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Higher metabolic plasticity in temperate compared to tropical lizards suggests increased resilience to climate change

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Abstract:

Patterns in functional diversity of organisms at large spatial scales can provide insight into possible responses to future climate change, but it remains a challenge to link large-scale patterns at the population or species level to their underlying physiological mechanisms at the individual level. The climate variability hypothesis predicts that temperate ectotherms will be less vulnerable to climate warming than tropical ectotherms, due to their superior acclimatization capacity. However, metabolic acclimatization occurs over multiple levels, from the enzyme and cellular level, through organ systems, to whole-organism metabolic rate (hereafter biological hierarchy). Previous studies have focused on one or a few levels of the biological hierarchy, leaving us without a general understanding of how metabolic acclimatization might differ between tropical and temperate species. Here, we investigate thermal acclimation of three species of *Takydromus* lizards distributed along a broad latitudinal gradient in China, by studying metabolic modifications at the level of the whole organism, organ, mitochondria, metabolome, and proteome. As predicted by the climate variability hypothesis, the two temperate species *T. septentrionalis* and *T. wolteri* had an enhanced acclimation response at the whole organism level compared to the tropical species *T. sexlineatus*, as measured by respiratory gas exchange rates. However, the mechanisms by which whole organism performance was modified was strikingly different in the two temperate species: widespread *T. septentrionalis* modified organ sizes, while the narrowly distributed *T. wolteri* relied on mitochondrial, proteomic and metabolomic regulation. We suggest that these two mechanisms of thermal acclimatization may represent general strategies used by ectotherms, with distinct ecological costs and benefits. Lacking either of these mechanisms of thermal acclimatization capacity, the tropical species is likely to have

increased vulnerability to climate change.

Keywords: climate warming, latitudinal pattern, mechanistic regulation, metabolic rates, *Takydromus* lizards, thermal acclimation

INTRODUCTION

Climate models predict that thermal variability will increase in the future (IPCC 2021), impacting species differentially depending on their sensitivity (or resilience) to environmental change (Williams et al. 2008, Huey et al. 2012). Thermal variability affects demography by impacting rates of mortality and by compromising net energy gain, leading to loss of performance and reproductive failure, and ultimately a decrease in fitness (Huey and Kingsolver 2019, Buckley et al. 2021). Resilience to environmental change is thus conferred in part by physiological plasticity in energy metabolism, with high levels of physiological plasticity associated with lower negative impacts of global change (Dillon et al. 2010, Magozzi and Calosi 2015, Rohr et al. 2018, Norin and Metcalfe 2019). Physiological plasticity is shaped by selection imposed by historical thermal regimes, and it is thus important to understand how physiological plasticity varies biogeographically, in order to predict potential vulnerabilities to global change (Tewksbury et al. 2008, Huey et al. 2012, Logan and Cox 2020).

The climate variability hypothesis (CVH) posits that tropical organisms should possess lower physiological plasticity, due to the reduced thermal variability under which they have evolved (Janzen 1967, Ghalambor et al. 2006, Gaston et al. 2009). Consistent with this theory, temperate compared to tropical species tolerate or thrive over a broader range of temperatures, as well as having a larger thermal safety margin, suggesting they may be more resilient to climate change (van Berkum 1988, Addo-Bediako et al. 2000, Deutsch et al. 2008, Dillon et al. 2010, Sunday et al. 2019). However, comprehensively testing the CVH requires that we explicitly consider interacting timescales of phenotypic plasticity. On the timescale of minutes to hours, rates of physiological processes change due to thermodynamic effects on molecular

reaction rates; also known as passive plasticity to acknowledge that it occurs without central regulation by the organism (Schulte 2015, Havird et al. 2020). Over days to weeks, individual ectotherms remodel their acute responses to temperature through the reversible process of seasonal acclimatization, leading to changes in the acute response to temperature (i.e., shifts in the thermal performance curve), which is also known as 'active plasticity' (Havird et al. 2020). It is unclear whether active plasticity is greater in temperate compared to tropical animals, with a number of recently published meta-analyses giving conflicting conclusions alternately refuting the hypothesis (Seebacher et al. 2014), partially supporting it (in small but not large animals; Rohr et al. 2018), and some calling out methodological issues that prevent a critical test (Einum et al. 2019, Havird et al. 2020). Because seasonal acclimatization is one of the primary adaptations to seasonality, answering the question of whether temperate animals possess greater active plasticity than tropical animals is an important research priority.

Thermal plasticity (both active and passive) can result from changes across levels of the biological hierarchy, from molecular level (e.g., gene or protein expression, metabolites, or enzyme activity) to whole organism morphology and physiology (body and organ sizes, rates of energy flux)(Logan and Cox 2020). Traits measured at lower levels of the biological hierarchy provide a higher-resolution assessment of the physiological state than an emergent property, such as metabolic rate, and yield data on how organisms may be partitioning energy differently in response to environmental change (Gaston et al. 2009). Traits at different levels of the biological hierarchy (e.g., organ, cellular, subcellular, and biomolecular levels) show different degrees of active plasticity (Seebacher et al. 2014). To answer how and why physiological plasticity varies biogeographically thus requires integration across the levels of biological

hierarchy. These sorts of mechanistic investigations are necessary not only to identify specific phenotypic targets of selection, but also to answer how evolutionary trajectories may differ in the face of either natural or anthropogenic environmental variation (Gaston et al. 2009, Bozinovic et al. 2011, Somero 2012, Logan et al. 2018). Seasonal acclimatization of metabolic rate is a multistep integration of regulation in biological processes (Somero 2012, Schulte 2015, Norin and Metcalfe 2019), which should involve modifications at organ-level, cellular/subcellular-level, and biomolecular level (Somero 2012, White et al. 2012, White and Kearney 2013, Schulte 2015). In particular, metabolic rate can be modified by modulating organ sizes, particularly of liver, heart, and brain (e.g., Konarzewski and Diamond 1995, Sparti et al. 1997, Vezina et al. 2006), mitochondrial properties, and activities of primary enzymes inner mitochondria (Dhillon and Schulte 2011, White et al. 2012, Sun et al. 2015). Global quantitation of protein and metabolite levels can give a biochemical fingerprint of the activity of metabolic pathways, facilitating identification of processes that are altered during thermal acclimation (Morowitz et al. 2000, Goodacre et al. 2004, Lin et al. 2006). However, while the metabolic rate is clearly regulated at all of the levels above, there is little evidence of how they collectively contribute to determining whole organism thermal acclimatization capacity (Norin and Metcalfe 2019). Therefore, to fully elucidate the mechanisms underlying latitudinal patterns in metabolic acclimation, we must integrate across the organ, subcellular, and biomolecular levels (Hochachka and Somero 2002, Seebacher et al. 2003, Somero 2004, Angilletta 2009).

As ectothermic vertebrates, lizards are strongly influenced by thermal environments (Huey 1982), and have been increasingly documented to be vulnerable to ongoing climate warming (e.g., Whitfield et al. 2007, Tewksbury et al. 2008,

Sinervo et al. 2010, Logan et al. 2013, Diele-Viegas and Rocha 2018, Sun et al. 2018a, Diele-Viegas et al. 2020, Rosso et al. 2020, Obregon et al. 2021). Widespread lizard congeners provide ideal systems to study latitudinal patterns of thermal adaptation (Huey et al. 2009, Huey et al. 2012, Aguilar-Kirigin and Naya 2013, Campbell-Staton et al. 2017). *Takydromus* represents a genus of small size Lacertid lizards (snout-vent length, SVL < 70 mm), mainly distributed in China and islands in Southeast Asia, among which *Takydromus sexlineatus* (mean SVL = 52.31 mm) is primarily distributed in tropical areas, *Takydromus wolteri* (mean SVL = 50.50 mm) in temperate areas, and *Takydromus septentrionalis* (mean SVL = 62.23 mm), is a widespread temperate species whose distribution spans subtropical and temperate areas (Zhao et al. 1999, Ota et al. 2002) (Fig. 1a). Thermal physiology has diverged among these species consistent with their latitudes and thermal histories: the optimal temperatures for sprint speed are around 34, 32, and 29□ for *T. sexlineatus*, *T. septentrionalis* and *T. wolteri*, respectively (Zhang and Ji 2004), and thermal sensitivity of metabolic rates differs among *Takydromus* lizards (Sun 2014). Therefore, *Takydromus* lizards provide an ideal system for studying the patterns and mechanisms underlying differences in active acclimation of metabolism in tropical compared to temperate species.

To reveal metabolic modifications to temperatures across latitudes at multiple levels of the biological hierarchy, we used *T. sexlineatus, T. septentrionalis* and *T. wolteri* from different climate areas, and acclimated each species under cold and warm treatments. We then measured aerobic metabolism-related responses to acclimation of each species at multiple levels from the whole organism to molecular, including organ sizes, mitochondrial respiration, COX activity, and proteomics and metabolomics. Based on the climate variability hypothesis (Janzen 1967, Ghalambor

et al. 2006, Gaston et al. 2009), we first predicted that both temperate species (*T. septentrionalis* and *T. wolteri*) would have greater active physiological plasticities in response to thermal acclimation compared to the tropical species (*T. sexlineatus*), at the levels of whole organism (metabolic rate), organ (liver, brain and heart size), organelle (mitochondrial function), and molecule (metabolome and proteome). Because physiological capacities can be related to the breadth of the species thermal niche (Bozinovic et al. 2011), we further hypothesized that narrowly distributed *T. wolteri* and widespread *T. septentrionalis* would depend on different mechanisms in regulating metabolic plasticity (Somero 2012, Norin and Metcalfe 2019).

MATERIALS AND METHODS

Lizard collection

From late April to early May of 2014, we captured 22 adult *T. sexlineatus* from Zhaoqing (22°53' N, 112°36' E) in Guangdong province, 45 *T. septentrionalis* from Wenzhou (28°36' N, 119°37' E) in Zhejiang provinces and 28 *T. wolteri* from Chuzhou (32°17' N, 118°17' E) in Anhui province. Their habitat covers a latitudinal range of ~1175 km, from the high-latitudinal species (*T. wolteri*) to the low-latitudinal species (*T. sexlineatus*). The natural habitats for these three species are similar, with mixtures of shrub and grasslands (Zhao et al. 1999, Sun et al. 2021a). The yearly average ambient temperatures of the sample sites decreased, and the yearly thermal fluctuation increased as latitude increased (Fig. 1a). We captured adult lizards by noose or hands, and transferred lizards to the laboratory in Beijing.

