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Physiological and regulatory underpinnings of geographic variation in reptilian cold tolerance across a latitudinal cline

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Abstract

Understanding the mechanisms that produce variation in thermal performance is a key component to investigating climatic effects on evolution and adaptation. However, disentangling the effects of local adaptation and phenotypic plasticity in shaping patterns of geographic variation in natural populations can prove challenging. Additionally, the physiological mechanisms that cause organismal dysfunction at extreme temperatures are still largely under debate. Using the green anole, *Anolis carolinensis*, we integrate measures of cold tolerance (CT_{min}), standard metabolic rate, heart size, blood lactate concentration and RNAseq data from liver tissue to investigate geographic variation in cold tolerance and its underlying mechanisms along a latitudinal cline. We found significant effects of thermal acclimation and latitude of origin on variation in cold tolerance. Increased cold tolerance correlates with decreased rates of oxygen consumption and blood lactate concentration (a proxy for oxygen limitation), suggesting elevated performance is associated with improved oxygen economy during cold exposure. Consistent with these results, co-expression modules associated with blood lactate concentration are enriched for functions associated with blood circulation, coagulation and clotting. Expression of these modules correlates with thermal acclimation and latitude of origin. Our findings support the oxygen and capacity-limited thermal tolerance hypothesis as a potential contributor to variation in reptilian cold tolerance. Moreover, differences in gene expression suggest regulation of the blood coagulation cascade may play an important role in reptilian cold tolerance and may be the target of natural selection in populations inhabiting colder environments.

KEYWORDS
adaptation, *Anolis*, metabolism, physiology, RNAseq, thermal tolerance

1 | INTRODUCTION

Ambient temperature has important limiting effects on biological function. The rates of biochemical reactions, structure of macromolecules responsible for catalysis and information processing, the speed and efficiency of ecologically relevant traits (locomotion, reproduction and growth), and geographic distributions of organisms all correlate with variation in the thermal environment (Hochachka & Somero, 2002). Because organisms are only able to maintain optimal performance over a limited range of temperatures, thermal fluctuations outside this optimal range can have profound consequences for individual fitness (Bradshaw & Holzapfel, 2001; Portner & Farrell, 2008; Sibley & Calow, 1986). Consequently, changes in the magnitude and variability of temperature extremes are powerful selective

forces that can have ramifications at all levels of biological organization (Brown & Brown, 1998; Rodríguez-Trelles, Tarrío, & Santos, 2013). Thus, local adaptation and acclimatization in traits that are central to thermal physiology are likely to play an important role in colonization of novel environments, adaptive divergence and resilience to environmental perturbation. Gaining a clearer understanding of the mechanistic basis of thermal limits will therefore improve our ability to predict organismal response to ongoing and predicted environmental changes.

For tropical reptiles, colonizing temperate environments poses a distinct set of physiological challenges. Many reptiles are dependent on behavioural thermoregulation to maintain optimal body temperature in the face of fluctuating thermal conditions. As a result, changes in behaviour can buffer individuals from natural selection on temperature-dependent physiological processes (Bogert, 1949). However, the overall reduction in minimum temperatures and longer nights during the extended winter seasons at higher latitudes may reduce the opportunity for behavioural thermal regulation. Without physiological changes to compensate for these environmental challenges, many ectothermic species are forced to become inactive in the face of decreasing temperatures. Thus, these environmental and biological constraints may necessitate physiological acclimatization and/or adaptation for long-term population survival in temperate environments, especially for species that remain active during the coldest parts of the year. This is supported by the observation that ectothermic species display increased thermal tolerance ranges at higher latitudes (Addo-Bediako, Chown, & Gaston, 2000; Snyder & Weathers, 2011; VanBerkum, 1988). Despite these observed latitudinal trends, we still lack a thorough understanding of the physiological constraints that set thermal limits in ectothermic species, and how adaptation can overcome these constraints to extend the limits of thermal tolerance (Schulte, 2015; Somero, 2012).

The oxygen and capacity-limited thermal tolerance (OCLTT) hypothesis offers a systems-level explanation for the functional limits of thermal tolerance in multicellular ectotherms; thermal limits are hypothesized to result from a mismatch between oxygen supply and metabolic demand at extreme temperatures (Claireaux & Lefrançois, 2007; Pörtner, 2001, 2002a, 2002b, 2010; Portner & Farrell, 2008; Pörtner & Knust, 2007). This hypothesis postulates that during cold exposure, decreased ability to uptake and transport oxygen outpaces decreasing metabolic rate, resulting in a mismatch between oxygen supply and demand (Frederich & Pörtner, 2000; Zielinski & Pörtner, 1996; Figure 1). The OCLTT hypothesis has been supported as a major limitation of thermal tolerance in marine ectotherms (Frederich & Pörtner, 2000; Portner & Farrell, 2008; Sommer, Klein, & Pörtner, 1997; Zielinski & Pörtner, 1996) and has been proposed as a unifying principle for understanding limits of thermal performance across ectothermic taxa (Pörtner, 2001). However, recent studies across a wide variety of ectotherms suggest the relative importance of oxygen transport and utilization compared to other processes (protein function, ion homeostasis, membrane permeability) may vary depending on the environments and lineages under consideration (Schulte, 2015). The evolutionary transition from marine to terrestrial environments is marked by higher oxygen availability and decreased energetic costs of oxygen uptake via ventilation, potentially decreasing the risk of experiencing oxygen limitation. Similarly, differences in respiratory physiology among ectothermic lineages may also have a dramatic impact on the importance of oxygen limitation in setting thermal limits. For example, oxygen limitation may play a larger role in determining the thermal tolerance limits of species that rely on convective oxygen transport (e.g., vertebrates and crustaceans) than in those that rely principally upon diffusion [e.g., insect tracheal systems (Klok, Sinclair, & Chown, 2004; Stevens, Jackson, Bester, Terblanche, & Chown, 2010)]. These considerations highlight the importance of further testing the OCLTT hypothesis to gain a better understanding of the environmental and physiological circumstances that govern thermal limits of ectothermic function. If systemic oxygen deprivation generally contributes to thermal limits, as expected under the OCLTT hypothesis, thermal acclimation and/or local adaptation should offset the effects of temperature-induced hypoxia (Pörtner, 2001) through increased oxygen transport capacity and/or decreased oxygen demand.

