStabilizingTransformation* function in DESeq2 and removed any genes with missing data or variance of zero. After these steps, one individual from the cool/wet treatment failed quality control and was removed from downstream analyses.

### Gene Ontology enrichment analyses

To identify functional enrichments associated with sets of genes, we used the R package *goseq* (Young et al., 2010) for Gene Ontology (GO) enrichment analyses. We excluded GO terms associated with fewer than 10 and more than 500 genes to reduce enrichment sensitivity and redundancy. To reduce positive sequencing bias for longer transcripts, transcript length for all genes was integrated into the analysis. That is, the *goseq* package fits a Probability Weighting Function of differential gene expression versus transcript length that

accounts for the chance of a given transcript being selected in the null distribution for a GO membership based on its length (Young et al., 2010). The entire set of genes in the *P. metcalfi* skin transcriptome consisted of 9,346 unique transcripts. However, we excluded any uncharacterized transcripts from the analysis, resulting in a background set of 6,953 genes.

## Results

### Water loss/oxygen uptake trade-off

In four of the five time points between May and September, salamanders in the seasonal acclimatization study exhibited a significant trade-off between skin resistance ($r_i$) and oxygen uptake ($VO_2$; Figure 1), indicated by a negative relationship between the traits. Each analysis also accounted for body mass, which was significantly related to oxygen uptake in each model ($p < .05$ in each analysis). However, salamanders collected in August did not show a significant relationship between the two traits ($\beta = –0.002$, $p = .97$). August was one of the warmest and wettest months of the year (Riddell et al., 2018b; Supplementary Figure S2). Although the trade-off seems mostly consistent throughout their active season, the lack of a trade-off in August suggests the two traits can be regulated independently under certain conditions.

Similarly, the trade-off between skin resistance and oxygen uptake in the laboratory acclimation study was significant in individuals prior to acclimation (Figure 2A), but not after (Figure 2B). We also found evidence for the trade-off in the plasticity between traits, with increases in skin resistance ($\Delta r_i$) being associated with reductions in oxygen uptake ($\Delta VO_2$) over the course of the experiment (Figure 2C; $p < .001$). These results indicate that the trade-off between the two traits constrains acclimation responses. The results also show that the strength of the trade-off changes in response to acclimation (Figure 2A, B). The temperature and humidity treatments had no significant effect on the trade-off or its change during the laboratory experiment (see Supplementary Table S1). We also note that the sequenced individuals ($n = 32$) exhibited the same patterns as all individuals in the acclimation experiment ($n = 120$, Supplementary Figure S3).