Thermal acclimation

After being weighed (± 0.001g) and measured (± 0.1mm), lizards from each species were randomly assigned to warm or cold acclimation treatment. The snout-vent length (SVL) and body mass between treatments were equal for *T. sexlineatus* (SVL: 5.2 ± 0.1 *vs.* 5.2 ± 0.1 mm; BM: 2.261 ± 0.133 *vs.* 2.215 ± 0.124g), *T. septentrionalis* (SVL: 6.0 ± 0.1 *vs.* 6.1 ± 0.1 mm; BM: 5.107 ± 0.224 *vs.* 5.028 ± 0.256g) and *T. wolteri* (SVL: 4.7 ± 0.1 *vs.* 4.8 ± 0.1 mm; BM: 2.278 ± 0.147 *vs.* 2.304 ± 0.212g), respectively. The thermal acclimation lasted more than 40 days (6th May to 16th June). After acclimation, we re-weighed and re-measured the lizards to calculate the growth rate in SVL and BM. The growth rate of SVL and BM were calculated as the daily change of SVL (mm/day) and BM (g/day), respectively.

Lizards in the warm treatment were kept in terraria in a 26°C room with 10h supplementary heating by 50W UVA+ UVB lamp (Qupa, Shanghai, China) per day (0800 to 1800), which mimicked the mean daily minimum temperature in Zhaoqing, the tropical locale (about 26 °C in May). Lizards in the cold treatment were kept in terraria at 16 °C with 6h supplementary heating per day (1000 to 1600), which mimicked the mean daily minimum temperature in Chuzhou, the northernmost temperate site (about 16 °C in May). Minimum temperatures in May were used as a baseline because they are near to the minimum temperatures the lizards would experience during the active season. Thermal environments in terraria were recorded hourly through the acclimation period (Fig. 1b). Food (crickets and larvae of *Tenebrio molitor* dusted with vitamins and minerals) and water were provided *ad libitum* for all lizards. During the acclimation stage (6th May to 16th June), the cloacal temperatures of a subset of 10 lizards from each species under each treatment were measured hourly from 0700 to 1900 on the 15th and 30th day (Fig. 1b).

Resting Metabolic Rate

After acclimation, the resting metabolic rate (RMR) of lizards were determined by the respiratory gas exchange at five temperatures (18°C, 23°C, 28°C, 33°C, and 38°C) in a repeated measures design (all individuals were measured at all temperatures in a random sequence), the measurements were conducted with males and un-gravid females every other day. Sample sizes were 11, 16, and 12 for the warm treatment and 11, 16, and 16 for the cold treatment of *T. sexlineatus*, *T. septentrionalis*, and *T.wolteri*, respectively. RMRs were measured with Sable system respirometry (Sable Systems, Henderson, NV), using a closed-circuit respirometry (Sun et al. 2018b). In brief, each lizard was acclimated within the chamber (Length × Diameter = 173 × 51mm) of the circuit in an incubator (Sanyo, MIR554-PC, Japan) at test temperatures for 2 hours. During the acclimation, the circuit system (volume = 286 mL) was open first, and the chamber was flushed with dry, CO_2-free air (generated by through Drierite-Ascarite-Drierite column) at a flow rate of 300 mL/min. After a temperature acclimation period of 2 hours, the circuit system was sealed, and the rate of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) in the closed□circuit system was continuously recorded secondly. The results from preliminary experiments indicated the record length within 30 mins does not affect the metabolic rates ($p = 0.724$), so the record length was set as 10 to 15 mins. All lizards were resting without any activity under the dark condition in the chamber during measurements. To eliminate the effects of circadian rhythms on metabolic rates, all the trials were conducted between 10:00 to 18:00. To minimize the effects of food digestion on metabolic rates, we removed the food and enabled the lizards were fasted for at least 12 hours prior to the trials started (Ma et al. 2018, Sun et al. 2018b). Oxygen consumption and carbon dioxide production were used for metabolic rate

calculation. The RMR was expressed as mL/g/h. We also calculated the respiratory exchange ratio (RER) of each lizard as the rate of carbon dioxide production divided by oxygen consumption. We calculated the thermal sensitivity (Q_{10}) of metabolic rate as follows:$Q_{10} = (\frac{R2}{R1})^{10/(T2-T1)}$. Where *R1* and *R2* are metabolic rates at *T1* (18°C) and *T2* (38°C), respectively. After measurement, lizards were released back to the terraria until the next measurement.

Organ size determination

After RMR measurements were completed at all temperatures, six lizards from each treatment of each species were sacrificed and dissected for organ size determination. The fresh livers, hearts, and brains were removed, cleaned by saline and weighed (±0.001g) immediately. Then the mitochondria of fresh livers were extracted with published methods for measurement of mitochondrial respiration and cytochrome *C* oxidase activities (Sun et al. 2015).

Mitochondrial respiration and cytochrome C oxidase (COX) activity

During isolation of mitochondria, one-third of each liver was homogenized immediately in ice-cold extraction buffer (250 mM sucrose, 5 mM Tris/HCl and 2 mM EGTA, pH7.4 at 4°C), the other parts were set aside for iTRAQ and metabolites analysis (see following). The homogenate was centrifuged at 1000×g for 10 min at 4 °C, and the supernatant was centrifuged at 12,000×g for 10 min at 4 °C. Then the sediment was resuspended with the medium, and the high-speed spin cycle was repeated twice. Finally, the mitochondrial suspension was made by resuspended pellets in isolation buffer (120 mM KCL, 3 mM HEPES, 5 mM KH_2PHO_4 and 1 mM EGTA, pH 7.2, at 4°C). Mitochondrial protein concentration of mitochondrial

suspension was determined according to the Folin phenol method, with bovine serum albumin (BSA) as a standard (Sun et al. 2015).

Mitochondrial respiration and COX activity were measured polarographically, using a temperature-controlled Clark electrode system (Hansatech Instruments, UK). The assays for each sample were carried out at 18°C, 23°C, 28°C, 33°C and 38°C with a random sequence, according to the established methods with minor modifications (Sun et al. 2015). In brief, the mitochondrial respiration system comprised 0.94 mL medium and 0.06 mL mitochondrial suspension for State 4 and State 3 respiration measurements. We measured oxidative phosphorylation, and we added succinate (4 mM) as the substrate for Complex II for State 4 respiration, while ADP (200 mM) was added for State3 respiration. The activities of COX were determined according to published protocols within a system comprised of 1.98mL medium and 0.01mL mitochondrial suspension, and then 0.01mL cytochrome *C* was added to establish reaction. The rate of oxygen consumption was used for mitochondrial respiration and COX activity calculation, respectively.

Proteomic analysis

For the isobaric tag for relative and absolute quantification (iTRAQ) analyses, three livers of congener lizards with the same treatment were mixed as test candidates (see details in Appendix S1). The extraction and analysis were conducted by the Beijing Genomics Institute. Briefly, total proteins were extracted from the liver tissue, and total concentration was determined using the Bradford method (Appendix S2: Table S1, Fig. S1, S2). An aliquot volume contained 100 μg protein was denatured and digested into peptides, then were labeled with the 8-plex iTRAQ reagents following the manufacturer's instructions (Applied Biosystems, USA). Then labeled

peptides were separated, equilibrated, and eluted. The fractions were monitored by the absorbance at 214 nm. Then the peptides were desalted and identified by Nano RP high performance liquid chromatography (HPLC, Agilent, USA) and Mass Spectrometry (MS, Thermo fisher Q-Exactive, USA).

The raw MS/MS file data were transferred, and then searched against the database of *Anolis carolinensis* from NCBI with Mascot (version 2.3.0; Matrix Science, Boston, MA) (Sun et al. 2021b, data S1) (Alfoldi et al. 2011). An automatic decoy database search was performed. All identified peptides were qualified for further data analyzed under a false discovery rate (FDR) < 0.01. The fold changes in protein abundance were defined as the median ratio of all significantly matched spectra with tag signals.

Metabolites analysis

Six fresh livers from each treatment of each species were used individually for metabolite extraction. Metabolite analysis was conducted by OE Biotech (Shanghai, People's Republic of China), in relative quantitation. The livers were homogenized and then ultrasound treated on ice. The homogenate was then centrifuged and the supernatant with polar metabolites of each sample was transferred to a 2 mL GC/MS vial, another 11 μL supernatant from each sample was pooled as a QC sample.

Then the samples were condensed, reconstituted in sequence for incubation, and cooled at room temperature. A 10 μl FAMEs (Standard mixture of fatty acid methyl esters, C8-C16: 1 mg/mL; C18-C24: 0.5 mg/mL in chloroform) was added to the QC sample, and then gas chromatography/time-of-flight mass spectrometry (GC/TOFMS) was conducted.

GC/TOFMS analysis was performed on a gas chromatograph system (Agilent

7890, Agilent, USA) coupled with a Pegasus HT time-of-flight mass spectrometer (LECO Chroma TOF Pegasus HT, LECO, USA). The mass spectrometry data were acquired in full-scan mode with the m/z range of 50-500 at a rate of 20 spectra/s after a solvent delay of 455s. Then analysis was performed with Chroma TOF 4.3X software (LECO Corporation, USA) and the LECO-Fiehn Rtx5 database. The RI (retention time index) method was used in the peak identification, and the RI tolerance was 5000. Metabolites features detected in <50% of QC samples were removed.

Statistical and bioinformatics analysis

IBM SPSS statistics 21.0 were used for data analysis. Prior to analysis, the normality of distributions and homogeneity of residuals were tested using the *Kolmogorov–Smirnov* test and *Levene's* test, respectively. A paired *t*-test was used to analyze thermal environments among-treatment differences for each species and inter-species differences for each treatment during the acclimations. ANCOVAs were used to analyze the difference of growth rate in SVL and BM in each species, with treatment as the main factor, and initial SVL and BM as covariates, respectively. ANCOVAs were used to determine the differences in the sizes of liver, heart, and brains between acclimation treatments with body mass as covariates for each species. Repeated-measures ANOVAs were used to detect between-treatment differences in active body temperatures with the time as the repeated measures for each species, respectively. To analyze between-treatment differences in metabolic rates, respiratory quotient, mitochondrial respiration (State 3 and State 4), and COX activities, we used repeated measures ANOVAs with test temperature as the repeated measures, and treatment as the main factor for each species. Repeated measures ANOVAs were also

used to distinguish the mean traits values if we found a significant interaction between treatment and test temperature for each species.

For proteomic analysis, we calculated fold changes as the expression in cold treatment/expression in warm treatment. Differentially expressed proteins (DEPs) were defined as at least a 1.2-fold increase or decrease in protein abundance, with a *P*-value of < 0.05 after correction for multiple testing. Functional annotations and bioinformatics were conducted with Gene Ontology Consortium (http://www.geneontology.org), and DEPs were assigned to peptides were mapped Kyoto Encyclopedia of Gene and Genomes (KEGG) database to identify the related pathways (see details in Appendix S1).