Tropical ectothermic lineages that have recently colonized temperate environments provide excellent opportunities to investigate the mechanistic underpinning(s) of enhanced cold tolerance. Green anoles (*Anolis carolinensis*) colonized the continental United States from Cuba during the Miocene–Pliocene (Campbell-staton et al., 2012) and subsequently expanded their range northward to occupy the highest latitudes of any member of the neotropical genus (~400 species; Michaud & Echternacht, 1995). Winter temperatures are hypothesized to limit the northern edge of the species' range (Williams, 1969), and cold tolerance of this species is highly correlated with winter temperature across its range (Campbell-Staton, Edwards, & Losos, 2016; Wilson & Echternacht, 1987). However,

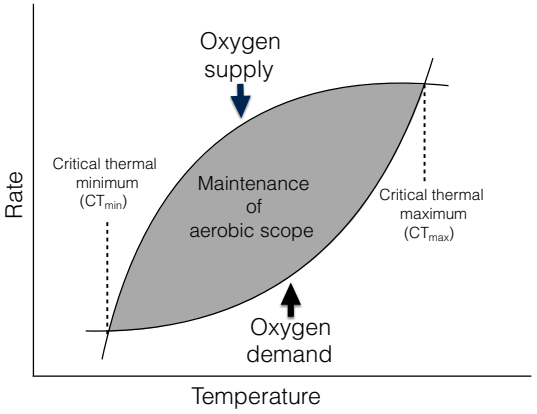

FIGURE 1 Theoretical depiction of the relationship between rates of oxygen supply and demand with respect to temperature. An assumption of the oxygen limitation hypothesis is that oxygen supply diminishes at cooler temperatures than metabolic demand, while oxygen demand exceeds supply at critically high temperatures. The intersection of these two rates results in the loss of aerobic scope, leading to systemic at the upper (critical thermal maximum, CT_{max}) and lower (critical thermal minimum, CT_{min}) extremes of temperature

the relative roles of seasonal acclimatization, genotypic specialization (constitutive genetically based differences) and evolution of plasticity in shaping geographic variation in thermal tolerance are not clear.

In this study, we investigate the roles of acclimatization, genotypic specialization and the interaction between the two in shaping geographic variation of cold tolerance in populations of green anoles distributed along a thermal cline. Additionally, we measure the effects of chronic and acute cold exposure on rates of aerobic metabolism, blood lactate concentration (a proxy for oxygen limitation induced glycolysis and key metabolic by-product of anaerobic metabolism) and indicators of blood oxygen carrying capacity (heart size and blood haemoglobin concentration). We test a key component of the OCLTT hypothesis, that oxygen limitation (as estimated by lactate concentration) functionally limits cold tolerance, under both acute and chronic cold stress in lizards sampled across the thermal cline. Finally, we utilize a functional genomics approach to identify co-expression modules within the liver associated with blood lactate concentration and to assess the effects of genotypic specialization and thermal acclimatization on regulatory responses to cold exposure.

2 | MATERIALS AND METHODS

2.1 | Study system and collection sites

We collected adult lizards from five localities along a latitudinal transect from the southern tip of Texas to southeastern Oklahoma during June–August of 2013 (Figure 2a). After capture, animals were housed in individual clear plastic containers (912 cm^3) lined with artificial turf and moss at 30°C under a 12-hr light cycle for 18–26 days to allow them to acclimate to captivity. We placed a wooden dowel and artificial foliage in each cage for perching and shelter. All individuals were fed ad libitum with ½ inch, 4-week-old *Acheta domestica* and were misted with water twice daily. After the initial acclimation period, we randomly split lizards from each population into two 14-day experimental acclimation treatments. One group was kept at 30°C (control treatment); the other was transferred to 20°C (chronic cold treatment) during this acclimation period. 20°C was chosen to identify physiological changes associated with a 10°C shift from our control treatment. 20°C is well above the minimum daily winter temperatures experienced across the focal thermal cline (−4.73°C–7.95°C, Campbell-Staton et al., 2016, 2017). A map of study locations and a schematic of experimental procedure can be found in Figure 2. All animal husbandry and experimental protocols described below were approved by the University of Illinois IACUC protocol 14049 and Harvard University IACUC protocol 26-11.

2.2 | Critical thermal minimum (CT_{min})

To assess the lower thermal limits of each lizard, we employed a common metric used to estimate thermal tolerance in ectotherms, critical thermal minimum [CT_{min}, (Campbell-Staton et al., 2016; Cowles & Bogert, 1944)]. During CT_{min} testing, internal body temperature (T_b) was monitored continuously via a 20-gauge digital thermocouple inserted approximately 5 mm inside the cloaca and secured with tape. Beginning from room temperature, we cooled the T_b of each lizard 1°C per min. We then flipped the lizard onto its back and stimulated it with forceps, allowing it 30 s to right itself. We repeated this protocol periodically until it was unable to right itself within the given window of time. The body temperature at which an animal could no longer right itself after 30 s was recorded as its CT_{min}.

To identify factors that may contribute to variation in CT_{min} across the latitudinal transect, we used a multivariate linear model to estimate the response of CT_{min} to experimental treatments. We built a model of effects on CT_{min}, using cooling rate during each trial, sex, acclimation condition, latitude of origin and an interaction effect between acclimation and latitude as predictor variables.

2.3 | Standard metabolic rate via oxygen consumption

After CT_{min} testing, lizards were returned to their acclimation temperature for 24 hr. Following this recovery period, half of the lizards from each acclimation treatment were weighed to the nearest 0.01 g, euthanized and measured for heart mass and haematological parameters as outlined below. On the remaining lizards, we measured standard metabolic rate on resting individuals during acute cold exposure using open flow respirometry. A Sable Systems (Sable Systems International, North Las Vegas, NV) open flow respirometry system was used to measure oxygen consumption during cold exposure. Each lizard was placed into an airtight chamber, which was then placed inside of a temperature cabinet. Ambient air was passed through a column of anhydrous calcium sulphate to remove atmospheric water vapour. Dried air was then passed through the airtight animal chamber at a flow rate of 208.9 ± 1.21 ml/min. Air leaving the chamber was pumped into a column of ascarite to scrub CO_2, followed by an additional column of anhydrous calcium sulphate to remove additional water from the air before being read by the oxygen analyser. We measured mass for each lizard to the nearest 0.01 g before being placed into the animal chamber. Lizards from the 30°C acclimation group were held at 30°C for 45 min, and oxygen consumption readings were taken continuously. The temperature was then decreased to 20°C and held constant for 45 min while oxygen consumption was continuously measured. This procedure was then repeated at 10°C. Lizards from the 20°C acclimation group were held at 20°C for 45 min then subsequently decreased to 15°C, then 10°C for 45 min each during oxygen consumption trials. This procedure ensured that animals from different acclimation groups were tested at the same number of temperatures, exposed to acute cold stress for the same total amount of time and experienced the same minimum temperature during testing. The first 15 min at each temperature during trials were used as an acclimation period for each animal, during which baseline oxygen measurements were taken on an empty chamber. Oxygen consumption measurements were then averaged for the final 30 min at each