### Gene expression associated with the trade-off

The intersection between $|r|_{\Delta ri} > Q_{99}$ and $|r|_{\Delta VO2} > Q_{99}$ was represented by 19 genes, 10 of which are characterized (Supplementary Table S2). Consistent with the underlying functional basis of the trade-off, oxygen uptake and resistance to water loss were inversely correlated with the expression of each gene (Figure 3A, B), suggesting antagonistic pleiotropy. The highest combined absolute correlation with both traits was for pyruvate dehydrogenase isozyme 4, which regulates
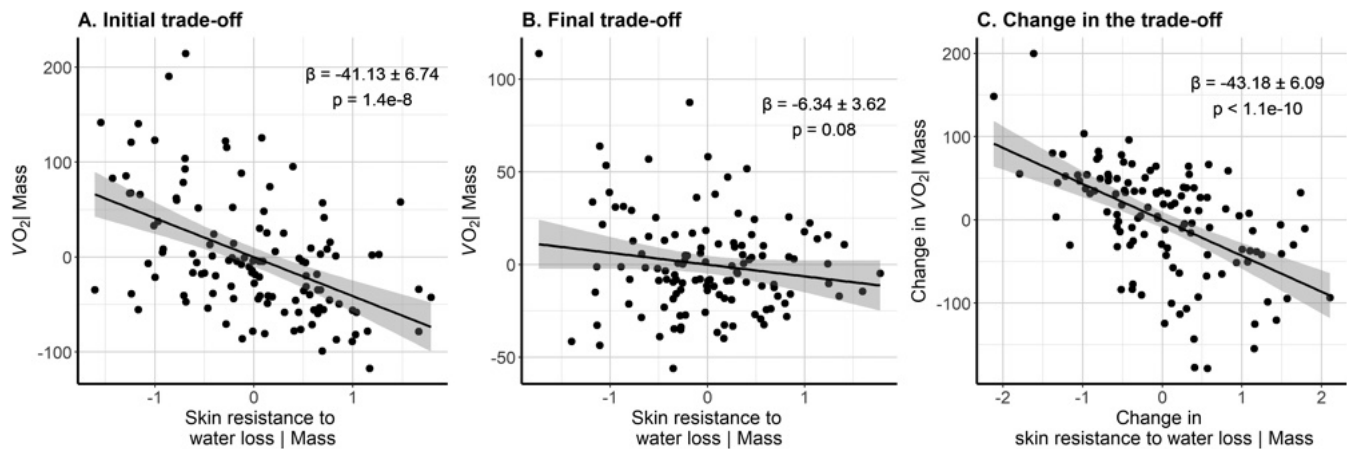
**Figure 2.** Partial residual plots accounting for body mass for the oxygen uptake/water loss trade-off during the laboratory acclimation experiment. (A) Skin resistance and oxygen uptake had a significant negative association at the beginning of the experiment. (B) After the acclimation experiment, the relationship between the two traits was marginally non-significant. (C) Increases in skin resistance were strongly associated with decreases in oxygen consumption after the acclimation experiment.

a shift from glycolysis to fatty acid metabolism as energy fuel in mammalian tissues and cell cultures (Pettersen et al., 2019). In the principal component analysis, PC1 explained 77.3% of the variation in the physiological traits (i.e., change in the two traits that is consistent with the trade-off, Supplementary Figure S4), with each trait loading with opposite signs on PC1 (Pearson's correlation, PC1 and $\Delta r_i$ = −0.88; PC1 and $\Delta VO_2$ = 0.88). The WGCNA found differentially expressed genes were distributed across 27 modules. The WGCNA package names co-expression modules after colors (e.g., *brown*, *lightcyan*). Among those, one co-expression module was significantly associated with PC1 (*magenta*; $r$ = −0.37, $p$ = .037). The gene ontology analysis of the *magenta* module (203 genes) resulted in 24 enriched terms (Supplementary Table S3), the most significant of which was *negative regulation of angiogenesis* (three overexpressed genes). Of the 19 genes most correlated with the trade-off, three were included in the *magenta* module (one of which is characterized).

Next, we investigated functional enrichments in the larger set of genes correlated with both traits ($|r|_{\Delta ri} > Q_{90}$ and $|r|_{\Delta VO2} > Q_{90}$; $n$ = 267; Figure 3C; Supplementary Table S4). Among the five most significant enrichments, at least three terms were associated with protein degradation and downregulation of aerobic metabolism (Figure 3D; *Ubiquitin protein ligase activity*, 7 genes; *autophagy of mitochondrion*, 3 genes; and *protein ubiquitination*, 7 genes). The third most significant enrichment was for *muscle contraction* (three genes). Furthermore, 8.9% of genes in this set (24 out of 267) also occurred within the *magenta* module.

### Genes associated with the independent regulation of physiological traits

Thirty genes had transcription levels with the highest absolute correlations with $\Delta r_i$ ($|r|_{\Delta ri} > Q_{99}$) while being uncorrelated with $\Delta VO_2$ ($|r|_{\Delta VO2} < Q_{90}$; Figure 4A; Supplementary Table S5). The strongest correlation with $\Delta r_i$ was with the expression of tyrosinase, *TYR* ($|r|$ = 0.58, $p$ = .001; Figure 4B), which is a precursor to melanin synthesis. Among functional enrichments associated with the larger set of 429 genes ($|r|_{\Delta ri} > Q_{90}$ and $|r|_{\Delta VO2} < Q_{90}$; Supplementary Table S6), the most significant was for the biological process *mesenchyme migration*

(5 genes). Furthermore, the cellular component *melanosome* was significant (four genes, $p$ = .02).