We followed published methods to conduct metabolomics analyses (Hu et al. 2018, Zhang et al. 2018a). The acquired MS data were analyzed by Chroma-TOF software (v 4.34, LECO, St Joseph, MI). The three-dimensional datasets, including sample information, retention time-m/z, and peak intensities were obtained first. After being normalized based on the total area of each test sample, the dataset was fed to SIMCA14.1 (V14.1, MKS Data Analytics Solutions, Umea, Sweden) for multivariate statistical analysis. First, the principal component analysis (PCA), and orthogonal projections latent structures-discriminate analysis (OPLS-DA) were conducted to identify the metabolic differences between treatments for each species. By PCA and OPLS-DA analysis, the dataset can arrive at a linear transformation, which facilitates preserving as much of the variance in the original data as possible in lower dimensionality output data (Worley and Powers 2013). The variable importance in the projection (VIP) ranks the overall contribution of each identified metabolite came from the validated OPLS-DA model, and those variables with VIP > 1.0 are considered relevant for treatment discrimination for each species. After that, Student's

t-test was performed to determine the difference in expressed compounds between cold and warm treatments in *T. sexlineatus*, *T. septentrionalis*, and *T. wolteri*, respectively. Then, the metabolites with a *P*-value of < 0.05 after correction by multivariate statistical method (i.e., $P < 0.05$) and with the first principal component of variable importance in the projection (VIP) of > 1.0 (i.e., VIP > 1) were selected as differential metabolites, and annotated with the aid of available reference standards. After, the unknown metabolites and analytes were discarded. Finally, the differential metabolites were used for hierarchical cluster analysis and mapped to relevant metabolic pathways according to the database of KEGG.

In pathway analysis, we mainly focus on DEPs and metabolites in carbohydrate, lipid, and amino acid metabolism, due to their central roles in energy production.

RESULTS

Thermal environments of acclimation and active body temperatures

During the acclimation stage, average hourly ambient temperatures were higher in the warm treatment (28.80 ± 0.06°C, with the coldest and warmest records of 23.81°C and 38.13°C) than in the cold treatment (18.78 ± 0.04°C, with the coldest and warmest records of 15.88°C and 26.25°C) ($t = 213.63$, $df = 1967$, $P < 0.0001$, Fig. 1b). Average ambient temperatures for *T. wolteri* ($t = 37.97$, $df = 23$, $P < 0.0001$), *T. septentrionali* ($t = 36.80$, $df = 23$, $P < 0.0001$) and *T. sexlineatus* ($t = 37.96$, $df = 23$, $P < 0.0001$) were higher in the warm treatment than for congeners in the cold treatment, respectively. However, the ambient temperatures under the same treatment did not differ among species (for cold treatments: all $P > 0.313$; for warm treatments: all $P > 0.449$). Correspondingly, average active body temperatures of lizards in the warm treatment were significantly higher than those in the cold treatment in all three

Takydromus species ($F_{1, 54}$ = 21032, P < 0.0001), but did not differ among species under the same acclimation treatments ($F_{2, 54}$ = 1.893, P = 0.160) (Fig. 1b).

Growth Rate

The growth rate (GR) of SVL or BM did not change between treatments in *T. sexlineatus*, whereas *T. septentrionalis* enhanced the growth rate of SVL and BM in the warm treatment, and *T. wolteri* increased the growth of BM but not the growth rate of SVL in the warm treatment (Table 1).

Resting Metabolic Rates

In all three species, the RMR increased with increasing test temperature ($\dot{V}CO_2$: *T. sexlineatus*: $F_{4, 80}$ = 210.57, P < 0.0001; *T. septentrionalis:* $F_{4, 120}$ = 60.970, P < 0.0001 and *T. wolteri*: $F_{4, 104}$ =252.86, P < 0.0001). The RMR was not different between treatments in *T sexlineatus* ($F_{1, 20}$ = 0.309, P = 0.584); but was higher in the cold compared to the warm treatment in *T. septentrionalis* ($F_{1, 30}$ = 39.725, P < 0.0001) (Fig. 2a, b). Interestingly, the RMR was more thermally sensitive in the warm compared to the cold treatment in *T. wolteri* ($F_{4, 104}$ = 13.163, P < 0.0001, Fig. 2c), such that lizards in the cold treatment had higher RMR at low temperatures ($F_{1, 26}$ = 13.921, P < 0.001), but lower RMR at high temperatures ($F_{1, 26}$ = 17.168, P < 0.001), compared to lizards in the warm treatment. This effect can be seen from the higher Q_{10} values in the warm compared to the cold treatment (cold vs. warm: 1.84 ± 0.07 vs. 2.76 ± 0.07; $F_{1, 26}$ =91.947, P < 0.0001). In contrast, Q_{10} were not different between cold and warm treatments in *T. sexlineatus* (cold vs. warm, 2.12 ± 0.10 vs. 2.25 ± 0.06; $F_{1, 20}$ = 1.259, P =0.275) or *T. septentrionalis* (cold vs. warm, 1.95 ± 0.34 vs. 1.76 ± 0.08; $F_{1, 30}$ = 0.288, P =0.595), confirming no alteration of thermal sensitivity

in response to thermal acclimation. The respiratory exchange ratio (RER) increased as test temperature rose in all species (*T sexlineatus*: $F_{4, 80} = 4.152$, $P = 0.004$; *T. septentrionalis:* $F_{4, 120} = 19.640$, $P < 0.0001$ and *T. wolteri*: $F_{4, 104} = 19.445$, $P < 0.0001$). The RERs were not different between treatments in *T sexlineatus* ($F_{1, 20} = 0.734$, $P = 0.402$) or *T. septentrionalis* ($F_{1, 30} = 0.741$, $P = 0.396$), however RERs were higher in the warm compared to the cold treatment at all test temperatures in *T. wolteri* ($F_{1, 26} = 36.173$, $P < 0.0001$; Fig. 2 d, e, f).

Organ sizes

The liver sizes of lizards were 2.7-fold greater in the cold treatment to the warm treatment in *T. septentrionalis*, but not different between treatments in *T. sexlineatus* or *T. wolteri*. The heart sizes or brain sizes of lizards were not different between treatments in any species (Table 1; Fig. 2g, h, i).

Mitochondrial respiration and Cytochrome C oxidase (COX) activity

In tropical *T. sexlineatus*, the state 3 mitochondrial respiration did not differ between treatments ($F_{1, 10} = 3.354$, $P = 0.10$). However, the state 4 mitochondrial respiration did not differ between the treatments from 18 to 28°C ($F_{1, 10} = 0.650$, $P = 0.439$), but significantly increased in the lizards of the cold treatment from 33 to 38°C ($F_{1, 10} = 12.391$, $P = 0.006$; Fig. 2j, m).

In widespread temperate *T. septentrionalis*, neither state 3 nor state 4 mitochondrial respiration differed between treatments ($F_{1, 10} = 2.648$, $P = 0.135$; $F_{1, 10} = 2.392$, $P = 0.153$; Fig.2k, n). In narrowly-distributed temperate *T. wolteri*, the state 3 ($F_{1, 10} = 35.703$, $P < 0.001$) and state 4 ($F_{1, 10} = 14.327$, $P = 0.004$) mitochondrial respiration was significantly higher in the warm treatment than the cold treatment.

Moreover, in *T wolteri*, the thermal sensitivity of state 4 mitochondrial respiration differed between treatments (test temperature × treatment: $F_{4, 40} = 12.12$, $P < 0.0001$). The state 4 mitochondrial respiration did not differ between treatments from 18 to 28°C ($F_{1, 10} = 3.258$, $P = 0.101$), but was significantly higher in *T. wolteri* from the warm treatment from 33 to 38°C ($F_{1, 10} = 20.521$, $P = 0.001$; Fig. 2l, o).

COX activities did not differ between treatments in *T. sexlineatus* ($F_{1, 10} = 1.465$, $P = 0.254$; Fig. 2 p) or *T. septentrionalis* ($F_{1, 10} = 3.959$, $P = 0.075$; Fig. 2 q). In contrast, COX activities were significantly higher in the warm treatment than the cold treatment in *T. wolteri* ($F_{1, 10} = 9.361$, $P = 0.012$), with an interaction between treatment and test temperature ($F_{4, 40} = 5.061$, $P = 0.002$). The lizards from the warm treatment had lower COX activities than those from the cold treatment at the low temperature of 18°C ($F_{1, 10} = 8.229$, $P = 0.017$), but higher COX activities from 23 to 38°C ($F_{1, 10} = 10.783$, $P = 0.008$; Fig. 2 r).

Proteome regulation

We identified a total of 660 proteins, among which the number of differentially expressed proteins (DEPs) were highest in *T. wolteri* (N=302), and lowest in *T. sexlineatus* (N=170), with *T. septentrionalis* (N=251) intermediate (Sun et al. 2021b, data S2). Three Gene Ontology (GO) categories were enriched with DEPs: molecular functions, biological processes, and cellular components (Appendix S3: Fig. S1; Sun et al. 2021b, data S3). Similarly, GO analysis of general and exclusive DEPs in three species after acclimation are also enriched in molecular functions, biological processes, and cellular components (Appendix S3: Fig. S2). Among DEPs, 91 proteins were involved in carbohydrate, lipid, amino acid or energy metabolism, with the numbers of DEPs being higher in *T. wolteri* (N=65) and *T. septentrionalis* (N=47)

than in *T. sexlineatus* (N=32) (Sun et al. 2021b, data S4). Hierarchical clustering analysis revealed that the patterns of regulation in these core metabolic pathways differed strikingly among species, with each species having one of three large clusters of proteins regulated in opposite directions in response to cold acclimation (Appendix S3: Fig. S3).

Conserved regulation of Glycolysis.

Enzymes in the Glycolysis pathway were conservatively regulated in response to acclimation across species. PFK, ACS, and LDH (see detail abbreviations in Table 2) in Glycolysis were regulated in the same way in all three species (max P =0.013, Fig 3a). PFK and ACS, which catalyze flux from glucose to acetyl-CoA, were coordinately down-regulated in the cold treatment in all three species (Fig 3a, Sun et al. 2021b, data S5). The temperate species *T. wolteri* and *T. septentrionalis* showed coordinated regulation in the Pentose phosphate pathway, including down-regulated RPE, PGM and PRPS, and up-regulated TKT in cold treatment, which facilitate the reactions from D-glucose-6P to D-glyceraldehyde-3P (Fig. 3b; Sun et al. 2021b, data S5). In contrast, the tropical species *T. sexlineatus* showed less DEPs as a result of acclimation, in any metabolic pathways besides Glycolysis. Several enzymes in the Citrate cycle (TCA) pathway were differentially regulated in response to acclimation in both *T. sexlineatus* and *T. wolteri*, with the only shared enzyme (fumarase) differentially regulated in conflicting directions (Fig. 3c; Sun et al. 2021b, data S5). Notably, no enzyme in the TCA was differentially regulated in *T. septentrionalis.*

Fatty acid metabolism is altered in T. wolteri.