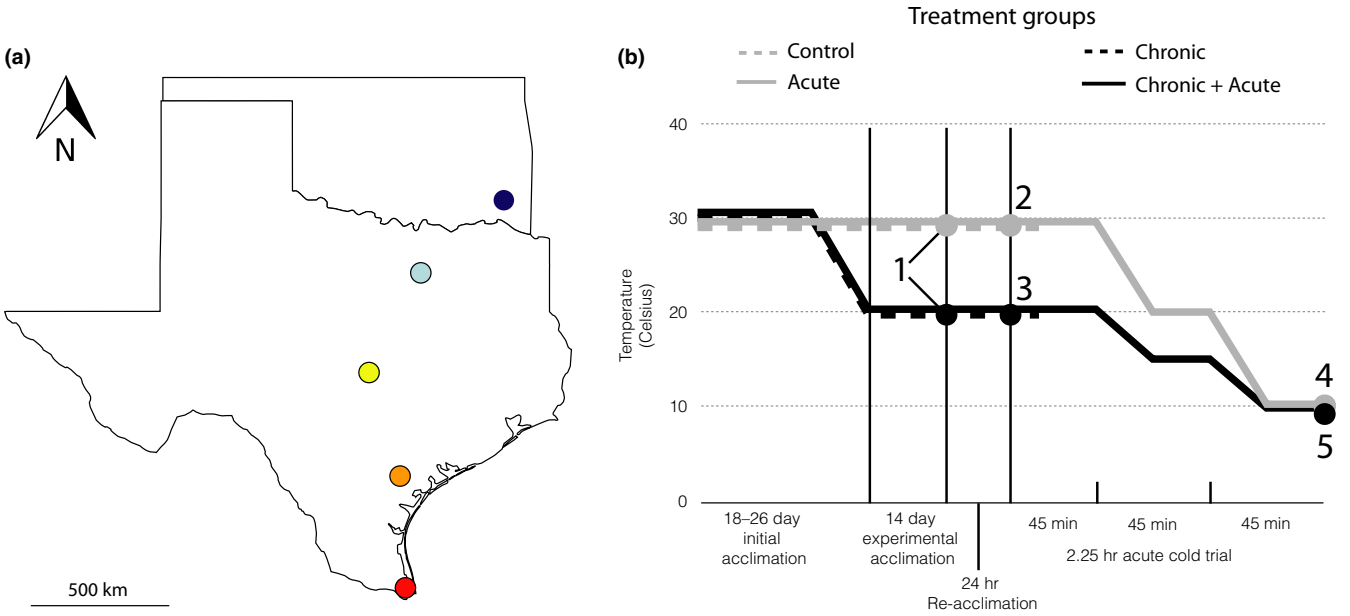

FIGURE 2 (a) Geographic sampling locations of green anoles. (b) Experimental testing procedure. Sampling Point 1: Cold tolerance (CT_{min}) experiments were performed on all lizards. Sampling Points 2 and 3: Lizards in the control and chronic cold exposure groups, respectively, euthanized and phenotyped. Sampling Points 4 and 5: Lizards in the Acute and Chronic + Acute exposure groups were euthanized and phenotyped immediately following oxygen consumption trials during acute cold exposure [Colour figure can be viewed at wileyonlinelibrary.com]

temperature for further analysis. These values were divided by body mass to calculate mass-specific rates of oxygen consumption.

A linear mixed-effects model was fit to the mass-specific O_2 consumption data, with sex, acclimation group, temperature during testing and latitude of origin designated as fixed effects. Random intercepts were modelled for each individual to control for repeated measures. Next, we built a set of subordinate models, iteratively removing each fixed effect, and compared each subordinate model to the full model using a likelihood ratio test. Due to the effects of acute but not chronic cold exposure on oxygen consumption rates (see below), we performed a post hoc analysis to estimate the effects of differences in daily temperature fluctuations at each site to test the hypothesis that variation standard metabolic rate is specifically associated with latitudinal differences in acute (daily) thermal fluctuations. We fit a linear mixed-effects models as outlined above, substituting mean diurnal range of temperature (Worldclim, Bio2) for latitude as a fixed effect (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005).

2.4 | Heart mass and haematological measurements

Lizards from all acclimation and experimental groups were anesthetized immediately upon the conclusion of experimentation using 5% vapourized isoflurane and euthanized via cervical dislocation. We measured blood haemoglobin concentration as a proxy for total blood oxygen carrying capacity using a Hemocue®Hb 201+ meter using blood samples drawn immediately following euthanasia. Additionally, we measured blood lactate concentration on samples from the same individuals using a Lactate Pro (Arkray) meter. As a by-product of anaerobic metabolism, lactate is a direct indicator of oxygen limitation in ectothermic vertebrates (Pörtner, 2001). Following these haematological measures, we also measure heart mass to the nearest 0.001 g. We then performed a linear regression of heart mass on body mass to obtain residual (mass-corrected) heart size, which served as an indicator of blood circulation capacity. A multiple linear model was run to estimate the effects of experimental conditions on each of the measurements above, using acclimation temperature (30°C vs. 20°C 2-week acclimation), experimental temperature and latitude as independent variables.

2.5 | Associations between blood lactate concentration, metabolic rate and cold tolerance

To estimate the association between rate of aerobic metabolism and cold tolerance, we used a linear regression. CT_{min} was designated as the response variable. We used oxygen consumption measurements taken at 10°C as an estimate of aerobic metabolism at the body temperature nearest critical thermal limits. Additionally, we estimated the relationship between blood lactate concentration and cold tolerance using linear regression. Blood lactate concentration following 45-min exposure to 10°C during the O_2 consumption trials was used to estimate the degree of oxygen limitation nearest critical thermal limits. These variables along with acclimation temperature were modelled as fixed effects. Cooling rate during each CT_{min} trial was added to the model as a random effect.

2.6 | Transcriptome sequencing

As a metabolically active tissue involved in a variety of physiological processes, we used liver as a focal tissue to investigate regulatory

changes in response to cold. Liver tissue samples were taken immediately following euthanasia, flash-frozen in liquid nitrogen and stored at −80°C. We sequenced the full liver transcriptomes of 26 individuals from the acclimation experiment outlined above. Eight individuals were sequenced from each end of the latitudinal transect (Brownsville, TX, and Hodgen, OK). For each of these sites, four animals were chosen from both the 30°C and 20°C acclimation control treatments. An additional ten animals collected from the mid-latitude sites and included in the 30°C control treatment were also sampled (Victoria, TX $N = 6$; Austin, TX $N = 4$). None of these animals were not included in oxygen consumption trials. The comparison of the 20–30°C acclimation treatments in lizards from Brownsville, TX, and Hodgen, OK, allowed us to examine the effects of chronic cold exposure on gene expression both within and between populations, whereas the additional sampling in Victoria, TX, and Austin, TX, at 30°C allowed us to investigate clinal variation in gene expression under controlled conditions. Total RNA was extracted from each sample using Trizol RNA isolation reagents. Transcriptome libraries were constructed using a Wafergen mRNA directional library preparation kit. Each library was sequenced as 75-bp reads using the Illumina Nextseq 500 platform. We used Trimmomatic (Bolger, Lohse, & Usadel, 2014) to trim the resultant sequences based on quality. Sequence quality was assessed for each read in a 4-bp sliding window. Sequences were trimmed once Phred33 quality scores fell below an average of 15 within the window. Adapter sequences, if detected, were removed. These quality controlled sequences were then mapped to the *Anolis carolinensis* genome (Alföldi et al., 2011) using Tophat2 (Kim et al., 2013). Raw reads counts were normalized by library size using the *cpm* function in EDGER (Robinson & Smyth, 2008). These values were quantile normalized and log-transformed for the gene co-expression network analyses described below.