Thirty-eight transcripts had the highest correlations with $\Delta VO_2$ ($|r|_{\Delta VO2} > Q_{99}$) while being uncorrelated with $\Delta r_i$ ($|r|_{\Delta ri} < Q_{90}$; Figure 4A; Supplementary Table S7). The highest correlations with $\Delta VO_2$ were for *CMTR1* ($|r|$ = 0.57, $p$ < .001; Figure 4B), associated with RNA cap formation (Liang et al., 2023), and *PLK2* ($|r|$ = 0.51, $p$ = .003; Figure 4B), which regulates the mitotic spindle orientation (Villegas et al., 2014). For the larger set of 429 transcripts ($|r|_{\Delta VO2} > Q_{90}$ and $|r|_{\Delta ri} < Q_{90}$; Figure 4C; Supplementary Table S8), the second most significant enrichment was for the term *heme binding* (nine genes; Figure 4D).

In the principal component analysis, PC2 explained 22.7% of the variation (i.e., phenotypic change orthogonal to the trade-off). One WGCNA module was significantly correlated with PC2 (*darkorange*, $r$ = −0.36, $p$ = .043). The two most significant enrichments in the GO analysis of the *darkorange* module were *immune system process* and *immune response*, with several other enriched terms associated with the regulation of unspecific cellular processes (Supplementary Table S9). None of the genes most correlated with the independent regulation of gas uptake and skin resistance were included in the *darkorange* module. Among the broader set of genes, 1.2% (5 out of 429) occurred within the *darkorange* module.