Seven out of eight enzymes in the Fatty acid degradation pathway were

differentially regulated in *T. wolteri* in response to thermal acclimation. Fatty acid degradation enzymes of CPT-1, ACO, EHHADH, HADH, and ACAA were up-regulated in the cold treatment relative to the warm treatment (Fig. 3d). In contrast, fewer enzymes associated with fatty acid metabolism were detected in *T. septentrionalis* (i.e., CPT-2, EHHADH and ACAA) or *T. sexlineatus* (i.e., CPT-1 and ACAA) (Sun et al. 2021b, data S5).

Specific regulation of amino acid metabolism pathways.

In total, fourteen, seven and three enzymes related to three pathways in amino acid metabolism were differentially regulated in response to thermal acclimation in *T. wolteri*, *T. septentrionalis* and *T. sexlineatus*, respectively. First, *T. wolteri* showed differential expression of enzymes in Valine, leucine and isoleucine degradation pathways. Enzymes of EchA, HADH, HSD17B10, ACAA (catalyzing acetyl-CoA production by Isoleucine degradation) was up-regulated in the cold treatment; whereas BCKDH, MCC and ACAT (catalyzed the pathway from Leucine to acetyl-CoA) were down-regulated in the cold compared to warm treatment (Fig. 4a). By contrast, only two enzymes were differentially expressed in *T. septentrionalis* (i.e., echA and ACAA), and no differential expression was observed in *T. sexlineatus* (Fig. 4a, Sun et al. 2021b, data S5). Second, enzymes in Cysteine and methionine metabolism were differentially expressed in all species, with coordinately up-regulated enzymes of TST and LDH, and down-regulated GOT1 in the cold compared to treatment in both *T. wolteri* and *T. septentrionalis*. In contrast, GOT1, TST and LDH were up-regulated in Cysteine and methionine metabolism along the direction of pyruvate production from L-cysteine and serine in cold compared to warm treatment of *T. sexlineatus* (Sun et al. 2021b, data S5). Lastly, Alanine, aspartate, and glutamate

metabolism was less regulated in *T. sexlineatus* with only GOT1 up-regulated in the cold compared to warm treatment. In *T. septentrionalis*, only GOT1, ADSL, and SSADH were down-regulated in the cold treatment. However, in *T. wolteri*, ALT, ADSL, GOT1, NIT 2 and SSADH were depressed in the cold treatment along the pathway from alanine, aspartate, and glutamate metabolism to produce fumarate, oxaloacetate, 2-oxoglutarate and succinate, which are the substrates for the reactions in the TCA cycle (Fig. 4b; Sun et al. 2021b, data S5).

Only a few proteins related to Oxidative phosphorylation were differentially regulated in three lizards in response to acclimation. The regulation only occurred in a few subunits of complex I and complex V (Sun et al. 2021b, data S5).

Metabolome regulation

Metabolic profiling identified 488 peaks, and a total of 462 metabolites were detected, among which 84 were unknown metabolites, 174 were undefined analytes, and 204 were confirmed metabolites (Sun et al. 2021b, data S6). Principal component analysis (PCA) showed the distribution of metabolites (Appendix S4: Table S1, Fig.S1). The parameters indicated that orthogonal projections to latent structures-discriminate analysis (OPLS-DA) provided stable fitness and prediction (Appendix S5: Table S1, Fig. S1, S2).

The number of differentially-abundant metabolites (between acclimation treatments) was higher in *T. wolteri* (N=40) than in *T. septentrionalis* (N=3) and *T. sexlineatus* (N=5) (Appendix S3: Fig. S4). After unknown metabolites and undefined analytes had been discarded, there were eight differentially abundant metabolites were associated with carbohydrate, lipid, amino acid, and energetic metabolism in *T.wolteri*, but none in *T. septentrionalis* or *T. sexlineatus* (Table 3). Among these eight

differentially-abundant metabolites in *T. wolteri*, seven were enriched in the cold compared to warm treatment, indicated by hierarchical cluster analysis (Appendix S3: Fig. S5).

Integration of proteomic regulation and metabolite analysis

To examine the co-regulation of proteins and metabolites, we conducted a pathway analysis on differentially expressed proteins and metabolites in *T. wolteri*, the only species with identified and differentially expressed metabolites. We detected two networks regulated in carbohydrate and amino acid metabolism of *T. wolteri* (Fig. 5). In *T. wolteri*, the enzyme of MDH and the metabolites of Malate and FAD degradation products were up-regulated in the cold compared to warm treatment. Furthermore, lizards in the cold treatment down-regulated enzymes of NIT2, GOT1, and ADSL that catalyzes L-asparagine transfer to oxaloacetate and fumarate respectively, which involves in the TCA (Fig. 5). Accordingly, intermediary metabolites (i.e., aspartate and alanine) involved in conversion of asparagine to pyruvate were enhanced in the cold treatment. ACS, which catalyzes the reaction from pyruvate to acetyl-CoA, was down-regulated in *T. wolteri* in the cold treatment, whereas LDH, which catalyzes the interconversion of pyruvate and L-Lactate, was up-regulated in the cold treatment (Fig. 5). When we take into account that L-lactamide was up-regulated, which is derived from L-lactate, we conclude that flux from pyruvate to L-Lactate (and thus to L-lactamide) was enhanced in the cold compared to warm treatment (Fig. 5).

DISCUSSION

Understanding geographical patterns in animals' physiological response to thermal variation is critical to determine adaptation, and thus to predict the

vulnerabilities to climate warming (Somero 2011, Huey et al. 2012, Logan and Cox 2020). We examined acclimation capacity across levels of the biological hierarchy in order to test the predictions of the climate variability hypothesis, which states that tropical species will have lower physiological plasticity (Janzen 1967, Ghalambor et al. 2006, Gaston et al. 2009). We found strong support for this hypothesis, and moreover uncovered two distinct integrated strategies of thermal acclimation in the two temperate lizard species.

Patterns of whole organism acclimation

In contrast to tropical species *T. sexlineatus*, both temperate species *T. septentrionalis* and *T. wolteri* modified the thermal responses in metabolic processes through acclimation when thermoregulation was limited; an example of active plasticity (Fig. 1b, Fig. 2a, b, c)(Havird et al. 2020). Interestingly, the direction of active plasticity differed between species: the higher metabolic rates of *T. septentrionalis* in the cold treatment (Fig 1b) is a typical case of thermal compensation (Hochachka and Somero 2002), whereby increased metabolic rates in cool conditions allow increased metabolic function in those conditions for cool-acclimated animals. In contrast, in *T. wolteri*, the more narrowly distributed temperate species, a decreased thermal sensitivity of metabolism in cool conditions allowed lizards to retain functions specifically at low temperatures, while avoiding high metabolic rates at warm temperatures, as the beneficial acclimation hypothesis predicts (Wilson and Franklin 2002).

Although the two temperate species modified their metabolism in different ways through active plasticity (Conover and Schultz 1995, Clarke 2003, Havird et al. 2020), the growth rates of *T. septentrionali*s and *T. wolteri* were potentially enhanced in the

warm treatment, which suggests metabolic acclimation was adaptive under warming temperatures (Huey and Kingsolver 2019). Both types of modification could give rise to advantages for individuals by either enhancing their performance across a wide range of temperatures (i.e., *T. septentrionalis*) or sustaining functions at low temperatures (i.e., *T. wolteri*), and for individuals from warm climates by avoiding energetic dissipation (i.e., *T. septentrionalis*) or facilitating functions at high body temperatures (i.e., *T. wolteri*) (Hochachka and Somero 2002, Wilson and Franklin 2002). As well as providing a potential benefit in increased energetic allocation to physiological demands, higher metabolic rates could also impose a cost by increasing energy requirements, which would not manifest in this study due to *ad libitum* food availability (Le Henanff et al. 2013, Glazier 2015). In addition to genetic differentiation among species, the differences in acclimation responses among species that we observed may result from developmental plasticity, since lizards were sampled from different latitudes after developing in the field (Bozinovic et al. 2011, Somero 2012, Somero et al. 2017).

Two distinct mechanisms of acclimation

The two temperate lizard congeners, *T. septentrionalis* and *T. wolteri*, achieved thermal acclimation of whole-organism metabolism through modifications to liver size and intensity, respectively. *T. septentrionalis* enlarged liver size in response to cold acclimation, and thus increased individual metabolic rates at all test temperatures with little change to underlying metabolic pathways; whereas *T. wolteri* substantially remodeled the function of metabolic pathways in the liver at the protein, metabolite, enzyme, and mitochondrial levels, leading to shifts in the respiratory exchange ratio (RER) and thermal sensitivity of metabolism. Our results highlight the liver as a

critical organ involved in active thermal plasticity in lizards, broadly consistent with observations in fish, reptiles, birds, and mammals; where changes in either size or metabolic intensity and fuel use of the liver have been documented in response to cold acclimation (e.g., Stone and Sidell 1981, Barré et al. 1987, Walter and Seebacher 2007, Seebacher et al. 2009, Orczewska et al. 2010). These distinct mechanisms of acclimation (modification of liver size or intensity) represent two possible outcomes of selection for enhanced thermal acclimatization in seasonal environments (Norin and Metcalfe 2019).

Strategy 1 – Modulate liver size

T. septentrionalis, the widespread temperate-tropical species from a mid-latitude population, enlarged liver size in response to cold acclimation, with few modifications to liver biochemical function. At the organ level, the size of primary organs of the brain, liver, and heart are the most significant contributors to whole organism standard or resting metabolic rate (e.g., Bundle et al. 1999). In this study, the liver size of *T. septentrionalis* in the cold treatment was increased 2.7-fold compared to livers of congeners in the warm treatments (Table 1), suggesting that the liver forms a substantial contribution to the increased metabolic rates of *T. septentrionalis* in the cold treatment, similar to other species in cold environments (e.g., Kent et al. 1988, Song and Wang 2006, Vézina et al. 2006, Orczewska et al. 2010). In contrast, in all three species in this study, heart and brain sizes failed to respond to thermal acclimation. Brain and heart sizes may be fixed after development, leading to limited plasticity in these organs within a single generation (Healy and Rowe 2007). As the major site of metabolism, the larger livers in cool conditions could be achieved by some combination of enhancement of cell size and number (Starostová et al. 2009),

higher mitochondrial density (Song and Wang 2006), higher enzyme concentration (White et al. 2012), and more fuel storage (Sowton et al. 2020), leading to up-regulation of whole-organism metabolic rates (e.g., Steyermark et al. 2005, White and Kearney 2013). A larger liver may contribute to resource assimilation, catabolism, and faster growth rates through increased rates of metabolism, but may become a cost in resource-limited environments (Glazier 2015). Down-regulated metabolic rates under warming environments would be helpful in energetic saving, providing an advantage for lizards under climate warming (Dillon et al. 2010, Norin and Metcalfe 2019) and potentially counteracting 'metabolic meltdown', wherein accelerating metabolic costs due to temperature increases occur simultaneously with declining energy intake due to reduced foraging times (Huey and Kingsolver 2019).