2.7 | Identification of biological functions associated with blood lactate concentration

We constructed gene co-expression networks using the *blockwiseModules* function in WGCNA (Langfelder & Horvath, 2008). Pairwise Pearson correlations between each pair of genes were used to create co-expression networks. An adjacency matrix was then computed by raising the correlation matrix to a soft threshold power β. The soft thresholding approach favours strong correlations over weak ones (Zhang & Horvath, 2005) and approximates a scale-free topological network. A soft threshold β value of 10 was chosen as the value for which improvement of scale-free topology model fit (as estimated by R^2) plateaus with increasing power (Figure S1). We then computed topological overlap from the resulting adjacency matrix for each gene pair. Dissimilarity was then calculated from topological overlap and used to create cluster dendrograms based on hierarchical clustering. We identified co-expression modules as branches of the resulting cluster tree using the dynamic tree-cutting method (Langfelder & Horvath, 2008). Highly correlated modules ($R^2 = .75$) were then merged for downstream analyses.

To identify co-expression modules associated with blood lactate concentration, we summarized module expression using a principal component analysis (PCA) of gene expression profiles for each module with the *blockwiseModules* function in WGCNA. Eigengenes (a summary of overall module expression) were calculated as the first principal component (PC1) of expression variation for each module (Cheviron, Connaty, Mcclelland, & Storz, 2014; Langfelder & Horvath, 2008; Scott, Elogio, Lui, Storz, & Cheviron, 2015; Stager, Swanson, & Cheviron, 2015; Velotta, Jones, Wolf, & Cheviron, 2016). We then used module eigengene values to test for associations between module expression and blood lactate concentration by Pearson correlation with the *cor* function of WGCNA. *p*-Values for the correlation were determined by a Student's asymptotic test using the function *corPvalueStudent*. Modules showing significant correlations with blood lactate concentration are hereafter referred to as lactate modules. For each module, we identified the gene with highest intramodular connectivity, the strength of correlation with overall module expression (referred to as a hub gene; Langfelder & Horvath, 2008). Hub genes may be central to the architecture of the regulatory network(s) represented by each co-expression module.

Next, we tested for significant impacts of acclimation treatment and latitude of origin on expression of blood lactate-associated modules. Lactate modules were grouped by positive or negative correlation with blood lactate concentration. These groups were analysed separately. We built linear mixed-effects models for each group, using eigengene value as the response variable. Latitude of origin and acclimation temperature were assigned as independent variables while sample, sex and module were designated as random variables. We then built subordinate models by iteratively removing one independent variable. We then compared each subordinate model to the full model using likelihood ratio tests, allowing us to estimate the significance of each independent variable. We then used the R package GPROFILER (Reimand, Kull, Peterson, Hansen, & Vilo, 2007) to identify gene ontology categories within in each group of positively and negatively correlated modules that occur more often than expected by chance; these are referred to as "enriched" categories. The list of genes in positively associated modules were hierarchically sorted by association with blood lactate concentration. This list was compared to a background list of all genes expressed in our liver transcriptome data set to identify enriched gene ontology categories. This procedure was then repeated for genes in negatively associated modules.

2.8 | Association between blood clot degradation pathway gene expression and blood lactate concentration

Based on the gene ontology enrichment results of our co-expression network analysis, activation of the fibrinolysis enzyme plasminogen shows a strong association with blood lactate concentration. To further investigate the results of our gene ontology enrichment tests, we explored expression variation among all sequenced individuals in candidate genes identified by gene ontology analyses as being involved in the plasminogen activation/plasmin system. We tested

the hypothesis that regulation of blood clotting is directly associated with blood lactate concentration. We focused our analyses on plasminogen (ENSACAG00000003225), a major contributor to clot reduction and prevention, and six genes associated with the positive (ENSACAG00000006272—coagulation factor XII, ENSACAG00000015714—melanotransferrin, ENSACAG00000017828—hepsin) and negative (ENSACAG00000001241—SERPINE1, ENSACAG00000004328—SERPINE2, ENSACAG00000028392—thrombospondin 1 orthologue) activation of plasminogen identified from the enrichment analyses outlined above. For each gene, we performed a linear regression analysis using cpm normalized read counts to test for associations with blood lactate concentration, which was measured at 10°C. All variables were log-transformed prior to analyses.

3 | RESULTS

3.1 | Critical thermal minimum (CT_{min})

The multiple linear model significantly explained observed variation in CT_{min} (multiple $R^2 = .59$, adjusted $R^2 = .57$, $F_{(5,100)} = 28.7$, $p \ll .001$). Within the model, experimental cooling rate had a significant impact on CT_{min} ($t = 5.44$, $p \ll .001$). Independent of this effect, 2-week acclimation temperature had a significant effect on CT_{min} ($t = -1.995$, $p = .049$; Figure 3). Exposure to a 10°C decrease in temperature over 14 days lowered CT_{min} by 4.5°C ± 2.4. Latitude at site of capture also contributed significantly to the observed variation in cold tolerance among individuals ($t = -4.797$, $p \ll .001$). An increase in latitude of 1° lowered CT_{min} by 0.27°C ± 0.06. No significant interaction between latitude and acclimation was observed ($t = 1.38$, $p = .171$). There were also no significant differences in cold tolerance between males and females ($t = 0.48$, $p = .63$).

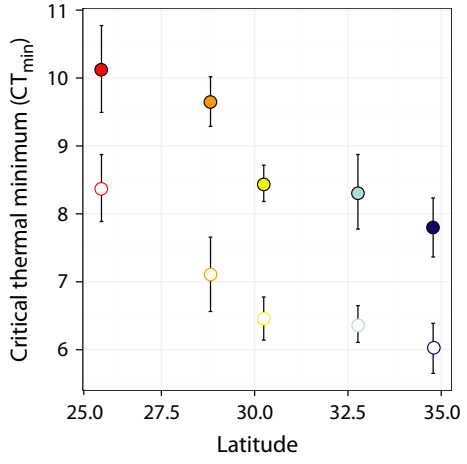

FIGURE 3 Association between cold tolerance and latitude of origin among sample sites of the green anole after 2-week acclimation at 30°C (closed circles) and 20°C (open circles). Values shown are mean ± *SE*. Multiple regression analysis reveals a significant effect of both acclimation ($p < .05$) and latitude of origin ($p < .01$) on variation in cold tolerance. Circle colours indicate sampling sites shown in Figure 2a [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Oxygen consumption rates

After the 30°C 2-week acclimation treatment, rates of oxygen consumption ranged from 0.048–1.21 ml O_2/hr/g at 10°C, 0.057–0.771 O_2/hr/g at 20°C and 0.146–0.889 ml O_2/hr/g at 30°C. Oxygen consumption rates after 2-week exposure to 20°C were generally lower: 0.014–1.113 ml at 20°C, 0.03–0.69 ml at 15°C and 0.012–0.532 ml O_2/hr/g at 10°C. The range of oxygen consumption rates reported here are generally higher than those reported from Gatten, Echternacht, and Wilson (1988), potentially due to differences in acclimation procedure and experimental design. However, the range of metabolic rates measured at 10°C after chronic cold exposure in this study encompass reported values for overwintering green anoles from other parts of the species' range (Jenssen et al., 1996).