## Discussion

The concept of pleiotropic genes generating fitness trade-offs plays a central role in evolutionary theory (Hughes & Leips, 2017; Wagner & Zhang, 2011; Williams, 2001; Zhang, 2023). Such antagonistic pleiotropy is thought to constrain the pattern of trait combinations, but the mechanisms giving rise to trade-offs between traits can impact how constrained or independent different traits are (Guillaume & Otto, 2012; Hansen, 2003; Paaby & Rockman, 2013). Because most traits linked to fitness have a polygenic basis, trade-offs are likely to arise from the pleiotropic effects of gene regulatory networks and the biological pathways that determine the genotype–phenotype relationships (Wagner & Zhang, 2011). Importantly, a general feature of these networks is redundancy and
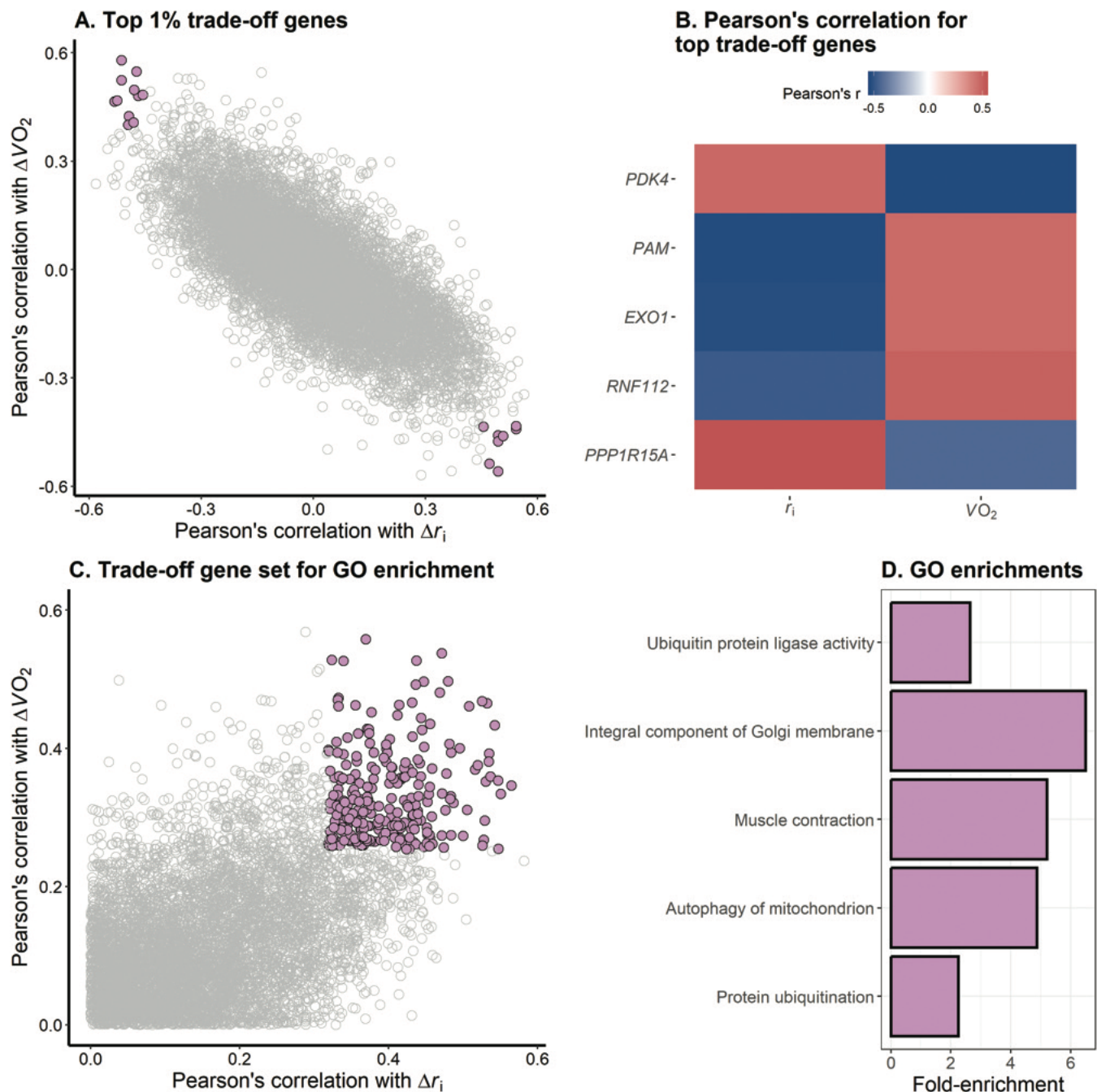
**Figure 3.** Genes and processes putatively involved in the trade-off between oxygen uptake and water loss. (A) Pearson's correlations with changes in skin resistance to water loss ($\Delta r_i$) and with changes in oxygen uptake ($\Delta VO_2$) throughout the laboratory acclimation experiment for 9,346 transcripts sequenced from *P. metcalfi* skin tissues. Highlighted points correspond to genes above the 99th percentile of absolute correlations with both $\Delta r_i$ and $\Delta VO_2$ ($n = 19$). (B) Heatmap for the genes with the highest combined absolute correlation across $\Delta r_i$ and $\Delta VO_2$. (C) Set of genes for functional enrichment analysis. Highlighted points correspond to genes above the 90th percentile of absolute correlations with both $\Delta r_i$ and $\Delta VO_2$ ($n = 267$) out of 6,953 characterized transcripts in the *P. metcalfi* skin transcriptome. (D) Five most significant functional enrichments for the set of genes in panel (C).