Strategy 2 – Modulate liver function

T. wolteri, the narrowly distributed temperate species collected from a high-latitude population, modulated liver function in response to warm acclimation, providing increased metabolic intensity (mitochondrial respiration and COX activity) and apparent shifts in fuel utilization without corresponding increases in organ size. Inter-individual or inter-population differences in mitochondrial function (e.g., mitochondrial respiration) or COX activities have been considered as fitness-related traits in ectotherms, and their modification contributes to variation of metabolic rates (e.g., Speakman et al. 2004, Ellison and Burton 2006, Seebacher and Wilson 2006, White et al. 2012). The suppressed thermal sensitivity of mitochondrial metabolism in cold-acclimated *T. wolteri* matched well with the pattern in respiratory gas exchange at the whole organism level, suggesting that actual energy turnover may be driven by liver mitochondrial capacities in this species, but not in the widespread temperate or

tropical species (e.g., Di Maria et al. 2009, White et al. 2012).

At the biochemical level, proteomics revealed *T. wolteri* in the cold treatment down-regulated key enzymes in Glycolysis, while enhancing key enzymes in Fatty acid degradation, Pentose phosphate pathway, Alanine, aspartate and glutamate metabolism (Fig. 3a, b, d; Sun et al. 2021b, data S5). Through these modifications, the supplement of acetyl-CoA from D-glyceraldehyde-3P and long-chain lipid were enhanced (e.g., Voet and Voet 1995, Hochachka and Somero 2002, Dijkstra et al. 2011). Correspondingly, lower RER in *T. wolteri* supported the conclusion that cold-acclimated lizards switched towards a greater reliance on lipid oxidation (RER is around 0.7). When encountering cold temperatures, lipid metabolism imposes advantages to facilitate cold resistance. Diapausing insects and hibernating mammals frequently rely on lipid metabolism during winter dormancy (e.g., Boyer and Barnes 1999, Carey et al. 2003, Kabine et al. 2003, Hahn and Denlinger 2007), and our results suggest that this may happen more broadly in ectothermic vertebrates during cold acclimation. In active ectotherms such as fish, increased lipid catabolism enhances cold resistance (Lu et al. 2019), suggesting additional potential benefits of lipid catabolism, beyond its high energy density. In contrast, the observed alterations to pyruvate, lactate and alanine metabolism suggest that amino acid metabolism may be enhanced in cold-acclimated *T. wolteri*, which could contribute to the reduced RER (Natali et al. 1990, Scott and Johnston 2012, Zhang et al. 2018b).

Shared regulation in responding to thermal variation in Takydromus lizards

Glycolysis in this study is down-regulated in all three *Takydromus* lizards in response to cold acclimation, as occurs in other ectothermic species (e.g., Shumway et al. 1983, Colinet et al. 2013). Interestingly, as a supplementary pathway in providing

pyruvate from D-glucose-6P (Voige 2012), enhanced Pentose phosphate pathway flux in *T. septentrionalis* and *T. wolteri* could add metabolic substrates by decreasing oxygen dependence in the cold treatment (Fig. 3b) (e.g., Dijkstra et al. 2011).

Implication for vulnerability to climate warming based on acclimation capacity

Comparisons of physiological proxies (e.g., thermal sensitivity and acclimation capacity) are fundamental and necessary to assess vulnerabilities of organisms with different latitudinal distributions (Williams et al. 2008, Huey et al. 2012, Logan et al. 2014, Pacifici et al. 2015). Species with significant metabolic plasticity are expected to be less vulnerable to climate warming (Seebacher et al. 2015, Rohr et al. 2018, Norin and Metcalfe 2019). The climate variability hypothesis (CVH) posits that temperate species will have higher plasticity than tropical species, due to historically higher thermal variability (Janzen 1967, Ghalambor et al. 2006, Gaston et al. 2009). Supporting this hypothesis, temperate *Takydromus* species have been shown to regulated physiological and biochemical traits through thermal acclimation to a greater degree than the tropical species. We assume that this acclimation response in the laboratory corresponds to a greater seasonal acclimatization capacity in the field (Fangue and Bennett 2003, Schou et al. 2015, Filatova et al. 2019) (but see Gatten et al. 1988). Thus, temperate *Takydromus* species likely have a greater ability to seasonally acclimatize to thermal fluctuations than tropical species, giving them an advantage as global climate change leads to increasing thermal variability (Bathiany et al. 2018). In addition, our warming experiments in outdoor mesocosms have demonstrated that tropical *Takydromus* species have lower survival rates when exposed to warming climate, but those temperate congeners do not (Sun et al., unpublished data). This adds to growing evidence that tropical ectotherms, and

particularly reptiles, will be more vulnerable to climate change due to low capacity for plastic responses (Dillon et al. 2010, Huey et al. 2012, Morley et al. 2019). Nonetheless, it is worth noting that reduced plasticity in tropical species could facilitate rapid adaptive evolution by permitting stronger selection on fitness-relevant traits, although this may also increase the chance of stochastic extinction (Hoffmann and Sgro 2011, Bell 2017, De Meester et al. 2018, Miller et al. 2018, Catullo et al. 2019).

In contrast, the two temperate species showed not only a significant ability to acclimate their metabolism, but also two distinct strategies for doing so, likely with different costs and limits (DeWitt et al. 1998). For the widespread temperate *T. septentrionalis*, which occupies across subtropical and temperate regions, modification of liver size may provide a rapid and reversible strategy, with limited acclimatory responses at cellular, subcellular and biomolecular levels. Modification of metabolic pathways in response to cold acclimation, as seen in *T. wolteri*, the narrowly distributed temperate specialist, may come with biochemical trade-offs (e.g., protein stability-flexibility, Somero et al. 2017) that reduce performance in warm temperatures (but are beneficial in a highly seasonal environment with consistently cold winters)(Wilson and Franklin 2002). These hypotheses require further testing with greater replication at the species level, but this demonstration of two distinct outcomes of selection imposed by seasonality in closely related congeners suggests that understanding the costs and limits to different strategies of seasonal acclimatization is an important research priority.

In summary, in three *Takydromus* lizards adapted to different thermal regimes from tropical to temperate latitudes, we found strong support for the climate variability hypothesis. The tropical species *T. sexlineatus* showed limited metabolic

acclimation compared to the two temperate species *T. septentrionalis* and *T. wolteri*, across levels of the biological hierarchy. This integrative investigation of active plasticity has also demonstrated two distinct strategies of seasonal acclimatization in the two temperate species, which support our predictions in mechanisms (Bozinovic et al. 2011, Norin and Metcalfe 2019). The widespread temperate/sub-tropical species *T. septentrionalis* showing a striking degree of plasticity in liver size, that was absent or less pronounced in the other species. In contrast, *T. wolteri*, with a narrow temperate distribution and originating from a cold-adapted population, relied on modifications at mitochondrial, proteomic and metabolomic levels, during metabolic acclimation. These strategies may represent two general outcomes of thermal acclimation, with distinct costs and benefits. Our work thus supports the conclusion that tropical species (*T. sexlineatus* in the present study) with limited acclimation capacity will be more vulnerable to increasing thermal variability due to lack of physiological and metabolic plasticity (Janzen 1967, Ghalambor et al. 2006, Gaston et al. 2009).

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AUTHOR CONTRIBUTIONS

Baojun Sun and Caroline Williams contributed equally to this study. W. G. Du, B. J. Sun, and T. Li conceived the ideas and designed the methodology. B. J. Sun, C. Williams, T. Li, Z. G. Jin, H. L. Lu, and L. G. Luo collected the data, B. J. Sun, C. Williams, W. G. Du and J. Speakman analyzed data, led the writing of the manuscript. All authors contributed critically to the drafts, revisions and gave final approval for publication.

CONFLICT OF INTEREST

We declare no conflict of interest associated with this publication.

SUPPORTING INFORMATION

Additional supporting information may be found online at: **

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Table 1. The growth rate (GR) in SVL and BM, and mass of liver, heart and brain in three *Takydromus* lizards after warm or cold acclimation. Data are expressed as least square mean ± SE, bold font indicates the significant difference, α=0.05.

	***T. sexlineatus* (tropical)**				***T. septentrionalis* (widespread temperate)**				***T. wolteri* (narrow temperate)**			
	warm	cold	Statistics		warm	cold	Statistics		warm	cold	Statistics	
			F	*P*			*F*	*P*			*F*	*P*
GR in SVL	0.00196 ± 0.00044	0.00085 ± 0.00044	3.213[a]	0.089	0.00506 ± 0.00062	0.00243 ± 0.00064	**8.612[c]**	**0.005**	0.00529 ± 0.00060	0.00366 ± 0.00053	4.115[d]	0.054
GR in BM	0.00674 ± 0.00162	0.00550 ± 0.00162	0.293[a]	0.595	0.00885 ± 0.00173	0.00060 ± 0.00176	**11.163[c]**	**0.002**	0.00702 ± 0.00118	0.00377 ± 0.00105	**4.253[d]**	**0.050**
Liver mass	0.073 ± 0.009	0.103 ± 0.009	4.980[b]	0.053	0.099 ± 0.026	0.272 ± 0.026	**17.148[b]**	**0.003**	0.048 ± 0.013	0.070 ± 0.013	1.378[b]	0.271
Heart mass	0.006 ± 0.001	0.006 ± 0.001	0.002[b]	0.965	0.010 ± 0.001	0.010 ± 0.001	0.773[b]	0.402	0.006 ± 0.001	0.006 ± 0.001	1.497[b]	0.252
Brain mass	0.019 ± 0.001	0.019 ± 0.001	0.090[b]	0.771	0.025 ± 0.001	0.028 ± 0.001	2.589[b]	0.142	0.018 ± 0.001	0.019 ± 0.001	0.217[b]	0.652

[a] $= F_{1,19}$
[b] $= F_{1,9}$
[c] $= F_{1,42}$
[d] $= F_{1,24}$

Table 2 Abbreviation of the enzyme and descriptions in pathway analysis.

abbreviation	Description
Glycolysis	
DEPs	Differentially expressed proteins
PFK	phosphofructokinase
ACS	acetyl-coenzyme A synthetase
LDH	L-lactate dehydrogenase
Pentose phosphate pathway	
PRE	ribulose-phosphate 3-epimerase
PGM	phosphoglucomutase
PRPS	ribose-phosphate pyrophosphokinase
TKT	transketolase
Fatty acid degradation	
CPT-1	carnitine O-palmitoyl transferase 1
ACO	acyl-CoA oxidase
EHHADH	enoyl-CoA hydratase
HADH	3-hydroxyacyl-CoA dehydrogenase
ACAA	acetyl-CoA acyltransferase
ACAT	acetyl-CoA C-acetyltransferase
Valin, Leucine and Isoleucine degradation	
EchA	enoyl-CoA hydratase
HADH	3-hydroxyacyl-CoA dehydrogenase
HSD17B10	3-hydroxy-2-methylbutyryl-CoA dehydrogenase
ACAA	acetyl-CoA acyltransferase
BCKDH	2-oxoisovalerate dehydrogenase
MCC	3-methylcrotonyl-CoA carboxylase
ACAT	acetyl-CoA C-acetyltransferase
Cysteine and Methionine metabolism	
GOT1	aspartate aminotransferase
TST	thiosulfate/3-mercaptopyruvate sulfurtransferase
Alanine, Aspartate and Glutamate metabolism	
ADSL	adenylosuccinate lyase
SSADH	succinate-semialdehyde dehydrogenase
ALT	alanine transaminase
NIT2	omega-amidase

Table 3. Metabolites enrichment in *Takydromus* lizards after warm or cold acclimation. Peak means the description of metabolites; mean-cold and mean-warm mean the means of enrichment in cold and warm treatment respectively; VIP means the variable importance in the projection of OPLS-D, *P*-Value means the significant difference according to Student's *t*-test, Q-Value is calibrated significant value. Fold change means the rates of cold compared to warm treatment.