The linear mixed model revealed a significant effect of temperature during acute cold exposure on variation in aerobic metabolism (Table 1). As temperature decreased by 10°C during acute cold challenge, rates of O_2 consumption were suppressed by ~0.19 ml O_2/hr/g ($df = 6$, $\chi^2 = 20.837$, $p = 5e^{-06}$). Neither sex ($df = 6$, $\chi^2 = 0$, $p = 1.00$) nor acclimation treatment ($df = 6$, $\chi^2 = 0.0229$, $p = .8798$) had a significant effect on variation in mass-specific oxygen consumption. These results suggest a plastic response of oxygen consumption to acute, but not chronic cold exposure during our experimental timeframe. Rates of oxygen consumption decreased with increasing latitude, but not to a significant degree ($df = 7$, $\chi^2 = 3.4022$, $p = .065$). However, linear mixed model analysis revealed a significant decrease in rates of oxygen consumption with increasing mean diurnal range of temperature ($df = 7$, $\chi^2 = 3.935$, $p = .047$).

3.3 | Heart mass and haematological measurements

Multiple linear regression ($F_{(4,94)} = 20.22$, multiple $R^2 = 0.46$, adjusted $R^2 = .44$, $p = 4.832e^{-12}$) revealed that blood lactate concentration at 10°C was significantly affected by sex (Effect = −1.78781, $t = -3.411$, $p = .001$), with females having higher lactate concentrations than males. Additionally, lactate concentration decreased under acute cold exposure (Effect = −4.214, $t = -8.001$, $p = 3.18e^{-12}$), increased with acclimation temperature (Effect = 1.053, $t = 2.014$, $p = .047$) and decreased with latitude (Effect = −0.176, $t = -2.046$, $p = .0436$; Table 2). Neither acute nor chronic cold exposure had a significant effect on blood haemoglobin concentration (chronic: Effect = 0.002, $t = 0.007$, $p = .99$, acute: Effect = −0.17, $t = -0.65$, $p = .52$) or residual heart mass (chronic: Effect = 0.0001, $t = 0.031$, $p = .975$, acute: Effect = 0.005, $t = 1.25$, $p = .26$) during our experimental timeframe. Latitude also had no effect on either phenotype ([Hb] = Effect = −0.03, $t = -0.68$, $p = .50$, residual heart mass: Effect = −0.0005, $t = -0.7$, $p = .49$). Sex did significantly affect haemoglobin concentration, with males displaying higher concentrations than females (Effect = 0.72, $t = 2.76$, $p = .007$).

TABLE 1 Results of a mixed-effects model to identify significant predictors of mass-specific oxygen consumption

	df	AIC	BIC	Loglikelihood	Deviance	χ^2 (1)	*p*
Full Model 1	7	−3.2234	13.9570	8.6117	−17.223	0.0229	.8798
Acclimation Null	6	−5.2005	9.5256	8.6002	−17.201		
Full Model 1	7	−3.2234	13.9570	8.6117	−17.223	20.837	≪.001
Temperature Null	6	15.6141	30.340	−1.8070	3.6141		
Full Model 1	7	−3.2234	13.9570	8.6117	−17.223	3.4022	.065
Latitude Null	6	−1.8212	12.905	6.9106	−13.821		
Full Model 1	7	−3.2234	13.9570	8.6117	−17.223	0	1
Sex Null	6	−5.8	9.3	8.9	−17.8		
Full Model 2	7	−3.7563	−13.424	8.8781	−17.756	3.935	.047
Mean diurnal temperature range Null	6	−1.8212	−12.905	6.9106	−13.821		

Full Model 1: oxygen consumption ~ (1|individual) + sex + acclimation + temperature + latitude
Full Model 2: oxygen consumption ~ (1|individual) + sex + acclimation + temperature + mean diurnal temperature

3.4 | Associations between rates of oxygen consumption, blood lactate concentration and cold tolerance

There was a significant effect of mass-specific rates of oxygen consumption (X *df* = 1, $p = 1.152e^{-13}$) and blood lactate concentration (X *df* = 1, *p* = .0026) on variation in cold tolerance (Figure 4). As lactate concentration decreased by 1 mM, CT_{min} fell by 0.069°C. Similarly, as oxygen consumption decreased by 1 ml/hr, CT_{min} decreased by 4.27°C.

3.5 | Co-expression modules and associated with blood lactate concentration

Weighted gene correlation network analysis identified 62 total co-expression modules across 26 *Anolis* liver tissue transcriptomes. Of these, the expression of nine modules was negatively associated with blood lactate concentration ($p \leq .05$; Figure 5b). These modules were enriched for 50 biological processes, nine cellular components and 11 molecular functions (Table S1). Several gene ontology categories within this data set are associated with oxygen transport, including heparin binding (GO:0008201, *p* = .03), negative regulation of plasminogen activation (GO:0010757, *p* = .001) and platelet activation (GO:0030168, *p* < .001). Linear mixed-effects models reveal a significant contribution of acclimation (*df* = 7, χ^2 = 14.38, *p* < .001), but not latitude (*df* = 7, χ^2 = 2.4837, *p* = .1143) on the expression of this set of modules. In addition to two unannotated genes, hub genes of these modules are involved in immune response (ZWILCH, PDZD8, PTX3), axon myelination (SH3TC2) chromatin remodelling (MORC3), proteasome function (PSMA3) and fucose degradation (FUCA1; Figure S1b).

TABLE 2 Results of a multiple linear model to identify significant predictors of blood lactate concentration

Model fit	Predictor variable	Reg. coef.	*SE*	*t*	*p*
Residual standard error (94): 2.6	Intercept	14.8137	12.670	5.548	≪.001
$F_{(4,94)}$: 20.22	Sex	−1.7878	0.5241	13.411	<.01
p-Value (*F*): ≪.001	Treatment (Acute cold)	−4.2142	0.5267	−8.001	≪.001
Multiple R^2: .4625	Acclimation (20°C)	−1.5030	0.5229	−2.014	.047
Adjusted R^2: .4396	Latitude	−0.1756	0.0859	−2.046	.044

Six co-expression modules (Module 9: 357 genes, Module 10: 243 genes, Module 12: 98 genes, Module 21: 49 genes, Module 29: 237 genes, Module 30: 97 genes) were positively associated with blood lactate concentration (Figure 5a). These modules are enriched for 30 biological processes, seven cellular components and 18 molecular functions (Table S2). Among these terms, several are associated with blood circulation and aerobic metabolism. Biological processes associated with regulation of blood coagulation (GO:0030193, *p* = .014) and positive regulation of plasminogen activation (GO:0010756, *p* = .038) are both enriched in this data set. Additionally, genes involved in oxygen binding (GO:0019825, $p \ll .001$) and haem binding (GO:0020037, $p \ll .001$) are strongly enriched. Oxidation–reduction processes (GO:0055114: oxidation reduction process, $p \ll .001$; GO:0016491: oxidoreductase activity, $p \ll .001$) are also enriched in positively associated modules as well as the mitochondrion as a cellular component (GO:0005739, *p* = .019). Linear mixed-effects models show that both acclimation (*df* = 7, χ^2 = 12.343, *p* < .001) and latitude (*df* = 7, χ^2 = 8.3443, *p* < .001) have a significant impact on the expression of these modules. Hub gene within these modules are involved in immune response (VSIG10), glycolate degradation (HAO1), morphogenesis