context-dependence, such that there is potential for traits to escape pleiotropic constraints (Pavličev & Cheverud, 2015). However, the degree to which organisms may leverage alternative pathways that mitigate a trade-off by allowing traits to occupy new phenotypic spaces and circumvent trade-offs remains unclear (Hashemi et al., 2024; Shoval et al., 2012). In this study, we demonstrate that the fundamental linkage between cutaneous gas exchange and water loss in a lungless salamander varies in strength across seasons (Figures 1 and 2), which suggests that salamanders have the capacity to

regulate functional conflicts related to this trade-off (Buehler et al., 2012). Similarly, we identified genes associated with the independent regulation of both gas uptake and skin resistance to water loss. In contrast to these independent effects, we also identified generally strong associations between gas uptake and skin resistance in the seasonal and acclimation studies as well as multiple genes expression patterns consistent with antagonistic pleiotropy (Figure 3), highlighting the strong interdependence of the two traits. Therefore, patterns of gene expression can not only identify the putative mechanisms that
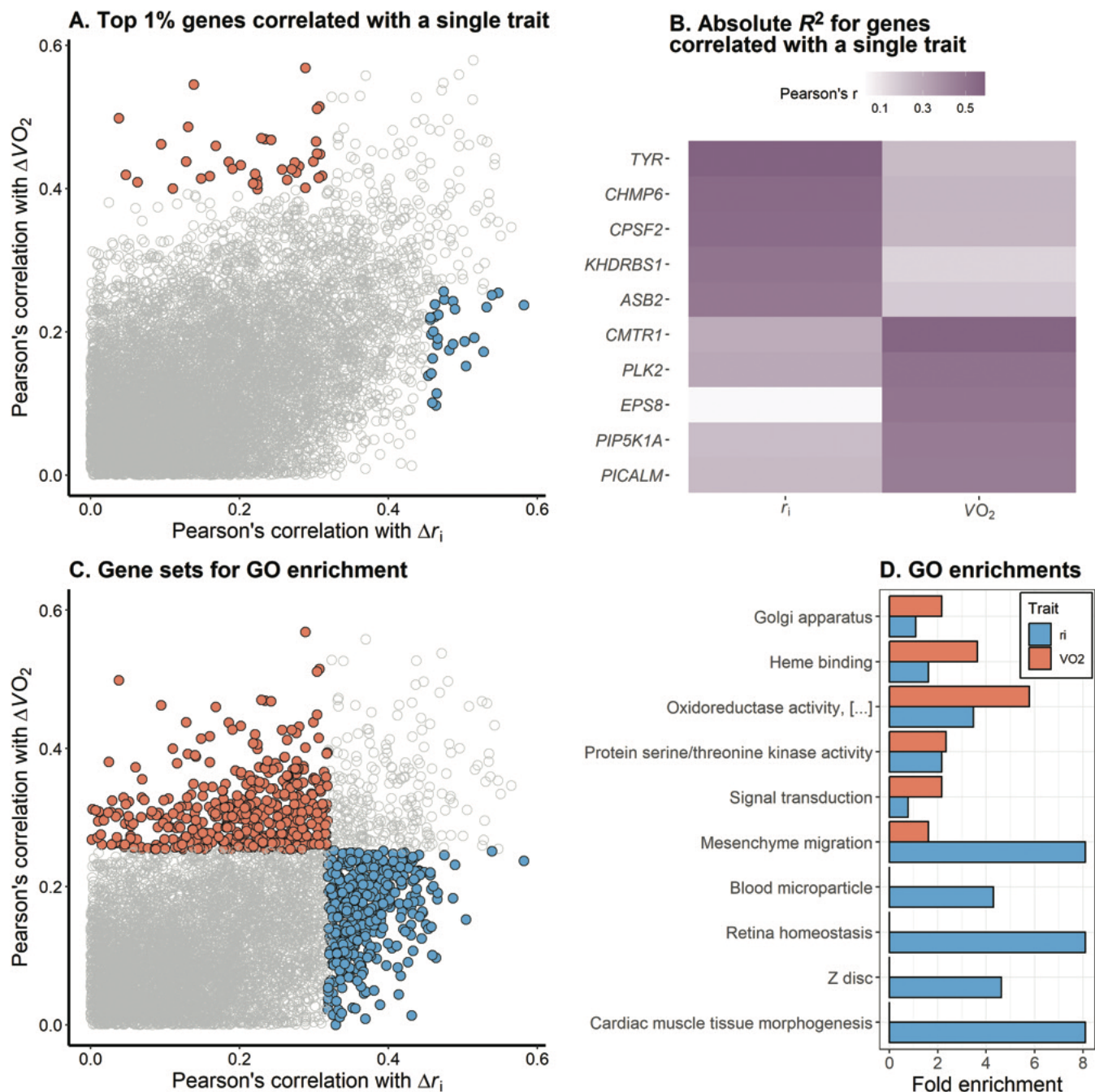
**Figure 4.** Genes and processes independently correlated with oxygen uptake and skin resistance to water loss. (A) Absolute values for Pearson's correlations with changes in skin resistance to water loss ($\Delta r_i$) and with changes in oxygen uptake ($\Delta VO_2$) throughout the laboratory acclimation experiment for 9,346 transcripts sequenced from *P. metcalfi* skin tissues. Highlighted points correspond either to genes above the 99th percentile of absolute correlations with $\Delta r_i$ and below the 90th percentile of absolute correlations with $\Delta VO_2$ ($n = 30$), or to the reciprocal set for $\Delta VO_2$ ($n = 38$). (B) Heatmap for the five genes with the highest absolute correlations for each of the two sets highlighted in (A). (C) Set of genes for functional enrichment analysis. Highlighted points correspond either to genes above the 90th percentile of absolute correlations with $\Delta r_i$ and below the 90th percentile of absolute correlations with $\Delta VO_2$ ($n = 429$) out of 6,953 characterized transcripts in the *P. metcalfi* skin transcriptome, or to the reciprocal set for $\Delta VO_2$ ($n = 429$). (D) Five most significant functional enrichments for each set of genes in panel C.