Species	Peak	Mean-Cold	Mean-Warm	VIP	*P*-Value	Q-Value	Fold change
T. sexlineatus	D-Arabitol	2.67E-08	0.429	2.004	0.011	0.213	6.24E-08
T. septentrionalis	Thymidine 2	2.21E-04	0.001	1.960	0.021	0.242	0.204
T. wolteri	4-Androsten-19-ol-3,17-dione 2	0.004	0.002	1.917	0.032	0.330	1.695
T. wolteri	5-alpha-Cholestan-3-one 1	0.405	1.280	2.045	0.019	0.257	0.316
T. wolteri	5-Aminovaleric acid 1	0.004	0.015	1.647	0.006	0.154	0.275
T. wolteri	Alanine 1	0.065	0.042	2.600	0.000	0.012	1.545
T. wolteri	Aspartic acid 1	1.127	0.700	2.079	0.007	0.164	1.610
T. wolteri	Flavin adenine degrade product	0.035	0.021	1.454	0.017	0.243	1.699
T. wolteri	Lactamide 2	0.039	0.027	1.759	0.033	0.333	1.452
T. wolteri	L-Malic acid	0.248	0.202	1.751	0.042	0.367	1.228
T. wolteri	Methionine sulfoxide 2	0.095	0.037	2.652	0.000	0.004	**2.592**

T. wolteri	Oxalic acid	0.047	0.030	2.296	0.003	0.107	**1.536**
T. wolteri	Putrescine 2	0.107	0.245	2.180	0.012	0.216	**0.439**

Figure legends:

Fig. 1 Sampling sites of *T. sexlineatus*, *T. septenrionalis* and *T. wolteri* (a), and thermal environment and active body temperatures of lizards during acclimation (b). (a) Lines with different colors indicate the distribution area of the species. The *T. sexlineatus*, *T. septenrionalis,* and *T. wolteri* were collected from Zhaoqing, Wenzhou, and Chuzhou, respectively. The gradient colors on the map indicate the annual fluctuations of thermal environments. (b) Orange and blue lines and symbols indicate warm and cold treatment, respectively. Data are shown as mean ± SE.

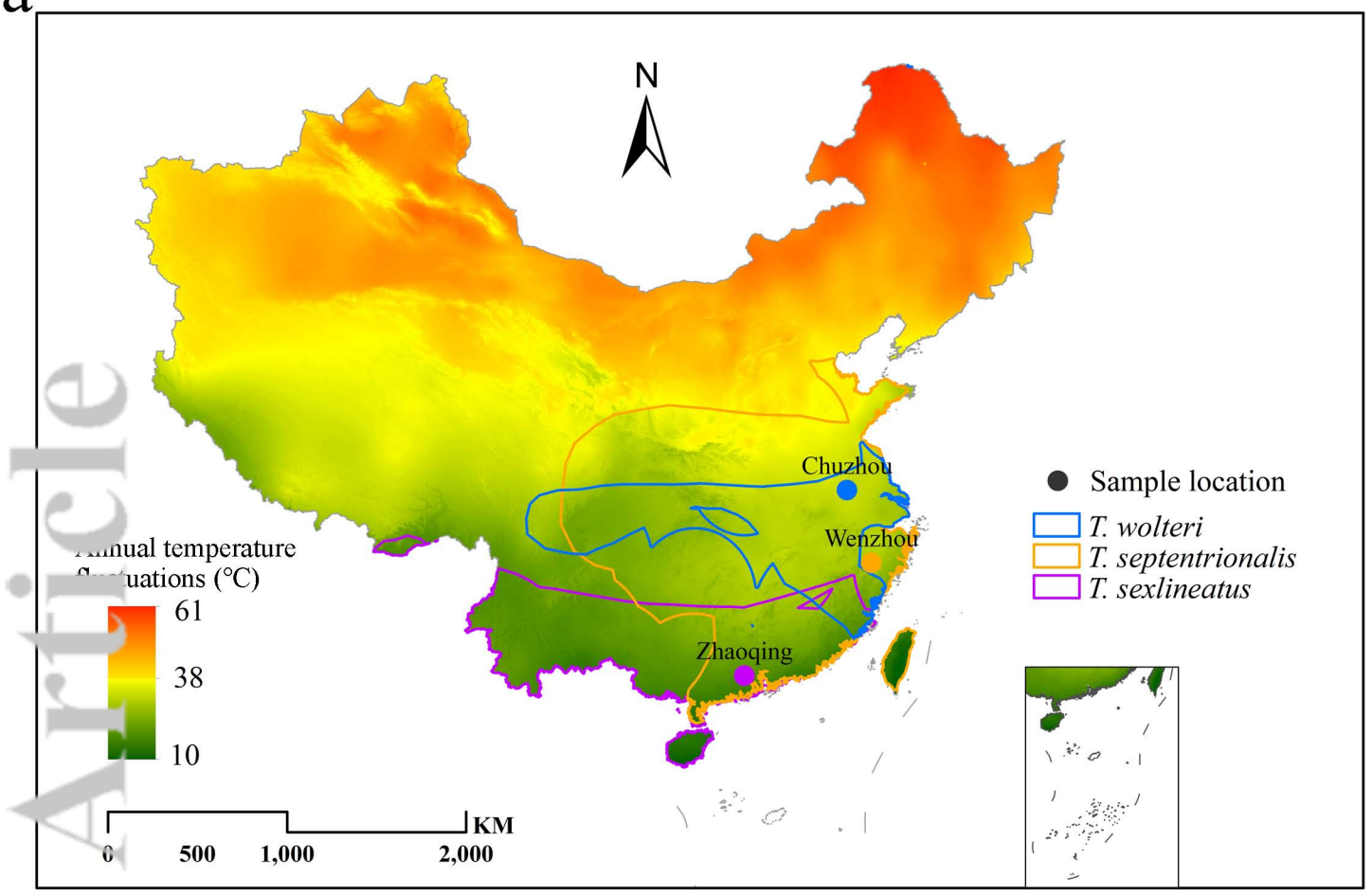
Fig. 2 The acclimation responses in respiratory gas exchange (a, b, c), respiratory exchange ratio (d, e, f), organ sizes (g, h, i), mitochondrial state 3 respiration (j, k, l), mitochondrial state 4 respiration (m, n, o) and cytochrome *C* oxidase (COX) activities (p, q, r) of *T. sexlineatus*, *T. septentrionalis* and *T. wolteri* respectively. Blue symbols and bars indicate cold treatments (16°C +6hr heating); orange symbols and bars indicate warm treatments (26°C +10hr heating). Asterisks indicate significant differences between treatments in (f) and (h).

Fig.3 Enzymes modifications in (a) Glycolysis, (b) Pentose phosphate pathway, (c) Citrate cycle, and (d) Fatty acid degradation of *Takyromus* lizards after acclimation. Upward and downward arrows indicate up- and down-regulation of enzymes in cold treatments relative to warm treatments, colors correspond to species (*T. sexlineatus*, purple, tropical; *T. septentrionalis*, yellow, widespread temperate; and *T. wolteri*, blue, narrow temperate). Compounds with full names are metabolites, and acronyms are the enzymes in the pathways. Acetyl-CoA which linked the pathways was expressed in red font.

Fig. 4 Enzymes modifications in (a) Valine, leucine and isoleucine degradation, and (b) Alanine, aspartate and glutamate metabolism of *Takyromus wolteri* after acclimation. Upward and downward arrows indicate up- and down-regulation of enzymes in cold treatment relative to warm treatment. Compounds with full names are elements in the processes (i.e., metabolites), and acronyms are enzymes in the pathways.

Fig. 5 Integrative regulation of differentially expressed proteins (DEPs) and metabolites in Alanine, aspartate and glutamate metabolism of *Takydromus wolteri*. Red squares indicate the enrichment of metabolites in the cold treatment, while red upward and blue downward arrows mean up-regulation and down-regulation of catalyzed enzymes, respectively. The comparison is conducted by cold-treatment relative to warm-treatment. Compounds with full names are elements in the processes (i.e., metabolites), and acronyms are enzymes in the pathways.