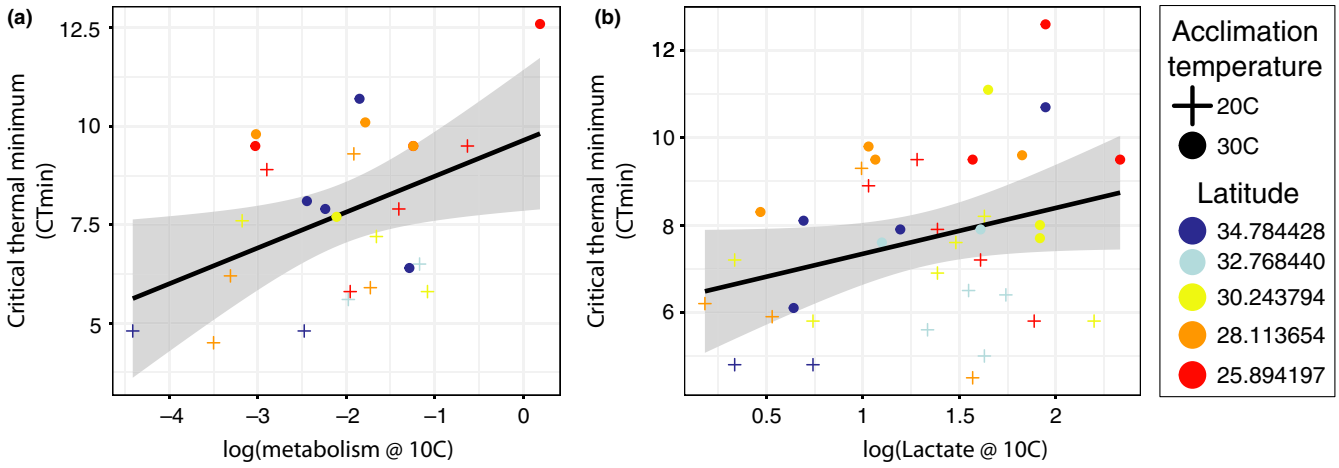

FIGURE 4 Significant associations ($p \leq .05$) of cold tolerance (critical thermal minimum, CT_{min}) with (a) Oxygen consumption at 10°C and (b) blood lactate concentration at 10°C. Individuals are coloured by latitude of origin. Lizard exposed to 30°C and 20°C 2-week acclimation treatments are identified by circles and crosses, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

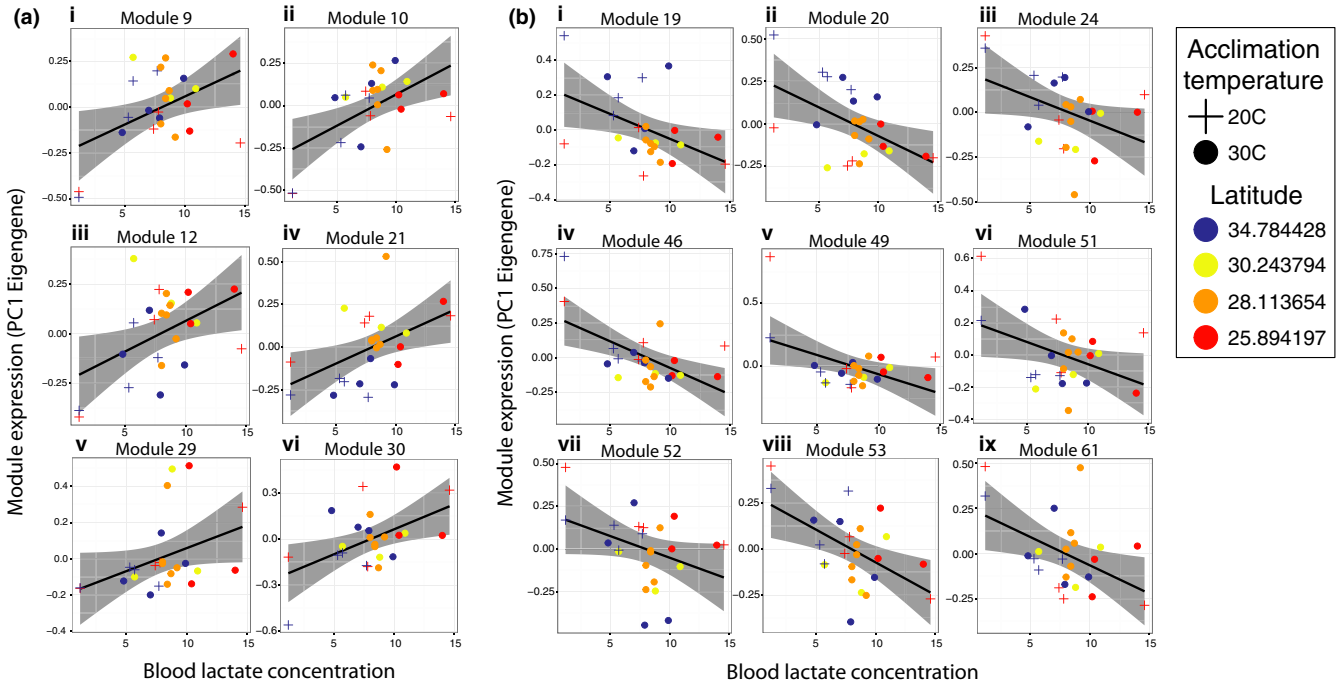

FIGURE 5 Co-expression modules identified from analysis of 26 green anole liver transcriptomes via WGCNA. (a) Six modules (Module 9: 357 genes, Module 10: 243 genes, Module 12: 98 genes, Module 21: 49 genes, Module 29: 237 genes, Module 30: 97 genes) were positively associated with blood lactate concentration. (b) Nine modules (Module 19: 155 genes, Module 20: 80 genes, Module 24: 136 genes, Module 46: 75 genes, Module 49: 242 genes, Module 51: 112 genes, Module 52: 288 genes, Module 53: 550 genes, Module 61: 408 genes) were negatively associated with blood lactate concentration). All associations shown are statistically significant ($p \leq .05$, Student's asymptotic test) [Colour figure can be viewed at wileyonlinelibrary.com]

(RSPO2, ALK, ZIC4) as well as steroid and sex hormone metabolism (HSD17B10; Figure S1a).

3.6 | Associations between blood clot degradation pathway genes and blood lactate concentration

Linear regression analyses reveal significant associations between expression of genes involved in the plasminogen activator/plasmin system and blood lactate concentration (Figure 6). Expression of plasminogen was positively associated with blood lactate concentration ($R^2 = .233$, $p = .017$). Among the three genes associated with negative regulation of plasminogen activation, SERPINE1 was significantly and negatively associated with lactate concentration ($R^2 = .32$, $p = .004$). SERPINE2 and thrombospondin 1 did not exhibit significant correlations with blood lactate concentration (SERPINE2: $R^2 = .104$, $p = .124$; thrombospondin 1: $R^2 = .057$, $p = .263$). All

three genes associated with positive regulation of plasminogen activity display significantly positive correlations with blood lactate concentration (coagulation factor XII: $R^2 = .464$, $p < .001$; melanotransferrin: $R^2 = .367$, $p = .002$; hepsin: $R^2 = .280$, $p = .008$).