drive the trade-off but also the mechanisms that mitigate the trade-off. These findings provide new insights into the mechanisms through which organisms can regulate trade-offs and the environmental contexts in which this may occur, but our findings also point to the new trade-offs that may arise as a consequence.

Many physiological functions share biochemical pathways that reflect underlying genetic, hormonal, and physical mechanisms that generate trade-offs between traits (Garland et al., 2022). Mechanisms that tie gas exchange and water loss together are often regulated at the respiratory surface (Woods & Smith, 2010). In many amphibians, gas exchange and water loss can be regulated by changes in blood flow (i.e., perfusion) to the dermal capillary beds (Burggren & Vitalis, 2005; Riddell et al., 2019) via contraction of smooth muscle around the vasculature, leading to vasoconstriction (Clark

& Pyne-Geithman, 2005). In our study, muscle contraction was among the five most significant enriched processes for genes correlated with both changes in resistance to water loss and oxygen uptake. In addition, the module associated with phenotypic change consistent with the trade-off (PC1) was enriched for negative regulation of angiogenesis (i.e., blood vessel growth). Therefore, regulation of the vasculature (such as perfusion, vasoconstriction, and vasoregression) is likely the primary mechanism that shapes the interdependency of resistance to water loss and gas uptake. The analysis also identified potentially novel ways in which regulation of gas uptake may affect resistance to water loss, as in the association between the trade-off and the terms protein degradation and mitochondrion autophagy. The gene with the highest correlation with both traits was *PDK4*, which is involved in the downregulation of glucose metabolism and promotion of aerobic fatty acid oxidation (Pettersen et al., 2019). Thus, processes related to the promotion of aerobic respiration may result in concomitant increases in water loss if more blood is delivered to the respiratory surface in the process (for instance, due to higher cardiac output). Furthermore, these top genes were also distributed across many modules, suggesting the trade-off is structured by pathways distributed across the transcriptome. Compensation from alternative pathways may explain the persistence of the trade-off in our focal species as well as many other taxa (Riddell et al., 2024; Woods & Smith, 2010). Despite the consistency of the trade-off, we also identified periods in which the trade-off was weaker and genes that were associated with independent regulation of both physiological traits.