a



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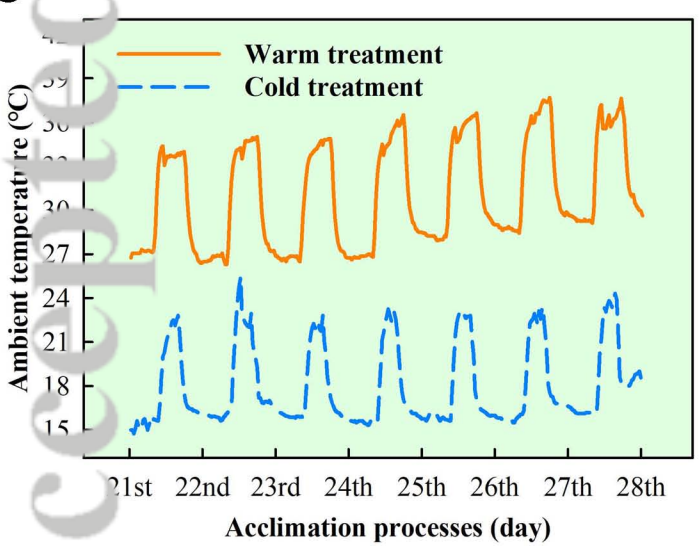
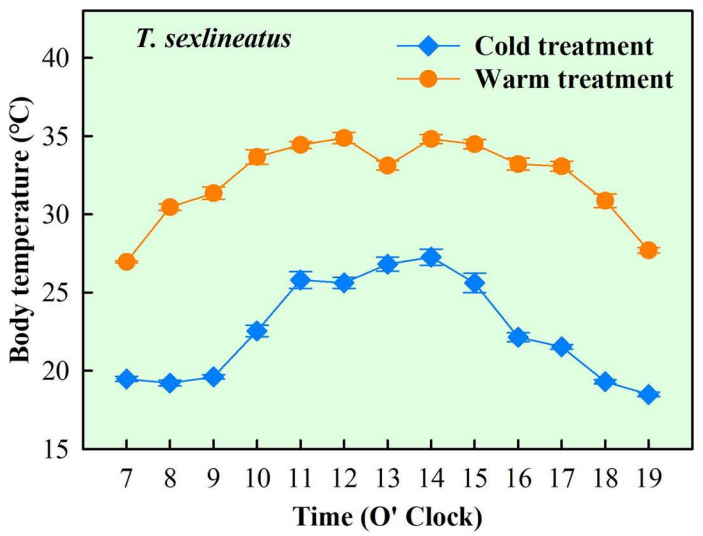
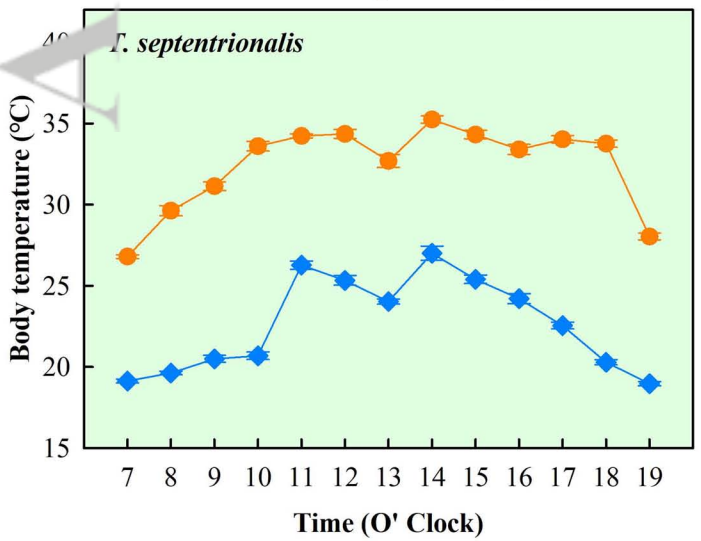
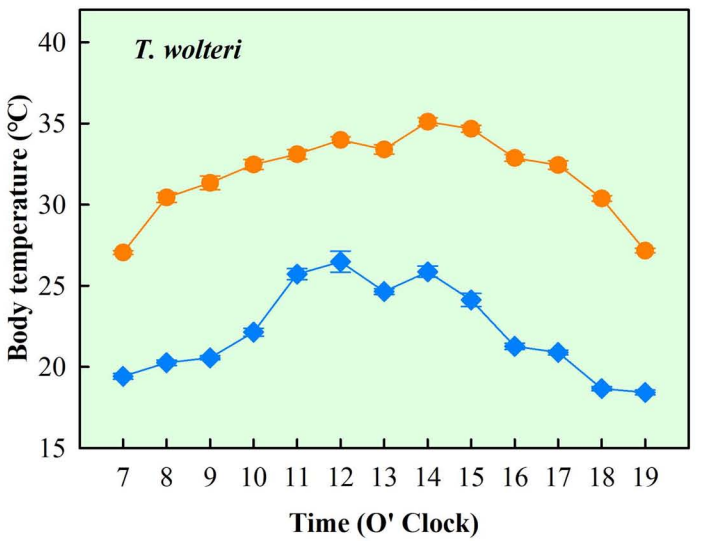
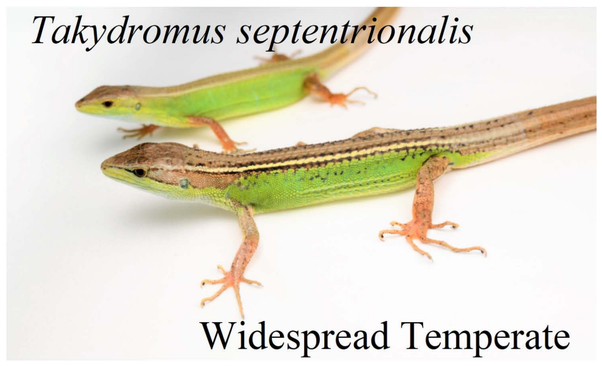