4 | DISCUSSION

4.1 | Adaptive plasticity and genotypic specialization shape geographic variation in cold tolerance

Phenotypic and genotypic shifts along environmental gradients can be indicative of local adaptation to recent or contemporaneous spatially varying selection pressures (Conover, Duffy, & Hice, 2009; Endler, 1977; Kawecki & Ebert, 2004; Savolainen, Lascoux, & Merilä, 2013). Studying such shifts can provide insights into species colonization and establishment in novel environments by identifying targets of natural selection. The results of this study indicate that both thermal acclimatization and genotypic specialization contributed to the successful migration of green anoles into temperate environments in mainland North America.

Tropical ectotherms are predicted to exhibit reduced thermal acclimation capacities (Janzen, 1967), and cold tolerance shows limited acclimation ability in tropical anole lineages (Kolbe, Ehrenberger, Moniz, & Angilletta, 2014; Muñoz et al., 2014). In contrast, our results suggest temperate populations of the green anole display significant adaptive plasticity, increasing cold tolerance after a period of acclimation to cooler temperatures. There was no significant interaction between latitude and acclimation, suggesting that acclimatization responses do not differ among populations. The correlation between latitude of origin and cold tolerance observed in this study is congruent with other studies of wild populations of green anoles (Campbell-Staton et al., 2016; Wilson & Echternacht, 1987). Given that cold tolerance is heritable in green anoles (Campbell-Staton et al., 2016), the correlation between cold tolerance and environmental variables along a latitudinal cline supports a hypothesis of local adaptation via genotypic specialization in the green anole (Campbell-Staton et al., 2016; Wilson & Echternacht, 1987). However, experimental validation is needed to explicitly test whether lower CT_{min} provides fitness benefits in colder environments. Additionally, we were unable to control for developmental plasticity in this experiment. Further study is needed to investigate the effects of embryonic and hatchling environment on adult cold tolerance in this species.

4.2 | Population differences in cold tolerance are associated with metabolic depression and reduced oxygen demand

Identifying the physiological mechanisms that contribute to geographic variation in thermal performance is key to understanding how environmental variation influences population differentiation. The OCLTT hypothesis provides a potential mechanistic explanation for variation in thermal tolerance among ectotherms, but its general applicability is still under debate. Studies that relate variation in rates of oxygen consumption to cold tolerance in ectotherms may shed valuable light in this regard, providing clear hypotheses about energy utilization strategies and their physiological consequences.

Our results show that green anoles from localities with greater thermal variability have constitutively lower metabolic rates than their conspecifics living in more thermally stable environments. This pattern of geographic variation in metabolic rate mirrors population differences in CT_{min}; individuals from colder, more thermally variable environments tended to have lower rates of oxygen consumption and greater cold tolerances. Together, these results support the OCLTT hypothesis as a viable explanation for this pattern of latitudinal variation in cold tolerance. Decreased metabolic rates may have at least two primary benefits in variable environments. First, reduced

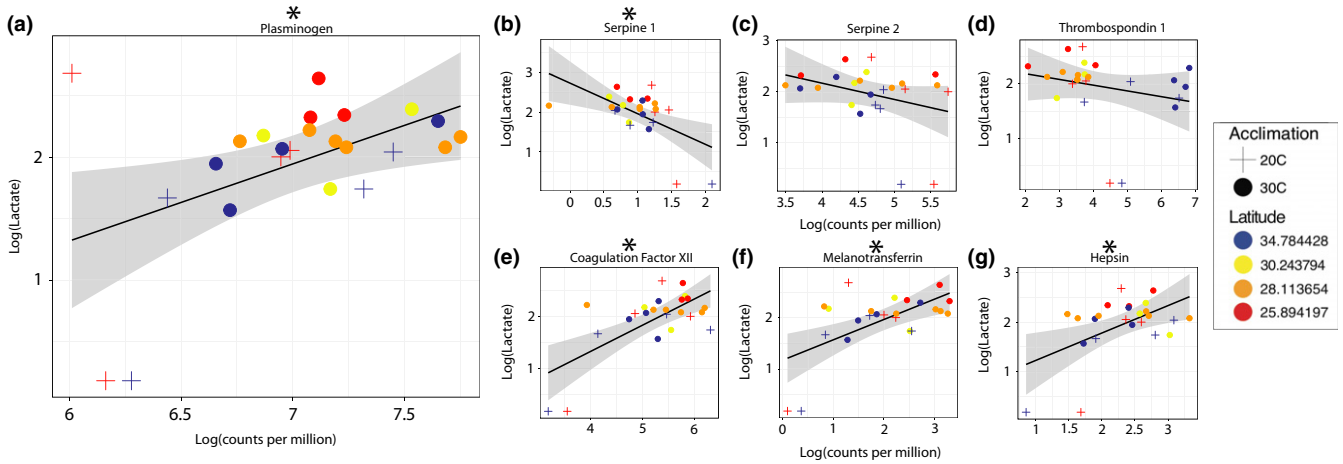

FIGURE 6 Associations between gene expression of plasminogen (a) and several of its regulators with blood lactate concentration. (b–d) indicate negative regulators of plasminogen while (e–g) indicate positive regulators of plasminogen. Asterisks indicate statistically significant associations (linear regression, $p \leq .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

rates of oxygen consumption may promote greater oxygen economy, guarding against cold-induced inhibition of cardiorespiratory capacity, and second, reduced metabolic rates may promote energy conservation during periods of reduced resource acquisition (Fitzpatrick, Bristol, & Stokes, 1971; Hazel & Prosser, 1974; Precht, 1958; Roberts, 1966; Tsuji, 1988). Northern green anole populations exhibit reduced movement during the winter months (<1 m from overwintering sites) that is limited to times of direct sun exposure (Bishop & Echternacht, 2004). These thermal constraints on movement are likely to impose severe limitations on resource acquisition during the winter months in locations with high thermal variability. While our results show only a marginal relationship between latitude and metabolic rate across the five populations studied here, we did find a significant association between metabolic rate and diurnal temperature range across the thermal cline, and mean diurnal range is strongly correlated with latitude across the distribution of green anoles (Campbell-Staton et al., 2016). Interestingly, plastic increases in cold tolerance in response to our 2-week temperature acclimation were not associated with a suppression of metabolic rate in cold-acclimated animals. These results suggest that the physiological mechanisms that underlie cold acclimation differ from those that underlie constitutive population differences in cold tolerance.