We observed the potential for independent regulation of the traits involved in the trade-off from gene-trait associations and co-expression modules (Hernández et al., 2022). The independent regulation of gas uptake and skin resistance, although transient, suggests that salamanders have access to phenotypic space outside of the trade-off through plastic adjustments, which may allow individuals to produce alternative ecological strategies (Bolnick et al., 2003; Layman et al., 2015; Østman et al., 2014). That is, when selective pressures on the costs of water loss are relaxed (such as due to saturated conditions), metabolic rates may become unconstrained and expressed more freely (Frédérich et al., 2014; Gianoli & Palacio-López, 2009; Pigliucci et al., 1995). Such scenarios may constitute viable substrates for selection to act on and promote alternative strategies and speciation (Levis & Pfennig, 2016). In this study, the trade-off was not detected in August during the seasonal acclimatization study, which was the most humid month of the year (Supplementary Figure S2). Under these more humid conditions, salamanders could adjust physiological traits more independently due to the lower threat of desiccation. Because we sampled unique individuals at each time point in the seasonal acclimatization study, variation in the trade-off could be due to genetically based differences among individuals rather than phenotypic plasticity. That said, we find this unlikely given the plasticity observed in the laboratory acclimation experiment. Although we did not find an effect of temperature or humidity treatment in the acclimation study, the lack of an effect may have been due to the relatively similar humidity treatments or brevity of the exposure to humidity (~ 3 hr per night). In the gene co-expression network analysis, we identified a module that was related to the independent regulation of gas uptake and skin resistance (i.e., orthogonal to the trade-off axis). This module

was enriched with processes mostly related to immune function and unspecific cellular regulatory processes, which likely indicates that these processes influence maintenance (metabolic) costs without affecting skin resistance. In contrast to the network analysis, the correlation analyses were better able to identify putative mechanisms underlying the independent regulation of gas uptake and skin resistance.

Organisms may decouple oxygen uptake from water loss by adjusting processes related to oxygen binding affinity. In our study, genes correlated to the change in oxygen uptake but not skin resistance were enriched for heme binding. Oxygen binding affinity to hemoglobin can be impaired by elevated temperatures due to the exothermic nature of the process (Gangloff & Telemeco, 2018; Pörtner, 2002; Verberk et al., 2016; Weber & Campbell, 2011). Consequently, aquatic and terrestrial organisms generally promote oxygen binding to hemeproteins under hypoxia or temperature stress in acclimation experiments (Burggren & Wood, 1981; Chung et al., 2017; Storz et al., 2010; Tufts et al., 2013). Generally, adaptive increases in oxygen binding affinity are localized to respiratory tissues via changes in temperature, pH, and $CO_2$ concentration, and balanced by decreased binding affinity in systemic tissues for oxygen delivery (Webb et al., 2022). In studies on salamanders, some species acclimate by adjusting oxygen binding affinity, whereas others do not (Bonaventura et al., 1977; Maginniss & Booth, 1995; Ultsch, 2012; Weber et al., 1985). Thus, regulating oxygen binding affinity may produce additional trade-offs, particularly if oxygen delivery to systemic tissues is compromised. Similarly, increasing hemoglobin content may promote oxygen-carrying capacity of the blood, but at the cost of increased viscosity of the blood (Wood, 1991). Nevertheless, regulation of oxygen binding affinity has the potential to independently adjust gas exchange rates without influencing water loss physiology.