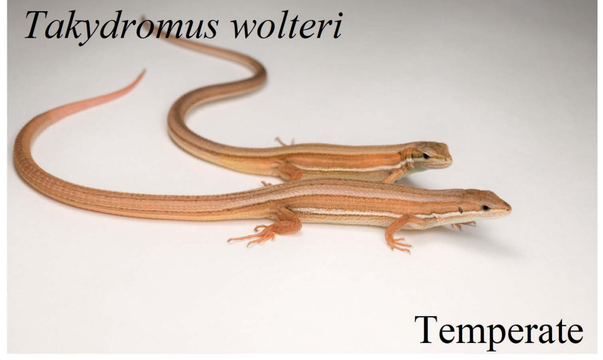




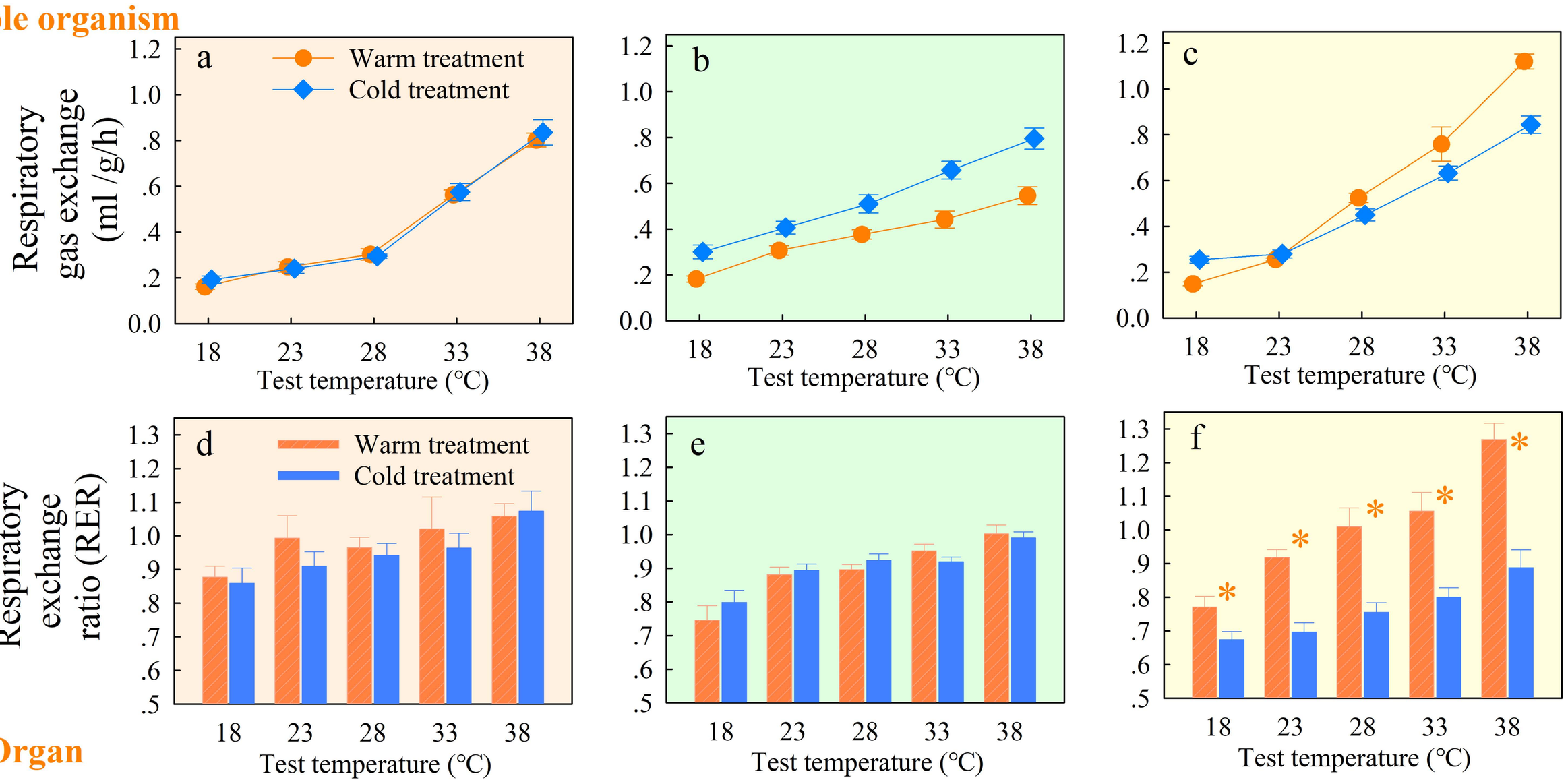


Level of Biol Hierarchy

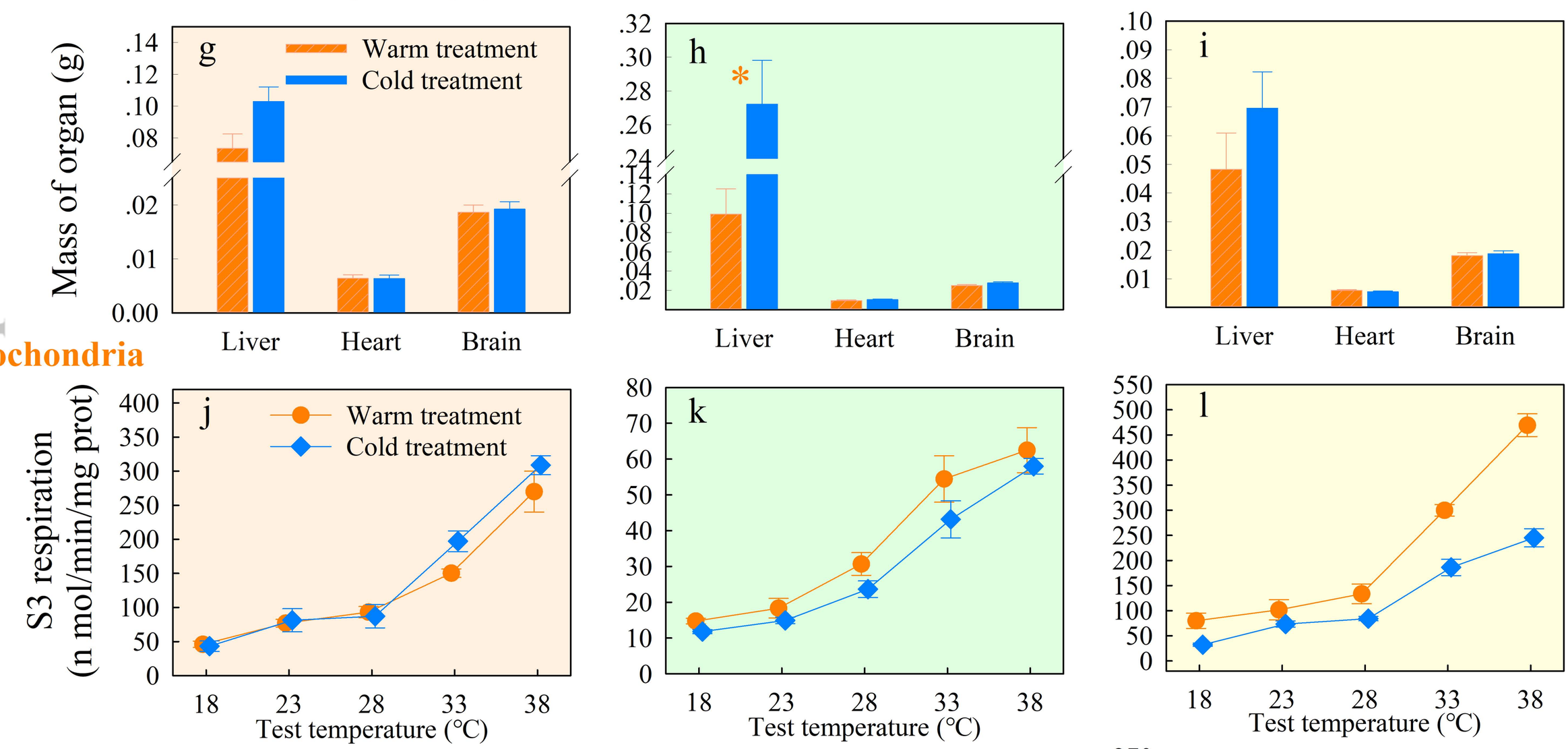




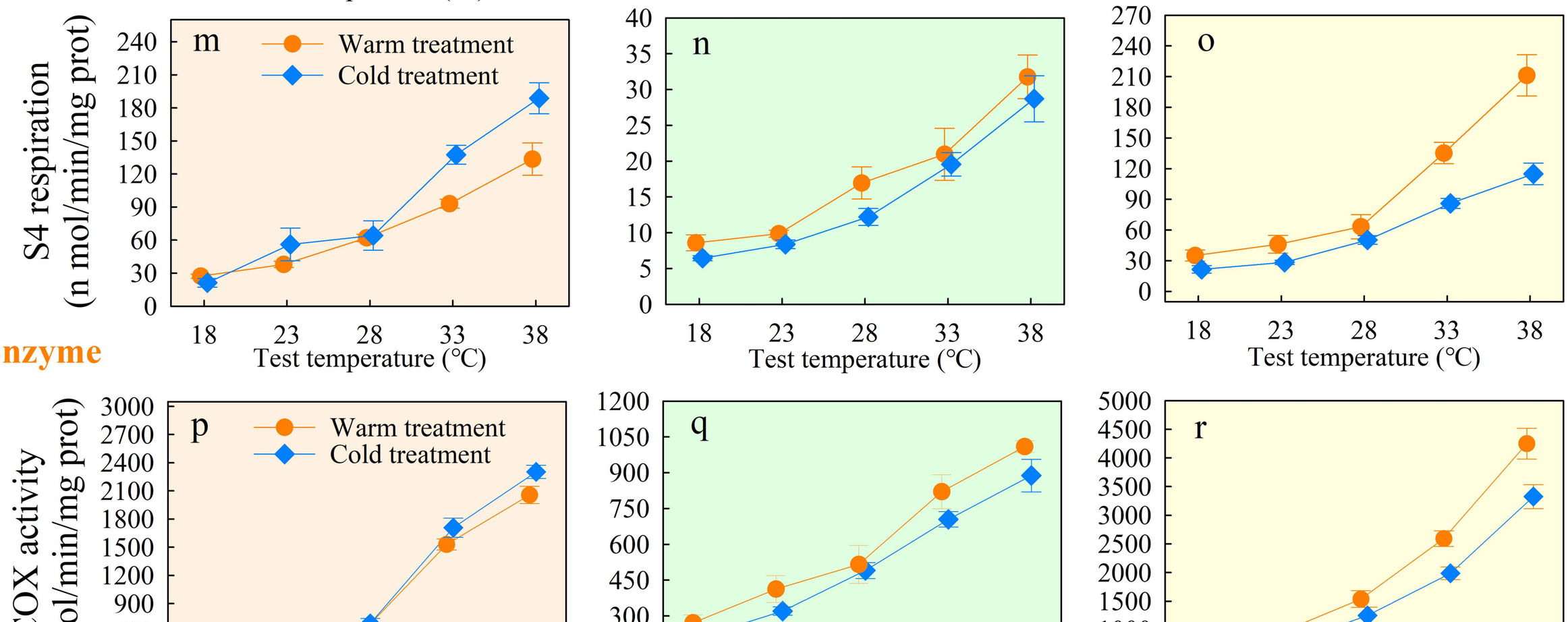
Whole organism



Organ



Mitochondria



Enzyme

T. sexlineatus

T. septentrionalis

T. wolteri

a Glycolysis

α-D-Glucose-1P

β-D-Fructose 6-P

PFK

β-D-Fructose 1,6-P2

D-Glyceraldehyde-3P

ACS

Pyruvate

Acetyl-CoA

LDH

L-Lactate

b Pentose phosphate pathway

D-Glyceraldehyde-3P

TKT

D-Ribose-1P

D-Ribose-5P

D-Ribulose-5P

β-D-Glucose-6P

PGM

PRPS

RPE

PRPP

D-Xylubose-5P

d Fatty acid degradation

Fatty acid

L-Palmitoyl

CPT-1

Hexadecanoyl-CoA

ACO

trans-Hexadec-2-enoyl-CoA

EHHADH

S)-3-Hydroxy-hexadecanoyl-CoA

HADH

3-Oxohexadecanoyl

ACAT

ACAA

Acetyl-CoA

Acetoacetyl-CoA

c TCA cycle

Acetyl-CoA

CS

Oxaloacetate

ACLY

Citrate

MDH

(S)-Malate

FUM

Fumarate

2-Oxoglutarate

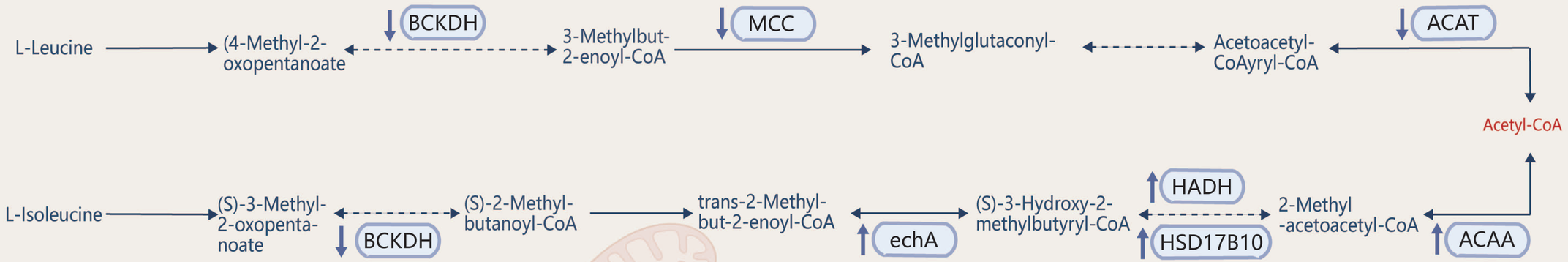
LSC

DLST

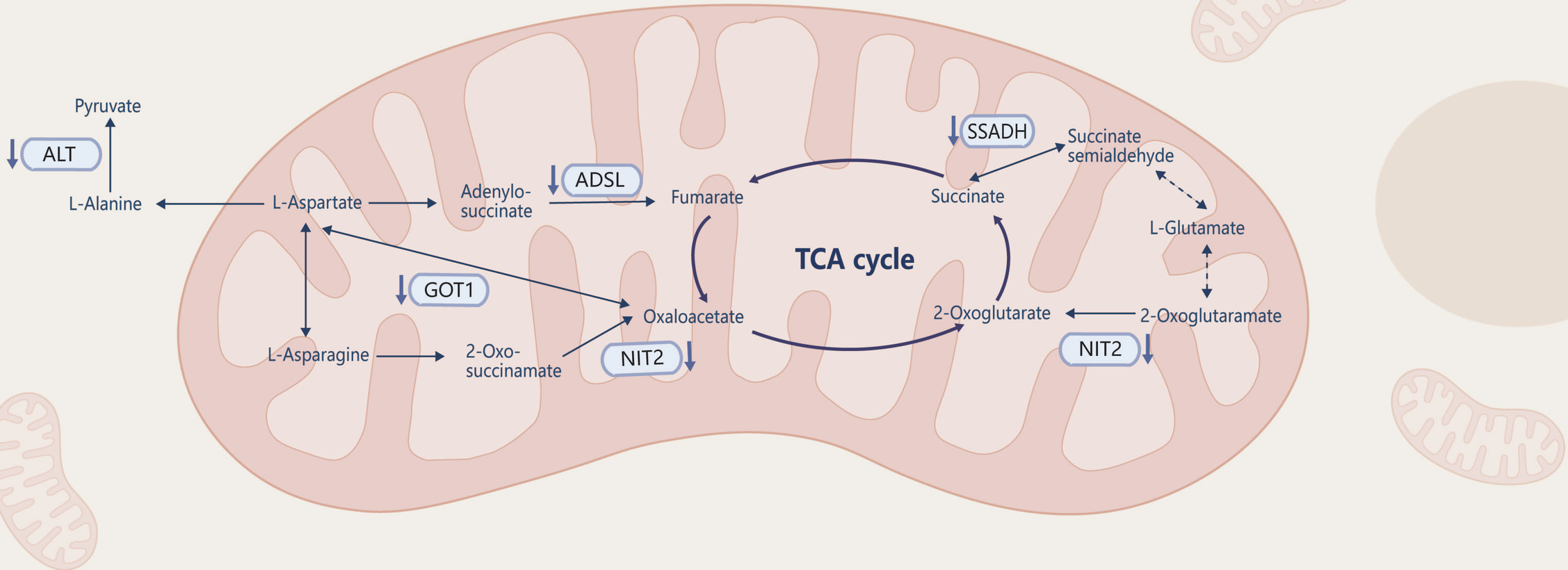
Succinate

Succinyle-CoA

a Valine, Leucine and Isoleucine Degradation



b Alanine, Aspartate and Glutamate metabolism



Alanine, aspartate and glutamate metabolism