If limits to oxygen uptake and utilization set the boundaries of thermal tolerance in reptiles, then physiological and metabolic adjustments to thermal extremes should relieve oxygen limitation. Blood lactate concentration is an indicator of oxygen limitation in ectothermic vertebrates (Pörtner, 2001), and the significant negative association between blood lactate concentration and cold tolerance in green anoles suggests that cold-tolerant animals are better able to alleviate oxygen limitation in the cold. Oxygen limitation results in diminished aerobic scope (the difference between minimum and maximum oxygen consumption rate) due to a mismatch between oxygen supply and demand. Maintenance of aerobic scope can therefore be accomplished through increasing oxygen supply or reducing oxygen demand. In either case, physiological adjustments allow oxygen supply to meet oxygen demand, preserving aerobic scope. The lack of population differences in relative heart mass and haemoglobin concentration, as well as a lack of acclimation responses in both traits, suggests these aspects of cardiac output and blood physiology do not contribute to population-level differences in CT_{min} or plastic increases in cold tolerance following cold acclimation, at least over our experimental time periods. If oxygen limitation constrains cold tolerance in green anoles, the apparent lack of improvements of circulatory O_2 delivery may necessitate suppressed rates of oxygen consumption to reduce oxygen demand and maintain function at cold temperatures.

Physiological modifications that increase oxygen transport capacity can be energetically expensive and may provide little benefit to species with limited activity and food availability during the winter months, whereas a decreased metabolic rate offers a means of maintaining aerobic scope while foregoing such costly physiological investments during periods of resource limitation (Tsuji, 1988). Alternatively, physiological constraint on upstream portions of the oxygen transport cascade may limit the ability of this species to upregulate metabolic rate in response to cold exposure, necessitating a strategy of oxygen conservation. Taken together, these data suggest that suppressed rates of oxygen consumption allow for the maintenance of function at cold temperatures via decreased oxygen demand. This further supports our hypothesis that local adaptation via genotypic specialization plays an important role in clinal variation of thermal physiology in this species (Campbell-Staton et al., 2016). The physiological mechanisms that result in plastic increases in cold tolerance following cold acclimation are unclear, and warrant further study. However, our transcriptomic results point to several candidate mechanisms.

4.3 | Transcriptional regulation of blood physiology may contribute to alleviation of oxygen limitation in the cold

Ectothermic species are faced with a suite of physiological challenges associated with oxygen transport and utilization under cold stress. Cold temperatures slow ventilation rate, diffusion times, blood flow and biochemical reaction rates that are necessary to fuel metabolic processes. Thus, physiological remodelling in response to cold exposure and local adaptation to different environments may be associated with regulatory changes that ameliorate these challenges. Our transcriptomic analyses revealed patterns of differential gene expression that suggest physiological stress and dysfunction associated with blood physiology and metabolism may be related to oxygen limitation under cold conditions and play a role in local adaptation to thermally variable environments.

Ectothermic species facing cold stress can suffer from decreased circulatory performance (Pörtner, 2001) and increased blood viscosity (Snyder, 1971). Additionally, cold temperatures can result in aberrant precipitation of blood plasma proteins such as fibrin (cryofibrinogenemia), leading to increased rates of clot formation and resulting in tissue ischaemia (Amdo & Welker, 2004). In association with these temperature-induced complications, several winter hibernating species (both endothermic and ectothermic) display modifications to coagulation cascade which decrease risk of clotting and facilitate microcirculation in face of diminished circulatory performance (Cullinan, 2015; Jacques, 1963; Srere, Belke, Wang, & Martin, 1995).

As a major blood anticoagulant, GO enrichment of heparin binding among modules negatively associated with lactate concentration suggests that regulation of blood coagulation and clotting pathways may reduce cellular oxygen limitation by maintaining some degree of oxygen delivery during cold exposure. This hypothesis is further supported by the GO enrichment of regulatory networks responsible for activation of plasminogen, which plays a role in anticoagulation (Hoover-Plow, 2010) and dissolving blood clots (fibrinolysis) as well as activation of platelets, which provide surfaces for coagulation to occur (Markiewski, Nilsson, Ekdahl, Mollnes, & Lambris, 2007). Negative regulation of platelet activation may act to diminish the magnitude of clot formation. Regulatory patterns of these three

components may be symptomatic of circulatory stress due to the severity of cold-induced clot formation and blood hyperviscosity at low temperatures, leading to oxygen limitation via tissue ischaemia. The significant effects of acclimation temperature on co-expression modules enriched for heparin and platelet regulation suggest these modules are involved in acclimatization processes that may relieve oxygen limitation. Similarly, expression of modules related to plasminogen activity may be involved in both acclimatization and local adaptation across the latitudinal distribution of the green anole. Further analyses of the plasminogen activation/plasmin system show a direct correlation between the expression of genes in this pathway and lactate accumulation. The positive correlation of plasminogen and its positive regulators with blood lactate concentration suggest that individuals experiencing greater oxygen limitation may also be under greater stress to manage aberrant cold-induced blood clot formation (cryofibrinogenemia). Thus, cryofibrinogenemia, a cold-induced pathophysiological condition that has been described in humans (Amdo & Welker, 2004), may also be an important physiological stress that contributes to oxygen limitation in terrestrial ectotherms during cold exposure. Regulatory variation of the genes associated with mounting a response to this stress may be the target of natural selection in cold environments. However, further experimentation is needed to corroborate direct cause and effect of this relationship, as it is possible that the correlated response is the result of indirect influence of other biotic and abiotic factors. Even so, under the OCLTT hypothesis, thermal limits are predicted to be set by the mismatch between oxygen supply and demand. Alteration of the plasminogen system may provide a means of decreasing the sensitivity of oxygen supply to low temperatures, allowing for the continued satisfaction of oxygen demand and delaying the onset of whole-organism dysfunction.

5 | CONCLUSION

Understanding the mechanisms that allow species to adapt to variation in their environment is a central goal of evolutionary biology. However, disentangling the effects of phenotypic plasticity and genotypic specialization in shaping variation in thermal tolerance can be difficult. The results of this study shed light on the environmental and physiological factors that shape geographic variation in thermal tolerance in reptiles at several levels of biological organization. Significant associations of cold tolerance with aerobic metabolism and lactate concentration support the hypothesis that oxygen limitation plays an important role in setting the limits of reptilian cold tolerance. Furthermore, regulatory modifications of the blood coagulation cascade may play an important role in physiological acclimatization and local adaptation to thermally variable environments at temperate latitudes. These findings provide fundamental insights into the mechanistic basis of thermal tolerance in terrestrial vertebrate ectotherms. While our results support a mechanistic role of oxygen limitation in defining the limits of cold tolerance of the green anole, these findings contrast with recent studies investigating the role oxygen limitation in reptiles and amphibians exposed to high temperatures (Fobian, Overgaard, & Wang, 2014; Overgaard et al., 2012), which have failed to find significant support for the OCLTT. Clearly, further study is needed to fully understand the physiological mechanisms involved in loss of function at extreme temperatures and how those mechanisms may differ at the upper and lower boundaries of thermal tolerance. Such a mechanistic understanding will be vital for predictions of how reptilian species will acclimate and adapt to ongoing shifts in the thermal environment due to anthropogenic climate change.

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DATA ACCESSIBILITY

Sequence data are available from the National Center for Biotechnology Information: SAMN06042490 to SAMN06042537. Cold tolerance data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.g500m. Samples are accessioned in the Harvard Museum of Comparative Zoology (MCZ Cryogenic 4448-4586).

COMPETING INTERESTS

The authors of this manuscript have declared no competing interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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