Our analyses also identified putative pathways for the independent regulation of skin resistance to water loss. The expression of tyrosinase (*TYR*) had the strongest correlation with changes in resistance to water loss, while also being uncorrelated to changes in oxygen uptake. *TYR* is the enzyme responsible for oxidizing the amino acid tyrosine, which can then be converted into various forms of melanin (Sánchez-Ferrer et al., 1995). Several recent studies have indicated that melanin acts as a barrier against water loss in the integument of insects (King & Sinclair, 2015; Rajpurohit et al., 2008; Ramniwas et al., 2013; Välimäki et al., 2015), fungi (Fernandez & Koide, 2013; Jiang et al., 2016), and humans (Elias et al., 2010; Man et al., 2014). The underlying process is related to an acidification effect of melanin in the epidermis which reduces water permeability (Man et al., 2014). The salamanders in our study range from gray to nearly black-pigmented, and being nocturnal, are unlikely to benefit from melanin protection from UV radiation (Nicolaï et al., 2020). Therefore, melanin synthesis may have an important role in water conservation in lungless salamanders. Melanin production, however, likely introduces alternative trade-offs as the melanocortin system is known to have wide-ranging phenotypic effects on organisms (Ducrest et al., 2008). Its synthesis can be limited by the availability of resources such as its amino acid precursor tyrosine or metals such as Cu, Zn, and Fe (McGraw, 2008). Therefore, upregulating the expression of tyrosinase may be costly to other processes (i.e., the Y-model; van Noordwijk & de Jong, 1986). More generally, the neuroendocrine components of the melanocortin system are associated with changes in oxygen consumption, cardiovascular

tone, immune function, and a suite of behaviors (Ducrest et al., 2008). Thus, we assume the fitness consequences for decoupling oxygen uptake from water loss are likely to be substantial, and such consequences highlight why escaping the trade-off is only observed under benign humidity conditions. In the absence of these constraints, skin resistance to water loss may be regulated more independently and evolve more freely.

In summary, we identified putative mechanisms that allow organisms to mediate functional conflicts between linked traits. However, while mitigating the trade-off between water loss and oxygen uptake may appear adaptive, alternative resource or functional constraints can emerge. Few studies have explored the mechanisms and fitness consequences of mitigating trade-offs, despite the expectation that escaping trade-offs can have beneficial fitness consequences (Guillaume & Otto, 2012; Pavlíček & Cheverud, 2015). Our insights from the potential fitness costs and alternative trade-offs associated with breaking the trade-off may ultimately preclude lungless salamanders from regulating oxygen uptake and resistance to water loss independently. Because we observed the trade-off persisting through most of the laboratory acclimation experiment and active season, it suggests that escapes from the trade-off may be ephemeral, at least in lungless salamanders. However, even the brief independent regulation of gas uptake and skin resistance may provide ecological advantages if they provide more time to forage and find mates or reduce energetic costs. These results provide new hypotheses on whether the independent regulation of physiological traits allows individuals to outcompete conspecifics or congeners by mitigating the inherent costs of a functional conflict. Future experiments might focus on manipulating the expression of tyrosinase, either through tyrosinase inhibitors (e.g., kojic acid) or RNA inhibitors (RNAi), to more directly evaluate the potential for these alternative pathways to mitigate the trade-off. In addition, short-term acclimation experiments could capture novel, acute processes that may also regulate the trade-off but may have gone undetected by our sampling design (Bonzi et al., 2021). From an evolutionary perspective, the trade-off could be more directly tied to fitness using mark-recapture methods to directly tie combinations of phenotypes to growth, reproduction, and survival. If combined with the animal model, these studies could evaluate whether the trade-off is heritable and therefore a potential target of selection (Wilson et al., 2009). Such alternative strategies and limitations define the phenotypic space in which natural selection may favor novel trait combinations and allow organisms to occupy new ecological niches and undergo speciation processes.

## Supplementary material

Supplementary material is available online at *Evolution*.

## Data availability

Data are available through the OSF repository at 10.17605/OSF.IO/YND79. R scripts for all analyses are available at https://github.com/braulioassis/Plethodon-tradeoff.

## Author contributions

B.A. and E.R. conceptualized the study. E.R. collected all data. B.A. performed analyses. B.A., C.G., and E.R. wrote the manuscript.

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